

**PERFORMANCE OF DYNABEAD TECHNIQUE COMPARED TO FACS
TECHNIQUES IN ENUMERATION OF CD4+ T CELLS IN HIV
POSITIVE PATIENTS ATTENDING MBAGATHI HOSPITAL, NAIROBI**

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*Performance of
dynabead technique*



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DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other university or any other award.

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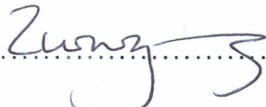
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DEDICATION

Dedicated to my husband Richard Maina, daughter Ruth Maina and son Timothy Maina.

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ACRONYMS AND ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
AZT	Zidovudine
BD	Beckton Dickson
CBC	Coulter blood count
CD	Cluster of differentiation
CDC	Centre for Disease Control
CTL	Cytotoxic T lymphocytes
DP	Dual Platform
FACS	Fluorescent Activated Cell Sorter
FDA	Food and Drug Administration
HIV	Human immuno-deficiency Virus
HTLV-III	Human T lymphocyte Virus -III
LAV	Lymphadenopathy Associated Virus
MSF	Medecins Sans Frontieres (Doctors without borders)
PCA	Personel cell analyzer
PCP	Pneumocytis carinii Pneumonia
PLG	Pan-leucogating
PMTs	Photomultiplier tubes
RNA	Ribonucleic acid
TLC	Total Lymphocyte Count
UNAIDS	United Nations Programme on HIV/AIDS
WHO	World Health Organization

ABSTRACT

Human immuno-deficiency virus and Acquired Immune Deficiency Syndrome (HIV and AIDS) is a major public health problem, a socio-economic burden and a serious threat to development particularly in developing countries. Human Immunodeficiency Virus (HIV) causes progressive impairment of the body's immune system, increased susceptibility to infections, tumors and Acquired Immune Deficiency Syndrome (AIDS). Enumeration of CD4+ T cells evaluates the strength of the immune system and is crucial in monitoring of HIV infected persons, as it is the best indicator of immuno-suppression. Flow cytometry (FACS count) is the accepted gold standard method for CD4+T cell counting. However the cost of the equipment and reagents of FACS count is often unaffordable for routine use in resource limited settings. This study evaluated the application of Dynabead technique using a light microscope for CD4 enumeration on HIV positive patients. Fifty four (54) EDTA blood samples from HIV positive patients attending Mbagathi District Hospital care clinic were used in the study. Parallel CD4+T cell count was carried out using BD FACS count and Dynabead techniques. The sensitivity, specificity, positive and negative predictive values of Dynabead technique was determined using a 2x2 table. Precision of Dynabead technique, as well as the effect of delay in sample handling was also determined. The results of the study revealed that CD4+T cell counts by FACS count and Dynabead technique were highly correlated $r= 0.962$, $p<0.01$. There was no significant difference in CD4+T cell count as enumerated by the two techniques at clinically relevant CD4+ T cell counts ($t=0.085$, $df=53$, $p>0.05$). The sensitivity and specificity of Dynabead technique at clinically relevant CD4+Tcell count (200 cell/ul of blood) was 87.5% and 96.7% respectively. The coefficient of variation for the precision of Dynabead technique varied between 3.4% and 7.5%. CD4+T cell count by Dynabead technique on fresh and four days old blood revealed a significant difference ($t=8.694$, $df =53$, $p<0.05$).The results showed that Dynabead technique is comparable to FACS count in CD4+T cell enumeration on fresh blood sample and therefore can be used as a reasonable substitute to FACS count in resource constrained areas in Kenya. However it was also noted that Dynabead technique was not accurate in CD4 enumeration for samples that had been preserved for four days at room temperature. It is recommended that Dynabead technique be adopted as a method for CD4+ T cell enumeration in resource constrained areas in Kenya.

CHAPTER ONE: INTRODUCTION

1.1 Background of the study

Acquired Immune Deficiency Syndrome associated with Human Immune-deficiency virus is a continuum of progressive damage to the immune system from the time of infection to the manifestation of severe immunologic damage by opportunistic infection, neoplasm, wasting or low CD4 lymphocyte count that defines AIDS (Dennis, 1998). In advanced HIV infection with severe immuno-suppression, there is a decrease in the number of CD4+ T cells to below 200 cells/ μ l in AIDS (Cheesbrough, 2000).

Studies of HIV infected persons have shown that nearly all infected persons have a CD4 lymphocyte count below the mean for sero-negative persons and show a progressive loss of these cells over time (Phillips *et al.*, 1991). The time from HIV infection to a CD4 lymphocyte count of less than 200 cells/ μ l is on average nearly 2 years less than to manifestation of an AIDS- defining opportunistic infection (Longini *et al.*, 1991). However with antiretroviral therapy survival time has increased among all HIV and AIDS patients over the age of 35 years with a CD4 lymphocyte count of greater than 100 cells/ μ l (Rachils *et al.*, 1998).

The number of people falling ill and subsequently dying from AIDS has a tremendous impact on society, demography, household, health and education, work place and economic aspects (Belhu, 1998; EFA, 2002). A study in several sub-Saharan countries has estimated that the combined impact of AIDS related absenteeism and productivity decline could cut profits drastically (Haacker, 2002). Sixty percent of the people living with HIV and AIDS are in sub-Saharan Africa.

One million of the nearly 32 million in Kenya are infected (UNAIDS, 2003). An estimated 24.5 million adults and children were living with HIV in Sub-Saharan Africa at the end of 2005. In Kenya people living with HIV and AIDS are 1.3 million, 740,000 women and 150,000 children. Acquired Immune Deficiency Syndrome (AIDS) deaths total to 140,000 while orphans due to AIDS are 1.1 million (UNAIDS/WHO, 2006). Sub-Saharan Africa by far remains the worst affected region with an estimated 22.5 million people living with HIV at the end of 2007, 68% of the global total. Based on a new study conducted in 2007 findings show an increase in HIV prevalence. An estimated 1.8 million people are living with AIDS in Kenya, 180,000 being children. Acquired Immune Deficiency Syndrome (AIDS) deaths total to 130,000 (UNAIDS, 2008)

The CD4+T lymphocytes are the hall mark and the apparent source of the central immune defect in HIV disease. Therefore, CD4+ T lymphocyte count and viral load have been used as the marker of disease progression (Dennis, 1998). Increase in HIV-RNA correlate with rate of CD4 lymphocyte decline (Mellors *et al.*, 1997). In addition to the CD4 lymphocyte count and the quantity of virus in peripheral blood, other laboratory tests have been used to predict AIDS in asymptomatic HIV infected persons. This include serum levels of Beta₂ α microglobulin, serum and urine levels of neopterin, soluble CD8, soluble interleukin -2 receptor, interferon-alpha and serum levels of IgA. These methods are however not conclusive (Lifson *et al.*, 1992).

The accurate determination of absolute and percentage values for CD4+T lymphocyte is crucial in monitoring and treatment of individuals infected with HIV (Liam *et al.*, 2002). In order to decide when to start treatment for HIV, CD4+T cell counts and CD4: CD8

ratio monitoring for children are necessary (WHO, 2003). These counts are also important in monitoring response to antiretroviral treatment in the absence of viral load determination and to decide on when it is safe to stop opportunistic infection prophylaxis (Nookhai *et al.*, 2002).

Flow cytometry is the accepted standard method for enumerating CD4+T cells in monitoring immune status. However, the cost of the equipment and reagents for flow cytometry is often unaffordable for routine use in resource-constrained settings (Pattanapanyasat *et al.*, 2004). With conventional CD4+T cell count techniques and viral load tests being unaffordable for routine use in resource constrained settings where low cost antiretroviral medication are now being offered, there is need for more reproducible and cost effective techniques for CD4+T cell monitoring and the costs for CD4 assays and viral load tests must be reduced for developing countries (Helene *et al.*, 1995; Bartholomew, 2001). The global struggle with HIV and the battle to develop affordable CD4+T cell counting technology are both unfulfilled goals in 2008. The need for such instrumentation is more critical now as implementation of ART is in progress in many resource limited regions (Manday *et al.*, 2008).

Several African countries have made progress in identifying practical solutions for improving HIV testing, prevention, monitoring and treatment in highly affected countries. Cheaper methods for CD4+ cell counts have already been introduced in countries such as Uganda, Malawi and South Africa. Pan-leucogating (PLG) whose cost is less than US \$5.00 per test compared to current cost of US \$ 20.00 for the Fluorescent Activated Cell Sorter (FACS) has been provisionally accepted by WHO as an

alternative for CD4+ cell counting in South Africa and in other resource poor settings (Mandy *et al.*, 2002). Cyflow another affordable CD4+count technique is in use in Malawi (Fryland *et al.*, 2004) and Rwanda (Servais *et al.*, 2004). Dynabead by fluorescent microscopy is a promising cost effective method for CD4+ cell enumeration (Carella *et al.*, 1995) and has demonstrated high correlation, compared to values yielded by flow cytometry (Jannosy *et al.*, 2002). Dynabead technique using acridine orange and fluorescent microscope is currently in use in Burkina Faso in resource limited areas (Diagbouga *et al.*, 2002).

In the category of manual methods such as Cytosphere system and Dynabead (magnetic bead) system for CD4+ cell counting, Dynabead system is the most promising method (George *et al.*, 2002). The Dynabead (magnetic beads) method uses two sets of beads CD14 and CD4. The CD14 beads bind to monocytes and are used to remove them from the blood. The CD4 beads bind to the CD4+T cells which are later stained with acridine orange to make the cells nuclei visible for counting under a fluorescent microscope (Diagbouga *et al.*, 2002). A modified Dynabead system using a different stain for CD4+ T cells – Turk solution can be used with an ordinary light microscope which is less expensive (MSF, 2004). George *et al.* (2002) recommended multi-centre studies to consolidate the tentative consensus that recommends manual method for CD4+ T cell counts hence the need to verify Dynabead technique using an ordinary light microscope.

In techniques verification and validation, there is need for laboratories from different geographic regions with high and low prevalence of HIV to compare the Dynabead technique with a gold standard. Test methods must be validated on accuracy, precision,

sensitivity, specificity, predictive values and efficiency before a decision to use them is made. The American college of physicians in 1997 noted that sensitivity and specificity could change if the population tested is dramatically different from other population tested earlier (Bailey and Scotts, 1998).

1.2 Study Justification

Efforts in blood bank HIV surveillance, genetic subtype and recombinants, plasma and cell samples to support vaccine research are on top gear in Kenya. However, research on affordable methods of monitoring HIV disease progression is yet to be considered. Currently enumeration of CD4 and CD8 cells is costly for most HIV and AIDS patients in developing countries. To ensure that antiretroviral (ARV) therapy is adequately monitored in resource constrained areas, breakthrough in cost effective CD4+ T cell monitoring methods in patients undergoing AIDS therapy must be a priority. Currently flow cytometry is used for HIV therapy monitoring in clinical trials. In order to increase access to the immunological monitoring among HIV patients, there is need to evaluate more affordable techniques such as Dynabead technique using light microscope. Less expensive, simpler protocols have been described but require more published validation data to gain widespread acceptance. It is against this back ground that this study was designed to determine the sensitivity, specificity, predictive values and precision of the Dynabead technique using a light microscope compared to flow cytometry. This will help in determining

the suitability of Dynabead technique as a provisional affordable and reliable alternative technique for immune status monitoring in resource limited settings in Kenya.

1.3 Research Questions

- a) What are the sensitivity, specificity and predictive values of Dynabead technique compared to the gold standard FACS count?
- b) What is the precision of Dynabead technique?
- c) What is the effect of storage of blood for four days at room temperature on CD4+T cell count using Dynabead technique?

1.4 Null Hypothesis

- a) There is no significant difference in the sensitivity, specificity and predictive values of Dynabead technique as compared to FACS count in CD4+T cell count.
- b) There is no significant difference in CD4+T cell counts by Dynabead technique in fresh blood samples compared to blood stored at room temperature for four days

1.5 Objectives of the study

1.5.1 General Objective

To evaluate Dynabead technique in CD4+ T cells enumeration as compared to the gold standard FACS count.

1.5.2 Specific Objectives

- a) To determine the sensitivity, specificity and predictive values of Dynabead technique in comparison to FACS count in CD4+ T cell enumeration among HIV and AIDS patients.
- b) To determine the precision of Dynabead technique.

- c) To determine the effect of storage of blood at room temperature on CD4+T cells count using Dynabead technique.

1.6 Limitations of the Study

Due to cost limitations, only fifty four (54) blood samples were used.

CHAPTER TWO: LITERATURE REVIEW

2.1 History of Human Immunodeficiency Virus and Acquired Immune Deficiency Syndrome (HIV and AIDS)

Centre for Disease Control (CDC) in Atlanta published a report about the occurrence without identifiable cause of *Pneumocystis carinii Pneumonia* (PCP) in five men in Los Angeles in June 1981. This marked the beginning of general awareness of AIDS in the USA (CDC, 1981). In 1982 doctors thought AIDS was an appropriate name for PCP and other rare life threatening opportunistic infections as they were acquired as a result of a weakened immune system and they showed a number of manifestations rather than a single disease (Connors and Kingman, 1988).

Doctors at the institute of Pasteur in France reported isolating a new virus, which they suggested, might be the cause of AIDS and named it Lymphadenopathy Associated Virus (LAV). In 1984, Dr. Robert Gallo of the National cancer institute also isolated the virus, which caused AIDS and named it HTLV-III (Marx, 1984). It was revealed that LAV and Human T lymphocyte Virus-III (HTLV-III) was one and the same virus (Max, 1985). In 1986, the International committee on the taxonomy of viruses ruled that both LAV and HTLV-III names be dropped and the virus was given a new name Human Immunodeficiency Virus (HIV; Coffin *et al.*, 1986). The first HIV case was officially recorded in the Soviet Union and massive HIV testing programme was conducted in 1987 (Headley, 1996). The HIV virus rapidly spreads through the host population who exhibit no sign of AIDS for the first ten years. Therefore by the time HIV infection was recognized as a specific disease in the gay male population of San Francisco, Los Angeles and New York in early 1980s, a substantial proportion of persons in those subpopulations were already infected with the virus (Koopman *et al.*, 1997). In the same

year, zidovudine (AZT) was approved as the first antiretroviral drug to be used as treatment for AIDS (FDA, 1987). By 1988 it was estimated that 5.1 million people were already infected with HIV and more than 400,000 lived with AIDS (WHO, 1989). In 1989 the second drug for the treatment of AIDS, dideoxyinosine (ddi) was made available to people with AIDS (Feudeinheim, 1989).

Towards the end of 1990 the estimate of the number of people with HIV world wide rose to 8-10 million, the highest number of cases being from Africa (Chin, 1990). In 1992, the food and drug administration (FDA) approved the use of ddi in combination with AZT for adult patients with advanced HIV infection and who were showing signs of clinical or immunological deterioration. This was the first successful use of combination drug therapy for treatment of AIDS (FDA, 1992).

In 1995, FDA approved the first potent new family of Anti-AIDS drug. The drug saquinavir belongs to a class of drug called protease inhibitors (Schwartz, 1995). In this same year it was estimated that worldwide 4.7 million new HIV infections occurred, of which 2.5 million occurred in South East Asia and 1.9 million in Sub-saharan Africa (Mann and Tarantola, 1996), The Food and Drug Administration (FDA) approved the drug viramune (Nevirapine), a non-nucleoside reverse transcriptase inhibitor and alongside it, viral load test was introduced to help provide information about the risk of disease progression (FDA, 1996).

Over the years HIV and AIDS cases have been on the rise. By 2005, 38.6 million were living with HIV and AIDS worldwide, 36.3 million being adults, 17.3 million women, and 2.3 million children. People newly infected with HIV in 2005 were 4.1 million while

AIDS deaths were 2.8 million. Over 25 million people worldwide have died of AIDS since 1981 and there are 12 million AIDS orphans in Africa (UNAIDS/WHO, 2006). By the end of 2005, women accounted for 48% of adults living with HIV worldwide and for 59% in sub-Saharan Africa. About 6,000 young people (15-24 years) become infected with HIV every day (UNAIDS/WHO, 2006). Of all the infectious diseases first recognized in the 20th century, AIDS has had not only the most profound effect on human illness and death, it also ended the developed world's complacency about infectious diseases (Korber *et al.*, 2000).

2.2 Impact of HIV and AIDS in Africa

During 2005 an estimated 2 million adults and children died as a result of AIDS in sub-Saharan Africa. The number of people falling ill and subsequently dying from AIDS has a tremendous impact on the society, demography, household, health, education sector, work place and economic aspects (Belhun, 1998 and EFA, 2002). In sub-Saharan Africa, annual direct medical costs due to AIDS (excluding ARV therapy) have been estimated at about 30 US dollars per capita. Combined impact of AIDS related absenteeism, productivity decline among others could cut profits drastically (Hacker, 2002). Rising mortality rates caused by HIV and AIDS in South Africa have substantial and lingering impact on poor households (Collins *et al.*, 2007). Health care and funeral expenses impact heavily on households, affecting income and expenditure (Max and Frederick, 2003). The importance of such costs have previously been shown in Rwanda (Nandakumar *et al.*, 2000), Ivory Coast (Topouzis, 2000), Uganda (Topouzis, 2000), Ethiopia (Bollinger and Stover, 1999) and Tanzania (Bollinger and Stover, 1999).

Life expectancy in sub-Saharan Africa has dropped from 62 years without AIDS to 47 years with AIDS (Stanecki, 2002). Together with other factors, HIV and AIDS have had a devastating effect on economies of many African countries. The annual, cost associated with sickness and reduced productivity as a result of HIV and AIDS varied from 17 US dollars per employee in a Kenyan manufacturing firm to 300 US dollars in the Ugandan Railway Corporation (Bolling and Stover, 1999). By 2002, more than 1 million of the 32 million Kenya's population were infected with HIV and more than 890,000 children had been orphaned by AIDS (UNAIDS 2003). Currently an estimated 39.5 million people are living with HIV world wide. There were 4.3 million new infections in 2006 with 2.8 million (65%) of these occurring in sub-Saharan Africa. In 2006, 2.9 million people died of AIDS related illnesses (WHO, 2007).

Sub-Saharan Africa remains the most affected region in global AIDS epidemic. More than 76% of global deaths due to AIDS-related illnesses in 2007 occurred in sub-Saharan Africa. This proportion is an evidence of the unmet need for antiretroviral treatment in the region. In sub-Saharan Africa an estimated 1.7 million people were newly infected with HIV in 2007. However adult (15-49 years) HIV prevalence declined from 5.8% in 2001 to 5.0% in 2007 (UNAIDS, 2007). In most countries in East Africa adult HIV prevalence is either stable or has started to decline. This is most evident in Kenya, where the HIV epidemic has been declining amid evidence of changing behavior. Besides behavioral change, mortality of people infected with HIV several years ago has also contributed to the declines in prevalence (UNAIDS, 2007).

2.3 Epidemiology and Pathology of HIV

Human Immunodeficiency Virus (HIV) belongs to the subfamily lentivirinae (slow virus) in the family of animal retroviruses. In general, retroviruses cause either proliferation (transforming viruses) or destruction (cytopathic viruses) of the cells they infect. Human Immunodeficiency Virus is a cytopathic virus. There are two sero types of HIV: Type 1 (HIV-1) and Type 2 (HIV-2). Type 1 and Type 2 share about 60% RNA sequence homology in some regions and 30-40% in other regions. Type 1 is by far the most common cause of AIDS in the western world. Type 2 produces a disease similar to that caused by HIV-1 and is present in high incidences in West Africa but is being found increasingly outside of Africa, in particular India (Stewart and Edward, 2001). Type 2 (HIV-2) is very closely related to Simian-Immunodeficiency Virus (SIV) which causes a form of AIDS with encephalitis in monkeys (Macaques) that is very similar to AIDS in humans. SIVmac the Macaque form of SIV has 75% sequence similarity to HIV-2 and 50% similarity to HIV-1 (Stewart and Edward, 2001).

Human Immune-deficiency Virus-1 (HIV-1) is the more predominant strain and it comprises three distinct viral groups termed M, N, and O. Group M is the major group within which eleven subtypes A-K have been identified (Harold, 2002).

Subtypes A and D predominate in Sub-Saharan Africa, subtype C in southern Africa and India while subtype E and F are found in Central Africa and South America respectively (Chemtai, 1998). Group O containing a distinct group of heterogeneous viruses which are of low prevalence have been found in West Africa, particularly Cameroon (Cheesbrough 2000). The HIV-2 strain is found mainly in West Africa, Europe, Asia and Latin America

(Stine, 1997; Cheesbrough, 2000). Type 2 (HIV-2) strain infecting humans consist of six evolutionary lineages; subtype A through F (Harold, 2002).

Human Immune-deficiency Virus is present in semen, vaginal/ cervical secretions and blood of infected persons and these are the main body fluids by which the virus is transmitted (Cheesbrough, 2000). Once HIV has entered the body, the immune system initiates anti-HIV antibody and cytotoxic T cell production. Within hours of exposure to HIV, CD4+T lymphocytes are found to be infected, showing active viral replication. This results in destruction of billions of CD4+T lymphocytes, eventually overwhelming the immune system's regenerative capacity (WHO, 2007).

Researchers also indicate that long before their numbers drop significantly, the CD4+ T lymphocytes of HIV positive individuals steadily lose their ability to respond to foreign antigens (Harold, 2002). Some researchers believe that HIV infection may trigger programmed cell death, known as apoptosis, in CD4+ T lymphocytes (UNAIDS/WHO, 2000). Others believe there is a chain reaction of CD4 lymphocyte activation by HIV's envelope protein (gp 120) which never stops, since HIV is always present in the body after infection. Eventually this constant CD4+T lymphocyte activation is thought to result in immune system collapse as seen in AIDS (Harold, 2002). Another hypothesis postulates that in HIV infection, TH2 cells become more common and the number of TH1 cells declines. Individuals with HIV infection show evidence of B cells over stimulation and therefore produce a large number of antibodies. A decline in TH1 cells seems to be linked with a drop in productivity of killer T cells that appear more necessary to control HIV (Harold 2002; UNAIDS/WHO, 2000). The infected CD4+ T cells release

virions by budding through the cell membrane or by lysis of infected cells. The released virus particles then infect healthy CD4⁺ T lymphocytes. CD4⁺ T lymphocytes also serve as important reservoirs of HIV (WHO, 2007; Cheesbrough, 2000). A study of HIV transmission suggest that transmission will not occur at all when the viral titers are less than 1,500 copies/ml. With successful multi-drug HIV therapy, viral titers of that level and lower can be expected and sustained for some time during the course of treatment (Quinn *et al.*, 2000).

Human immune deficiency virus (HIV) infection has four distinct stages. The first stage is the establishment by the virus which enters and colonizes the host. In the second stage, the primary infection, the virus proliferates to high densities and the host sero converts, causing an abrupt decline in the titer of circulating virus. In stage three, the circulating viruses remain at low levels, and disease symptoms are absent. Finally in stage four AIDS is obvious (Bruce *et al.*, 2001). New infections transmitted by recently infected persons, in stage 1, contribute much more to the spread of HIV than infections from persons in stage three (Levin *et al.*, 1996). The high virulence of HIV-1 might be due to an accident of evolution. It's believed that gene function lost during the course of viral evolution predisposed HIV-1 to spur the fatal immune system failures that are hall mark of AIDS. HIV is unusually lethal compared to similar viruses such as Simian immunodeficiency Virus (Kate, 2006).

During the primary HIV infection, the number of CD4⁺ T lymphocytes in the blood stream decreases by 20-40%. In acute HIV-1 infection, in addition to the decline in CD4⁺ T lymphocyte counts, qualitative impairment of CD4⁺ T lymphocyte functions are

detected (WHO, 2007). It can however, take one to six months for an individual exposed to HIV to produce measurable quantities of antibodies. The immune response is weakened as memory T cells (CD4+, CCR5+) are destroyed. Reduction in HIV specific helper T cell numbers leads to decreased activation and survival of cytotoxic CD8+ cells that can remove the HIV infected cells. The rapid loss of memory helper T cells and the inability to replace these cells leads to increasing immuno-deficiencies (Altifield *et al.*, 2001; Lange *et al* 2003). The progressive loss of CD4+ lymphocytes results in the loss of ability to mount desirable immune response to any pathogen and therefore vulnerability to opportunistic pathogens and tumor development characteristic of AIDS (WHO, 2007).

Following acute primary HIV infection, one may remain free of HIV related illness, often for years despite ongoing replication of HIV in the lymphoid organs and relentless destruction of the immune system (WHO, 2007). The median incubation period from HIV infection until development of AIDS is estimated at approximately 10 years for young adults but the estimate varies with age (Bachett and Moss, 1989).

With antiretroviral therapy it has been possible to reverse the decline in CD4+ T cells numbers and also control viraemia (WHO, 2007). Treatment extends the survival time of AIDS patients (Anderson, 1991).

Diagnosis of asymptomatic HIV infection in a person does not necessarily signify recent infection. On average, 8-11 years elapse before a person has onset of symptoms of HIV infection. Knowledge of newly acquired (e.g < 6 months) HIV infections will enable more accurate monitoring of trends among persons recently infected (Longini *et al.*,

1989). Centre for Disease Control (CDC) and its partners conduct supplemental studies to monitor clinical outcomes of HIV and AIDS cases, including integrating laboratory technologies with HIV and AIDS surveillance to monitor variant, atypical and drug resistant strains of HIV (Bennet, 2005).

2.4 Roles of T helper (CD4+T) and cytotoxic T cells (CD8+T) cell in immune responses.

The T helper and cytotoxic T cells play important roles in the body's immune responses. Cytotoxic lymphocytes kill target cells using multiple effectors mechanisms including damage to the target cell membrane by perforin released from Cytotoxic T Lymphocytes (CTL) granules and induction of apoptosis through interaction of Fas ligand with the Fas molecule on the target cell membrane (Lever, 1996). A massive, oligoclonal expansion of CD8+T cells responses has been described during acute HIV-1 infection (Pantaleo *et al.*, 1994). Virus specific CD4+T lymphocytes are clearly essential to provide help for the generation of virus-specific antibody responses. Cytokines secreted by CD4+ T cells may also enhance NK cell activity and the maturation of virus-specific CTL (Lever, 1996). Impairment of HIV-1 specific CD4+T cells function occurs very early in acute infection (Altfield *et al.*, 2001). This is followed by a functional impairment of CD4+T cell responses to other recall antigens as well as reduced responsiveness to novel antigens (Lange *et al.*, 2003). Immunological changes in HIV-1 infection include a decrease in CD4+ T cells, a transient increase in CD8+ T cells, total lymphocyte and inversion of the CD4 and CD8 ratio (Cooper *et al.*, 1988; Pedersen *et al.*, 1990).

A normal CD4+T cell count is 600-1500/ μ l of blood while a normal CD8+T cell count is about 300-1000 cell/ μ l (Highleyman, 2003). There are two types of CD8+ T cells, the T suppressor cells and the T killer cells which suppress immune responses and attacks

cancerous or cells infected with virus respectively (Highleyman, 2003). A CD4 count of above 500 cells/ μ l indicates a fairly normal immune function and low risk of opportunistic infections. A count below 350 cells / μ l indicates risk of opportunistic infections while below 200 cell/ μ l is diagnosed as having AIDS (Highleyman, 2003).

2.5 Parameters used in Monitoring Antiretroviral Therapy

Access to ARV therapy is affected by the cost of the drugs and laboratory monitoring tests (UNAIDS/WHO 2003). Introduction of affordable monitoring tests such as CD4+T-lymphocyte counts and viral load assay will go along way in helping to realize the proposal by the world health organization for the treatment of 3 million HIV-positive patients by the year 2005 (Janossy *et al.*, 2000; Janossy *et al.*, 2002; Crowe *et al.*, 2003). Several parameters have been used in monitoring the effectiveness of antiretroviral therapy in various countries. When absolute lymphocyte counts were used as indicators of antiretroviral therapy in Indonesia, 39% of HIV infected persons were misclassified (Donega *et al.*, 2004). Weight gain has also been used as a surrogate marker for response to antiretroviral therapy (ART). Research by CDC in Atlanta Georgia concluded that weight gain was not correlated with viral load reduction (Teshale *et al.*, 2004). Following a comparison of clinical findings and CD4+T cell counts by flow cytometry techniques, the sensitivity and specificity of the clinical criteria was 39% and 91% respectively. Positive and negative predictive values were 76% and 68% respectively. These findings suggested that clinical findings were not accurate in determining when to start antiretroviral therapy or to monitor response to therapy once it is started (Solberge *et al.*, 2004).

Researchers in Kampala, Uganda evaluated paired total lymphocyte count (TLC) and CD4 counts along side clinical features. The correlation between TLC and CD4 counts was significant ($r=0.72$; $p<0.0001$). Total lymphocyte counts less than 1200 cells / μ l predicted CD4 count <200 with a positive predictive value of 100% and negative predictive value of 32%. These researchers concluded that despite good correlation between the two methods, combination of TLC and clinical features is required to identify patients with CD4 cell counts, less than 200 cells/ μ l (Akinola *et al.*, 2003). Total lymphocyte count (TLC) is therefore a poor substitute of CD4+ cell counts in the pre-symptomatic phase of HIV disease and is not useful for monitoring ART (Jacobson, 2003; Mane, 2003; Akinola *et al.*, 2003; Semitala *et al.*, 2004 and WHO., 2007). Kimani *et al.* (2003) suggested that efforts should be directed towards ensuring affordability of proper CD4+T cell counting.

Viral load is the other laboratory marker of HIV disease progression. Increases in HIV RNA correlate with rate of CD4 lymphocyte cell decline (Mellors *et al.*, 1997). In a study by Kannangai *et al.* (2001) it was revealed that there was a significant negative correlation between the HIV-1 load and CD4+ T cell counts estimated by flow cytometry ($r=-0.63$, $p<0.001$) as well as between the HIV-1 load and CD4+ T cell counts estimated by Capcellia ($r=-0.61$, $p<0.001$). In addition to CD4 count and viral load determination in peripheral blood, other assays have been shown to predict AIDS in asymptomatic HIV infected persons. These assays include cytokine induction, antigen induced proliferation and measurement of activation markers which assess the function of lymphocytes. However, the total CD4 T lymphocyte numbers remain the most reliable marker of immune competence (WHO, 2007).

2.6 CD4+T Lymphocyte Count and Antiretroviral Therapy

Antiretroviral treatment for HIV infection consists of drugs which work by slowing down the replication of HIV in the body. The decision about which drugs to take depends on a number of different factors. These include the availability and price of drugs, the number of pills, the side effects of the drugs, the laboratory monitoring requirements and whether there are co-blister packs or fixed dose combinations available (WHO, 2006 and Renaud-Thery *et al.*, 2007). The increase in availability of HIV-1 antiretroviral drugs in resource limited settings has led to the urgent need to develop systems and technologies for accurate and cost effective measurements of CD4+T cells (O’Gorman *et al.*, 2008). In the absence of facilities for CD4+ T lymphocyte count, all patients with WHO stage 3 and 4 disease should start Anti Retroviral Therapy (ART). Those with WHO stage 1 and stage 2 disease should not start therapy but should be monitored carefully, with a minimum of three-monthly clinical reviews at any time if new symptoms develop (Cheesbrough, 2000; WHO, 2007 and Renaud *et al.*, 2007). The proposed WHO staging system comprises about 32 clinical conditions grouped into four clinical stages. These stages include: Stage 1, primary infection and sero-conversion; Stage 2, clinical latent infection; Stage 3, early HIV disease; Stage 4, late HIV disease and full blown AIDS (Chemtai, 1998; Cheesbrough 2000). The clinical conditions include persistent generalized lymphadenopathy, recurrent respiratory tract infections, weight loss, chronic diarrhea, Kaposi sarcoma among others (WHO, 2006).

The WHO recommends that one particular combination of drugs should be chosen to be provided for most people when they start treatment. Additional drugs should be available for people who need to substitute one drug for another because of toxicity or for use in

special situations (WHO, 2006). In Clinical studies, CD4 count and plasma viral load are predictors of the estimated risk of progression to AIDS, which is a factor in determining when to start treatment (Ian and Williams, 2001). With the advent of HAART, the overall progression of HIV infection to AIDS and from AIDS to death has slowed (Palella *et al.*, 1998). Consequently AIDS surveillance no longer serves as a reliable surrogate for monitoring HIV-infection trends. Relevant clinical and laboratory information (e.g CD4 count, viral load), is critical for monitoring HIV and AIDS (CDC, 1999). Due to the relatively high cost of virus load estimation, the CD4+T lymphocyte count remains the most important key indicator for initiation and monitoring of ART and a measure of the effectiveness of the treatment in clinical trial evaluations (WHO, 2007). Clinical practices across Europe and North America vary, but most clinicians would consider initiating therapy when the CD4 count is $200-350 \times 10^6 / l$ and in all patients who are symptomatic (Ian and Williams, 2001). Table 2.1 shows recommendations for starting antiretroviral therapy in adults.

Table 2.1 Recommendations for starting antiretroviral therapy in adults according to British HIV Association (BHIVA) and United States Department of Health and Human Services (USDHHS)

Disease stage	BHIVA	USDHHS
Symptomatic	Treat	Treat
Asymptomatic		
CD4 $< 200 \times 10^6 / l$	Treat	Treat
CD4 $200-350 \times 10^6 / l$	Therapy depending on rate of CD4 counts decline and symptoms.	Therapy should generally be offered
CD4 $> 350 \times 10^6 / l$	Defer	Defer or consider therapy if high viral load

Adapted from BHIVA, USDHHS (2001)

According to WHO, someone who has WHO stage 4 disease should start ARV treatment whatever the results of their CD4 test. They should also start if they have stage 1 and 2 disease and a CD4 count below 200. If the person has stage 3 disease then whether they should start depends on their clinical symptoms and whether they have a CD4 count below 350 (WHO, 2006). Initiation of treatment is recommended for all HIV infected pregnant women who have a CD4 count below 350 and have reached stage 3 as well as for all HIV- infected people who have CD4 counts below 350 and have pulmonary TB or severe bacterial infection (WHO, 2006; Renaud *et al*, 2007). It is now accepted that falls in plasma viral load combined with increased CD4 count are predictive of the clinical treatment responses on different combination regimens at 1-2 years (Ian and Williams, 2001).

World Health Organization (WHO) recommends that in most cases a first line regimen should consist of two drugs from nucleoside/nucleotide (NRTI) group and one drug from the non-nucleoside (NNRTI) group. Drugs from protease inhibitor (PI) group are generally less suitable for starting treatment in resource limited settings for a number of reasons including cost, the number of pills which need to be taken and the particular side effects that occur with the protease drugs (WHO, 2006). Choice of the antiretroviral regimen will depend on efficacy, tolerability, and adherence and resistance profile of regimen (Chemtai. 1998). The preferred firstline regimen consists of Zidovudine (ZDV/AZT) or tenofovir (TDF) combined with Lamivudine (3TC) or emtricitabine (FTC) combined with (efavirenz) EFV or nevirapine (NVP). Alternatively, AZT or TDF can be replaced by either stavudine (d4T) or abacavir (ABC). Rapamycin is also known to suppress HIV type 1 replication (Roy *et al*, 2002; WHO, 2006). The use of potent

combination antiretroviral therapy has also been linked to the development of adverse consequences (e.g metabolic complications and viral resistance), which can pose challenges to clinical management (Rockville, 2006). Other immunotherapy approaches include CD4 decoys and interferon administration among others. Soluble CD4 binds to the CD4+T cell site receptors and intercepts viral gp 120 preventing its binding to CD4+ expressing target cells (Chemtai, 1998).

2.7 CD4+ T cell Enumeration Techniques

Measuring CD4+ counts in HIV positive patients remains the single most important immunological parameter measured in HIV infected individuals for evaluation of their prognosis, immune status, response to therapy and diagnosis of AIDS (Maurice *et al.*, 2000; Pattanapanyast *et al.*, 2005). A CD4+ cell count is less expensive than viral load determination and may provide additional information of value to the clinician with respect to prognosis and need for prophylaxis and treatment (Lynn *et al.*, 2005).

2.7.1 Flow Cytometry

Flow cytometry is the accepted gold standard method for CD4+ T cell enumeration due to its accuracy, precision and reproducibility (Pattanapanyasat *et al.*, 2005). Flow cytometry differentiates and quantifies properties of labeled cells by Flow Cytometers (Janeway *et al.*, 1999). The three main instruments for flow cytometry are FACS Calibur, FACS Scan and FACS count (MSF-Belgium, 2004). The first two instruments are intended for research purposes and can access several surface markers. FACS count enumerates CD4+, CD8+ and CD3+ T cells and is intended for routine use (Janeway *et al.*, 1999). Cells to be analyzed by flow cytometry are first labeled with fluorescent dyes

(immuno-phenotyping). The cells are forced through a nozzle in a single-cell stream that passes through a laser beam. Photomultiplier tubes (PMTs) detect the scattering of light which is a sign of cell size, granularity and emission from different fluorescent dyes (Turgeon, 1993; Janeway *et al.*, 1999; WHO, 2007).

CD4+T cell counts by FACS machines are expensive because of the following reasons: machines and the antibodies are expensive and highly trained persons are necessary to perform the tests and maintain the equipment (Crowe *et al.*, 2003; Bob, 2004; Pattanapanyasat *et al.*, 2005). The FACS machine also requires well-established infrastructure hence unaffordable for routine use in resource-constrained settings (Pattanapanyasat *et al.*, 2004). Moreover, flow cytometry requires fresh blood samples for accurate results, which is a problem in areas with no refrigeration and efficient transport. Table 2.2 shows a summary of standard flow cytometry (method of choice) with different gating strategies.

Table 2.2 Summary of flow cytometry based assay for CD4+ T cell count using standard flow Cytometry (method of choice) with different gating strategies

System	Parameters measured	Advantages	Disadvantage
1. Standard flow cytometry (method of choice)	Absolute count and percentages of CD4, CD3 and CD8+Tcells and other lymphocyte subsets for example T,B and NK cells	(a) Percentages from the flow cytometer. (b) Absolute counts using a dual platform system with hematology analyzer. Different gating strategies can be used for gating the population of interest. (c) Can be used as a single platform system when the bead containing tubes are used to obtain the absolute count from the flow cytometer. (d) Can be automated.	(a) High cost of instruments and reagents systems. (b) Technically demanding (c) Requires controlled utilities and environmental conditions. (d) Need good technical support. (e) Requires hematology analyze for dual platform approach

(e) High throughput system

(f) Open system
(can be used with reagents from various sources)

2. Gating on the basis of size and granularity (light scatter gating)	Same as above	Gating can be universal since no fluorescence marker is used for gating	(a) Can not be used on aged samples. (b) Purity for the gated population is questionable
3. T cell (CD3) gating (lineage gating)	CD4, CD8 and CD3 percentages and absolute count (a) CD4+ T cell and lymphocyte percentage. (b) Absolute CD4+ T cell count and lymphocyte with trucount (BD) or flowcount (Coulter) tubes	Accurate gating of the population of interest (a) Single tube approach can be used accurately on the aged sample (up to 5 days). (b) High throughput (upto 300-400) sample per day on a single instrument - 8 hour/ day.	Can not be used on aged samples
			(c) Most widely

used approach.
Can be
performed with
no lysis
protocol

Adapted from WHO (2007)

2.7.2 Cost effective CD4+ T cell Enumeration Techniques

With the increasing access to antiretroviral therapy and the increasing numbers of HIV and AIDS patients, a cost effective CD4 and CD8 enumeration tool must be made a priority. As ART becomes more affordable, valid, and reliable, inexpensive laboratory tests are also needed to monitor the progress of disease in people with HIV infection (Kannangai *et al.*, 2001). The urgent need for affordable and technically simple CD4 diagnostics is widely recognized. Introduction of affordable monitoring tests will go along way in helping realize the proposal by WHO for the treatment of over 5 million HIV positive patients in future (Janossy *et al.*, 2002 and Crowe *et al.*, 2003). Progress has been made in identifying practical and cost effective CD4+ T cell monitoring techniques (Greve *et al.*, 2003). One of the responses is by finding ways of delivering flow cytometry tests more cheaply while maintaining or even improving the quality. One of such ideas is Cyflow system from Partec Germany, which can be delivered as a compact unit that can be powered from a car battery (Greve *et al.*, 2003; Cassen *et al.*, 2004). Cyflow is designed for use in resource-limited areas. It is less expensive, uses less expensive reagents and is able to produce an absolute CD4+ T cell count without additional instrumentation (Teav *et al.*, 2004). Cyflow is however vulnerable to trapped air in the system and the capital equipment cost is high, 20,000 U.S dollars. A commercially available density-based negative selection assay (Rosette Sep) has also been modified to make it applicable for low-cost cell enumeration. The technique has

potential to deliver an accurate, precise, low-cost test to monitor HIV positive patients (Bold *et al.*, 2007).

Panleucogating (PLG) is another promising cost effective technique. Using CD45 antibodies it identifies CD4+ markers on white blood cells and the number of CD4+ T cells in the blood is expressed as a percentage of the total leucocyte population, CD45+ (Mandy *et al.*, 2002). Panleucogating (PLG) with generic reagents could reduce the cost per test from 11.50 U.S dollars to 2.30 U.S dollars. However while these savings are significant, this method still relies on an initial investment in equipment costing up to 20,000 U.S dollars as well as expensive yearly maintenance contracts (Pattanapanyasat and Sippy, 2004 ; Pattanapanyasat *et al.*, 2004). Capcellia is yet another cost effective, user friendly assay that correlates well with HIV-load determination for individuals both with and without treatment (Kannangai *et al.*, 2001). Table 2.3 is a summary of flow based assays for CD4+ T cell count enumeration using a single platform approach.

Table 2.3 Flow cytometry based assay for CD4+ T cell count using single platform approach

System	Parameters measured	Advantages	Disadvantages
1. FACS Count with microbead-based system (Becton Dickinson)	(a)CD4,CD8 andCD3+Tcel l absolute count (b)CD4/CD8 ratio	(a)Fully automated (b) No RBC lysis required (c) Low-level expertise required (d) US FDA approved. (e)Accuracy and precision validated (f) Can be used on blood up to 48 hours after collection. (g)100µl whole blood / test	(a)No CD4 percentage capability, important for monitoring infants and children. (b)Dedicated and closed system. (c)Process time (1 hour 30 minutes) (d) High cost
2. Guava Easy CD4 Volumetric system	CD4,CD8 and CD3+Tcell absolute count	(a)Robust (b)Open system (c)US FDA approved	(a)Not validated adequately (b)Information on stabilized samples in not available.

3. Partec cyflow counter volumetric system	(a) CD4,CD8 and CD3+Tcell absolute count (b)CD4/CD8 ratio (c) Other subsets depending upon reagents	(a)No lyse system for absolute counts. (b)Volumetric cell counting , no bead calibrators required (c) Open software architecture for menu expansion (d)15 minutes turn around time (e)Lyse no wash system can give percentage (f)Open system	(a)No CD4 percentage capability if no-lyse method is used (b)No independent validation of performance. (c)No model distinction for various Cyflow models (d) Still under validation performance with stabilized sample is questionable
4. Point CARE system	Absolute CD4+,T cell absolute count and percentage, lymph% and absolute count	(a)No technical skill required (b)Robust (c) Useful in remote places (d)US FDA Approved	(a)Closed system (b)Not validated adequately (c) Information on the performance on stabilized samples is not available

Adapted from WHO (2007)

The third option of reducing cost of CD4+ T cell enumeration is the use of microscope-based CD4+ counting systems. These alternatives include the Cytosphere system and Dynabead (magnetic beads) systems. The Cytosphere system (Beckman Coulter) depends on being familiar with the shapes of cells. The monocytes are not removed but appear different under the microscope so the bead covered CD4+ cells can be counted. There is however a tendency to overestimate CD4+ cell counts (Karcher, 2003). Table 2.4 shows a summary of alternate technologies for CD4 enumeration.

Table 2.4 Summary of alternative technologies for CD4 enumeration

System	Parameters measured	Advantages	Disadvantages
1. Beckman counter cytosphere kit	CD4 absolute count	(a)Simple: microscope and counting chamber(0.1 mm deep) is needed (b)Can be performed at remote sites (c)FDA approved (d)100ul whole blood sample/ test (e)Good correlation with standard flow assays	only (a)No CD4 percentage hence could not be used in infants and children (b)Low throughput (c)Moderate cost/ test (d)Labour intensive
2. Microchip-based systems	Absolute CD4 count	(a)No technical skill required (b)robust	(a)Still under development (b)Cannot give CD4

		(c) Useful in remote places	
		(d) Can be upgraded with CCD camera for image analysis at distant central laboratory	
3. Semi-bio manual slide technology	Absolute CD4 count	(a) No technical skill required	(a) Still under development
		(b) Robust	(b) cannot give CD4 Percentages
		(c) Useful in remote places	
4. Total lymphocyte count	WBC count and differential lymphocyte count and percent	(a) Automated system	(a) Correlation to clinical assessment of drug efficacy unknown
		(b) Low cost	(b) Needs fresh sample for analysis to ensure accuracy
		(c) Low expertise required	

Adapted from WHO (2007)

2.7.3 Dynabead Technology

Dynabead systems include the manual microscope-based CD4⁺ cell counting (DynaL Biotech) and the CD4⁺ cell magnetic bead depletion with automated cell counting (Buranapraditkun *et al.*, 2004). The CD4⁺ magnetic bead depletion technique uses anti CD4⁺ dynabeads monoclonal antibodies for CD4⁺ cell depletion and automated Coulter Blood Cell (CBC) counter. An automated CBC counter measures pre and post depleted absolute lymphocyte counts. Subtraction of absolute lymphocyte counts before and after CD4⁺ cell depletion by magnetic Dynabeads gives the value of the absolute CD4 cell

count. Correlation and regression between the CD4+ cell counts of FACS based technique and the Magnetic bead CD4+ cell depletion is 0.92 and 0.84 respectively. The sensitivity and specificity at the CD4+cells cut-off point at 200 cells/ μ l is 88% and 86% respectively. This is a simple and reliable method but the availability of an automated CBC counter in resource-limited areas is still a challenge (Buranapraditkun *et al.*, 2004).

The manual microscope-based CD4+counting system uses two sets of beads. One type (CD14 beads) binds to monocytes and is used to remove them from the blood. The second type (CD4 beads) is then used for counting CD4+ cells. These CD4+ cells can be stained with acridine orange to make the cell nuclei visible for counting under a fluorescent microscope. The cells can also be stained with Turk's solution for use with a light microscope, which is even less expensive. A modified system can use a mirror and daylight as a light source (Diagbouga *et al.*, 2002). The Dynal magnet is used to separate Dynabeads from diverse liquid sample matrices. Dynabeads are attracted to the magnet adjacent to the tube wall when the tube is inserted into the Dynal MPCR.M housing. This enables easy removal of supernatant. The Dynabeads are left isolated to the side of the tube (Dynal-MPCR-M, 2003). Fig 2.1 illustrates sample preparation for CD4 T lymphocyte count by light microscope.

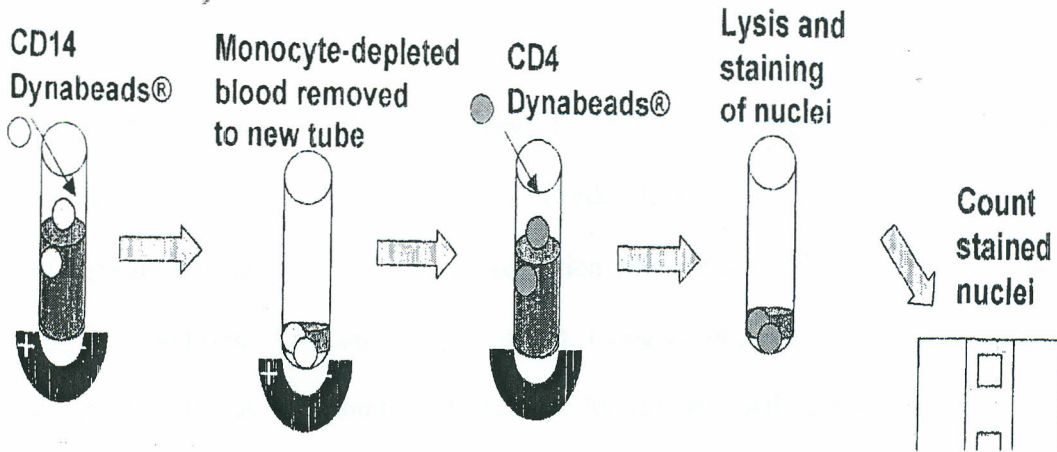


Figure 2.1 Sample preparations for CD4+T lymphocyte count by light microscope (MSF-Belgium, 2002).

2.8 Evaluation of HIV Diagnostic Tests

The validity of a diagnostic test is represented by sensitivity and specificity as well as positive and negative predictive values (Noordhuizen and Frankena, 1997). A simple diagnostic test for a particular disease or outcome classifies patients into two groups: those with the outcome and those without (Bewick *et al.*, 2004). A test is assessed by its ability to diagnose the outcome correctly, whether positive or negative. To help decide the presence or absence of disease, a cut off point for 'normal' or 'abnormal' is chosen (Akobeng, 2007). If the actual outcome is not evident then it may be supplied by the 'gold standard' test (Bewick *et al.*, 2004). Sensitivity and specificity are the basic measures of accuracy of a diagnostic test; however, they depend on the cut off points used to define 'positive' and 'negative' test results (Obuchowski, 2003). The sensitivity and specificity of a test vary according to the level that is chosen as the cut off point (Akobeng, 2007). A discriminating test would have sensitivity and specificity close to

100%. However a test with high sensitivity may have low specificity and vice versa (Bewick *et al.*, 2004).

Diagnostic sensitivity is the percentage of persons who have a given disorder who are identified by the test as positive for the disorder. Diagnostic specificity is the percentage of persons who do not have a given condition who are identified by the test as negative for the condition (La canna *et al.*, 2000). Craig *et al.*, (1991) defines specificity of diagnostic test as the conditional probability the test will classify an individual as belonging to the diseased-free or control population. Sensitivity of a test is the conditional probability that the test will classify an individual as belonging to positive, diseased or case population (Craig *et al.*, 1991). Sensitivity and specificity may not be invariant for a diagnostic test but may depend on characteristics of the population, for example age profile or severity of disease (Bewick *et al.*, 2004). Sensitivity and specificity define the operating characteristics of an assay, but it is the positive and negative predictive values of the assay that are generally of diagnostic importance to the clinician and patient (La canna *et al.*, 2000). Sensitivity and specificity are not affected by the prevalence of the disease (Bewick *et al.*, 2004).

Sensitivity and specificity do not address the problems of prevalence of disease in different population, hence the need to understand positive and negative predictive values. Positive predictive value is the probability that a person whose test result is positive truly has the disease or condition of interest. Negative predictive value is the probability that a person whose test result is negative does not have the disease or condition of interest (La canna *et al.*, 2000). Although positive predictive values (PPV)

and negative predictive values (NPV) give a direct assessment of the usefulness of the test, they are affected by the prevalence of the disease. Increase in prevalence leads to an increase in PPV and a decrease in NPV. When the prevalence is low, the PPV will be low irrespective of the sensitivity and specificity of the test (Bewick *et al.*, 2004)

Precision which refers to the reproducibility of a test when it is run repeatedly is of importance for a test to be acceptable. The precision of an accepted CD4+ T cell count technique, ELISA using TRAx CD4 test kit ranged between 4.6% and 5.9% (Helene *et al.*, 1995). The range of coefficient of variation of cyflow technique for CD4+ T cell count was 2.8% -4.9 % (Teav *et al.*, 2004). Intra laboratory coefficient of variation for Tru- count technique using samples held overnight for both CD4 and CD8 was observed to be 2% and 3% respectively (Carol *et al.*, 2000). Analysis shows that testing with the Easy CD4 assay is accurate and exhibits excellent reproducibility (Hayward, 2004). When Guava personnel cell analyzer (PCA) was evaluated against both single platform (SP) using flow count beads and Dual platform (DP) it compared well with a small bias (-16 cells/ μ l). The coefficient of variation (CV) was 8.49% (Scott *et al.*, 2004)

2.9 Effect of delay in sample handling on CD4+ T cell count

Specimen stability studies have established that whole blood can be held for up to 22 days at 4⁰C and for at least 3 days at room temperature before TRAx results are affected. This could be due to the fact that the principle of TRAx assay is based on detection of total CD4 protein in a whole-blood lysate, hence results are not dependent on intact or viable cells as with other cytometric techniques (Helene *et al.*, 1995). Samples that are

stored up to 7-8 days can be used with pan-leucogating when maintained at 25⁰C or less (Glencross, 2002).

For whole blood held more than 3 days at room temperature (22-26⁰C), the coefficient of variation of TRAx CD4 results double and after 10 days they increase dramatically over the expected variability for the test suggesting that the specimens may be deteriorating. At 4⁰C, the coefficient of variation exhibits no increase until after day 23. Whole blood lysates are stable at -70⁰C for 12 months. The ability to store specimens and repeat measurement for them has important implications for improving quality control procedures in clinical laboratories that perform CD4 testing routinely (Helene *et al.*, 1995).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Site

Mbagathi District hospital is located south west of Nairobi, the capital city of Kenya. It is a Nairobi District government hospital and serves as a referral hospital for HIV/AIDS cases are referred there. Mbagathi District Hospital was conveniently selected as it houses a comprehensive care clinic, funded by the government of Kenya and supported by Medecins Sans Frontieres (MSF)-Belgium. The hospital uses FACS count for CD4+T cell enumeration.

3.2 Test samples

Test samples used were obtained from HIV infected persons attending Mbagathi District Hospital comprehensive care clinic. The clinic provides medical and counseling care to HIV infected patients. An average of sixty HIV positive patients attends the clinic every week. Fifty four (54) samples were used in this study. A total of 126 tests by Dynabead technique were carried out and reported. The tests conducted involved fifty four (54) tests for fresh blood samples, fifty four (54) for stored blood samples and eighteen (18) tests for precision determination.

3.3 Inclusion Criteria

All HIV infected persons attending Mbagathi District Hospital Care Clinic were included in the study.

3.4 Exclusion Criteria

HIV infected persons not attending Mbagathi District Hospital Care Clinic.

3.5 Ethical Considerations

Clearance to carry out this study was obtained from the Board of Post Graduate Studies, Kenyatta University. Approval of the study was granted by the Medical Officer in Charge Mbagathi District Hospital. Blood samples for this study were obtained from diagnostic samples and were not linked to patient's name hence the patient's consent was not required.

3.6 Blood Sample Collection

Five milliliters (5 mls) of patient's whole blood was collected aseptically by venipuncture into a sterile K3 EDTA (Ethylene Diamine Tetra Acetic acid) vacutainer blood collection tubes. Each patient's sample was split into three aliquots for analysis. The first and the second aliquots were subjected to CD4+cell count by FACS count and Dynabead technique respectively within twelve hours of specimen collection. The third aliquot was stored at room temperature for four days and then subjected to CD4+ cell count by Dynabead technique.

3.7 Fluorescent Activated Cell Sorter (FACS) count in enumeration of CD4+ T cells

This was carried out as described in Becton Dickson manual (1999). Briefly, for the control sample, two reagent pairs were labeled CD4-zero, CD8-low and CD4- medium, CD8- high. The pairs were vortexed for ten seconds. Fifty microlitres of well mixed EDTA normal blood was reverse pipetted into each reagent tubes. Upon vortexing the tubes for five seconds, they were incubated for 60-120 minutes at room temperature in the dark. Fifty microlitres of fixative solution was then added into each reagent tube, the tubes were vortexed and then incubated for a further 30 minutes at room temperature in the dark. Fifty microlitre (50 μ l) of zero, low, medium and high control beads was

reverse pipetted into reagent tubes labeled “zero”, “low”, “medium” and “high” respectively. All the reagent tubes were then run on the FACS count within 2 hours of adding the control beads. When the screen displayed “control passed” it was an indication that the FACS count machine was ready to read the patients samples. The reagent pair tubes were discarded in an appropriate biohazard container.

Briefly, for the patient’s sample, a reagent pair tube labeled with the patient’s number was vortexed for 10 seconds. The reagent pair tubes were opened using a coring station and 50µl of well mixed EDTA patients’ whole blood was reverse pipetted into each tube. Upon vortexing for 10 seconds the reagent pair tube were incubated for 60-120 minutes at RT in the dark. Fifty microlitres of fixative solution was then added to each tube, the pair of tubes was vortexed for 5 seconds and incubated for a further 30 minutes at room temperature. The reagent pair tube was then ran on the FACS count within 24 hours of preparation.

3.8 Dynabead Technique in CD4+T cell Enumeration

This was carried out as described in MSF-B, manual (2003), a modification of Dynal’s procedure (Dynal Biotech-Oslo Norway, 2002). The patient’s whole blood was treated with Dynabead CD14 solution alongside the positive and negative controls for CD4+T cells. Upon mixing on a tilt and rotate mixer for ten minutes at room temperature and placing the tube in the magnet (Dynal MPCR-M; ASA, Oslo, Norway) for 2 minutes, the blood was depleted of monocytes. The monocyte depleted blood was then treated with CD4 solution, mixed on a tilt and rotate mixer for ten minutes and placed in the magnet for isolation of CD4 cells. The isolated cells were washed free of contaminating cells

using phosphate buffered saline (PBS; Appendix 2). The cells were then lysed using a lysis solution, treated with Turk solution (Appendix 2) and then cells were counted using a conventional light microscope in a cell counting chamber with cover slip. Cells were counted in the four quadrants in the corners of the counting chamber and multiplied by a factor of five (MSF-Belgium, 2002; Appendix 3).

3.9 Calculation of Sensitivity, Specificity and Predictive Values of Dynabead Technique

The characteristics (sensitivity and specificity), parameters (positive and negative predictive values) and proportion of discrepant results were calculated at different levels of CD4+T cells in blood. These levels were 100, 200, 350, 500 cells/ μ l. This was carried out using a 2x2 table (table 3.1) as described by Frankena *et al.* (1990) and Thrusfield *et al.* (2001).

Table 3.1 Two by two (2x2) Table for determining sensitivity, specificity and predictive values.

		Reference test (FACS count)		Total
		<200	>200	
Dynabead	< 200	a	b	a+b
	>200	c	d	c+d
Totals		a+c	b+d	a+b+c+d

- a. True positive CD4 counts less than thresholds by both methods
- b. False positive CD4 counts greater than thresholds by FACS count but less than thresholds by Dynabead.

- c. False negative CD4 counts greater than thresholds by Dynabead but less than thresholds by FACScout
- d. True negative CD4 counts greater than thresholds by both methods.
- Adapted from Bailey and Scotts (1998)**

Table 3.2 Attributes of diagnostic tests as applied in this study

Attributes	Definition
Sensitivity	Percentage of patients with CD4+ T cells less than 200 cells/ μ l and identified by Dynabead test as having less than 200 CD4+ T cells.
Specificity	Percentages of patients with CD4+ T cells greater than 200 CD4+ T cells/ μ l and are identified by Dynabead test as having greater than 200 CD4+ T cells.
Positive predictive value	Probability that a patient whose tests result is less than 200 cells/ μ l by Dynabead is truly less than 200 cells/ μ l.
Negative predictive value	Probability that a patient whose test results is greater than 200 cells/ μ l by Dynabead is truly greater than 200 cell/ μ l.
Proportion of discrepant results	Proportion of results where the two methods results do not agree in classifying the patients at a given threshold

The sensitivity, specificity, positive and negative predictive values and proportion of discrepant results were calculated as follows:

$$\begin{aligned}
 \text{(a) Sensitivity (\%)} &= \frac{a \times 100}{a+c} \\
 \text{(b) Specificity (\%)} &= \frac{d \times 100}{b+d} \\
 \text{(c) Positive predictive value \%} &= \frac{a \times 100}{a+b} \\
 \text{(d) Negative predictive value \%} &= \frac{d \times 100}{c+d} \\
 \text{(e) Proportion of discrepant results} &= \frac{b+c \times 100}{a+b+c+d}
 \end{aligned}$$

(Frankena *et al.* (1990) and Thrusfiel *et al.* (2001))

3.10 Determination of Precision of Dynabead Technique

To determine precision, fresh samples of patient's blood whose CD4+T cell counts had been determined using FACS count were subjected to CD4+T cell enumeration by Dynabead technique. These samples had CD4+T cell counts of 191, 495 and 2000 cells/ μ l by FACS count. These samples were conveniently selected to represent low, medium and high CD4 counts respectively. Each of these samples was split into three aliquots and CD4+T cells of each of these aliquots were enumerated in duplicate. The duplicate counts of each aliquot were averaged to give an individual result. The same analyst handled sample preparation and cell count for each aliquot. The mean and the standard deviation of the three individual results for each sample were calculated.

The co-efficient of variation for each sample was determined by expressing the standard deviation as a percentage of the mean (Cheesbrough, 1998).

3.11 Determination of the Effect of Delay on Sample Handling on CD4+ T Cell Count by Dynabead Technique

To determine the effect of delay in sample handling on CD4+T cell counts using Dynabead technique, fresh samples whose CD4+T cell counts were determined by Dynabead technique were stored at room temperature for 4 days. Four days was deliberately selected since studies on CD4+T cell counts by Cyflow technique on EDTA fresh blood samples and those stored at room temperature for 4 days showed high correlation (Teav *et al.*, 2004). After four days, CD4+T cell count was carried out on the samples using Dynabead technique.

3.12 Data Analysis

The data was analysed using Scientific Program for Social Sciences (SPSS) version 11.5. Bivariate data analysis was carried out using Pearson coefficient correlation to find the relationship between the two techniques. A mean separation to establish the mean difference in CD4+ cell counts using the two methods and within Dynabead fresh and stored blood counts was done. A probability value of < 0.05 was considered significant. Precision of the Dynabead technique was also determined at different CD4+T cell thresholds.

CHAPTER FOUR: RESULTS

4.1 Positive isolation of CD4+T cells with pre-depletion of monocytes for reliable CD4+T cell nuclei counting

Upon adding lysing solution to the isolated CD4+T cells and later adding Turk solution and observation under a light microscope, the beads appeared as small dark circles with a central shiny spot while the CD4+T cell nuclei appeared as larger shiny yellow circles under the microscope (Plate 4.1)

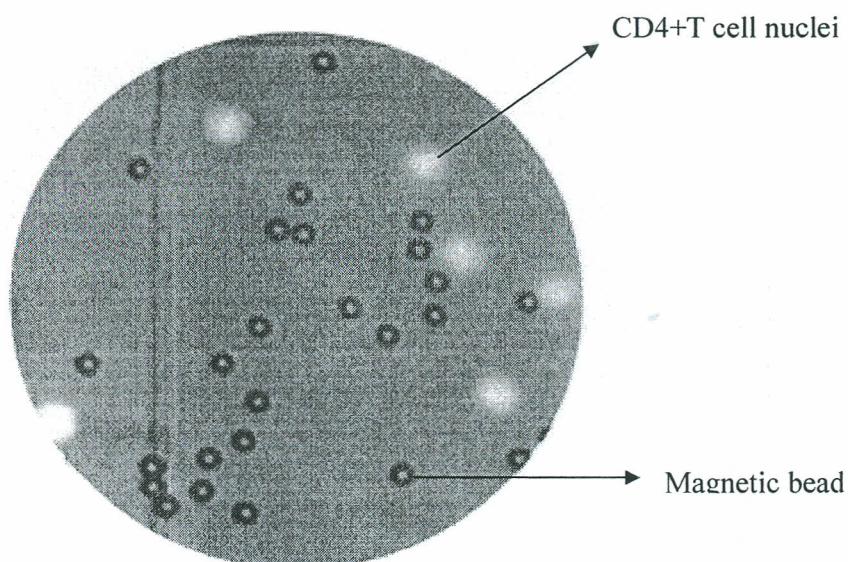


Plate 4.1 Microscope view of magnetic beads and CD4+T cell nuclei

4.2 Comparison of FACS count and Dynabead techniques in enumeration of CD4+ T cells

When CD4+ count was determined using FACS count and Dynabead technique, forty samples out of fifty four (74.1%) showed higher CD4+T cell counts with FACS count compared to Dynabead technique while fourteen samples (25.9%) showed higher CD4+T cell counts with Dynabead compared to FACS count (Table 4.1).

Table 4.1 Comparison of FACS count and Dynabead techniques for enumeration of CD4+T cells in HIV positive patients

Sample number	FACS count	Dynabead (Fresh)
1	410	380
2	190	130
3	185	135
4	134	85
5	73	65
6	180	120
7	267	150
8	494	475
9	328	255
10	427	355
11	166	165
12	47	40
13	513	440
14	386	350
15	241	235
16	512	485
17	129	114
18	51	45
19	101	75
20	369	225
21	105	100
22	764	700
23	349	300
24	229	225
25	495	460
26	441	405
27	422	300
28	355	250
29	384	300
30	1527	1100
31	652	600
32	644	585
33	418	370
34	1044	825
35	1524	1020
36	180	120
37	489	485
38	162	135

39	688	605
40	317	315
41	104	105
42	100	110
43	180	385
44	12	20
45	10	15
46	192	240
47	35	40
48	215	300
49	52	60
50	160	210
51	29	35
52	10	15
53	297	315
54	485	645

Sample 41- 54 shows CD4+T cells counts higher with Dynabead technique compared to FACS count.

The mean CD4+T cell count using FACS count for all samples tested was 338.39 while mean CD4+T cell count using Dynabead technique was 296.66 (Figure 4.1). When the means of the two techniques of CD4+T cells enumeration were compared using t-test there was a significant difference between the two methods ($P < 0.05$, $t = 2.884$, $df = 53$).

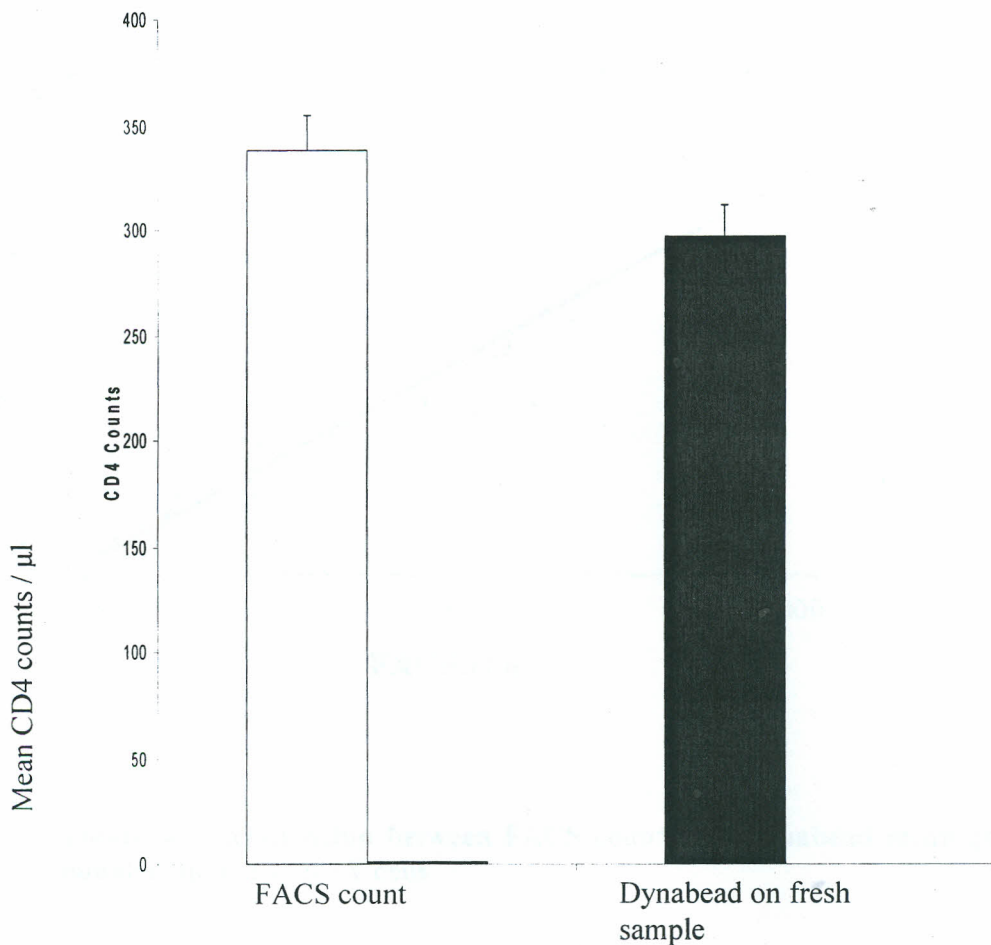


Figure 4.1 Comparison of mean CD4+T cell counts using FACS count and Dynabead techniques for all samples tested.

When the detection levels between FACS count and Dynabead techniques were compared across the 54 samples, it was evident that the two assays were highly correlated (Fig. 4.2: $r=0.962$, $p < 0.01$).

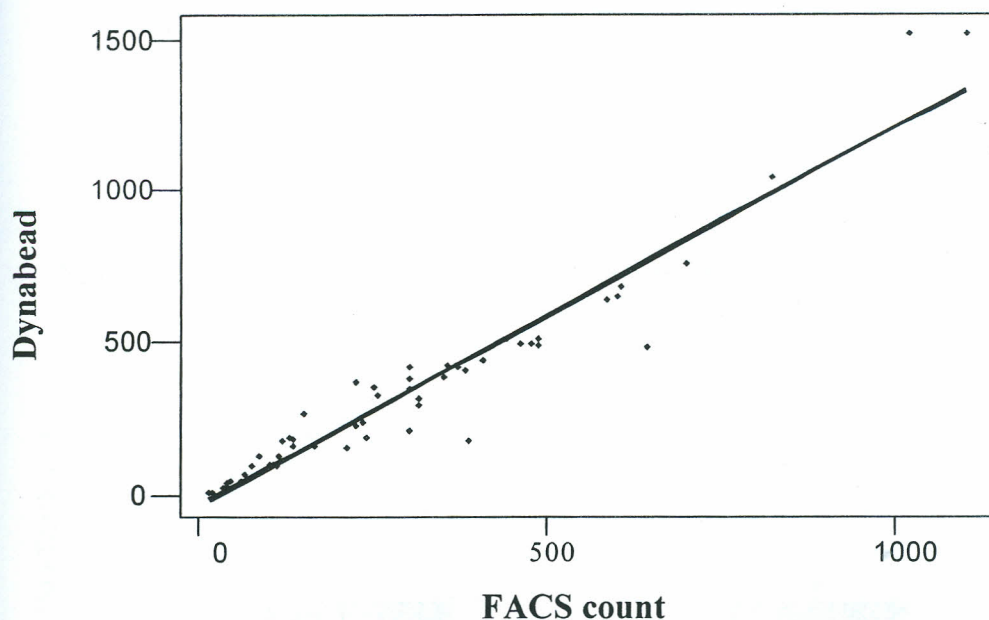


Figure 4.2 Relationship between FACS count and Dynabead techniques in enumeration of CD4+T cells

4.3 Comparison of mean CD4+ T cell counts at different ranges of CD4+ T cell count using FACS count and Dynabead techniques.

The CD4+ T cell counts were compared when determined by FACS count and Dynabead techniques at different ranges of CD4+ T cell counts/ μl of blood. At the range of 1-200 the mean CD4+ T cell count by FACS count and Dynabead were 107.79 and 106.88 respectively (Figure 4.3). At 201-400 the mean CD4+ T cell count by FACS count and Dynabead technique were 311.42 and 268.33 respectively (Figure 4.4) while at 400 and above it was 633.83 by FACS count and 568.61 by Dynabead technique (Figure 4.5).

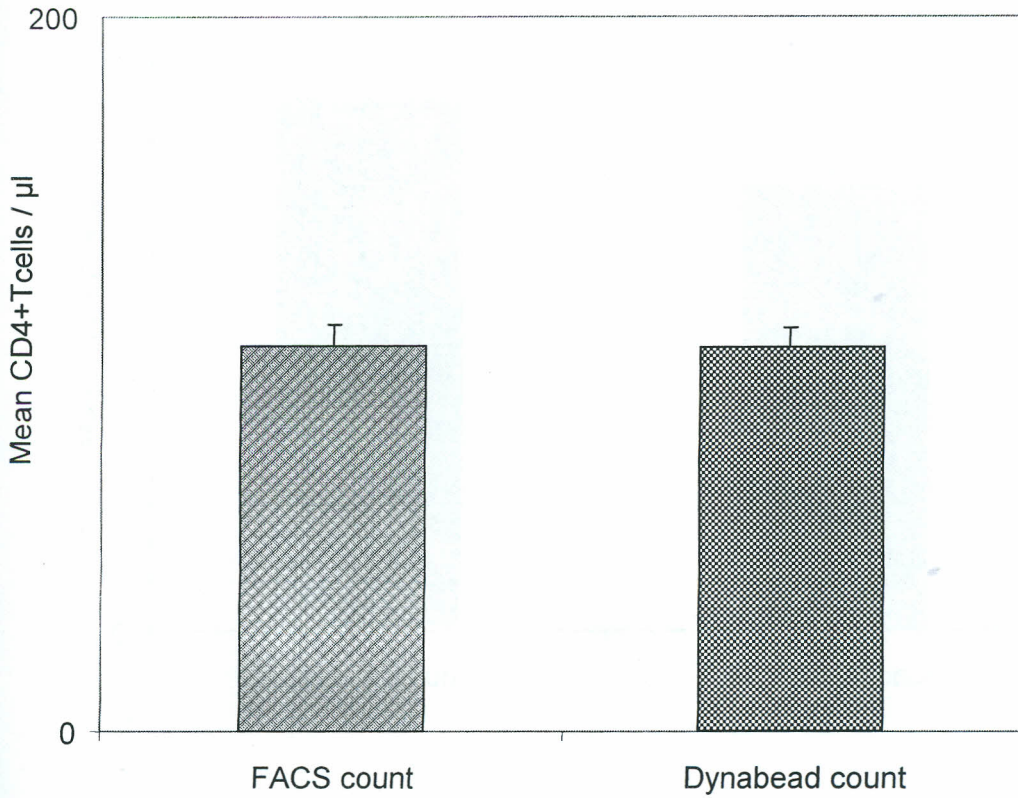


Fig. 4.3. Comparison of mean CD4+T cell counts using FACS count and Dynabead techniques at a range of 1 – 200 cells / μ l of blood.

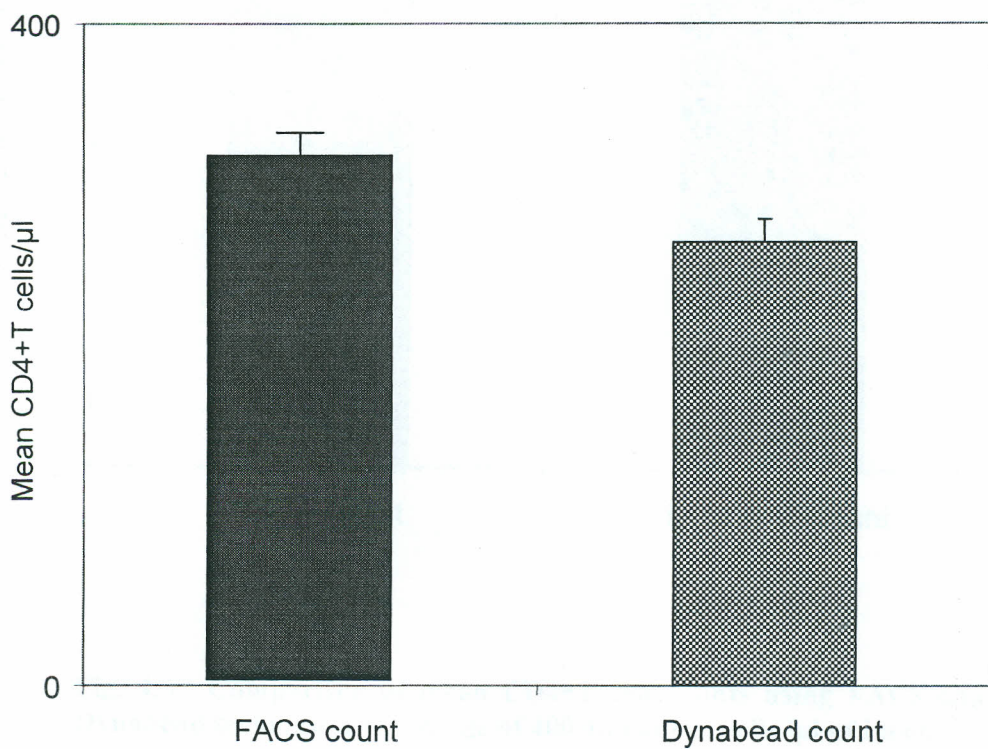


Fig. 4.4. Comparison of mean CD4+T cell counts using FACS count and Dynabead techniques at a range of 201 – 400 cells / μ l of blood.

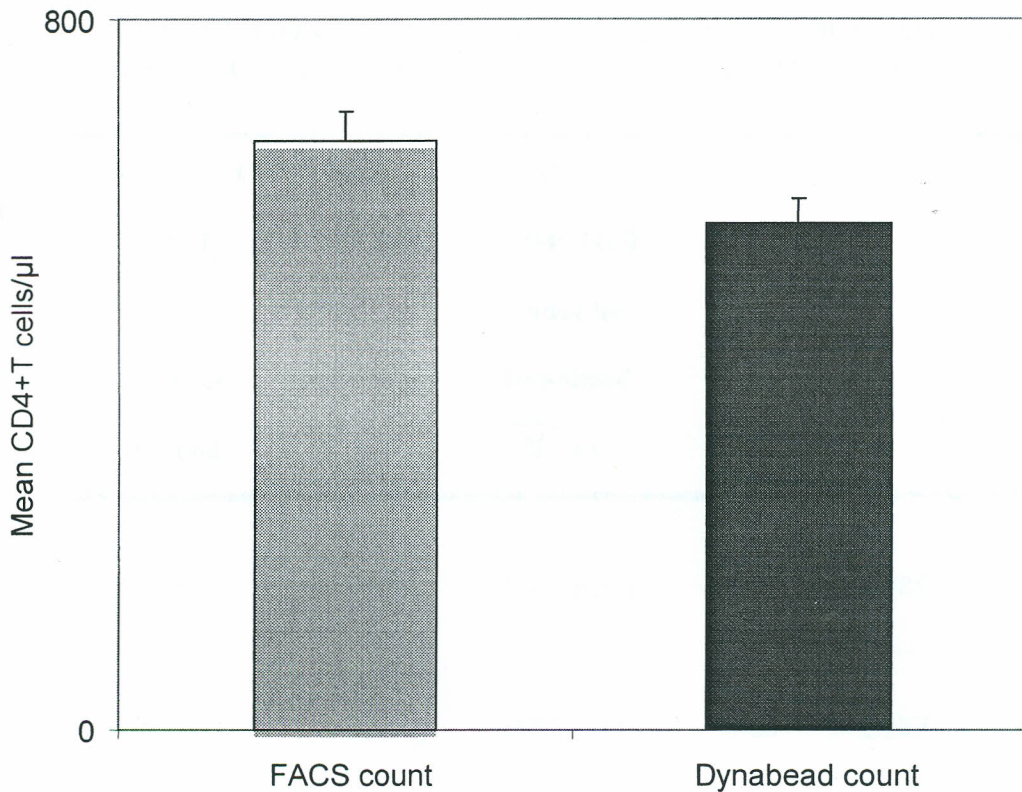


Fig. 4.5. Comparison of mean CD4+T cell counts using FACS count and Dynabead techniques at a range of 400 and above cells /μl of blood.

When the two techniques of CD4+ T cell enumeration were compared at different levels of CD4+T cell counts, there was no significant difference between the two methods at CD4+ T cell count level of 1-200 cells/μl of blood ($p>0.05$, $t=0.085$, $df=23$). However at CD4+ T cell counts levels of 201- 400 cells/μl of blood and above 400 cells/μl of blood there was significant difference between the two methods. At 200-400 cells/μl ($p<0.05$, $t=2.287$, $df=11$) while at above 400 cell/μl ($p<0.05$, $t=2.647$, $df=17$: Table 4.2)

Table 4.2 Statistical analyses comparing CD4+ T cell counts using FACS count and Dynabead techniques across different CD4+ T cell ranges

Level of CD4+T cell count/ μ l of blood	Mean CD4+Tcell count by FACScount \bar{X} (n)	Mean CD4+Tcell count by Dynabead \bar{X} (n)	P value	t value	df
1 – 200	107.79(24)	106.68(24)	>0.05	0.085	23
201– 400	311.42(12)	268.33 (12)	<0.05	2.287	11
400– and above	663.83(18)	568.61(18)	<0.05	2.647	17
Total No. of samples (N)	N = 54	N = 54			

4.4 Sensitivity, Specificity and Predictive Values of Dynabead Technique

Sensitivity, specificity, positive and negative predictive values of Dynabead technique at different levels of CD4+T cell/ μ l of blood was determined across the 54 samples used in this study (Tables 4.4, 4.6, 4.8 and 4.10).

4.4:1 Sensitivity, specificity and predictive values of Dynabead technique at the threshold of 100 CD4+T cells/ μ l

Of the 54 samples, 9 had a CD4+ count of less than 100 cells/ μ l by both FACS count and Dynabead technique. 3 samples showed CD4+ count greater than 100 with FACS count while the same samples showed CD4+ cell less than 100 by Dynabead technique. 1 out of the 54 samples showed CD4+ count less than 100 with FACS count but was greater than 100 with Dynabead technique while 41 samples showed CD4+ counts of greater than 100 by both the techniques. The number of discrepant results was 4 (Table 4.3). Based on these data, sensitivity, specificity, positive predictive value, negative predictive value and proportion of discrepant results was 90%, 93%, 75%, 97.6% and 7.4% respectively (Table 4.4).

Table 4.3: Number of samples detected at 100 cells/ μ l threshold using FACS count and Dynabead techniques

		FACScount		Totals
		<100	>100	
Dynabead	<100	9	3	12
	>100	1	41	42
	Totals	10	44	54

Bold number represents discrepant results = 1+3 = 4

Table 4.4: Sensitivity, specificity, predictive values and proportion of discrepant results of Dynabead technique at 100cells/ μ l threshold

Parameter	Percentage
Sensitivity	90%
Specificity	93%
Positive PV	75%
Negative PV	97.6%
Proportion of discrepant results	7.4%

Positive PV: Positive predictive values

Negative PV: Negative predictive values

4.4:2 Sensitivity, specificity and predictive values of Dynabead technique at the threshold of 200 CD4+T cells/ μ l

At threshold of 200 CD4+ T cell/ μ l of blood, 21 samples recorded a CD4+ count less than 200 cell/ μ l by both methods. One (1) sample recorded greater than 200 cells/ μ l with FACS count but less than 200cells/ μ l with Dynabead. Three (3) out of the 54 samples recorded less than 200 cells/ μ l with FACS count but greater than 200cells/ μ l with Dynabead. Twenty nine (29) samples showed CD4+ counts of greater than 200cells/ μ l by both techniques. The number of discrepant results was 4 (Table 4.5).

Table 4.5: Numbers of samples detected at 200 cells/ μ l threshold using FACS count and Dynabead techniques

		FACS count		
		<200	>200	Total
Dynabead	<200	21	1	22
	>200	3	29	32
	Total	24	30	54

Bold number represents discrepant results = $1+3 = 4$

Based on these results, sensitivity, specificity, positive predictive value, negative predictive value and proportional of discrepant results was 87.5%, 96.7%, 95.5% and 7.4% respectively (Table 4.6).

Table 4.6: Sensitivity, specificity, predictive values and proportion of discrepant results of Dynabead technique at 200cells/ μ l threshold

Parameter	Percentage
Sensitivity	87.5%
Specificity	96.7%
Positive PV	95.5%
Negative PV	90.6%
Proportion of discrepant results	7.4%

Positive PV: positive predictive values

Negative PV: negative predictive values

4.4:3 Sensitivity, specificity and predictive values of Dynabead technique at the threshold of 350 CD4+ cells/ μ l

At a threshold of 350 CD4+ cells/ μ l of blood, 31 of the 54 samples recorded a CD4+ count less than 350 cells/ μ l by both techniques while 5 samples read greater than 350cells/ μ l by FACSCount and less than 350cells/ μ l by Dynabead technique. One sample recorded less than 350 cells/ μ l with FACSCount and greater than 350cells/ μ l by Dynabead technique. Seventeen (17) of the 54 samples read greater than 350 cells/ μ l by both the techniques. The number of discrepant results was 6 (Table 4.7).

Table 4.7 Number of samples detected at 350 cells/ μ l threshold using FACS count and Dynabead techniques

FACSCCount

		<350	>350	Total
Dynabead	<350	31	5	36
	>350	1	17	18
	Total	32	22	54

Bold number represents discrepant results = 1 +5 = 6

Based on these results, sensitivity, specificity, positive predictive value, negative predictive value and proportion of discrepant results was 96.8%, 77.3%, 86.0%, 94.4% and 11.1% respectively (Table 4.8).

Table 4.8: Sensitivity, specificity, predictive values and proportion of discrepant results of Dynabead technique at 350cell/ μ l threshold.

Parameter	Percentage
Sensitivity	96.8%
Specificity	77.3%
Positive PV	86.0%
Negative PV	94.4%
Proportion of discrepant results	11.1%

Positive PV: positive predictive values

Negative PV: Negative predictive values

4.4:4. Sensitivity, specificity and predictive values of Dynabead technique at the threshold of 500cells/ μ l.

At the threshold of 500 CD4+ Tcell/ μ l of blood, 44 of the 54 samples recorded a CD4+ cell count of less than 500 cells/ μ l by both techniques. 2 samples recorded a CD4+ cell count of greater than 500 cells/ μ l by FACS count and less than 500 cells/ μ l by Dynabead technique. One sample read less than 500cells/ μ l with FACS count and greater than 500cells/ μ l with Dynabead. Seven samples showed CD4+ counts of greater than 500cells/ μ l by both the techniques. The number of discrepant results was 3 (Table 4.9).

Table 4.9: Number of samples detected at 500 cells/ μ l threshold using FACS count and Dynabead techniques

		FACS count		
		<500	>500	Totals
Dynabead	<500	44	2	46
	>500	1	7	8
	Total	45	9	54

Bold number represents discrepant results = 1 + 2 = 3

Based on these results, sensitivity, specificity, positive predictive value, negative predictive value and proportion of discrepant results was 97.7%, 77.8%, 95.6%, 87.5% and 5.5% respectively (Table 4.10).

Table 4.10 Sensitivity, specificity, predictive values and proportion of discrepant results of Dynabead technique at 500cells/ μ l threshold.

Parameter	Percentage
Sensitivity	97.7%
Specificity	77.8%
Positive PV	95.6%
Negative PV	87.5%
Proportion of discrepant result	5.5%

Positive PV: Positive predictive value

Negative PV: Negative predictive value

4.4. 5 Trends of sensitivity, specificity and predictive values of Dynabead technique at different levels of CD4+T cell counts

The highest sensitivity (97.7%) was obtained at a threshold of 500 cells/ μ l, highest specificity was (96.7%) at 200cells/ μ l, highest positive predictive value (95.6%) at 500cells/ μ l and highest negative predictive value (97.6%) at 100 cell/ μ l (Table 4.11). The proportion of discrepant results was 7.4%, 7.4%, 11.1% and 5.6% for 100, 200, 350 and 500 cells/ μ l thresholds respectively (Table 4.11). The sensitivity ranged from 87.5% to 97.7%, specificity 77.3% to 96.7%, positive predictive value from 75.0% to 95.6% and negative predictive value from 87.5% to 97.6% (Table 4.11). The sensitivity was relatively high (97.7 %) in higher CD4+T cell counts (500 cells/ μ l) as compared to clinically relevant threshold CD4+T cell counts (200 cells/ μ l) where it was 87.5%. Specificity was high (96.7%) in clinically relevant threshold (200 cells/ μ l) as compared to higher CD4+Tcell counts (500 cells/ μ l) where it was 77.8%. The general observation was that when sensitivity was high the specificity was lower and vice versa. However, the CD4+T cell counts at 100 cell/ μ l sensitivity and specificity were both high. It was also observed that when the positive predictive value was high, the negative predictive value was lower for each level of CD4+Tcell counts except for 200 cells/ μ l threshold where both positive and negative predictive values were high (Table 4.11).

Table 4.11: Sensitivity, specificity, predictive values and proportion of discrepant results of Dynabead technique at different threshold values.

Threshold cells/μl	Sensitivity (%)	Specificity (%)	Positive predictive Value (%)	Negative predictive value (%)	Proportion of discrepant
100	90.0	93.0	75.0	97.6	7.4
200	87.5	96.7	95.5	90.6	7.4
350	96.8	77.3	86.0	94.4	11.1
500	97.7	77.8	95.6	87.5	5.6

4.5 Precision of Dynabead technique

Precision is the reproducibility of a test when it is run several times. Precision is expressed in terms of coefficient of variation (CV). Coefficient of variation expresses sample variability relative to the mean of the sample. The three individual results for each level of CD4+ T cell counts for determination of precision were as follows : Samples with a count of 191 CD4 cells/ μ l by FACS count gave counts of 220, 200 and 190 CD4+T cell count by Dynabead technique; those with a count of 495 CD4cells/ μ l by FACS count gave counts of 460,430 and 450 cells/ μ l by Dynabead technique while those with a count of 2000 cells/ μ l by FACS count gave counts of 2075 , 2190, 1885 cells / μ l by Dynabead technique (Table 4.12).

Table 4.12: CD4+ T cell counts at low, medium and high levels of CD4+ T cell counts

Level of CD4+ T cellcount	Repeated CD4+T cell counts by Dynabead technique	Mean CD4+ T cell cōunt/ μ l
Low	220, 200, 190	203
Medium	460, 430 ,450	447
High	2075, 2190, 1885	2050

Based on this data, the Standard deviation (SD) for low, medium and high CD4+ T cell counts was 15.28, 15.28 and 154.03 while the CV was 7.5%, 3.4% and 7.5% respectively (Table 4.13).

Table 4.13: Precision of Dynabead technique at low, medium and high CD4+T cell count levels.

Level of CD4+T cell count	SD	Mean CD4 count	CV (%)
Low count	15.28	203	7.5
Medium count	15.28	447	3.4
High count	154.03	2050	7.5

4.6 Effect of Delay in Sample Handling on CD4+T cell Count by Dynabead Technique

All the 54 samples showed a decrease in the number of CD4+ T cells after storage for four days at room temperature (Appendix 1). Out of the 54 samples, six (11%) samples showed a decrease of $\leq 30\%$ of the total CD4+T cells while thirty four (62.9%) samples showed a decrease of 30-60% of the total CD4+ T cells while fourteen (25.9%) showed a decrease of $\geq 60\%$ of the total CD4+ T cells (Table 4.14)

Table 4.14: Numbers and proportions of samples exhibiting a decrease in CD4+T cell count after four day's storage at room temperature

Range of percentage decrease of CD4+ T cells	Number of samples exhibiting a decrease in CD4+ T cells	Percentage exhibiting a decrease in CD4+T cells
≤ 30	6/54	11.0
30-60	34/54	62.9
≥ 60	14/54	25.9

Based on the CD4+ T cell counts indicated in appendix 1, the mean CD4+T cell count of fresh sample as determined by Dynabead technique was 296.67. The mean CD4+T cell count of the samples when stored for 4 days at room temperature was 144.35. When the two means were compared, there was a significant difference between CD4+T cell count in fresh samples and samples stored for 4 days at room temperature ($p > 0.05$, $t = 8.694$, $df = 53$; figure 4.6).

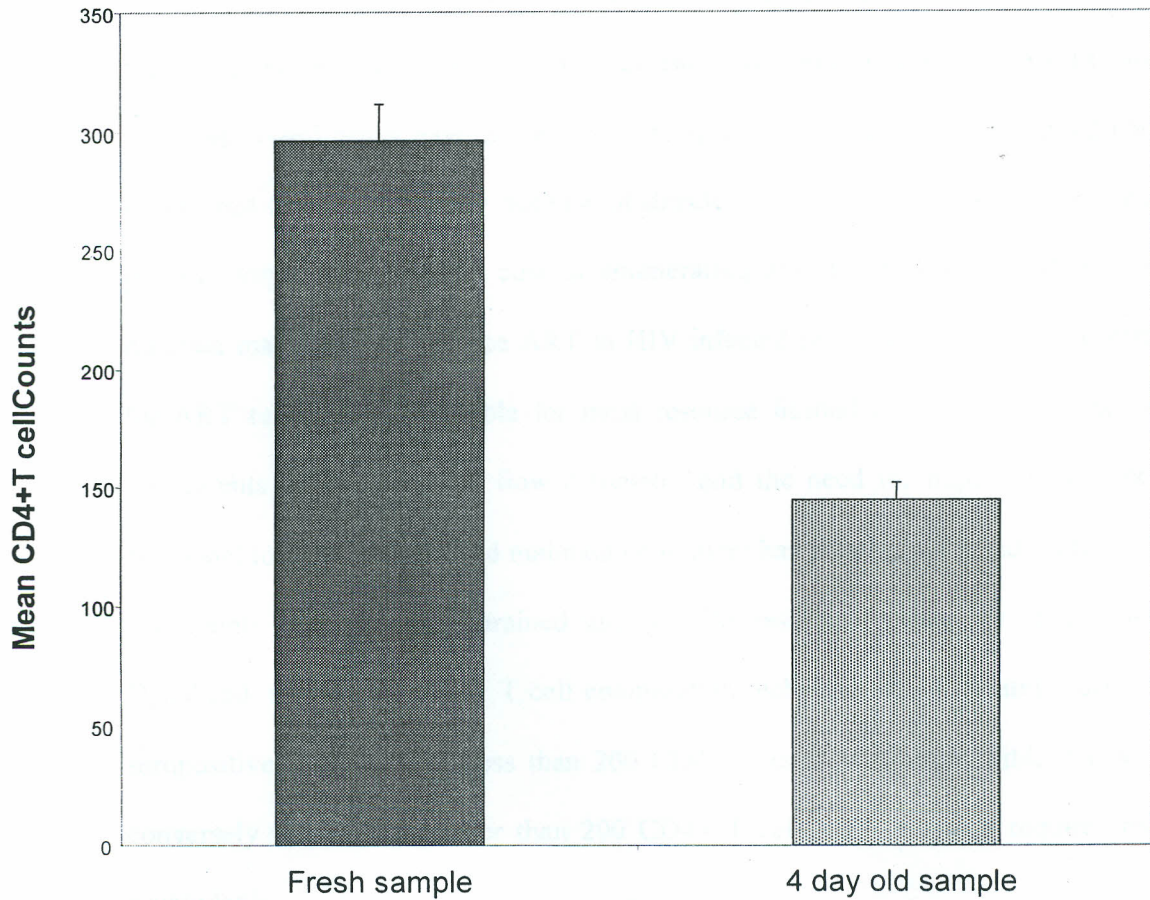


Figure 4.6 Comparison of mean CD4+ T cell count in fresh and stored blood samples by Dynabead technique.

CHAPTER FIVE: DISCUSSION

5.1 Comparison of Dynabead Technique and FACS count in CD4+T Cell Count

Anti- retro-viral drugs have become increasingly available in resource-limited countries in the past few years mainly because of drastic reductions in prices of proprietary and generic drugs. However the cost of enumerating absolute CD4+ T cells required for decision making to commence ART in HIV infected persons and monitoring responses for ART remains unaffordable for most resource limited countries. The high cost of instruments and reagents of flow cytometry and the need for highly trained technical personnel to perform tests and maintain equipment has greatly hampered routine CD4+ T cell counts in resource-constrained settings. The results of this study have shown that Dynabead, a low cost CD4+ T cell enumeration technique can accurately identify HIV seropositive subjects with less than 200 CD4+ T cells who are eligible for ART and conversely those with greater than 200 CD4+ T cells who may not require continued prophylaxis.

The results of this study have also shown that Dynabead can provide accurate CD4+ T cells count for patients who are on ART. These results are consistent with results from several other studies on correlation coefficients of various techniques compared to the standard method Flow cytometry. Researchers in Japan compared Dynabead (by conventional light microscope) with Flow Cytometry and obtained a correlation coefficient of 0.91 (Bi, 2004). In Sagamu, Nigeria comparison of FACS and Dynakit revealed a correlation coefficient of 0.831 (Osho, 2004). In Burkina Faso in 89% of cases, both FACS count and Dynabead technique (by epifluorescent microscope) consistently classified patients at the threshold of 200 cells/ μ l. The correlation coefficient

for the two methods was 0.89 (Diagbouga, 2003). According to Crowe *et al.*, (2003), Dynabead and flow cytometry assay results revealed excellent association, $r = 0.97$ as opposed to manual coulter assay and flow cytometry, $r=0.69$. This association between Dynabead and flow cytometry is in close agreement with data generated in current study where a correlation coefficient (r) = 0.962 was observed. Similar multicentre studies conducted in Nigeria showed high correlation between Dynabead and flow cytometry $r=0.89$ in Diagbouga *et al.* (2003). Lamuhaya *et al.* (1996) had reported a correlation coefficient (r) of 0.9.

Correlation coefficient reported in this study revealed that Dynabead technique (by light microscope) compared well with other cost effective techniques that are already licensed for use. According to Godwin *et al.* (2005) Cyflow and Dynabead technique produced comparable and well correlated CD4+ T lymphocyte counts ($r=0.93$ $p=0.057$; $n=40$) in CD4+ T-cell enumeration which is in close agreements with this current study's results where $r=0.962$. In a study carried out by Crowe *et al.* (2003) it was found that the Dynal assay was easier to correlate with data from Flow Cytometry ($r=0.96$) than the manual coulter assay was ($r= 0.69$). Similar multicenter studies also showed a high correlation ($r=0.9$) of Flow Cytometry (FACS count) with Dynabead CD4 T-lymphocyte count by Fluorescent microscope (Diagbouga *et al.*, 2003).

Wilja *et al.* (2003) noted that the correlation coefficient of Pan leucogating (PLG) as compared to Flow Cytometry was 0.96. Pan-leucogating is already in use in Uganda and South Africa. Researchers in Barbadoes, Caribbean islands also compared Panleucogating to sophisticated four-color method of cell counting using commercial

monoclonal reagents and obtained a correlation coefficient of 0.97 (Sippy *et al.*, 2004). Based on this correlation coefficient the Pan-leucogating technique has been adopted as an accurate method for enumerating CD4+Tcells and has major cost implication for the sustainability to the National HIV containment program in Barbadoes in the Caribbean Islands. The correlation coefficient of PLG in the studies reported by Wilja *et al.* 2003 ($r=0.96$), Sippy *et al.* (2004) ($r=0.97$) and Teav *et al.*, 2004 ($r=0.997$) are in very close agreement with the current study's results. Storie and Colleagues in 2003 also reported a high correlation between the state-of-art Single-Platform (SP) protocol and PanLeucogating ($r=0.9928$).

In Malawi where HIV-1 subtype C predominates, field evaluation of Cyflow reported a correlation coefficient of 0.92 (95% CI 0.89-0.95) when compared to FACS count (Fryland *et al.*, 2004) while a study in Cameroon on comparison of Cyflow and FACS count revealed a correlation coefficient of 0.989 (Crowe *et al.*, 2003). In Senegal where subtype A and D HIV-1 predominate, an excellent correlation was observed between bead based CD4 measurement on the Cyflow and CD4 measurement on the FACS scan ($r=0.99$) and FACS count ($r=0.99$). Rigid internal and external quality control monitoring and adequate training of technicians was considered to generate accurate volumetric CD4 measurement of the Cyflow (Dieye *et al.*, 2005). In Zimbabwe a strong correlation $r=0.995$ between Cyflow and FACS count was observed (Lynn *et al.*, 2006). All these results are in close agreement with the results recorded in this study. Magnetic bead CD4+ cell depletion with automated CBC and Flow Cytometry revealed a correlation coefficient (r) = 0.92 (Buranapraditkun *et al.*, 2004), while Point Care technique compared to dual platform Flow Cytometry, showed a correlation coefficient (r) = 0.98

(Crowe *et al.* (2003). These results compare well with the correlation coefficient of this study $r = 0.962$. The accuracy of a Microchip CD4+ cell counting method validated through testing in the United States and Botswana showed close agreement with standard Flow Cytometry, $r = 0.95$, over a range of absolute CD4+T cell counts. It also showed the ability to discriminate clinically relevant CD4 count threshold with high sensitivity and specificity (William *et al.*, 2005). In 2003, Cao demonstrated the correlation between Biochip and Flow cytometry to be 0.95. Flow Cytometry and Capcellia upon comparison has also shown a good correlation of $r=0.70$; $p<0.001$ (Kannagai *et al.*, 2001).

Results from the present study showed that proportions of discrepant results at threshold 100cells/ μ l, and 200cells/ μ l was 7.4%, and that of 350 cells/ μ l was 11.1% while at 500 cells/ μ l it was 5.6%. These findings are in agreement with reports by Diabougou *et al.* (2003) where a range of 4.7% to 11.3% was demonstrated. Diabougou *et al.* (2003) concluded that the ability of the Dynabead technique (by epifluorescent microscopy) to consistently classify results in agreement with Flow Cytometry at thresholds of CD4+ cell counts relevant for clinical care, was high. The implementation of the Dynabead technique (by epifluorescent microscopy) was easy and successful in the West Africa context (Diabougou *et al.*, 2003). With the close comparison of the correlation coefficients and proportions of discrepant results, Dynabead technique (by light microscope) evaluated in this study stands a chance of adoption in Kenya.

In the present study the sensitivity and specificity of Dynabead technique at the clinically relevant threshold 200cell/ μ l was 87.5% and 96.7% respectively. At 350cells/ μ l sensitivity was 96.8% while specificity was 77.3%. This is in agreement with studies by

Bi *et al.* (2005) who reported sensitivity and specificity for CD4+ cell count at 200cells/ μ l threshold as 79% and 94% respectively. For CD4+ cell counts at 350 cells/ μ l, the sensitivity and specificity are 95% and 88% respectively. Comparative studies by Buranapraditkun *et al.*,(2004) on Dynabead depletion with automated cell counting compared to Flow Cytometry showed sensitivity and specificity of CD4+T cell count at 200cells/ μ l as 88% and 86% respectively. This compares well with results of this study of sensitivity, 87.5% and specificity, 96.7%. The sensitivity and specificity at 100cells/ μ l in this study was 90% and 93% respectively. These percentages were higher compared to those of a study on magnetic bead depletion with automated cell counting, and Flow Cytometry by Buranapraditkun *et al.*, (2004) where the sensitivity and specificity were 88% and 85% respectively.

Comparison of results from Microchip assay and Flow Cytometry by William and colleagues in 2005 revealed the following: CD4<250, sensitivity=86%, specificity= 81%; CD4<350, sensitivity=97%, specificity=83%; and CD4<500, sensitivity=96%, specificity=85%. This is in close agreement with the results of this study of sensitivity=87.5%, specificity=96.7% at CD4<200; sensitivity=96.8,

specificity=77.3% at CD4<350 and sensitivity=97.7%, specificity=77.8% at CD4<500.

However researchers in Cambodia recorded a higher sensitivity (100%) with Cyflow technique and a positive predictive value of 97% to detect CD4 < 200 (Teav *et al.*, 2004).

This could be attributed to the fact that Cyflow is “simplified Flow Cytometry” which is not prone to cell counting error as the most promising manual method for CD4+ cell counting, the Dynabead technique.

The general observation was that sensitivity and specificity varied according to the level that was chosen as the threshold or cutoff points. This is in agreement with reports by Akobeng, (2007) and Obuchowski.(2003) which pointed out that sensitivity and specificity vary according to the chosen cut-off points. In this study it was also observed that when sensitivity was high at a given threshold, the specificity was relatively lower and viceversa. This compared well with a report by Bewick *et al.* (2004).

Since most of the provincial hospitals and some district hospitals in Kenya have a FACS count, further validation of Dynabead technique (Using light microscope) is warranted to give room for implementation of this technique in resource constrained areas for antiretroviral therapy monitoring.

5.2 Precision of Dynabead Technique

Precision is the reproducibility of a test when it is run repeatedly several times. In the current study coefficient of variation of Dynabead technique at different CD4+T cell threshold count varied depending on CD4+T cell threshold. For medium CD4+T cell count, the coefficient of variation for Dynabead technique (3.4%) was in close agreement with that of intra assay studies on precision of an accepted CD4+T cell count technique, ELISA using TRA x CD4 test kit which ranged between 4.6 to 5.9% (Helene *et al.* ,1995). The 3.4% obtained in our study is also within the range of coefficient of variance observed for precision of Cyflow technique for CD4+T cell count, 2.8%-4.9% (Teav *et al.*, 2004) and for FACScan, 3.2-3.3% (Becton, 1999).

Clinically relevant CD4+T cell count and high CD4+ cell count in this study revealed a coefficient of variation (CV) of 7.5%. This is in close agreement with coefficient of

variation (CV) observed for Dynabead technique, 8.4% by Diagbouga *et al.* (2003). In their study Diagbouga *et al.*, (2003) also observed CV of FACS technique of 8.3%, which was close to their Dynabead technique value of 8.4%. In this study the CV of 7.5% is within the range of the CV value (7.0%-7.8%) for the inter assay studies of the precision of ELISA using TRA x CD4 test kit (Helene *et al.*, 1995). The analysis of CD4 T-lymphocyte data obtained from 10 separate determinations performed on the same sample with Cyflow and Dynabead showed CV% of 1.9% and 7.8% respectively. The marked difference in CV% observed for Dynabead and Cyflow is because manual microscopic techniques such as Dynabead suffer limitations in accurately estimating cell counts in a sample with extremely low and high cell population compared to automated techniques (Carella *et al.*, 1995 and Godwin *et al.*, 2005)

Coefficient of variation in this study did not agree with that of lyse and no lyse Flow Cytometry procedures whose coefficient of variation (CV) was observed as 1.9% and 1.5% respectively (Greve *et al.*, 2003). George *et al.* (2002) notes that precision of manual methods such as Dynabead technique can vary widely since it is subject to operator's fatigue.

5.3 Specimen stability

Temperatures greater than 37°C may cause cellular destruction that affects both hematology and flow cytometry measurements (Paxton and Bendele, 1993). Therefore for optimal results by flow cytometry, tests should be performed within 30 hours but not later than 48 hours after drawing the blood specimen (Nicholson *et al.*, 1984; Weiblen *et al.*, 1984). The mean of CD4+T cell count of samples stored for four days at room

temperature prior to enumeration by Dynabead technique in this study was lower as compared to the mean of CD4+T cell count of the same samples while freshly obtained giving a clear difference in detection between fresh and stored samples. These results suggest that the samples CD4+T cells deteriorated upon storage at room temperature for four days. This is in agreement with the results of CD4+T cell count by TRA x CD4 test kit where by the fourth day at room temperature the coefficient of variation (CV) of the assayed samples was twice the expected variability of the assay, increasing dramatically by day 10 suggesting specimen deterioration (Helene *et al.*,1995).

Studies by Diagbouga *et al* (2003) on impact of delay in sample handling on Dynabeads technique showed that samples exhibiting decrease in CD4+ cell counts increased with time of storage of sample at room temperature. At eight hours of storage, 3 out of 28 samples (10.7%) showed a decrease in CD4 cell count, at twelve hours, 5 of the 28 samples (17.9%) showed a decrease in number of CD4 count while at 24 hours 14 of the 28 samples (50%) showed a decrease in CD4+ T cell count (Diagbouga *et al.*,2003). Since the principle of Dynabead technique is based on counting stained cells nuclei, the degenerated cell's nuclei may not take up the stain and therefore missed out during counting.

Results in this study however were not in agreement with those of researchers in Cambodia who compared CD4+T cell counts obtained by Cyflow on fresh samples and EDTA preserved samples kept at room temperature for four days. For the Cyflow technique study, in 27 samples tested, the correlation between CD4+ T cell counts on fresh blood and four days old blood was high, with this aged blood samples tending to

come in about 5 cells lower in a mean CD4+T cell count of 241 (Teav *et al.*, 2004). According to Glencross (2002), samples that are up to 7 to 8 days old can be used with Pan-leucogating when maintained at 25⁰C or less. Crowe (2003) reported that a study on correlation between fresh and 5 days old blood samples using Pan-leucogating technique revealed high correlation. Panleucogating has been recommended for testing aged blood in developing world (Scott, 2003). The results of this study revealed a significant difference between CD4+ T cell count in fresh blood samples and four days old samples, an indication that Dynabead technique is probably only good for fresh samples. This is in agreement with Dennis, (2005) report that complete blood counts were acceptable unfrozen for between 25 and 72 hours.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- i) Based on the results of this study the following conclusions were drawn.

There is no significant difference between the two methods in CD4+ T cell enumeration at CD4 cell counts level of 1-200 cells/ul of blood. However there is a significant difference between the two methods at CD4 T cell levels greater than 200 cells/ul of blood. The results of this study show that Dynabead technique can accurately identify HIV sero- positive patients with less than 200 CD4 + T cells who are eligible for ART.
- ii) The sensitivity, specificity positive and negative predictive values of Dynabead technique at clinically relevant threshold (200 cells/ μ l of blood) are as follows; 87.5%, 96.7%, 95.5% and 90.6% respectively which compares well with FACS count.
- iii) Dynabead's technique precision at clinically relevant threshold is acceptable and compares well with other tests in the market.
- iv) Dynabead technique is only acceptable for CD4+ T cell enumeration when using fresh EDTA blood samples.

6.2 Recommendations

6.2.1 Application of the findings

- i. Implementation of use of Dynabead technique for CD4+ enumeration in resource constrained areas in Kenya should be considered. This will support small-scale programmes, supplement and extend access to CD4+T and CD8+ T cell counting in places and at times when other options are not available. Constant internal and external quality control of Dynabead technique is necessary.
- ii. Dynabead technique should only be used for CD4+ T cell enumeration of fresh blood but not blood stored at room temperature.

6.2.2 Further studies

- i. Multi-centre studies using a large number of samples for comparing Dynabead technique to FACS count in different areas in Kenya should be carried out to consolidate tentative consensus that would recommend use of Dynabead technique for CD4+ enumeration in resource constrained count.
- ii. Further work should be done to help establish the acceptable time limit within which CD4+ T cell count should be carried out on stored blood samples to give valid results.

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APPENDICES

Appendix 1. Comparison of CD4+ T cell counts on fresh samples and four days old samples by Dynabead technique.

Sample number	Fresh sample	4 days old sample	Sample number	Fresh sample	4 days old sample
1	105	85	28	210	115
2	110	65	29	35	20
3	385	95	30	75	30
4	380	120	31	225	140
5	130	85	32	100	75
6	135	95	33	700	300
7	85	75	34	300	170
8	65	35	35	15	0
9	120	75	36	315	160
10	20	5	37	225	110
11	15	10	38	460	160
12	150	110	39	405	150
13	475	280	40	300	110
14	255	160	41	250	105
15	355	160	42	300	185
16	165	70	43	1100	660
17	40	20	44	600	220
18	240	95	45	585	300
19	440	210	46	370	150
20	350	125	47	825	285
21	235	30	48	1020	740
22	485	165	49	120	50
23	40	25	50	645	340
24	300	120	51	485	295
25	60	20	52	135	80
26	115	50	53	605	280
27	45	30	54	315	150

Appendix 2. Reagents and Solutions

2.1 Prepared reagents

Dynabead (R) M450 CD14

Dynabead (R) M450 CD4

Dynal lysis solution

Phosphate Buffered Saline (PBS)

All above reagents stored at 2-8⁰C

2.2 Reagents preparation

Turk solution: 2 mls of glacial acetic acid and 0.0128g gentian violet was dissolved in 100mls of deionised water.

Appendix 3. Dilution factor

Total volume: 125 μ l blood + 350 μ l PBS + 25 μ l Dynal = 500 μ l.

Whereas 200 μ l is transferred $200\mu\text{l}/500\mu\text{l}=40\%$

40% of 125 μ l blood = 50 μ l

Final volume of counting = 50 μ l lysis solution + 50 μ l suspension = 100 μ l hence dilution factor;

$100/50\mu\text{l}=2$.

N cells in 0.4 μ l (volume of 4 quadrants on counting chamber).

How many cells in 1 μ l?

CD4⁺ T cells/ μ l = $n \times 1/0.4 \times$ dilution factor.

= $n/4 \times 10 \times 2$

= $n \times 5$