MICROFLUIDIC TECHNOLOGIES FOR CAPTURING AND CONCENTRATING HUMAN IMMUNODEFICIENCY VIRUS-1 (HIV-1) PARTICLES

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Acknowledgements

I remember being with my dad in the berg climbing a mountain and moaning how tired I was and how I couldn't climb this mountain anymore. To this day I vividly remember his words, "if you lift up your foot, God will put them down." Little did he know how much those words would mean to me, but here I find myself, I kept lifting my feet and have found them placed firmly in a blessing.

I would like to use this opportunity to express my heartfelt gratitude to my dad, Howard Mᶜ Arthur, who gave me the opportunity to go to university and gain an education. To my mom, Dominique Mᶜ Arthur, for being there emotionally when I phoned her at times, in tears.

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"Everybody is a genius. But if you judge a fish by its ability to climb a tree, it will live its whole life believing that it is stupid"- Albert Einstein

Declaration

I**, Chanelle Crystal M^{c} Arthur,** hereby declare that this thesis entitled

"MICROFLUIDIC TECHNOLOGIES FOR CAPTURING AND CONCENTRATING HUMAN IMMUNODEFICIENCY VIRUS-1 (HIV-1) PARTICLES."

This work is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use was made of the work of others, it is duly acknowledged in the text.

Name: **Signature:**

Supervisor:…… **…….**

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Date:1st November 2016**…….**

Abstract

HIV-1 RNA assays are routinely used in developed countries to monitor the effectiveness of antiretroviral therapy (ART)[1]. These assays require well-trained operators, expensive equipment and reagents, and established laboratory infrastructure[1]. These requirements limit their usefulness in resource-limited settings where people are most afflicted by the HIV-1 epidemic[1]. Recent advances in microfluidics and nanotechnology offer new approaches for rapid, low-cost, robust and simple HIV-1 viral load monitoring systems[1]. Here we describe an approach within a microfluidic device to directly detect HIV-1 virus particles using an immune sandwich assay that includes anti-gp120 antibodies - conjugated to polystyrene microspheres and fluorescently labelled goat anti-HIV-1 FITC detection antibodies. The anti-gp120 antibody-conjugated microspheres were employed to capture and concentrate HIV-1 particles, whereas the FITC detection antibodies were used to generate fluorescent signal that represented the number of captured viruses. In the presence of HIV-1 particles, addition of microspheres and FITC detection antibody led to the formation of a microsphere/HIV-1 particle/FITC detection antibody complex. This complex was measured by analysing the fluorescence intensity produced by the FITC detection antibody bound to the HIV-1 particle within the complex. We demonstrated the utility of an in-house microfluidic device and assay in detecting $1x10^6$ virus particles/μl with a significance of ($p \leq 0.01$). This assay was completed within 3.8 hours, without any pre- or post- treatment of reagents.

Contents

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List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
ARV	Antiretroviral
ART	Antiretroviral therapy
ASSURED	Affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users
Bnabs	Broadly neutralizing antibodies
BSA	Bovine serum albumin
cDNA	Complementary DNA
CAD	Computer-aided design
CCR5	CC-chemokine receptor 5
CXCR4	CXC-chemokine receptor 4
DPI	Dots per inch
dsDNA	Double-stranded deoxyribonucleic acid
DBS	Dried blood spots
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration

GFP	Green fluorescent protein
GDP	Gross domestic product
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
LSI	Large scale integration
µl	Microliter
MTB	*Mycobacterium tuberculosis*
mg	Milligram
mL	Milliliter
ms	Milliseconds
MTCT	Mother-to-child transmission
MSL	Multilayer soft lithography
ng	Nanogram
nm	Nanometer
NA	Numerical aperture
pg	Picogram
PBS	Phosphate-buffer saline
POC	Point-of-care
POCT	Point-of-care testing
PDMS	Poly-dimethyl siloxane
PCR	Polymerase chain reaction
PMTCT	Prevention of mother-to-child transmission

rtLC	Real-time LightCycler
RT	Reverse transcriptase
RT-qPCR	Reverse transcriptase quantitative PCR
RNA	Ribonucleic acid
SIV	Simian immunodeficiency virus
TMCS	Trimethylchlorosilane
TB	Tuberculosis
ssRNA	Single stranded RNA
UV	Ultraviolet
vpc	Virus particles
WHO	World Health Organisation

Table 1: List of Abbreviations.

INTRODUCTION

Human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) has caused approximately 25 million deaths since it was first discovered in 1981[2]. In addition, there is an estimated 40 million people currently living with HIV-1 worldwide[3]. Of them, 97% are individuals living with HIV-1 in developing and moderate-income nations[4]. Of these 97% infected people, sub-Saharan Africa makes up 70%[5]. Closer to home, South Africa remains one of the most burdened countries with one in every 10 South Africans living with HIV-1[6] compared to only 2 in every 1000 individuals living with HIV-1 in the United Kingdom[7]. Typically, patients in heavily burdened areas have very limited resources and insufficient access to ART which can help suppress HIV-1 replication cycle and delay disease progression[2]. To curb this pandemic and to save lives, the World Health Organization (WHO) has rapidly started expanding access to ART in developing countries, originally aiming at universal access by the year 2010[2]. However, this has more challenging than originally envisioned since out of 15 million people needing ART in 2011, only 9.7 million had access to ART in low- and middle-income countries by the end of 2012, partly due to the lack of cost –effective ART administration and monitoring[8].

Currently, there are two major approaches to monitoring the effectiveness of HIV-1 ART. HIV-1 viral load in plasma, which indicates viral replication in the infected individual and CD4+ T lymphocyte count, which provides information on the functionality of the patient's immune system[2]. In developing areas, for individuals undergoing ART therapy, these parameters are not as regularly monitored compared to developed areas. These parameters are typically monitored once a year, whereas in developed areas, they are monitored every 3-6 months[2]. HIV-1 viral load is often monitored using commercial ribonucleic acid (RNA) assays, such as the reverse transcriptase-quantitative polymerase chain reaction (RT-PCR) for example Roche COBAS®, whereas CD4+ cell count is monitored using flow cytometry[2]. However, the application of such assays requires highly skilled personnel, high cost equipment (e.g., thermal cyclers) and expensive reagents [US$ 50-100 (ZAR 765-1,530) per test], which is not feasible for widespread application in resource-limited settings[2]. Due to the lack of viral load monitoring in developing areas, the WHO recommended a CD4+ cell count guideline for when to initiate ART in resource-limited settings. ART should be initiated when an individual has a CD4+ cell count of 500 cells/mm^3 or less[9]. Unfortunately, this WHO guideline has often lead to the late identification of virological ART failure. This may then allow for the accumulation of drug-resistant strains and reduce the efficacy of subsequent ART regiments[2]. Therefore, there is a need for affordable viral load monitoring to manage AIDS patients on ART in developing countries.

Some inexpensive alternatives to RNA viral load assays have been developed such as ExaVir™ RT viral load assay version 2.0 and in-house real-time reverse transcription quantitative-polymerase chain reaction (RT-qPCR) such as the BioMerieux NucliSens® [2]. However, these assays still have some drawbacks that limit their widespread use. For example, despite the reduced costs [approximately US$ 20 (ZAR 313) per test] the assays still require skilled operators and air conditioned laboratory conditions. These methods may be appropriate for centralized laboratories, but not for district clinics in resource-limited settings. The ExaVir™ RT viral load assay version 2.0 demonstrated good acceptability in a district hospital laboratory in Botswana, but there were some limitations[2]. The throughput of this assay was low, with a turnaround time of 2 days and up to 180 samples per week analysed. Other approaches resource-limited setting utilize to test patient's viral load include; sending samples elsewhere for analysis, dried blood spots (DBS) and viral load pooling. All of these assays still require the use of polymerase chain reaction (PCR)[10,11]. Therefore, recent advances focus on developing portable detection systems towards the point-of-care (POC) setting[2].

Recently, nano/microfluidic technologies have emerged as a powerful tool for the diagnosis and monitoring of infectious diseases, such as HIV-1, in both the developed and developing regions. Miniaturized microfluidic platforms enable the manipulation of small fluid volumes that can be useful for diagnosing patients in a more rapid and accurate manner that may also be more affordable. In particular, microfluidic diagnostic technologies are applicable within the global health sector, since they are typically inexpensive, portable, disposable, and easy-to-use[2].

Within this dissertation we review HIV/AIDS and HIV-1 viral load assays found in developed and developing countries. We also review how microfluidic technology is emerging as a potentially valuable approach for monitoring HIV-1 viral load of patients. Lastly, we demonstrate a pioneering effort that involved the construction, use and investigation of a microfluidics device for capturing, concentrating and quantifying HIV-1 particles.

The dissertation is composed of the following chapters;

Chapter 1: The core purpose of this chapter is to provide a concise literature overview of HIV/AIDS, ART, viral load monitoring techniques used in both developed and developing regions, and the advancement of microfluidics.

Firstly, the history of the development of HIV/AIDS via successful transmission of the simian immunodeficiency virus (SIV) to humans is discussed. From here, a description of how HIV infection leads to the immune system becoming vulnerable to opportunistic infections through the destruction of

the CD4+ immune cells leading to AIDS. Backgrounds on both HIV-1 and HIV-2 is explained, outlining the different phylogenetic lineages. Next presented, is the structure and life cycle of HIV-1. Then, how the development of ARV drugs exploit the life cycle of HIV-1, prohibiting certain stages of the life cycle. Thereafter, the relationship between HIV-1 burden and GDP of particular regions and countries is shown, bringing to light that those settings most highly burdened by HIV-1 have the least amount of resources to purchase ARV drugs and monitor the disease. Furthermore, we review the literature on how the lack of ARV monitoring availability in developing regions in particular, has led to the high risk of mother-to-child transmission (MTCT). Next, we define viral load monitoring and the production of diagnostic markers at certain stages during the natural course of HIV-1 infection. These markers, through the use of particular monitoring assays such as ELISA, PCR and flow cytometry assays are vitally important to measure. Thereafter, we discuss scientific techniques viral load monitoring assays often employ to determine viral load. These techniques include either; enzyme-linked immunosorbent assays (ELISAs), PCR or flow cytometry, providing both advantages and disadvantages of these techniques. From here we provide a review of current HIV-1 viral load assays for developed countries such as; COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test and RealTime HIV-1 assay. We then discuss alternative HIV-1 screening methods and approaches in resource-constrained settings such as DBS, sample pooling, Cavidi ExaVir™ RT assay, PerkinElmer Ultrasensitive p24 assay and lastly in-house RT-qPCR assays. The final section of the literature review discusses microfluidics. This chapter explains the fabrication process of microfluidics, advantages, limitations, current microfluidic technology applications and advances in HIV-1 POC viral load in resource-limited settings using microfluidics. To finish, Chapter 1, the aims and objectives of the dissertation is outlined.

The remainder of the chapters, **Chapters 2-5** are devoted solely to the materials, methods, results, discussion and conclusion.

CHAPTER 1

1. Literature Review

1.1. HIV/AIDS

In 1981, the first cases of an AIDS were reported among young, homosexual American men[12], heralding what we now know to be the beginning of the HIV/AIDS epidemic. AIDS can be caused either by HIV-1 or HIV-2, both of which are derived from a clade of the *Lentivirus* genus (SIV) of the *Retroviridae* family found naturally in more than 40 species of nonhuman primates in sub-Saharan Africa. SIV predominantly falls into host-specific clades, but has occasionally "jumped" species and successfully spread to new hosts[3]. Successful transmission of one particular strain of SIV from the chimpanzee subspecies (*Pan troglodytes troglodytes)* to humans lead to the development of HIV[3].

HIV infection causes an acquired immunodeficiency, primarily as a result of the depletion of CD4+ T cells. CD4+ T cells are a type of white blood cell that plays a crucial role in protecting the body from infection[13]. The mechanism by which the virus depletes these CD4+ cells, however, is not quite clearly understood[14] although apoptosis has been proposed as a key mechanism[15]. A study done by Gilad Doitsh *et al*., showed that apoptosis of the CD4+ T cells only accounts for the death of a very small fraction of the CD4+ cells that are both activated and infected. The remaining 95% of quiescent CD4+ T cells die due to pyroptosis, triggered by the viral infection[15]. Pyroptosis corresponds to an inflammatory form of cell death whereby inflammatory cytokines are released. This release of cytokines leads to two signature events in HIV infection, CD4+ T cell depletion and chronic inflammation. This further creates a vicious cycle whereby dying CD4+ T cells release inflammatory signals that further attracts more cells to die[15]. Over time the CD4+ cell count declines and eventually there are not enough CD4+ cells remaining to defend the body from opportunistic infections[16]. The patient at this point becomes highly susceptible to any opportunistic infection such as pneumonia, bacterial sepsis and tuberculosis[17]. Once a patient has reached this stage, the patient is said to have AIDS. Both the virus and the syndrome together are sometimes referred to as HIV/AIDS[18].

HIV is categorized into two types, HIV-1 and HIV-2. HIV-1 evolved from a SIV found in African apes[3] while HIV-2 came from an SIV in a type of monkey called the sooty mangabey[19].

HIV-1, the causative agent of AIDS, is composed of three major phylogenetic groups[20]. These groups include groups M, O and N, each of which resulted from an independent cross-species transmission event of the SIV[3]. Group M, the cause of the AIDS pandemic, has spread across Africa and throughout the rest of the world infecting more than 40 million individuals[3]. More than 90% of all HIV-1 infections are derived from group M. Group O is mainly restricted to west central Africa, having spread through Gabon, Nigeria, Cameroon, and other neighbouring countries. Group O has been estimated to have infected approximately 100,000 individuals[3] and represents 1-5% of HIV-1 infections[20]. Lastly, group N viruses have been identified in a limited number of individuals from Cameroon.

HIV-2, was first discovery around 1989[21]. Initially confined to West Africa, specifically Senegal and Guinea-Bissau[21], has since spread to Europe, India, and the United States[22]. An estimated 1-2 million HIV-2 infected people are found in West Africa[22]. However, in recent years the overall prevalence rate of HIV-2 has started declining, and in most West African countries, HIV-2 is being replaced by HIV-1. For example, in a rural area of north-western Guinea-Bissau in West Africa, the prevalence of HIV-2 dropped from 8.3% in 1990 to 4.7% in 2000. During the same time period, the HIV-1 prevalence in this region increased from 0.5% to 3.6%[22]. This trend may be related to the 5-fold lower rate of sexual transmission of HIV-2 compared to HIV-1, making HIV-2 less infectious than HIV-1[23]. HIV-2 viral load levels are also typically lower compared to HIV-1 infected individuals[21]. In untreated patients, viral load levels of HIV-2 tend to be approximately around 10,000 copies/mL, versus sometimes millions of copies/mL in HIV-1 infected individuals[24]. Lastly, it has been documented that most individuals infected with HIV-2 typically do not progress to AIDS[21], due to the aforementioned characteristics of HIV-2.

A detailed understanding of HIV-1 structure and how it establishes infections and causes AIDS is crucial not only to be able to identify and develop new effective vaccines and drugs, but also to define strategies for laboratories to diagnose HIV-1 infection. Diagnostic strategies have to be continuously reviewed according to new discoveries on the replication characteristics of HIV-1 infection[25].

1.1.1. HIV-1 Structure

The retrovirus genome is composed of two identical copies of single-stranded RNA molecules and is characterized by the presence of the following structural genes; *gag, pol and* env. In addition to these structural genes, there are regulatory genes; *tat* and *rev* and accessory proteins; Vpr, Vpu, Vif and Nef (Figure 1)[25].

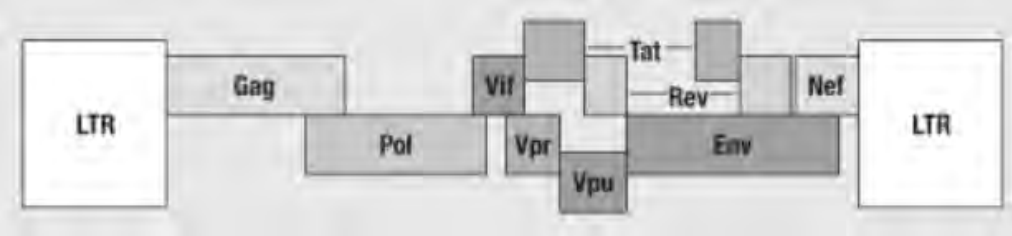

Figure 1: Organisation of the HIV-1 genome, highlighting the structural genes; *gag, pol and env,* the regulatory genes; *tat* and *rev* and the accessory proteins; Vpr, Vpu, Vif and Nef[25].

The *gag* gene encodes the structural proteins of the core (p24, p7, p6) and the matrix (p17). The *env* gene encodes the viral envelope glycoproteins gp120 and gp41, which recognize the host cell's surface receptors[25]. Lastly, the *pol* gene encodes for enzymes that are crucial for replication. These enzymes include the reverse transcriptase (RT) enzyme that converts viral RNA into DNA, the integrase enzyme that incorporates the viral DNA into the host cells chromosomal DNA (known as the provirus) and lastly, the protease enzyme that cleaves large Gag and Pol protein precursors, which is necessary for the generation of infections viral particles[25].

The regulatory genes *tat* and *rev* and the accessory proteins; Vpr protein, Vpu protein, Vif protein and Nef protein (Figure 1) play a key role in modulating viral replication[25]. The *tat* gene encodes for the protein (Tat) which is expressed soon after infection and promotes the expression of HIV genes. The *rev* gene encodes for the Rev protein, which helps exports processed genomic RNA from the cell nucleus to the cytoplasm. The accessory protein Vpr is involved in the arrest of the cell cycle, enabling the reverse transcribed DNA to enter the nucleus of the host cell[25]. Vpu protein is necessary for the correct release of virus particles, whereas the Vif protein enhances the infectiveness of the progeny virus particle. Lastly, the Nef protein is involved in the down regulation of the CD4+ cell receptor on the cells surface in order to allow virus budding in the late stages of the virus replication cycle[25].
HIV-1 particles have a diameter of 100 nm and are surrounded by a lipoprotein-rich membrane (lipidic membrane) see Figure 2.

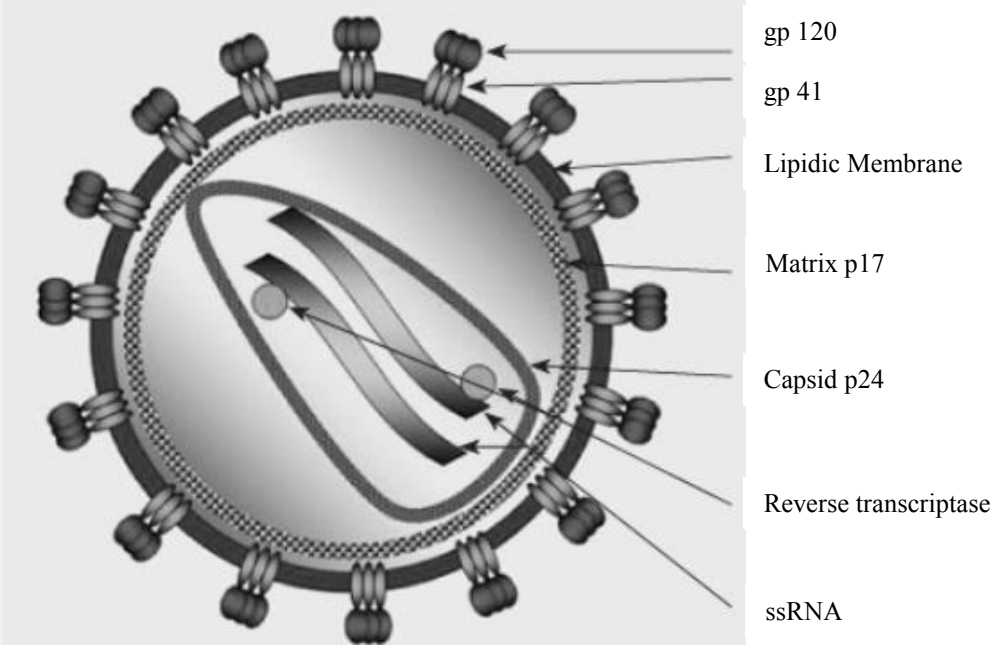

Figure 2: Structure of HIV-1 particle highlighting the core protein (p24) encoded by the *gag* gene, glycoproteins gp120 and gp41 encoded by the *env* gene and reverse transcriptase enzyme encoded by the *pol* gene. ssRNA: single strand RNA[25].

The lipidic membrane consists of glycoprotein complexes composed of triers of the external surface gp120 and the transmembrane spanning gp41 glycoproteins bound together[25]. The binding between the gp120 and gp41 is not covalent and so gp120 may shed spontaneously and can be detected in the serum. Anchored within the inside of the viral lipidic membrane is the matrix protein (p17). Both this membrane and matrix protein house the viral capsid, which is composed of polymers of the core antigen (p24). The capsid contains two copies of HIV RNA and the enzymes RT, integrase and protease[25].

1.1.2. HIV-1 Life Cycle

The HIV-1 life cycle, as shown in Figure 3, can be described in six steps; 1: binding and entry; 2) uncoating; 3) reverse transcription; 4) provirus integration; 5) virus protein synthesis and assembly and lastly, 6) budding[25].

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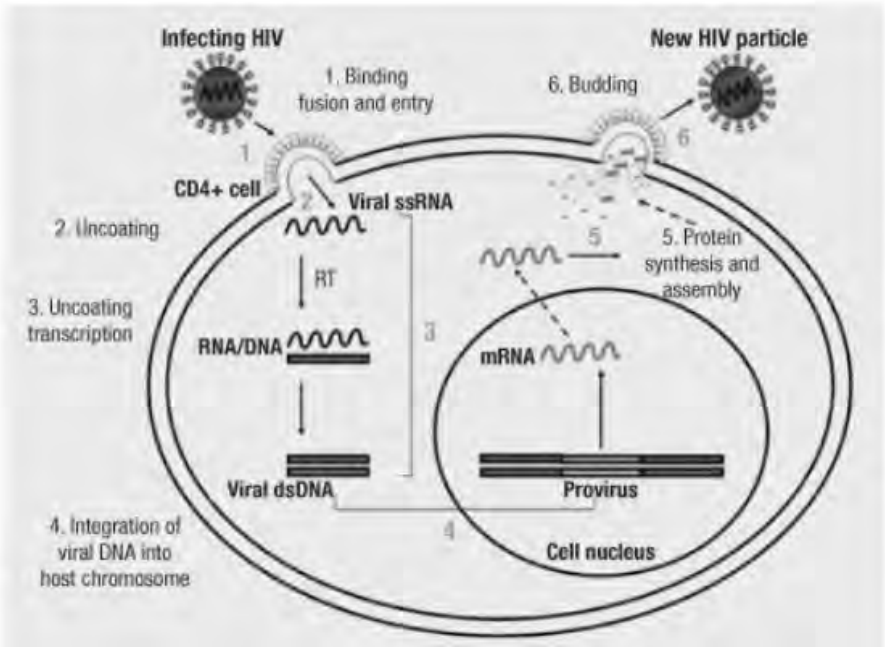

Figure 3: The 6 steps of the HIV-1 replication cycle; 1: binding and entry; 2) uncoating; 3) reverse transcription; 4) provirus integration; 5) viral protein synthesis and assembly and lastly, 6) budding[25].

1) Binding and Entry

The entry pathway of HIV-1 is divided into three major events: the virus binding to the cell, fusion and entry. The viral envelope trimeric complex, composed of gp120 and gp41, are vital for the virus recognition and entry into target cells (CD4+ T cells)[25]. The gp41 subunit contains a fusogenic peptide that is essential for fusion of the viral and host cellular membrane. HIV gp120 binds to a glycoprotein CD4, which is expressed on the surface of approximately 60% of the circulating T-lymphocytes[25]. Upon gp120 binding with the CD4 protein, the virus envelope complex undergoes a structural change. This change, exposes a specific domain in the gp120 that is capable of binding to chemokine receptors (coreceptors) on the host cell membrane. The coreceptors used by HIV-1 are either CXCR4 or CCR5. Some strains of HIV-1 bind to the coreceptor CCR5, which are mainly presented in macrophages and CD4+ T cells expressing CCR5 and are therefore known as macrophagetropic (M-tropic)[25]. CCR5 is the mostly commonly used corecptor. Other viral strains of HIV-1 use CXCR4 corecptor in primary CD4+ T-cells that express CXCR4, therefore are known as T-lymphocyte-tropic (T-tropic) viruses. Interestingly, some HIV-1 isolates are able to bind to both CCR5 and CXCR4 receptors and are known as dual tropic or X4R5 viruses see Figure 4[25].

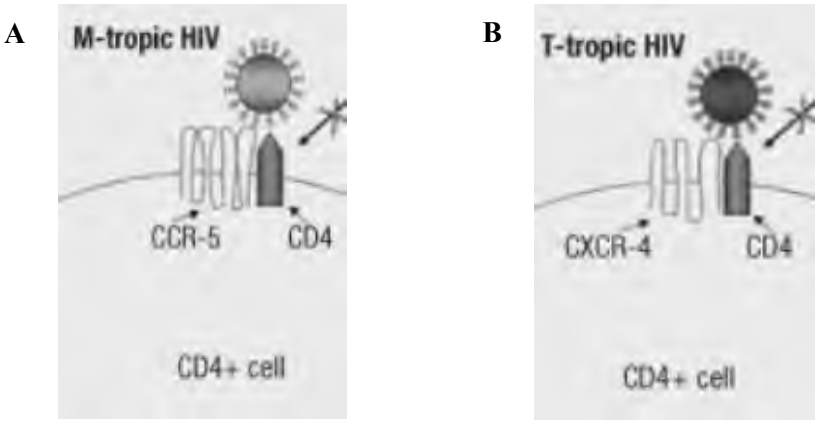

Figure 4: HIV tropism. A) M-tropic HIV-1 uses corecptor CCR5 and B) T-tropic HIV-1 uses corecptor CXCR4[25]

The binding of gp120 to both the CD4 and one of the coreceptors allows a more stable attachment of the virus. This in turn allows the gp41 fusogenic peptide to penetrate the host cell's membrane and bring both the virus and cell membrane closer together. This then allows fusion of the membranes and subsequent entry of the viral capsid into the host cell[25].

2) Uncoating

Once membrane fusion has occurred and the viral capsid has been injected into the host cell, the capsid uncoats in the cytoplasm of the host cell and releases the viral RNA[25].

3) Reverse Transcription

The viral RNA is then converted into proviral double helix DNA molecule by the RT enzyme[25].

4) Provirus Integration

Next, the integrase enzyme cleaves the ends of the double helix DNA creating sticky ends and transports the modified provirus DNA into the cell nucleus. From here, integrase helps facilitate the DNA into the host genome[25].

5) Viral Protein Synthesis and Assembly

Once the provirus is within the host genome, it will only be expressed when or if the host cell becomes activated. Only upon activation will the transcription of proviral DNA into messenger RNA occur. Transcription results in the synthesis of regulatory HIV-1 proteins (Tat and Rev) as well as the production of the structural proteins from the structural genes for the new virions[25].

The assembly of a new viral particle by is a stepwise process. The two viral RNA strands associate together with the viral enzyme, while core proteins assemble over them, making the virus capsid. At this point the virus is termed an immature particle. The immature particle migrates towards the cell surface, whereby the protease enzyme cleaves molecules on this immature particle, resulting in a new infectious viral particle. This infectious viral particle will then bud through the host cell membrane[25].

6) Budding

During this final stage, the newly assembled viral progeny bud from the cell by fusing with the host cell membrane. This often results in the virus lipid membrane incorporating some host cell proteins and it becomes enriched with cholesterol and lipids. The budding process doesn't kill the host cell immediately, but over time, the more damaged the cell membrane becomes from all the budding processes, the cell will die[25].

By having a detailed understanding of the above HIV-1 structure and life cycle, it enables scientists to identify and develop new effective vaccines and drugs. The advancement in the management of HIV-1 infection has been the treatment of patients with ARV drugs. These drugs are capable of suppressing the HIV-1 replication cycle to undetectable viral load levels[26]. From the time when the first HIV-1 specific ARV drugs were given as monotherapy in 1990, the standard of HIV-1 care has since evolved to include a cocktail of ARV drugs[26]. This cocktail of ARV drugs otherwise known as HAART was pivotal in reducing morbidity and mortality associated with HIV-1 infection[26]. From once being considered a death sentence, HIV-1 patients who have access to effective HAART now have a near to normal life expectancy.

1.2. HIV Disease Burden and the Motivation for more Effective Viral load Testing

1.2.1. Antiretroviral Therapy

The development of drugs for HIV-1 infection commenced soon after the virus was discovered 35 years ago. Since then, monumental steps towards the development of drugs that interfere with certain stages of HIV-1 life cycle have transpired with unprecedented success. Nearly, 6.6 million HIV/AIDS related deaths worldwide have been prevented due to HAART[27]. HAART consists of a combination of at least three drugs[8]. The most routinely prescribed HAART combination is NNRTI, NRTI and PI as a single pill or in various pill combinations[28].

Currently, there are 6 major classes of ARV drugs. These classes include; nucleoside analogue reverse transcriptase inhibitor (NRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI) and protease inhibitor (PI). The last 3 classes of ARV drugs includes; fusion inhibitor, chemokine coreceptor antagonist (including 2 subclasses: CCR5 and CXCR4 antagonist) and integrase inhibitor[29], see Figure 5 for a view of where these drug classes act during HIV-1 life cycle.

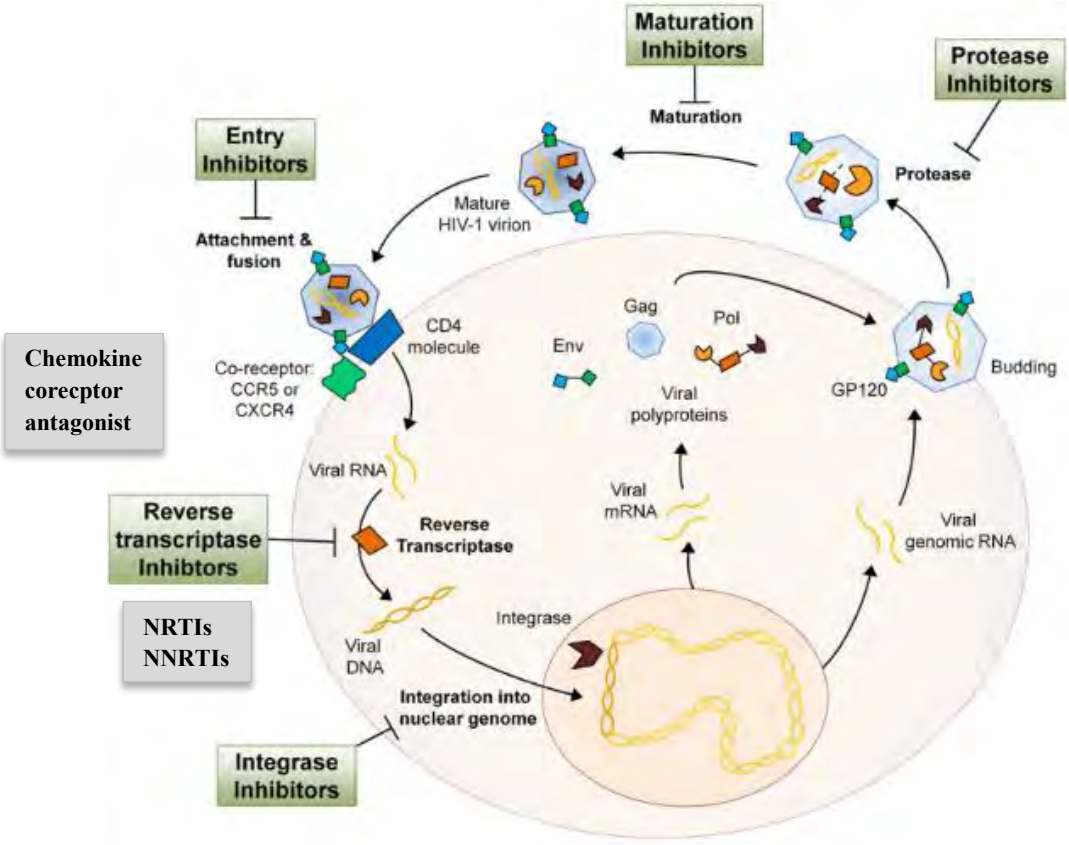

Figure 5: The HIV-1 life cycle and the ARV drug classes that disrupt certain processes in the life cycle[30]. Nucleoside analogue reverse transcriptase inhibitor (NRTI) inhibits the synthesis of DNA by

acting like structural analogues of natural nucleosides. Non-nucleoside reverse transcriptase inhibitor (NNRTI) completely suppresses the reverse transcriptase enzyme. Protease inhibitor (PI) interferes with the final stage of the replication cycle by not allowing the protease enzyme to cleave the gag precursor polyprotein, blocking formation of the virus particles. Fusion inhibitors block HIV from entering into target cells. Integrase inhibitors prevent the integration of the viral cDNA into the CD4+ cells genome by binding to the integrase enzyme and prevents the enzyme functioning. Lastly, chemokine coreceptor antagonists disrupt the initial binding of HIV-1 to the CD4+ cell. The antagonists bind to either the CCR5 or CXCR4 coreceptor on the surface of CD4+ cells and blocks a necessary step needed in viral entry, preventing HIV-1 entering the cell[30].

NRTIs function by inhibiting the synthesis of DNA by reverse transcriptase. The nucleoside analogues have a structural resemblance to the natural building block of DNA (the nucleosides adenosine, thymine, guanosine and cytidine)[29]. The nucleoside analogues under modification within the cell becomes phosphorylated. The reverse transcriptase enzyme of HIV-1 cannot distinguish the difference between the natural phosphorylated nucleosides and the phosphorylated NRTIs, and tries to use the drugs in viral DNA synthesis[29]. When the NRTI is incorporated into the strand of DNA being synthesised, any further nucleotide additions is prevented and a full-length copy of the viral DNA is unable to be produced[29]. Examples of NRTI drugs include; Zidovudine and Tenofovir[31]. NNRTI completely suppresses the reverse transcriptase enzyme[32]. It does so by binding to the reverse transcriptase in a way that inhibits the enzyme's activity, thereby inhibiting the synthesis of viral DNA[29]. Examples of NNRTI drugs include Nevirapine and Efavirenz[31]. PIs interfere with the final stage of the replication cycle by not allowing the protease enzyme to cleave the gag precursor polyprotein, blocking formation of the virus particles[32]. PIs do this by binding to the active site of the viral protease enzyme. This prevents processing of the viral proteins into functional conformations. The viral particles are still produced despite the protease enzyme being inhibited, but they are not effective at infecting new cells[29]. Examples of PIs are Ritonavir and Indinavir[31]. Fusion inhibitors (entry inhibitors) block HIV-1 from entering into target cells. Drugs within this class bind to the HIV-1 envelope protein gp41. Envelope protein gp41 is involved in viral entry, but when fusion inhibitors block gp41, they interfere with the conformational change of the envelope molecule needed for fusion with the host cell membrane[29]. Examples of fusion inhibitor drugs are Enfuvirtide and Maraviroc[31]. Integrase inhibitors prevent the integration of the viral cDNA into the CD4+ cells genome[32] by binding to the integrase enzyme and preventing the enzyme from functioning[29]. Examples of integrase inhibitors include Raltegravir and Elvitegravir[31]. Lastly, there are chemokine coreceptor antagonists that disrupt the initial binding of HIV-1 to the CD4+ cell. These antagonists bind to either CCR5 or CXCR4 coreceptor on the surface of CD4+ cells, blocking a necessary step needed in viral entry thereby preventing HIV-1 entering the cell[29]. Examples of a chemokine coreceptor drug is Selzentry[33].

Even with the advancement in ARV drugs, the development of drug resistance is a natural biological process that will occur over time[34]. With the on-going use of HAART, the rate of HIV-1 infection can be dramatically reduced. However, HAART is not a cure for HIV[17] as there are numerous reasons why HIV-1 cannot be eradicated with current drug therapy. Reasons such as high HIV-1 mutation rates, production of thousands of virion progeny and viral reservoirs, which all play a role in the inability of HAART from becoming a cure. The extensive heterogeneity observed in the HIV-1 epidemic originates from the rapid viral turnover of $1x10^{10}$ viral particles produced per day in an infected individual. This causes a high rate of incorrect nucleotides being substituted during HIV-1 reverse transcription, leading HIV-1 having a high mutation rate of 3×10^{-5} mutations per base pair, per replication cycle[35] (which occurs every 2-3 days[36]). Comparing this mutation rate to that of the Ebola virus, which has a nucleotide substitution rate of $1.3x10^{-3}$ substitutions per year[37]. Besides from the high HIV-1 mutation rate which poses a challenge for ARV therapy, the virus targets and replicates within many different immune-system cell types[17]. Treatments that can inhibit HIV-1 replication in one particular cell type may not be able to inhibit replication in another type[17]. Once an immune cell is infected by a single HIV particle, thousands of copies of the virion can be produced before the cell is destroyed. The virus also hibernates in a variety of reservoirs throughout the body and long-term remission requires continued control over the immune cells that are vulnerable to infection for the rest of the patient's life[17]. If the patient stops therapy, HIV-1 within the reservoirs becomes active again. These natural biological process of resistance development have also been accelerated due to human practices such as non-adherence, treatment interruptions, lack of HIV-1 monitoring and the prescription of incorrect drug regimens[34]. The main aim for current HIV-1 therapy is to break the replication cycle, which should lead to diminished replication events that occur and thereby lowering the viral load levels. This should aid the therapy regime to remain effective in the long run.

There has been evidence that the rate of transmitted drug resistance of HIV-1 has become less detected in more developed countries compared to developing countries. For example, the rate of transmitted drug resistance of HIV-1 to NNRTI in Canada is 1.6%[38], whereas the rate of transmitted drug resistance of HIV-1 to NNRTI in KwaZulu-Natal, South Africa is 5-15%[39]. There has also been a trend observed, that areas with a higher disease burden also have a lower literacy rate. For example, in 2011 Canada had a literacy rate of 99%, the highest in the world[40] and 71 300 people living with HIV-1[41]. This is in contrast to Chad (a Sub-Saharan Africa country) which only has a literacy rate of 34.5 %[42], but has 210 000 individuals living with HIV-1 in 2011[43]. The reason for Canada, a developed country, having less HIV-1 infected individuals, is due to both a high standard of healthcare systems and seemingly a more advanced educational system, whereby the importance of ARV and HIV-1 may be more widely known, compared to resource-limited settings. Most developed countries have implemented routine use of viral load testing in order to monitor how effective a patients HAART combination is and if it is successfully keeping the patient's viral load low[11]. Despite the rapid increase and efforts of the WHO to provide

access to ART worldwide, in low-and-middle income countries, viral load testing is still fairly rare due to the high costs. This often leads to a patient remaining on a standard fixed-dose HAART combination[11] for a prolonged period of time, until their viral load can be tested. Despite this approach allowing for a higher chance of a patient adhering to their therapy regime[44], it can also lead to drug resistance. Lastly, the price of ARVs poses another hurdle within the disease management system. In the year 2013 for low-and-middle income countries, the cost of ARV therapy varied depending on which regimen the patient was receiving. For first-line, second-line and third-line treatment, the price of therapy was US$ 115 (ZAR 1,753), US$ 330 (ZAR 5,031) and US$ 1 500 (ZAR 22,871) per year, per patient, respectively[45]. Due to the high cost of HAART, in sub-Saharan Africa countries with low GDPs, such as Nigeria, only 20% of HIV-1 patients have access to ARV treatment[46]. This in contrast to a country which has a higher GDP, such as Australia, whereby 100% of HIV-1 positive patients have access to ARV treatment[47].

1.2.2. Relationship between HIV-1 Burden and GDP (per capita)

Low-and-middle incomed countries struggle to cope with the HIV-1 disease burden and this can be seen through the GDP of these areas. By analysing the number of HIV-1 infected individuals in particular places around the world relative to the GDP, an inversely proportional relationship becomes apparent, see Figure 6.

Figure 6: Bubble plot highlighting HIV-1 burden of a specific countries and their corresponding GDP per capita. The size of the bubbles represents the amount of people currently living with HIV-1 (millions) in red or the GDP (per capita) (USD) (thousands) in blue. Some regions around the world are heavily burdened with HIV-1, having little GDP to manage, such as Sub-Saharan Africa. In contradiction, countries such as Australia where the HIV-1 burden is less, the income is high. (Graph made from information collected from UNAIDS[46].

From Figure 6, one can see a large disproportionality of incomes which adds to the HIV/AIDS epidemic. Poorer countries are most heavily disease burdened, but cannot afford the necessary interventions. For instance, Australia has one of the highest GDP per capita but, has one of the lowest HIV-1 burdens. This is in stark contrast to sub-Saharan Africa which represents nearly 70% of the total number of people living with HIV/AIDS globally[5] and has a low GDP per capita, thus scarcer resources to cope with the disease burden.

Found amongst the low GDP and heavily HIV-1 disease burdened sub-Saharan Africa were 2.9 million children living with HIV-1 in 2013. Only 24% of these children received ART, resulting in 190 000 children passing away from AIDS-related illnesses[48]. However, due to the lack of funds and resources

for adequate testing, paediatric ARV drugs and prevention of mother to child transmission (MTCT) programs, this results in children having to continue suffering from the epidemic.

1.2.3. Prevention of Mother to Child Transmission (PMTCT)

Pregnant women are an important group to target for HIV-1 prevention[49]. With early diagnosis and viral load levels quantified, appropriate management of MTCT through HAART usage can reduce MTCT by approximately 40%[50].

MTCT refers to the transmission of HIV-1 from a woman who is HIV-positive to her child during pregnancy, labour, delivery or breastfeeding. MTCT is the main means by which most children acquire HIV-1. Over 90% of new HIV-1 infections in infants and young children occur through MTCT in low- and middle- income countries[51]. With approximately 37 million individuals living with HIV-1 at the end of 2014, sub-Saharan Africa accounted for approximately 26 million out of the 37 million people living with HIV-1. Amongst the 26 million HIV-1 individuals in sub-Saharan Africa, 55% were women[52], which increases the risk of MTCT. In Uganda alone, 96 700 babies were born to HIV-positive mothers in 2011 and 20 600 (21%) of them contracted HIV-1 during birth from their mothers via MTCT[53]. In the absence of medical intervention, prior to the birth of the child by an HIV-positive mother, the risk of HIV-1 transmission to the child, without breastfeeding is between 15-30%. If the mother breastfeeds the child after birth, the transmission risk increases to 20-45%[54]. The risk of MTCT at birth can be dramatically reduced to fewer than 2%, if the necessary interventions are implemented.

These interventions are collectively termed as PMTCT. PMTCT strategies include; HIV-1 testing and antiretroviral prophylaxis, which is given to the HIV-positive mother during pregnancy and labour, as well as to the new-born infant in the first few weeks of its life. Other PMTCT strategies include an elected caesarean delivery before the onset of labour (prior to the membranes rupturing) and total avoidance of breastfeeding. These interventions are routinely practiced in high-income countries, lowering the risk of new HIV -1 infections in children dramatically[54]. This was evident between the year 2009 and 2013, whereby PMTCT interventions averted a total of 900 000 new HIV-1 infections in children, mainly in high income countries[55]. However, PMTCT interventions such as breastfeeding avoidance are seldom feasible in resource-limited settings due to many deterring factors such as, safe running water and the cost of infant formula -breast milk substitute- itself is exorbitant. If we consider women in South Africa, where the unemployment rate is more than 30%, most cannot afford breast milk substitutes. Another deterrent for preventing the practice of breastfeeding is the access of formula. For instance, women in rural areas often have to travel 10 to 15 kilometres with little to non-transport being available to access the infant formula[56]. One of the most crucial points of PMTCT to be decreased, is the deficiency of ARV coverage in rural areas. As previously stated, out of 15 million people needing

ART in 2011, only 9.7 million had access to ART in low- and middle-income countries by the end of 2012, partly due to the lack of cost –effective ART monitoring[8]. With little testing and ARV coverage, the MTCT rate during labour and delivery accounts for two-thirds of overall transmissions in infants in resource-limited countries[54]. To make PMTCT effective on a long-term basis, HIV-1 testing needs to be carried out more extensively and regularly for pregnant women. This will enable timely administration of PMTCT interventions for HIV-positive mothers. PMTCT efforts in resource-limited areas increases the need for drug susceptibility and HIV-1 testing for high-risk mothers prior to delivery.

As seen, for ART therapy to be useful for both individuals and mothers-to-be, monitoring viral load is of utmost importance to evaluate if treatment is effective by keeping a low viral load. For viral load testing to be carried out, certain markers produced during the natural course of HIV-1 infection must be measured.

1.2.4. Markers for HIV-1 Viral load monitoring.

The natural course of HIV-1 infection can be divided into 3 stages. These stages are, seroconversion, asymptomatic and symptomatic stages. At each of these stages, different diagnostic markers are found that can be used to gauge ART efficacy. These diagnostic markers include; HIV-1 RNA, DNA, antigen, antibody, RT and CD4+ cells[2], (Figure 7)[57].

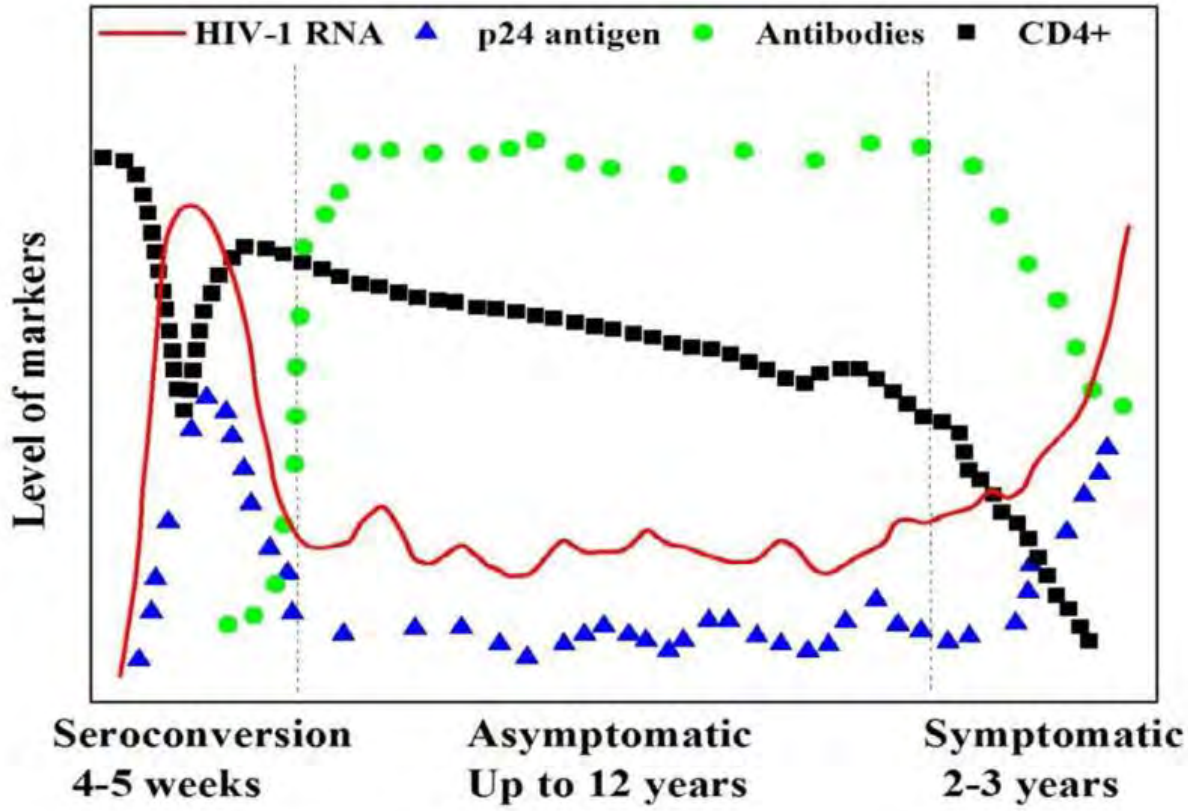

Figure 7: Diagnostic markers during the natural course of HIV-1 infection[57]. During the three stages of HIV-1; Seroconversion, asymptomatic and symptomatic, certain markers are produced that can be measured. During seroconversion, HIV-1 RNA (red line) peaks, but then decreases and remains at a low level, showing no clinical symptoms. HIV-1 p24 antigen (blue triangle) follows the same trend as HIV-1 RNA. HIV-1 specific antibodies (green circles) are produced during seroconversion, reaches a plateau during the asymptomatic stage and decreases in the symptomatic stage.CD4+ cells (black squares) drip rapidly and rebounds during seroconversion. Slowly over the course of infection the CD4+ cell number decreases as AIDS develops[2].

Viral load can be defined as the level of HIV-1 RNA in plasma (copies/mL)[2]. Soon after infection, the level of HIV-1 RNA increases significantly and reaches a peak of approximately 10^7 copies/mL (Figure 7). Almost immediately after infection, the immune system starts producing antibodies to control the virus. This stage is known as seroconversion and can take between three weeks to three months. During this stage, the host immune system is still intact, represented by a high CD4+ cell count. After seroconversion is the asymptomatic stage. This is when the individual shows no signs or symptoms that they are infected and they may even have at this point. An undetectable viral load means that there is little HIV-1 in their blood for a diagnostic device to detect[58]. This stage can last many years and during

this stage, the HIV-1 viral load is kept at a low level throughout. However, HIV-1 towards the end of this stage starts to slowly compromise the host immune system, indicated by a decrease in the CD4+ cell count[2]. HIV-1 p24 antigen has also been used as a surrogate marker for viral load monitoring in developing countries. As shown in Figure 7, the level of p24 antigen is highly correlated with HIV-1 RNA (viral load) throughout the natural course of HIV-1 infection. In addition to p24 antigen levels, RT levels have a close correlation with HIV-1 RNA in patients as well and can be used as a marker for viral load monitoring[2]. After the asymptomatic stage, people move into the symptomatic phase. Within this stage, early symptoms start developing such as, fatigue, weight loss and thrush. Over time, the CD4+ cell count continues to drop and the individual develops AIDS, whereby they are being infected with opportunistic infections[58].

CD4+ cell count has also been termed as a surrogate marker for HIV-1 disease progression. A study done by Alison. M. Morris *et al.,*2000; showed that as viral load decreased, CD4+ cell count increased (Figure 8)[59]. In another study done by Eaton C *et al.,*2011 the opposite relationship was shown, whereby an increase in viral load, resulted in a decrease in CD4+ cell count (Figure 9)[60].

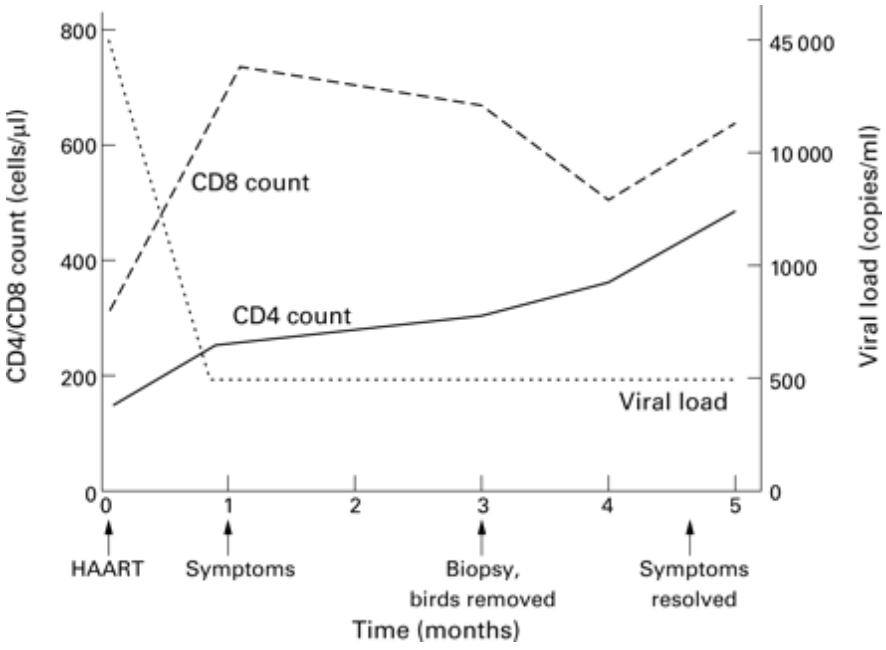

Figure 8: Time course comparing viral load with CD4+ count and CD8+ Count. As viral load decreased, CD4+ and CD8+ counts increased[59].

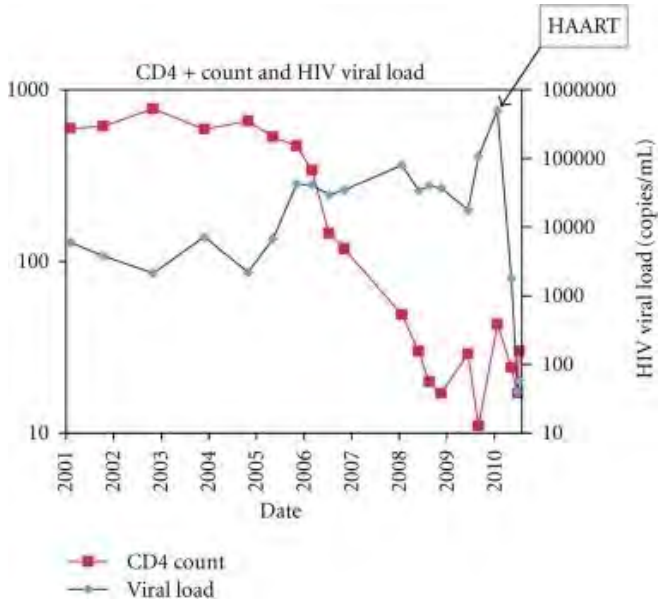

Figure 9: Graph of CD4+ cell count and HIV-1 viral load. As viral load increased over the years, the CD4+ cell count decreased[60].

These above mentioned markers are measured by specific assays in order to continuously monitor a patient receiving therapy. These specific assays vary though, depending on the location they are carried out in. Developing areas often cannot afford the assays that are performed in developed areas and therefore have had to adapt their assays or techniques for monitoring of HIV-1 patients. The accurate and timeous detection of HIV-1 infection is vital in allowing the patient's receiving of timely access to both care and drug therapy. This ultimately helps to improve overall treatment outcome[61]. There is a growing demand within the global health community for ways to both simplify and improve the efficiency of HIV/AIDS diagnosis without lessening the quality of patient care[61]. At the same time, there is a need to increase the level of access to high-quality and robust diagnostic solutions in resource-limited settings. This facilitates the prevention of HIV-1 transmission through early detection and treatment[61].

1.3. Viral load monitoring

Viral load monitoring is the measurement of either; HIV-1 RNA in a patient's plasma; the number of virus particles in a patient's blood (particles/mL)[62] or even the CD4+ cell count (number of cells/mm^3) can be used to monitor patients. The viral load measurement indicates how effective the HIV-1 patient's HAART therapy is. Typically viral load is measured in copies/mL. For a patient who is on HAART therapy, a high viral load is considered to be between 5 000 to 10 000 copies of RNA/mL or anything

above, whereas a low viral load is usually between 40 to 500 copies of RNA/mL[63]. For CD4+ cell count, a normal CD4+ cell count ranges from 500 – 1 500 cells/mm[64]. Anything below 500 mm^3 and the individual, if HIV-positive, must be placed on HAART. Anything below 200 cells/mm^3 indicates serious immune damage and is a sign of AIDS within individuals who are HIV-positive[65].

Viral load monitoring assays will often use one of the following scientific techniques to determine viral load. These techniques include either; enzyme-linked immunosorbent assay (ELISA), PCR or flow cytometry[10]. These techniques are mostly utilized within developed settings and are often modified for specific usage in developing settings. The next section describes these techniques with both their advantages and disadvantages.

1.3.1. ELISA

ELISA is a method typically used to detect antibodies against HIV-1. ELISAs are the most widely applied tests in immunodiagnostics. Commercial ELISA kits are used as the initial HIV-1 screening assay when an individual is being tested for the presence of HIV-1[66]. The principle of ELISA is illustrated in Figure 10. It is based on the interaction between the viral antigens and the antibodies that the patient's immune system has produced in response to the presence of HIV-1. The main reason why ELISA is the most widely applied assay for initial HIV-1 screening is due to its simplicity[67].

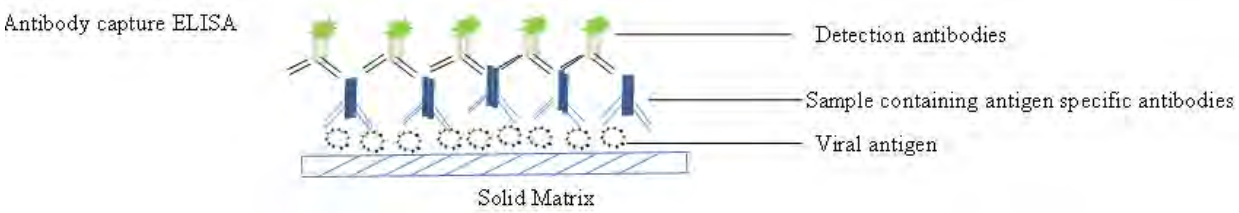

Figure 10: A schematic representation of an antibody capture ELISA principle used to capture HIV-1[67]. Viral antigens are immobilized to matrix, capturing any antigen specific antibodies within the patient's sample. Thereafter, detection antibodies are flown through the system, binding to any captured antigen specific antibodies. The detection antibodies produce a signal which can be detected as either a positive or a negative result.

The advantages ELISAs include, high sensitivity (>99.5%), high specificity and amenability with automation, therefore ease of use[67]. However, ELISA assays do have some drawbacks that may limit their widespread use. These drawbacks include, costly and sometimes unaffordable reagents and cross-reactivity with non-viral protein targets which may cause variability in the results. Other limitations

include that the test only provides qualitative results, thus rendering it unsuitable for viral load monitoring[66,67]. Furthermore, the ELISA reader machine itself is very expensive and estimated to cost US$ 9 500 (ZAR 152,429.0). As a result tests are generally not being performed at the point-of-care setting due to the unavailability of the ELISA reader at most clinics. To circumvent this problem, patients' samples are often sent for analysis at specialized laboratories. For example, in South Africa, ELISA tests for rural clinics are commonly carried out by the South African Medical Research Institute (SAMRI). This means that patient samples are sent to the institute, resulting in up to a weeklong lag until the results are sent back to the clinic[68]. This process creates difficulties in following up with patients[68]. Despite these disadvantages, ELISA assays have still managed to maintain popularity. This is primarily due to them being a relatively cost-effective screening tool compared to most alternatives. ELISA as a screening tool costs (ZAR 126)[70] per patient, per assay in South Africa.

1.3.2. Polymerase Chain Reaction

PCR has emerged as one of the most powerful tools for the amplification of genes or their RNA transcripts[66]. PCR is carried out in a machine known as a thermal cycler. The thermal cycler contains a thermal block with holes, into which a 96-well plate (containing all PCR reagents) is placed upon. Within the machine, the temperature is raised and lowered at specific times throughout the PCR cycle[71]. Each PCR cycle has three steps per 1 cycle. Each cycle includes the following steps; denaturation, annealing and extension, (Figure 11). These cycles are repeated to produce many copies of a specific product.

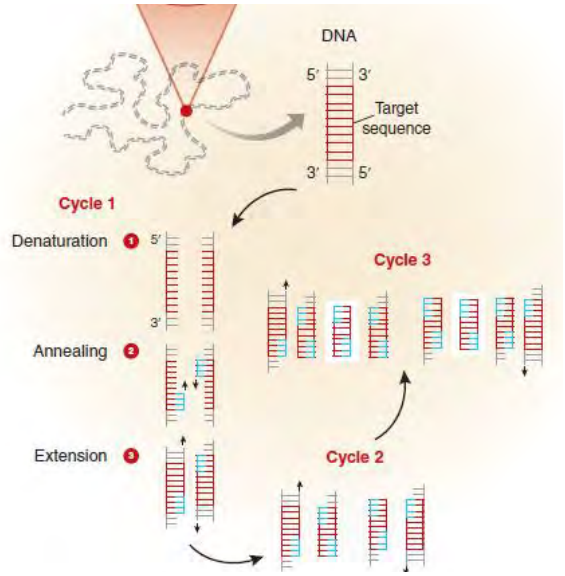

Figure 11: Schematic representation of the PCR Principle[71]. A specific DNA target will repeatedly undergo many PCR cycles, each involving denaturation, annealing and extension. These cycles produce many copies of the initial DNA target.

The first step within the PCR cycler is a denaturation step which involves the double stranded cDNA being separated by heating the solution above the DNA's melting temperature[71]. The thermal cycler is then cooled to a temperature that allows for specific primers to bind to the target DNA in a process known as annealing. The temperature is then raised again to allow the polymerase enzyme to extend the primers (extension phase) by adding the nucleotides in order to create the developing DNA strand[71].

The standard of care for monitoring a patient who is on HAART involves quantitative viral load testing based on plasma HIV-1 RNA concentrations. The favoured method for monitoring HIV-1 patients once they have been placed onto HAART is RT-qPCR viral load testing[61]. RT-qPCR measurements enable care providers to determine whether patients have a high or low level of HIV-1 circulating in their bloodstream. A high level of HIV-1 circulating in the bloodstream is an indication that the virus is actively replicating and the treatment regime is no longer effective[61]. RT-qPCR is a technique that is commonly used to detect RNA expression. It is used to qualitatively and quantitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from the HIV-1 RNA using fluorescent probes, such as Taqman probes[72]. The use of these fluorescent probes is what allows RT-qPCR to measure amplification as it occurs during the experiment. As it can be seen in Figure 12, RT-qPCR is utilized to clone expressed genes by reverse transcribing the HIV-1 RNA, released by HIV-1 virus into the CD4+ cells, into cDNA through the use of the RT enzyme. This creates newly synthesized cDNA that can be amplified using PCR cycles[72].

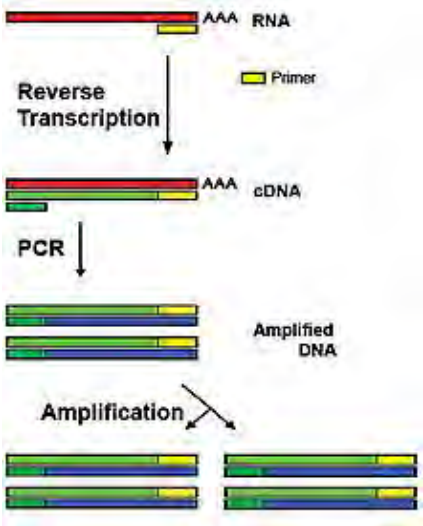

Figure 12: Schematic diagram of the RT-PCR principle[72]. HIV-1 RNA is reverse transcribed to cDNA via the reverse transcriptase enzyme. This produces newly synthesized cDNA, which can then be amplified within a thermal cycler machine to produce products of the cDNA.

RT-qPCR is characterized by the point in time during the cycle when the amplification of a PCR product is first detected. The higher the starting copy number of the sample, the sooner the fluorescent signal is detected. Each PCR assay requires the DNA sample, DNA polymerase and primers that detect and bind to the sample. Also required are four base deoxynucleotides including adenine, guanine, cytosine and thymine, which are the building blocks used by the polymerase enzyme to create the PCR products[71]. These components, including reverse transcriptase are mixed into a 96-well plate and placed in the thermal cycler.

The approach of PCR is a very important process that monitors a patient's responsiveness to HAART therapy with many advantages that include[73]; rapid production of results, high sensitivity and high specificity of 97-98%. The Amplicor HIV-1 monitor Test 1.5 is an HIV-1 RNA PCR test which has a lower detection limit of 400 copies/mL and an upper limit of detection of 10,000,000[74]. If a patient's viral load is below the level of detection, an "undetectable" result is reported, meaning the assay was not sensitive enough to be able to detect the virus[74]. The use of PCR also has the ability to produce billions of copies of a specific product and the ability to quantify the amount of product synthesized in order to monitor disease progression. Lastly, there should be relatively low start-up costs for equipment as most laboratories have the necessary equipment for performing PCR assays. As with every technique, there are disadvantages which include; high sensitivity that implies vulnerability to any form of contamination. This could yield misleading results. Furthermore, designing of primers for PCR which requires prior knowledge of the sequence of the target DNA. This knowledge is essential to prevent non-specific annealing of primers to sequences that are similar, but not completely identical to the target sequence[1,14]. There is also a possibility of incorrect nucleotides being incorporated into the PCR sequence by the polymerase enzyme. The RT-qPCR process requires a suitable detection chemistry in order to report the presence of PCR products. The instrument needs appropriate software for real time quantitative analysis of the amplification process. Reagents are expensive and the overall assay requires trained technicians. Furthermore, the entire PCR process is time consuming with certain PCR steps requiring dedicated laboratory space in a sterile laboratory space[1,14].

1.3.3. Flow Cytometry

CD4+ count through flow cytometry is considered an essential part of HIV-1 care. CD4+ cell count analysis has been described as the best surrogate marker in comparison to viral load assays[49], see previous figures, Figures 8 and 9. A CD4+ cell count can be used to monitor a patient's immune system in order to assess if HAART treatment needs to be initiated or if HAART treatment is effective for the patient[75]. If the CD4+ cell count continues to decrease, it means treatment is failing and the patient needs to be placed on new therapy. On the other hand, if a patient's CD4+ count begins to increase in

numbers, this is an indication that the treatment regime is successful. Therefore, even though this method is not a viral load assay in the sense that it is not measuring virions or RNA in the patient's plasma, it is discussed here given that it is used in certain areas, when viral load assays cannot be performed.

Flow cytometry has been termed the gatekeeper for assessing the immune status and establishing eligibility for treatment and care due to its ability to provide rapid analysis and ability to quantitatively measure the number of CD4+ cells a patient has[76]. As HIV-1 progresses, the number of total CD4+ cells decreases, allowing the absolute number of CD4+ cells to provide a powerful laboratory measurement for predicting, staging and monitoring progression as well as a patients response to HIV-1 treatment[77]. Unfortunately, the reality is that resource-limited countries lack the facilities to measure CD4+ counts, which stands in sharp contrast to the urgent need for implementing effective ART in these countries[78].

A flow cytometer is used to perform flow cytometry and consists of four basic components. The four components include laser(s), a fluidic system, optics and electronics (or an external computer system) and measures fluorescence and optical characteristics of cells, depending on the angle of light scattered[77]. When fluorescently labelled CD4+ cells pass by the light source, the fluorescent molecules are excited to a higher energy state and when they return to their resting state, the fluorochrome emits light energy at a higher wavelength. The measurement of this higher wavelength is analysed to provide a count of the number of CD4+ cells present in the sample[77]. The flow cytometry process requires multiple steps[79], as seen in Figure 13. In principle, CD4+ cells in the patient's sample are labelled with fluorescent detection antibodies. The sample is then loaded into the collection stage of the flow cytometer, drawn up into the fluidic system and then pumped into the flow chamber. Within the flow chamber, the CD4+ cells are combined with sheath fluid and passed through a laser beam which shines and excites the cells as they flow through. This excitation results in an emitted fluorescence, which is converted into an electrical signal via photodetectors and analysed by an external computer. The information is then displayed as a scatter plot (Figure 16) and quantitatively analysed to determine the CD4+ count.

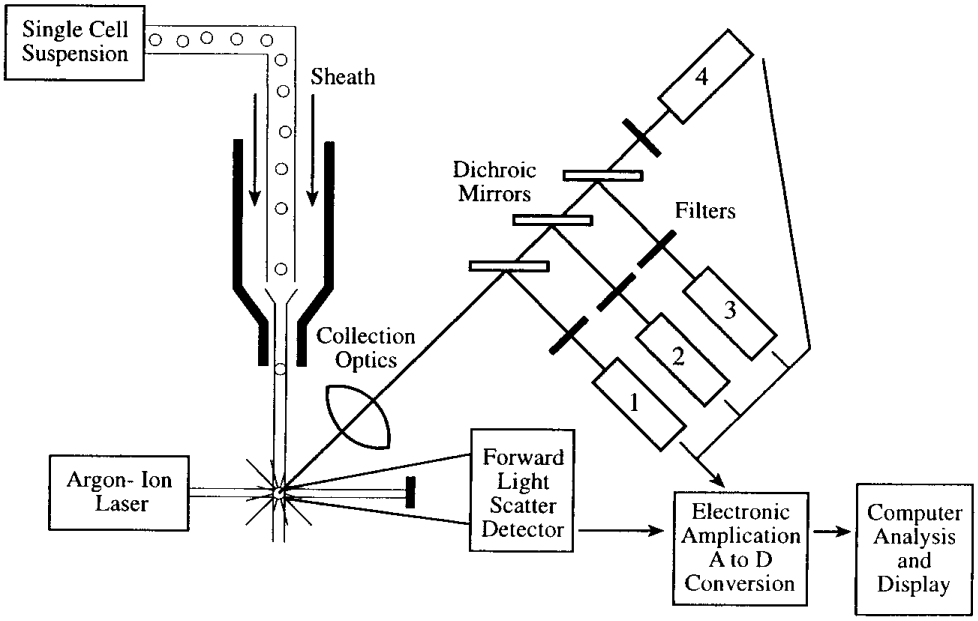

Figure 13: Schematic representation of a flow cytometer[77]. A patients CD4+ cells are labelled with fluorescent detection antibodies. The sample is then loaded onto the collection stage of the flow cytometer, drawn up into the fluidic system and pumped into the flow chamber. The CD4+ cells are then combined with sheath fluid and passed through the argon-ion laser, exciting the CD4+ cells as they flow through. The excitation of the cells results in an emitted fluorescence, which is converted into an electrical signal via photodetectors and analysed.

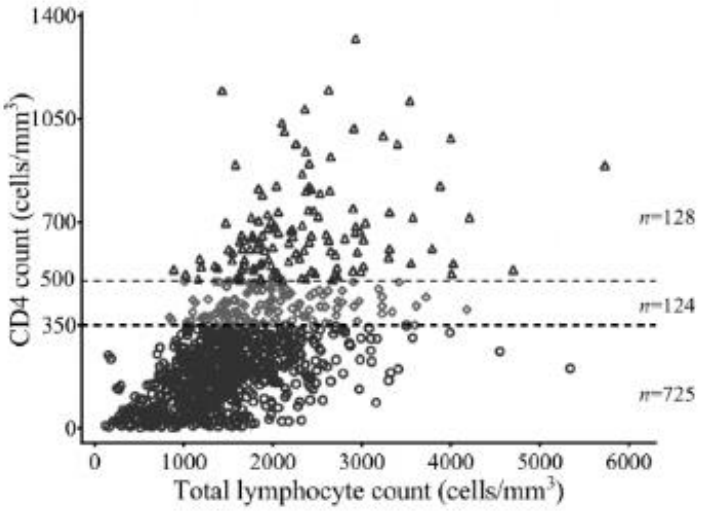

Figure 14: Scatter plot of CD4+ cell count found in a total lymphocyte population[79]. Labelled CD4+ cells emit a fluorescence as they pass through the argon-ion laser within the flow cytometer. This emitted fluorescence is converted into an electrical signal and displayed as a scatter plot.

Flow cytometry has remained one of the most popular tests to evaluate CD4+ cell counts due to the following advantages; the assay is extremely quick and thousands of cells are counted in seconds compared to 250 cells per minute counted by a scientist using a hemocytometer. The assay also provides quantitative and qualitative information[77,11]. Flow cytometry does have some limitations which include[79], the high cost of the actual machine, which second hand can cost approximately US$ 30 000 (ZAR 477,447) and brand new can cost as much as US$ 300 000 (ZAR 4,774,470). Flow cytometers are extremely large in size, taking up a lot of space, and a well-trained personnel is required to operate the flow cytometer. There are additional operational costs for the sheath fluid, servicing of machine, fluorochromes and analysis software that may make flow cytometry an even more expensive assay. However, flow cytometers are not used as initial screening tests due to being overall more costly to run[77] and a standard CD4+ test costs US$ 38 (ZAR 576) up to US$ 67 (ZAR 1,015)[80].

Given the availability of multiple techniques, which ones are used in developed settings and which are used or modified in developing settings? The factors needing to be taken into consideration are resources, both financial and structural, time and labour intensiveness of the technique and assay.

1.4. Review of Current HIV-1 Viral Load Assays for Developed Countries

Commercial RNA viral load assays (PCR) have been widely used as the "gold standard" to quantify HIV-1 RNA in plasma in developed settings. Companies such as Roche and Abbott have developed HIV-1 viral load assays. These companies have adapted real-time technology based on fluorescence in or to achieve simultaneous amplification and detection, instead of just an end-point detection result[2]. The real-time technology approach offers more accurate quantification, wider dynamic range and a high throughput. Roche and Abbott have further included automated sample preparation to prevent carry-over contamination and reduce manual time.

The device, COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, Food and Drug Administration (FDA) approved, produced by Roche is an example of a PCR product utilized in developed settings. Within this system, an extremely conserved region within the Gag gene is first transcribed into cDNA by the RT enzyme and then amplified during PCR[2]. During the amplification step, the use of TaqMan probes enables real-time detection. The Taqman probe is a dual-labelled sequence of DNA that is 25-30 nucleotides in length. There is a fluorescent reporter (Taqman) at the 5′ end and a quencher at the 3′ end. Due to the Taqman probe and quenchers close proximity, the Taqman probes fluorescence emitted is negatively suppressed by the quencher. During the amplification step, Taq DNA polymerase

elongates the forward primer and cleaves the TaqMan probe, which then hybridizes to the amplified HIV-1 cDNA[2]. When the TaqMan probe is cleaved, the distance between the quencher and the TaqMan probe is increased, resulting in detectable fluorescence emission, see Figure 15A. When the fluorescence intensity exceeds the background signal, the cycle number of PCR at this point is recorded and utilized for quantification. The cycle number is compared to a standard curve, whereby the HIV-1 RNA viral load is extrapolated from the cycle number (Figure 15 B and C)[81]. This assay is capable of detecting and quantifying a viral load range from 48 – 10^7 copies/mL as well as detecting HIV-1 subtypes A-D and F-H[2].

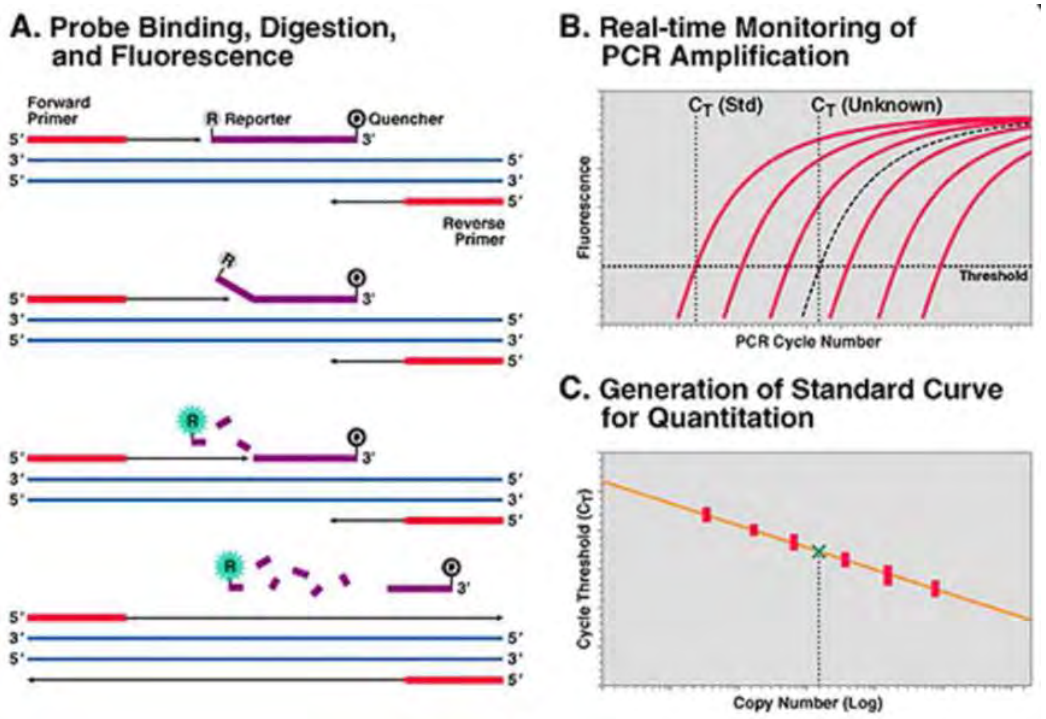

Figure 15: A) Principle of the TaqMan (reporter) probe. Due to the TaqMan probe and quenchers close proximity, the TaqMan probes fluorescence emitted is negatively suppressed by the quencher. During the amplification step, Taq DNA polymerase elongates the forward primer and cleaves the TaqMan probe, which then hybridizes to the amplified HIV-1 cDNA. When the TaqMan probe is cleaved, the distance between the quencher and the TaqMan probe is increased, resulting in detectable fluorescence emission[2]. B) Due to the TaqMan probe, real-time monitoring during the PCR amplification can occur. When the fluorescence intensity exceeds the background signal, the cycle number of PCR at this point is recorded and utilized for quantification[2]. C) Standard Curve for quantification. The cycle number is compared to a standard curve, whereby the HIV-1 RNA viral load is extrapolated from the cycle number[2,81].

Another FDA approved assay, similar to COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, is the RealTime HIV-1 assay produced by Abbott[2]. Within this assay, HIV-1 RNA undergoes RT and is then quantified by PCR. However, the difference between RealTime HIV-1 assay and COBAS® is that RealTime HIV-1 targets a highly conserved integrase gene within *pol,* unlike COBAS® which targets *gag.* The RealTime assay has a much wider subtype coverage including A-D, F-H and groups N and O, which are not detected by COBAS® [2].

1.5. Alternative HIV-1 Screening methods and approaches in Resource-Constrained Settings

The previous section discussed the current viral load detection and monitoring methods that are widely used in developed settings with well-funded laboratories. These methods require finances, a high level of expertise to perform the tests and interpret data, equipment, laboratory space and adequate infrastructure[66]. These requirements are abundantly available in developed countries and as a result, their HIV-1 treatment approach involves routine use of viral load measurements and CD4+ cell counts[82]. However, these monitoring methods are difficult to adapt in resourced-constrained regions, where there are limited resources and multiple challenges. Firstly, viral load monitoring is only available in reference laboratories, where they are financially supported by national and international efforts[2]. Secondly, the provincial hospitals and local clinics both lack basic laboratory infrastructure including reliable power and water supply, refrigeration and air conditioning[2]. Without stable electricity, it is impractical to maintain air conditioning and a temperature-controlled environment necessary for long term storage of patient samples and reagents[82]. Thirdly, technicians and staff members in such clinics and hospitals are rarely trained to perform technologically complex molecular techniques[2] or to even operate and maintain equipment[82]. Therefore, robust equipment and methods are needed to be able to withstand harsh conditions in the rural clinics. However, most often, viral load measurement machinery is completely absent from routine equipment lists due to the challenge of running, setting up and servicing them[82]. Lastly, fluctuations of environmental humidity and temperature cannot be controlled during transportation and storage of samples[2].

There are a few strategies and less expensive monitoring tools that have been developed for resource-constrained areas. Strategies employed include, DBS and sample pooling. Less expensive monitoring tools developed and utilized include; Cavidi ExaVir™ RT assay, PerkinElmer Ultrasensitive p24 assay and lastly, in-house RT-qPCR assays[2].

1.5.1. DBS

DBS is a technique that has gained popularity in rural clinics as it does not require electricity or specialized equipment for long term storage of samples[10]. DBS does not need cold-chain transport of liquid plasma samples, which would be far too costly and logistically complicated to perform for rural areas[10]. DBS involves sending patient's samples elsewhere for testing by PCR, which unfortunately has a slow turnaround time from sample–to-answer of up to several weeks[2]. This can lead to high loss-to-follow up. Briefly, performing a DBS involves a single drop of a patient's blood being placed on filter paper. Once the blood has dried (4 hours), the card is packaged and sent at ambient temperature to another laboratory. This DBS is then analysed[83] via RT-qPCR, providing an HIV-1 viral load[10]. A study done by Nishaki Mehta *et al.,*2009; performed on DBS samples using a real-time LightCycler (rtLC) PCR assay was costed at US$ 4.55 (ZAR 34)[84]. Within the study, 50 µl of whole blood was spotted onto filter paper, the filter paper was then allowed to dry for a minimum of 3 hours at room temperature and then placed within a ziplock bag with silica desiccant (reduce humidity within the bag)[84]. Within the study, DBS stored at -20°C and 37°C were analysed and it was shown that there was no statistically significant difference in viral load for those samples stored at -20°C and 37°C[84]. However, besides the cost of taking the sample and placing it onto a card, the cost of transport to send the sample to the relevant laboratory needs to be taken into account, therefore the cost per test will be higher taking transport into consideration.

1.5.2. Viral Load Pooling

A second strategy employed by resource-limited settings includes viral load sample pooling. This strategy is a very common practice in order to cut costs within these regions[11]. It involves mixing together up to 5 patients' blood samples and testing them as one sample via RT-PCR. If the combined average viral load of the pooled specimen is above 1000 copies/mL, it is concluded that HAART treatment is failing for a particular patient. It is only after this observation when each patient within that sample pool will be tested individually in order to identify the patient whose treatment is failing[44]. This method has the advantage of being both time and cost-effective[9], reducing costs of screening to up to 70% compared to individually testing patient samples[85].

The inexpensive alternative monitoring tools developed for viral load monitoring in resource-limited settings include; Cavidi ExaVir™ RT assay, PerkinElmer Ultrasensitive p24 assay and lastly, in-house RT-qPCR assays[2]. The ExaVir™ RT assay and PerkinElmer Ultrasensitive p24 assay are both based on an ELISA principle, whereby the activity of RT and the level of p24 antigen are measured. In-house RT-qPCR utilizes the same TaqMan technology as described by Roche, however this method targets

another highly conserved region within the long terminal repeat (LTR) of HIV-1[2]. These alternative techniques have significantly reduced cost US$ 13 - US$ 20 (ZAR 201–ZAR 310), compared to the commercial viral load assays US$ 50 – US$ 100 (ZAR 776-ZAR 1,552). In addition, the Ultrasensitive p24 assay and the ExaVir™ assay are both technologically simpler compared to commercial RNA viral load assays and therefore do not need highly skilled operators[2]. However, both these assays are still time-consuming and labour-intensive. The in-house RT-qPCR method, although highly correlated with commercial viral load assays, still require expensive thermal cyclers and trained operators, which hinders their use in de-centralized laboratories and clinics[2].

1.5.3. Cavidi ExaVir™ RT Assay

This assay is an ELISA-based assay measures the activity of the RT enzyme. Briefly, the first step within this assay is the separation part of the plasma. During this step, the plasma is treated to inactivate cellular enzymes[86]. From here, HIV-1 virions are purified from the plasma by the use of a gel which binds the virions. All antibodies and ART drugs are washed away during this stage[86]. Purified HIV-1 virions are then lysed to release RT. The second step is the RT-assay. During this stage, the released RT is analysed in an ELISA set-up. An RNA template is bound to the bottom of a 96-well RT reaction plate. A reaction mixture, containing primers and RT substrates are added to the plate with the lysate (lysed HIV-1 virions). If there is RT within the lysate, the enzyme will synthesise a DNA-strand from the bound RNA template within the well[86]. After incubation at 33°C for 40 hours, the synthesis of a DNA-strand is detected by the addition of an alkaline phosphatase (AP) – conjugated anti-BrdU antibody and a colorimetric AP substrate. The colour intensity correlates with the RT activity. Unknown samples are compared to known standards[2]. The ExaVir™ version 3.0, has a low detection limit of 200 RNA copies/mL, increased throughput (180 samples per week per operator) and shortened turnaround time (48-72h). More importantly, the cost of this assay can be reduced to US$ 13.66 (ZAR 213) per test[2].

1.5.4. PerkinElmer Ultrasensitive p24Assay

The PerkinElmer Ultrasensitive p24 assay is another ELISA based method, whereby the level of HIV-1 p24 antigen is measured. HIV-1 p24 antigen has a strong correlation with HIV-1 RNA levels (Figure 7). Firstly, specimens are lysed by the kit buffer and incubated at 100 °C for 5 minutes to release p24 molecules to a maximum degree from HIV-1 virions[2]. Next, the lysate is added to specific capture antibody-coated 96-well plate and p24 antigen are captured by the capture antibodies. Captured p24 antigen is recognized by a biotinylated anti-p24 antibody, which is followed by the colorimetric detection via streptavidin-horseradish peroxidase conjugate[2]. Emitted colour intensity of a sample is

compared to intensities emitted from known standards of known concentrations[2]. However, despite this assay showing good correlation with commercial RNA assays in some settings, there has been discordant results observed in some studies. For example, this assay detected 66.7% of the specimens with a viral load less than 10 000 copies/mL, but 87% detection with a viral load of between 10 000 and 100 000 copies/mL[2]. This assay can also not reflect the response to ART well as nucleic acid viral load assays, therefore further improvement and evaluation are needed so that this assay can be used for viral load monitoring in resource-limited settings[2].

1.5.5. In-House RT-PCR Assay

RT-qPCR are commonly used in commercial viral load assays, but recently the development of low-cost in-house RT-qPCR for HIV-1 RNA quantification has come about. This assay targets a highly sequence-conserved region within HIV-1, LTR[2]. This allows greater subtype coverage, which is more diverse in resource-limited setting compared to developed countries. By utilizing a probe that is a shorter minor groove binding probe and a shorter forward primer, viral load values obtained by this assay were highly correlated to values obtained by Roche assays[2]. In-house RT-qPCR is able to detect Group N and O, which are not detected by some Roche assays. The major benefit of this new approach, is the cost. The cost to perform this assay has been lowered to US$ 20 (ZAR 313) [2]. However, despite the cost per assay being significantly reduced, this assay still requires high-cost equipment, good laboratory infrastructure and well-trained technicians, restricting its use in district laboratories and rural clinics[2].

Although these inexpensive alternative viral load assays can be performed at centralized laboratories, it would still be very difficult to implement within rural areas. Despite the reduction in cost per assay, the limitations for the need of basic instruments such as 96-well plate washers, a reader for the RT assay and well-trained technicians still prevent theses assays being used in resource-limited settings. This is why often strategies such as DBS and sample pooling are resorted to, in order to further cope with these limitations, as it may still be a more cost effective approach. Ideally, portable, instrument-free systems that can be used by health care workers with minimal training and that can provide a readout in 30 minutes or less would be preferred[2]. Such a system would facilitate clinical decisions whilst patients are on site. Such an approach is known as point-of-care testing (POCT). POCT is a strategy whereby testing is performed near to or at the site of patient care, where the results may lead to an immediate change to the patients ART regime[76]. HIV-1 POC viral load assays need to be performed using a low-cost disposable device, less than US$ 10 (ZAR 156). This is feasible due to the small consumption of reagents in the miniaturized system. Due to the low cost, the self-contained device with preloaded reagents can be discarded after use to prevent cross contamination.

To address current viral load challenges for resource-limited settings, new approaches based on microfluidics, have been developed towards viral detection in a POC testing format. A microfluidic POC device approach contains the following characteristics, low cost, short turnaround time, high sensitivity and simple procedure. This device can also be easily run by less-trained health care workers at the POC setting.

1.6. Microfluidic Technology

Microfluidics is the science and technology of systems which process or manipulate small amounts of fluids (10^{-9} – 10^{-18} litres), using channels with dimensions of tens to hundreds of micrometres[87]. The diameter of these channels are about the thickness of a human hair.

Microfluidic large scale integration (LSI) arose in 1993 and describes a microfluidic channel circuitry composed of chip-integrated microvalves. These microvalves are based on flexible membranes between two layers, a liquid guiding layer and a pneumatic control-channel layer[88]. The microvalves close or open in correspondence to the pneumatic pressure that is applied to the control-channels. By combining multiple microvalves, features such as micropumps and mixers can be incorporated within the device[88]. During the same time microfluidic LSI arose, a novel fabrication technology for microfluidic channels, called soft lithography made its appearance[88]. Soft lithography describes a set of techniques for fabricating microstructures in an elastomeric material[89], such as PDMS, and uses replica molding of the elastomeric material to fabricate stamps as well as microfluidic channels[90]. Advantages of soft lithography, include the ease of fabrication, rapid prototyping and biocompatibility[90]. Microfluidic devices are typically created using multilayer soft lithography (MSL), which is the combination of multiple layers of soft elastomeric material made via the process of soft lithography and bonded together[91]. The most common materials for the fabrication of microfluidic devices during soft lithography includes, poly-dimethyl siloxane (PDMS) and silicon. In this dissertation, PDMS device fabrication using a method known as multilayer soft lithography (MSL) will be focused on, see Appendix A for MSL method. The manufacturing of microfluidic PDMS chips consist of three processes. These first process involves, creating the device using a computer-aided design (CAD) program (AutoCAD in particular), followed by photolithography and the last process is, MSL.

1.7. Microfluidic Fabrication Process

The first process to construct a microfluidic device is designing the device on AutoCAD. Once this is completed, the design is printed onto a high-resolution transparency with 40 000 dots per inch (DPI).

Once this transparency has been printed, it now serves as a photomask in the process of photolithography[92].

Photolithography refers to the use of light to create a pattern onto a silicon wafer using a photo-reactive material (photoresist)[93],. At the end of the photolithography process, the resultant patterned silicon wafer is known as the master mold. This master mold is used as the template for MSL see Figure 18 for the photolithography procedure.

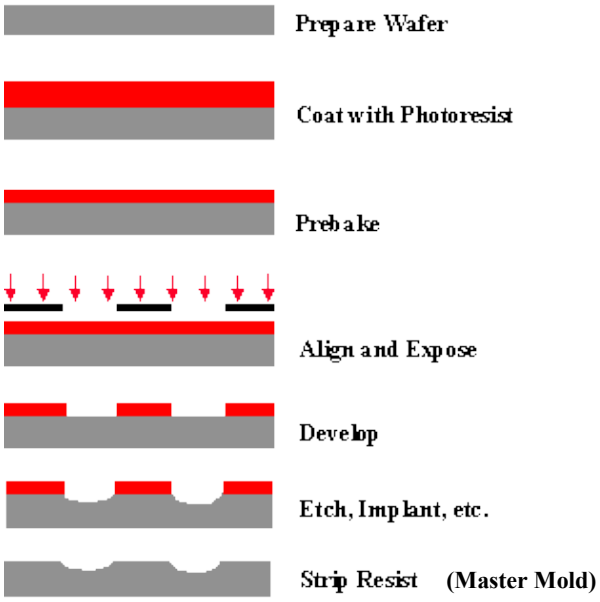

Figure 16: Steps of a typical photolithography process for the development of a patterned silicon wafer. The process starts with the preparation of the silicon wafer by a dehydration bake. Next, photoresist is coated onto the wafer via spin coating. The wafer is then baked to remove any excess photoresist solvent. Once the wafer has dried, it is aligned and exposed by UV light. From exposure, a post-exposure bake is completed which is then followed by the development of the pattern that has been transferred to the wafer. The final step involves stripping. This removes the remaining photoresist on the wafer by using a phenol – based organic solvent[94].

Briefly, the typical process of photolithography involves the following steps; firstly, silicon wafers are preparation by dehydration baking and coating of photoresist onto the wafer. This is done by pouring photoresist onto the wafer and spin coating[94]. This is then followed by a prebake, which removes excess photoresist solvent. Once the wafer has dried, it is aligned with the photomask on a mask aligner and exposed by using UV light in the range of 350 – 450 nanometer (nm). This exposure causes the transfer of the pattern on the photomask onto the wafer. After exposure, a post-exposure bake is completed which is then followed by the development of the pattern that has been transferred to the wafer. Most

commonly used solutions in the development process are aqueous bases developers[94]. Development is one of the most critical steps in the photoresist process as it determines the shape of the photoresist profile. The development process removes any photoresist that was not exposed to the pattern, creating etches within the wafer. Once the development solution has been applied for a certain time (subject to design) it has to be rinsed off in order to stop the development process[94]. The final step in photolithography involves stripping, a process which removes the remaining photoresist on the wafer by using a phenol – based organic solvent[94].

Once the process of photolithography is completed, the master mold now serves as the mold for casting PDMS patterns in the soft lithography process and subsequently the construction of a microfluidic device by MSL, see Figure 17.

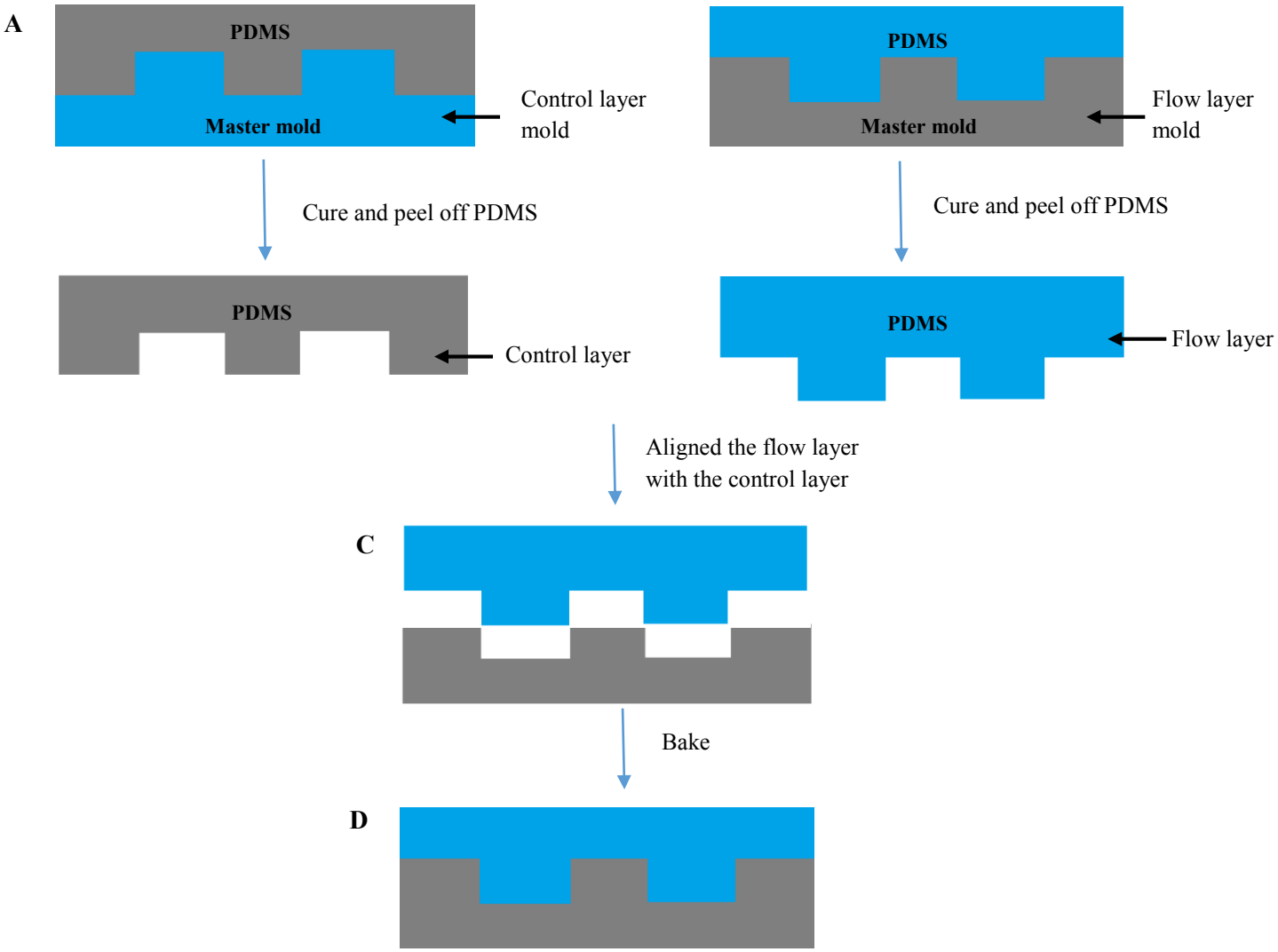

Figure 17: Principle of MSL. A) PDMS is poured onto the control layer master mold, cured and then peeled off. B) PDMS is poured onto the flow layer master mold, cured and then peeled off. C) The control layer and flow layer are aligned together. D) Once the two layers are aligned, they are baked together creating an irreversible bond[90].

The process of MSL for the dissertation can be found in more detail in Appendix A. Briefly, all molds are primed using TMCS vapour. Next, PDMS is mixed and poured onto the control layer mold. The flow layer was then made by mixing PDMS together. Once mixed, the mixture was poured onto the flow layer mold and spun at 1800g. Both the thick layer and flow layer molds were placed into a convection oven and baked. Once baked, the thick layer was peeled from the mold and. aligned onto the flow layer mold, aligning all valves. Once aligned, it was baked in the oven to bond the two layers together. Once baked, the bonded layer was peeled off from the mold.

1.8. Advantages and Limitations of Microfluidics

Microfluidics has the potential to become a global resource both in biological and medical science for a variety of reasons. These include high throughput processing as well as low reagent consumption (economy of scale). Economy of scale is achieved by carrying out reactions on microfluidic devices with length scales ranging from tens to hundreds of microns, resulting in up to six orders of magnitude smaller volume than is possible with the current standard HIV-1 viral load assays[95]. It is due to these small volumes and length scales that many hundreds to thousands of microfluidic assays can be performed simultaneously on a single device taking up a few centimetres. By combining both low reagent volumes and small length of scale, this reduces the cost per assay[96]. Other advantages include, complex fluidic manipulations, such as metering and mixing which can be performed on chip, making it possible to scale down current bench-top assays[97]. Lastly, another key advantage lies in the PDMS material itself. PDMS is an inexpensive material that offers several advantages such as being a rubber-like elastomer that has good optical transparency and biocompatibility[88]. With these aforementioned advantages, microfluidics aims at overcoming the current technological burden that the world is facing. One main advantage of microfluidics includes the ability for the chip to be redesigned on AutoCAD. This enables the device to house multiple patient runs on one chip. A chip design could easily be made to have 100 or 1000 reactors, meaning 100 - 1000 patient samples could be processed and analysed via automation simultaneously. The cost-effectiveness of microfluidics can be seen in the long run, once the system has been established. With both low reagent usage and the possibility of high number of patient samples being processed simultaneously, the cost per assay reduces. The ability for microfluidics platforms to accomplish all assay steps such as sample pre-treatment and detection on a single easy-to-use platform, via accurate and automatic means makes them an ideal future diagnostic

tool to provide real clinical value for HIV-1 monitoring in low-resource, laboratory free-settings[98]. However, like most technology, microfluidics possesses certain limitations. Limitations of the platform mainly relate to the properties of PDMS, for example, chemicals which the elastomer is not inert to cannot be processed[88]. PDMS also shrinks by 1% during curing and the cured PDMS can also swell when introduced to certain solvents[99]. Increased temperatures is not feasible for the PDMS chip as this results in the PDMS chip expanding. The softness of the PDMS elastomer limits the aspect ratio (height, length and width) of microstructures in PDMS. If the aspect ratio of the height and length are too high or too low, this causes the microstructures in PDMS to deform, generating defects in the resulting pattern. Other limitations include; for now, difficulties in implementing microfluidic technology in the field of POC, as hand-held devices are required. Lastly, external components such as pressure sources and valves would have to be downsized, which is technically feasible, but the costs would be high[88]. Microfluidics, as most technology requires an initial start-up cost. A microfluidics system requires a microscope, pressurized control board and computer system. The ongoing operation of microfluidics itself requires tygon tubing, Eppendorf tubes, the microfluidic chip itself and the necessary reagents.

1.9. Current Applications of Microfluidic Technology

Presently there are a number of microfluidic applications underlying current technology. The most abundant application that uses microfluidics, are rapid lateral flow immunoassays, often termed as rapid diagnostic test (RDT). The most familiar RDT is the home pregnancy test strip and for HIV-1 screening, the OraQuick Rapid HIV-1/2 Antibody Test has been rated highly by the WHO due to its 100% sensitivity and 100% specificity when utilized in southern Africa[100].

Another example of a well-known device that utilizes microfluidics, is the GeneXpert. These applications and assays will be discussed in more detail below.

Rapid Lateral Flow Immunoassays

The first RDTs, with principles of latex agglutination (a diagnostic to detect antibody using latex particles such as polystyrene), were developed in the 1950's and 1960's[101]. Today, RDTs for infectious diseases are supplied by more than 200 manufacturers worldwide as cards, strip tests or pads, and are usually easy-to-use and inexpensive, US$ 1 (ZAR 15) to US$ 10 (ZAR 158). In global health, the RDT HIV-1 antibody test are the most widely used, each being performed at least 100 million times per year worldwide[101].

The basic principle of lateral flow tests is capillary action[88]. Capillary action is when liquid moves along the surface of a solid, due to the attraction of the liquid's molecules to the surface of the solid. This in

turn causes the liquid to move up, down or across the solid[88]. For example, a sample is introduced into a RDT device through the test inlet and transported into the conjugate pad, via capillary action. The conjugate pad contains antibodies, which when the sample passes through, the antibodies are rehydrated and bind to the antigens in the sample. This reaction continues as the sample flows into the incubation and detection pad. On the test line, there is a second antibody which catches the particles coated with antigens, while a third type of antibody catches particles that did not bind to an analyte on the control line, (Figure 18). The result is visible after 2 to 15 minutes[88].

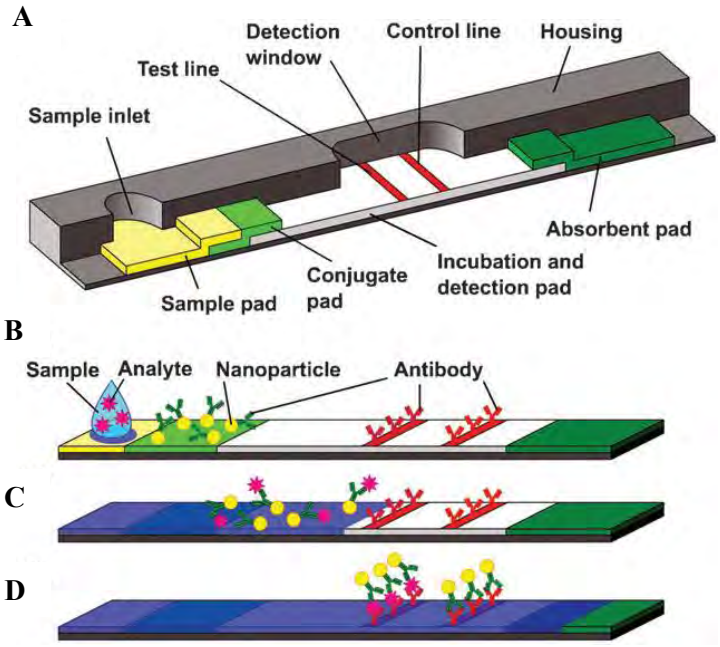

Figure 18: Schematic design of a lateral flow test, A) Sample pad (sample inlet and filtering), conjugate pad (reactive agents and detection molecules), incubation and detection zone with test and control lines (analyte detection and functionality test) and final absorbent pad (liquid actuation). B) Start of assay by adding liquid sample. C) Antibodies conjugated to colored nanoparticles bind the antigen. D) Particles with antigens bind to test line (positive result), particles w/o antigens bind to the control line (proof of validity)[88].

An example of a RDT for HIV-1 screening, is the Oraquick ADVANCE® Rapid HIV-1/2 Antibody Test from OraSure Technologies, Inc. The OraQuick Rapid HIV-1/2 Antibody Test is a lateral flow test that is used in resource-limited settings. This test is capable of utilising saliva or whole blood from a patient in a way that prevents or minimises stigma against the individual being tested. The OraQuick assay detects both HIV-1 and HIV-2 antibodies found in whole blood, oral fluid or plasma[102]. The method for using this device to detect HIV-positive patients utilizing whole blood is shown in Figure 19[102].

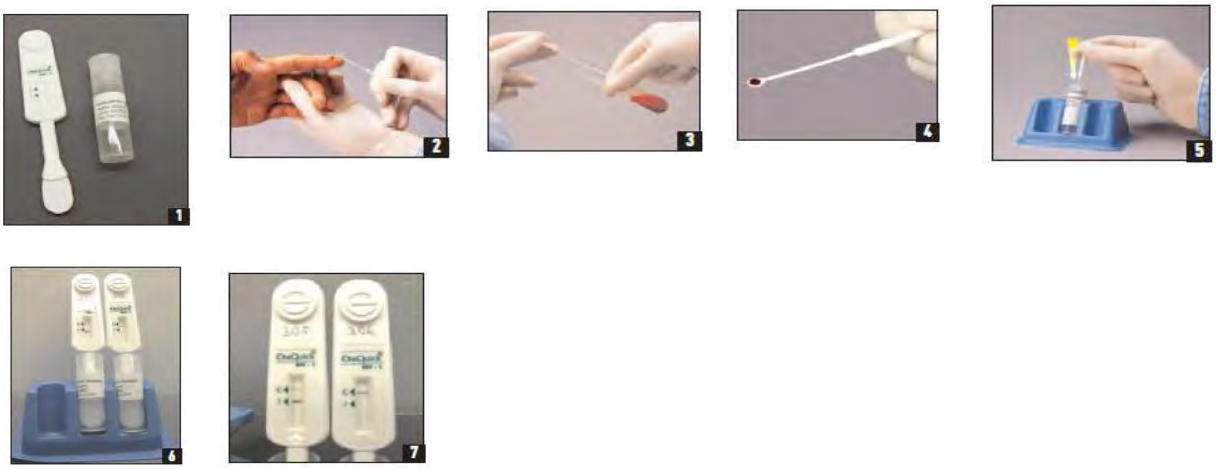

Figure 19: Performing the OraQuick Rapid HIV-1 Antibody Test using whole blood. The OraQuick Rapid HIV-1 Antibody Test (Image 1), collection of specimen by touching the collection loop to the finger prick blood droplet (Image 2), dipping of the collection loop into the test tube (Image 3), a filled collection loop (Image 4), insertion of the loop and stirring of the specimen in the developer solution vial (Image 5) and insertion of the OraQuick device into the developer vial (Image 6). Test results must be read after 20 minutes but no later than 40 minutes (Image 7)[102].

The advantages of the OraQuick device includes the following[102]; high sensitivity (99.3%), high specificity (99.8%), results produced in minutes and ease of sample collection. Other advantages include that the test is not time consuming, labour intensive, requiring skilled technicians or specialized equipment[103]. A slight drawback of the system, using oral fluid is that oral fluid typically has a lower antibody concentration compared to whole blood. This is due to whole saliva containing bacterial and salivary proteases which may lead to degradation of the HIV-1 IgG antibodies[104]. Another disadvantage of this device is that any HIV-positive samples need to be confirmed via a second confirmatory test[10] such as a western blot. However, comparing the ELISA assay which is a two day labour intensive assay, the OraQuick Rapid HIV-1/2 Antibody device is a better choice as it provides results within 40 minutes[102].

Other commercially available lateral flow test strips for diagnostic purposes include; Binax NOW which detects Malaria, Alere Determine HIV ½ Ag/Ab combo that detects AIDS and OncoE6 Cervical test, which detects cervical cancer. Another commercially available test includes StatusFirst CHF NT-proBNP which detects congestive heart failure[98]. It is evident that lateral flow tests serve a wide field of applications including health biomarkers, pregnancy, heart attacks, glucose testing and infectious diseases. Other applications include, identifying drug abuse, toxins and infectious agents such as anthrax[88].

RDT technology is also one of the most simplistic and successful diagnostic testing platforms[98] available in the rural areas of developing countries. Lateral flow tests have contributed enormously to the identification of diseases without the need for any modern laboratory set-up[98]. The global growth of this technology is attributed to many factors. These factors include the need for early stage detection of diseases such as HIV-1 and the demand for screening tools for the preventative management of infectious diseases. Another factor is the requirement for cost-effective devices with little to no maintenance[98]. Lateral flow tests may be seen as the gold-standard microfluidic platform in terms of handling, cost, simplicity and robustness, but there are a few limitations. The simplicity of the test is a major drawback as there are a limited number of unit operations imprinted in the microfluidic channel. Highly precise liquid handling and metering is also extremely challenging[88]. Other limitations include, poor reproducibility, poor accuracy and low sensitivity[98]. Lateral flow tests are also only qualitative in that they provide yes or no answers. They would not be able to be suitable for viral load monitoring.

Another device which uses microfluidics, is the GeneXpert MTB/RIF assay. This device is a rapid molecular assay that can be utilized at the point-of-care interface, requiring minimal technical expertise. Xpert simultaneously enables diagnosis of *Mycobacterium tuberculosis* (MTB) with the identification of rifampicin resistance within 2 hours[105]. Rifampicin resistance is caused by a mutation in the rpoB gene of MTB, therefore this resistance can be detected by identifying mutations within this region. A major advantage of the Xpert, is that unprocessed sputum samples can be used almost directly into the system. Xpert has been successful in combining both sample preparation and detection with fully-automated RT-PCR amplification[105]. Within the Xpert, there is a cartridge-based system (Figure 20) that integrates microfluidic technology, allowing almost fully automated nucleic acid analysis[105]. This cartridge contains multiple chambers preloaded with liquid buffers and reagent beads needed for sample processing, DNA extraction and RT-PCR. The cartridge alone is able to purify, concentrate, detect and identify specific nucleic acid sequences from unprocessed clinical samples[105].

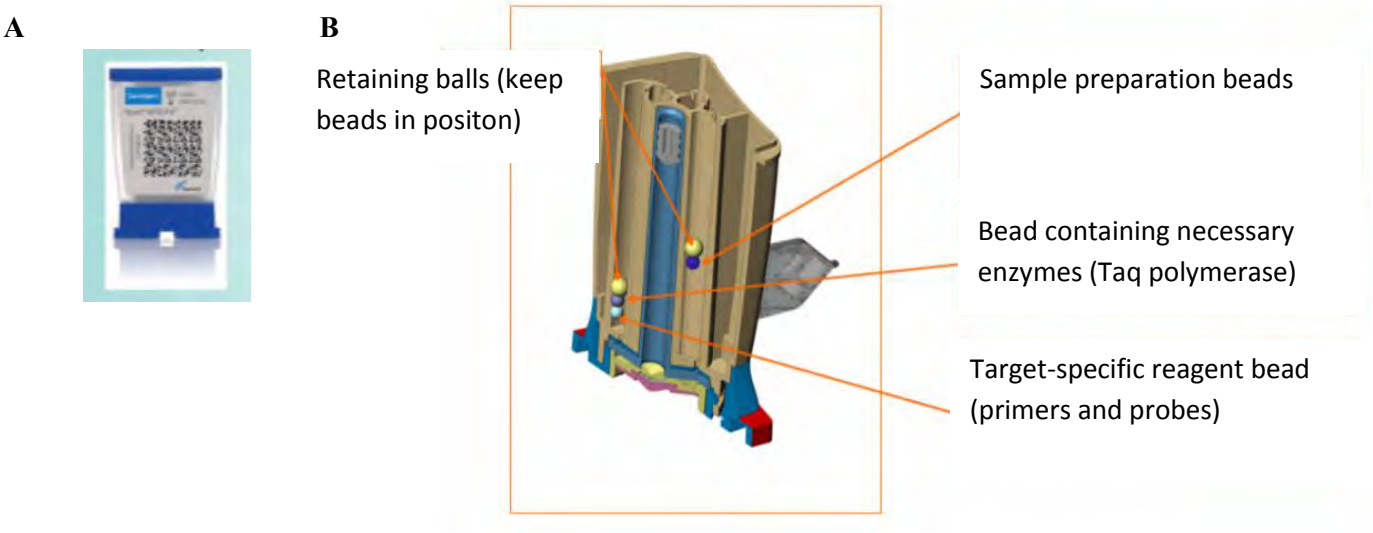

Figure 20: A) GeneXpert MTB/RIF cartridge[105]. B) Inside side view of cartridge, highlighting all the beads that enable the assay to be carried out via automation[106].

Microfluidic approaches and applications have shown to provide key advantages over current standard laboratory techniques used within developed settings. So far discussed are the HIV-1 viral load assays in developed countries (COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test and RealTime HIV-1 assay) and the alternative HIV-1 viral load assays for resource-limited settings (Cavidi ExaVir™ RT, PerkinElmer Ultrasensitive p24 and lastly, the in-house RT-qPCR assays). Unfortunately, despite the alternative HIV-1 assays used in resource-limited settings having reduced costs, they still are time-consuming and labour intensive. However, with the advantages of microfluidics, there has been advancement in HIV-1 POC viral load assays using microfluidic technology.

1.10. Advances in Developing HIV-1 POC Viral Load Assays In Resource-Limited Settings.

Two major microfluidic approaches that are able to quantify HIV-1 viral load or provide a surrogate for viral load at the POC setting include; microfluidics-based virus count and POC CD4+ cell count respectively.

Microfluidic-based virus count

More recently, HIV-1 whole virus particles have been utilized as a viral load marker, in microfluidic systems. Assays quantifying the HIV-1 particle, in theory, are more accurate compared to RNA viral load assays, because they decrease the quantification bias that arises from RNA degradation and

amplification inhibition in nucleic acid testing. The degradation of HIV-1 RNA is due to freeze-thaw cycles and ubiquitous RNases. With the progress in the area of microfluidics, direct virus counting has become more feasible. This can be seen in a paper by[107]. Within this paper, 10µl of whole blood was pumped into a microfluidic chamber, previously coated with anti-gp120 antibody. HIV-1 particles present, were captured by the anti-gp120 antibody. The captured HIV-1 particles were then sandwiched by a biotinylated anti-gp120 antibody that binds to a streptavidin-conjugated green quantum dot[107]. Furthermore, biotinylated ConA lectin was also added, which enabled co-recognition of captured HIV-1 particle, due to the biotinylated ConA lectin red quantum dot binding to the streptavidin-conjugated green quantum dot[107]. HIV-1 particles were indicated by co-location of the both the green and red fluorescent signal. This method eliminates the process of nucleic acid isolation, potentially cutting the cost of one device to US$ 5 (ZAR 78) and also uses such a small volume of sample, all that is required is a fingerpick[2]. However, the disadvantage of this approach is the detection of fluorescence via microscopy is not practical at the POC setting. The end goal would be using a portable lensless imaging system[2].

Microfluidic POC CD4+ cell count

As previously highlighted, the number of CD4+ T cells is necessary to determine the immune state of HIV-infected individuals and their HAART effectiveness. Functionalized microfluidic devices have been developed that are capable of capturing CD4+ cells from 10µl of whole blood[2]. In brief, anti-CD4+ antibodies were immobilized by biotin-streptavidin interactions on the surface of the microfluidics flow channel. Any present CD4+ cells in the whole blood, were captured and then tagged by a fluorescently labelled antibody[2]. The fluorescence was counted under fluorescence microscopy, which again, is not feasible at the POC setting. A light microscope can be used, but this would involve tedious counting manually. Alternative methods for CD4+ counting can be done using lensless ultra-wide-field cell array based shadow imaging (LUCAS), done by Ozcan and Demirci, 2008, which allowed CD4+ cell counting within 10 minutes using a battery-powered camera. More recently, portable cell counting technologies, such as the Alere Pima™ CD4 test have emerged. In 2012, the Alere Pima™ CD4 test made in onto the list of WHO prequalified diagnostics[101]. This device requires 25µl of whole blood and produces a CD4+ count in less than 20 minutes at the POC without a lab[108]. This assay requires very minimal time or skilled operators. Briefly, a finger prick amount of blood is collected into the Alere Pima™ CD4 cartridge, the cartridge is then place into the Alere Pima™ Analyser and within 20 minutes, the number of CD4+ cells are displayed on the analysers screen[108]. The reaction within the cartridge is as follows, two specific monoclonal antibodies (CD3 and CD4) each labelled with a different fluorescent dye dissolve into the blood sample once the cartridge is inserted into the analyser. T helper cells are detected by cells with both the CD4 and CD3 antibodies found[108], see Figure 21.

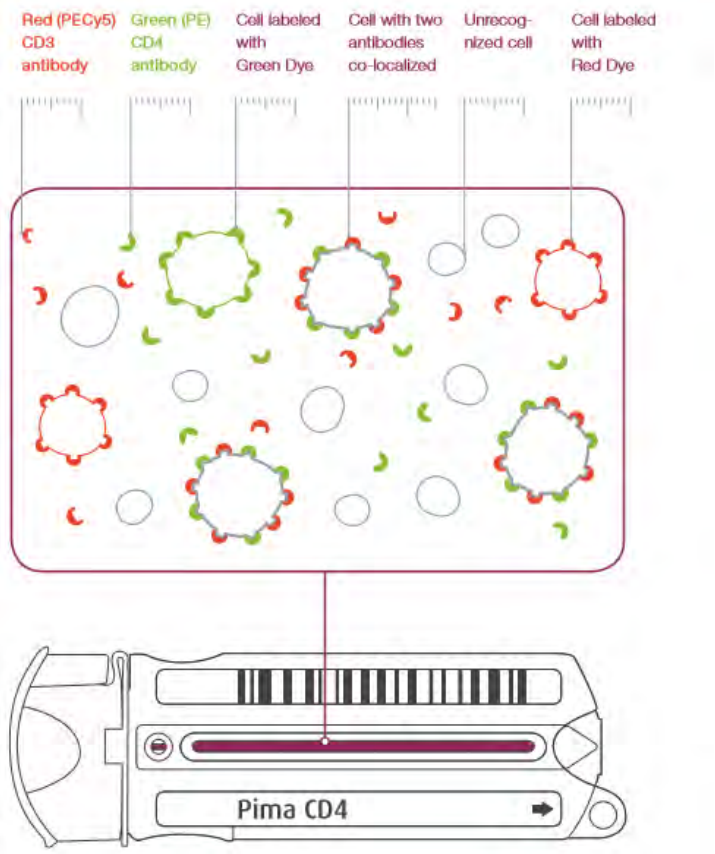

Figure 21: The Alere Pima™ CD4 cartridge that is inserted into the Alere Pima™ CD4 analyser, with the reaction that occurs within the cartridge, allowing CD4 cell count. Within the cartridge, there are CD4 antibodies labelled green and CD3 antibodies labelled red. When both of these antibodies are co-localized, this is denoted as a CD4 cell and is counted[108].

The main challenges for POC assay are to reduce overall cost, reduce sample-to-answer output time, requirement of instruments, reduce the need for human involvement and lastly, the assay requires high tolerance to variable storage conditions, whilst still maintaining high sensitivity. An assay is accepted for POC when it meets all the ASSURED[10] criteria as well as having a detection limit of 1000-5000 vpc/mL[1]. ASSURED is an acronym for affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users[10]. Microfluidic devices potentially have the capability of being able to provide the solution to these challenges, as this technology has enabled clear progress in HIV diagnostics, with already commercially available microfluidics-based CD4+ cell count using a drop of blood from a finger prick. However, despite remarkable progress in this areas, very few commercial POC viral load assays are readily available as HIV-1 viral load POC assays[101].

1.11. Dissertation Aim and Objectives

1.11.1. Problem Identification

Monitoring of a patient's viral load within developing regions still to this day poses many hurdles. Most rural areas rely on CD4+ cell counts or sending samples to other laboratories that can perform the necessary assays to determine a patient's viral load. This remains largely unaffordable due to the transportation costs and often leads to loss to follow up of patients due to the turnaround time of up to several weeks.

1.11.2. Research Aim

To design, develop and investigate the utility of an automated microfluidic device system capable of capturing, concentrating and quantifying whole HIV-1 particles through an ELISA approach.

1.11.3. Research Objectives

A) Design and create a microfluidic device.

B) Automate operations of the microfluidic device, whereby the experiment would run with minimal human involvement.

C) To test the ability of the microfluidic system to quantify whole HIV-1 virus particles.

D) Create a standard curve of numerous known HIV-1 viral concentrations with resulting fluorescence intensity observed at each concentration.

CHAPTER 2

2. Materials and Methods

2.1. Reagents

2.1.1. Chip Fabrication

Number 1 glass coverslip slides (25mm X 75mm) from Electron Microscopy Science [Hatfield, USA (CAT #72192-75)]; trimethylchlorosilane (TMCS) from Sigma, [St. Louis, MO, USA, (CAT# 92361)] and PDMS Part A and Part B obtained from RS Hughes (Sunnyvale, CA as Momentive RTV615 Clear 044).

2.1.2. Antibodies and Antigens

HIV-1 (IIIB strain) Purified Virus was obtained from Advanced Biotechnologies Inc [Progress Way, Eldersburg MD (CAT #10-118-000)]; NL4-3 virus was donated by Sigal laboratory (South Africa, KwaZulu-Natal, K-RITH); rabbit IgG antibody purchased from Cell Signalling Technology [USA (CAT # 2729S)] and goat IgG polyclonal-isotype control was obtained from USBiological [Swampscott, Massachusetts (CAT # ab37373)].

2.1.3. Fluorescent Probes and Buffers

Goat anti-HIV-1 (Human Immunodeficiency Virus-1) FITC (fluorescein isothiocyanate) from USBiological [Swampscott, Massachusetts (CAT #H6001-10C)]; casein sodium salt from bovine milk obtained from Sigma Aldrich [St Louis, MO, USA (CAT#C8654-500G)]; PBS (phosphate-buffer saline) was obtained from Sigma [USA (CAT#P3619)] and albumin from bovine serum (BSA) from Sigma [South Africa (CAT#C5679-500g)].

2.1.4. gp120 Conjugated Microspheres

Polystyrene goat anti-HIV-1 conjugated gp120 microsphere with a diameter of 6.1 µm were obtained from Solulink [San Diego, California (CAT # APX-60-10)].

2.1.5. Reagent Preparation

The polystyrene goat anti-HIV-1 conjugated gp120 microsphere were washed multiple times with PBS and centrifugation, see Appendix C**.** 10mg/mL of BSA and casein were prepared by taking 0.1g of BSA/casein and mixing it into 10mL of PBS, see Appendix D. Lastly, HIV-1(IIIB) was made into certain stock concentrations ($1x10^6$ and 500 000 vpc/µl), see Appendix E. All fluorescent probes and buffers were filtered through a cellulose Acetate Membrane syringe filters, 0.20 µm – 25 mm, obtained from Lasec [South Africa (Lot # 14140062307)].

2.1.6. Specific HIV-1 Product Information

Anti-HIV-1 conjugated gp120 antibodies bound to the polystyrene microsphere were raised against recombinant gp120 from HIV-1 IIIB. The goat anti-HIV-1 FITC detection antibodies were generated in goats using a mixture of two immunogens. Lastly, HIV-IIIB was propagated in H9 cells.
Please see Appendix B for further information.

2.2. Microfluidic Device Fabrication

2.2.1. Initial Stage of Microfluidic Designing

The first step in the microfluidic chip design was to design the device using AutoCAD 2013 Student Version. Once the microfluidic design was completed on AutoCAD, the design was sent off to the Stanford Microfluidics Foundry at Stanford University (Stanford, California) where the master molds required for MLS were manufacture. Once the master molds were received, the microfluidic chips were fabricated.

2.2.2. Chip Fabrication by MSL

Molds were exposed to TMCS vapour. PDMS in 5:1 and 20:1 ratios were mixed and degassed using a planetary centrifugal mixer obtained from Crystal Corporation [Japan (CAT # ARE-250CE)]. The 5:1 PDMS ratio was poured onto the control mold in a plastic Petri dish wrapped with aluminium foil followed by de-bubbling in the All Clear vacuum desiccator obtained from Wirsam Scientific & Precision Equipment [South Africa (CAT #PE/22461)]. The 20:1 ratio of PDMS was poured and spun over the flow mold at 1800 g for 60s using the spin processor from Laurell Technologies Corporation

[North Wales, U.S (CAT #WS650Mz)]. Both were baked at 80°C for 40 minutes in a Digital convection oven obtained from Scientific [South Africa (80L Oven Model 221)]. The control layer was peeled off its mold and cut into the respective chip piece. Control line ports were punched using the ACCU-PUNCH MP 10-UNV obtained from Syneo (Texas, USA). The Control layer was then aligned on top of the flow layer mold under a stereoscope. The result was baked at 80°C for 40 minutes. Chip pieces were then cut out and peeled off the flow layer mold. Flow line ports were punched. Lastly, a dummy layer on a glass coverslip was created by mixing a ratio of 30:1 of PDMS. Once mixed, the mixture was poured onto the glass coverslip and spun at 1800g and baked in the oven. Once baked, the bonded control and flow chip were place onto the glass coverslip. The now assembled chip underwent a final bake at 80°C for 8 hours. The chip fabrication can be seen in more detail in Appendix A.

2.3. Experimental Setup

An inverted Olympus CKX41 microscope was equipped with an Olympus UPlanFl 100x/1.30 numerical aperture (na) oil objective; a green (480-550 nm excitation, 590 nm emissions) and blue (460-490 nm excitation, 520 emissions) filter set and an ultraviolet lamp from Olympus (South Africa) and a Nikon DS-Ri1 Colour digital camera with a 400 millisecond (ms) exposure time and a 4.0x analogue gain for imaging from Nikon (South Africa). We then plugged steel tubes from New England Small Tube (Litchfield, NH, USA) into the chip's control channel ports. Their other ends were connected through Tygon® tubing from Cole-Parmer (Vernon Hills, IL, USA). This Tygon® tubing was then filled with deionized water, connected to Festo manifolds and operated via automation using Lab View software on a personal computer with a graphical user interface. When the manifold was pressurized, the deionized water filled the chips control lines, enabling control of the device valves.

2.3.1. Testing Experimental Setup and Reagents

To test both the imaging, microscope components and if the fluorescently labelled antibodies could be detected by the filter set within the microscope, we did the following. Firstly, the polystyrene goat anti-HIV-1 conjugated gp120 microsphere were flown into the microfluidic device and illuminated by bright light (microscopes light bulb) and secondly under excitation light originating from the UV mercury lamp, through the appropriate filter set. Next, to determine if the FITC detection antibody could be detected by the microscope filter cube set, a channel was filled with goat anti-HIV-1 FITC and imaged.

2.3.2. Data Analysis of Fluorescent Microsphere Images

Raw image data was analysed using Matlab R2012a Student version blob analysis, based on fluorescence intensity. Within microfluidics, if one reactor trapped 10 microsphere, all microspheres were subjected to the same experimental procedure separately, such that each microsphere is considered replicates of the same experiment.

2.3.3. Statistical Analyses

All data were expressed as mean ± standard deviation. Statistical analyses was performed on GraphPad Prism version 6 (GraphPad Software, San Diego, California USA). Statistical analyses of certain tests and conditions were performed using one-way analysis of variance (ANOVA). Values of $p \leq 0.05$ were considered statistically significant. P value representation provided by GraphPad Software, see Table 2 for P value representation.

Not significant (NS)	$P > 0.05$
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
****	$P \leq 0.0001$

Table 2: P value representation.

2.4. Experimental Assays

2.4.1. Photobleaching Autofluorescence

The first challenge encountered was the autofluorescence of the microspheres. The presence of autofluorescence needed to be resolved in order to enhance the systems sensitivity in being able to detect the fluorescence produced by the FITC detection antibody upon HIV-1 detection. The concern about the auto fluorescence was that it could possibly mask any subtle or slight signal of the detection antibody binding to the HIV-1 virions captured on the microsphere. Osborne *et al. 2000* [109] stated that non fluorescent microspheres exhibited residual fluorescence, which could be removed via photo bleaching. Therefore, a polystyrene goat anti-HIV-1 conjugated gp120 microsphere was loaded into the

microfluidic device and exposed to an excitation wavelength between 460-490 nm (Ultraviolet) for an extended period of time. An image was taken every 30 seconds through LabVIEW.

Once this experiment had been concluded, the microspheres needed to be further investigated, which was not initially thought of. The next experiment to analyse the microsphere was to test if there was non-specific binding between the microsphere and the FITC detection antibody.

2.4.2. FITC Detection Antibody and gp120 Microsphere Non-Specific Binding

A microsphere, previously having been photobleached was trapping in the microfluidic chip and exposed to multiple 4mg/mL FITC detection antibody incubations. After each 15 minute incubation, the FITC detection antibody was washed away with PBS and the fluorescence signal was measured.

2.4.3. Evaluating Efficient Blocking Agents

The identification of non-specific binding occurring between the microsphere and the FITC detection antibody is important. If a signal is detected, it should be due to the presence of the HIV-1 virions and not non-specific binding. The next step was to try eliminate or reduce the non-specific binding via testing different known blocking agents. Blocking agents are primarily utilized to prevent non-specific binding by taking up any available space that another reagent would bind too. In this case the FITC detection antibody binding to the microsphere. Different blocking agents (10mg/mL BSA, 1mg/mL rabbit IgG, 1mg/mL Goat IgG antibody and 10mg/mL casein) and their effect in blocking available binding sites on the microsphere were investigated. The first experiment conducted involved the recording of a microsphere's natural auto fluorescence and subsequently, the fluorescence signal when the microsphere had been incubated with FITC detection antibody. Next, the blocking agents were tested as follows; a microsphere was incubated in the respective blocking agent for 15 minutes, next FITC detection antibody was incubated with the microsphere for 15 minutes and lastly, PBS was flushed through the channel, removing unbound FITC detection antibody, see Appendix I for further information. An increase or lack thereof of fluorescence intensity would prove the effectiveness of the blocking agent.

2.4.4. Removing Debris within the Microfluidic System

Thus far during the experiments, debris was appearing within the microfluidic system. To try prevent the presence of debris, both the PBS and the FITC detection antibody were filtered through a cellulose syringe filter. The microspheres were rinsed and washed multiple times to remove any possible debris.

2.4.5. Off-Chip Viral Stock Validation

Before on chip experimentation of detecting HIV-1 virus particles, the validation of the $1x10^6$ vpc/µl stock solution needed to be done. This was done as follows; Rev-CEM cells were placed at different dilutions into two identical well plates. 200µl of the virus NL4-3 was placed into the different Rev-CEM cell dilutions in plate 1. 200 µl of the virus HIV-1 III B was placed into the different dilutions in plate 2. After a 4 day incubation at 37 ºC Cells were collected in an Eppendorf tube and spun at 3500 g for 5 minutes. The supernatant was removed and cells were re-suspended in 900 µl PBS and spun at 3500 g for 5 minutes. Supernatant was removed and re-suspended in FACS buffer. Each sample was flown through the FACSCalibur and % infection was generated and subsequently calculations were done to validate the viral stock concentration, see Appendix F for further information.

2.4.6. On-Chip Detection of Virus Particle Concentrations

In sandwich immunoassays, a monoclonal antibody (anti-HIV-1 antibodies on the microsphere) that is specific to the antigen (virus particle), is bound to a surface (microspheres). Next, the sample is put in contact with that surface, whereby the antibody captures the antigen. Next, a labelled polyclonal antibody (FITC detection antibody) attaches to the captured antigen to complete the sandwich immunoassay. The label is a linked enzyme that creates a fluorescent signal when under UV light. The basis of this dissertation HIV-1 virus particle capture is built on a sandwich assay as seen in Figure 22.

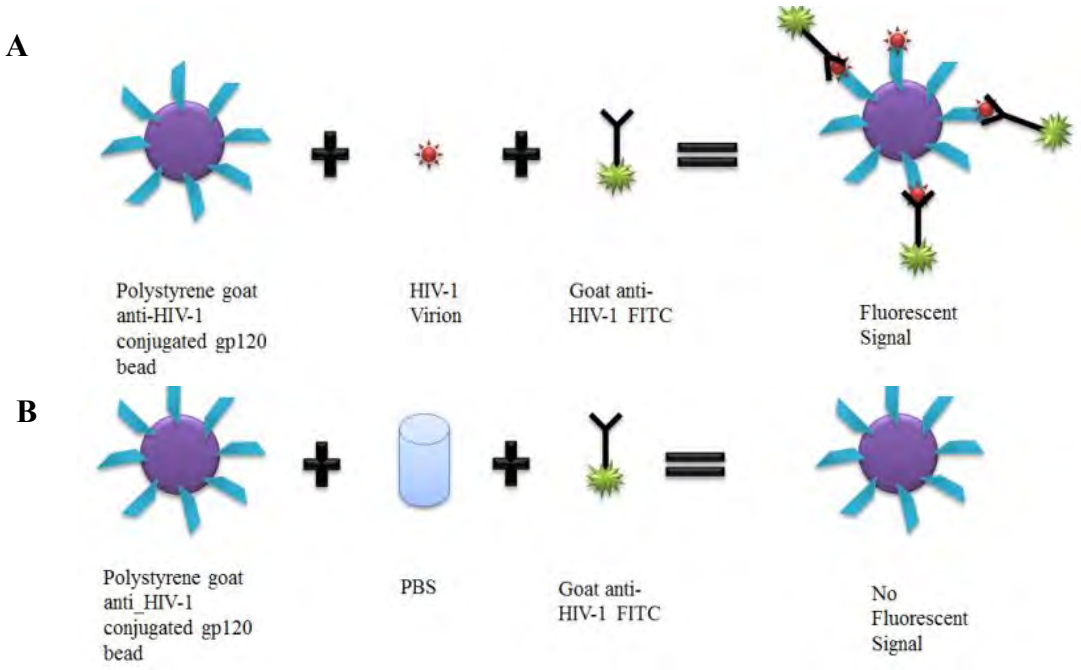

Figure 22: Diagram highlighting the basis behind the virus detection sandwich assay. A) In the presence of HIV-1, the anti-HIV-1 antibodies on the microsphere bind to the gp120 antigens of HIV-1. This allows for the FITC detection antibodies to bind to the microsphere - HIV-1 complex. This produces a fluorescence intensity which can be detected by ultraviolet (UV) light. B) In the absence of HIV-1, no HIV-1 binds to the anti-HIV-1 antibodies on the microsphere. This results in the FITC detection antibodies not being able to due to the absence of the microsphere-HIV-1 complex. No fluorescence intensity is produced.

In the experiment, 10mg/mL BSA was flown through the channel from A (input) to L (output) (Figure 23) and incubated. BSA binds to the microfluidic channel walls, reducing subsequent reagents binding to the microfluidic channel walls. Next, microspheres were flowed from C (input) to L (output) into the desired experiment channel and captured by structural sieve valves (Figure 24). Thereafter, either a virus particle concentration of 0 vpc/µl, 500 000 vpc/µl or $1x10^6$ vpc/µl was flown through the channel from G (input) to L (output) and incubated. After incubation, any unbound virus particles were flushed away with PBS that flowed from D (input) to L (output). 10 mg/mL BSA was then flown through the system again and incubated with the microsphere-virus complex to reduce non-specific binding. PBS was flushed through the system to remove any unbound BSA. Next, 4 mg/mL FITC detection antibody was introduced from F (input) to L (output) and incubated. Lastly, PBS was flushed through the system to remove any unbound FITC detection antibody. A fluorescent image was then taken. A more detailed step by step method for the biological assay procedure can be seen in Appendix G.

CHAPTER 3

3. Results

3.1. Initial Stage of Microfluidic Designing

The first stage in creating a microfluidics device, was to design the device on AutoCAD. The result of this step can be seen in Figures 23 and 24, with an illustration of the device layers from a side view as shown in Figure 25.

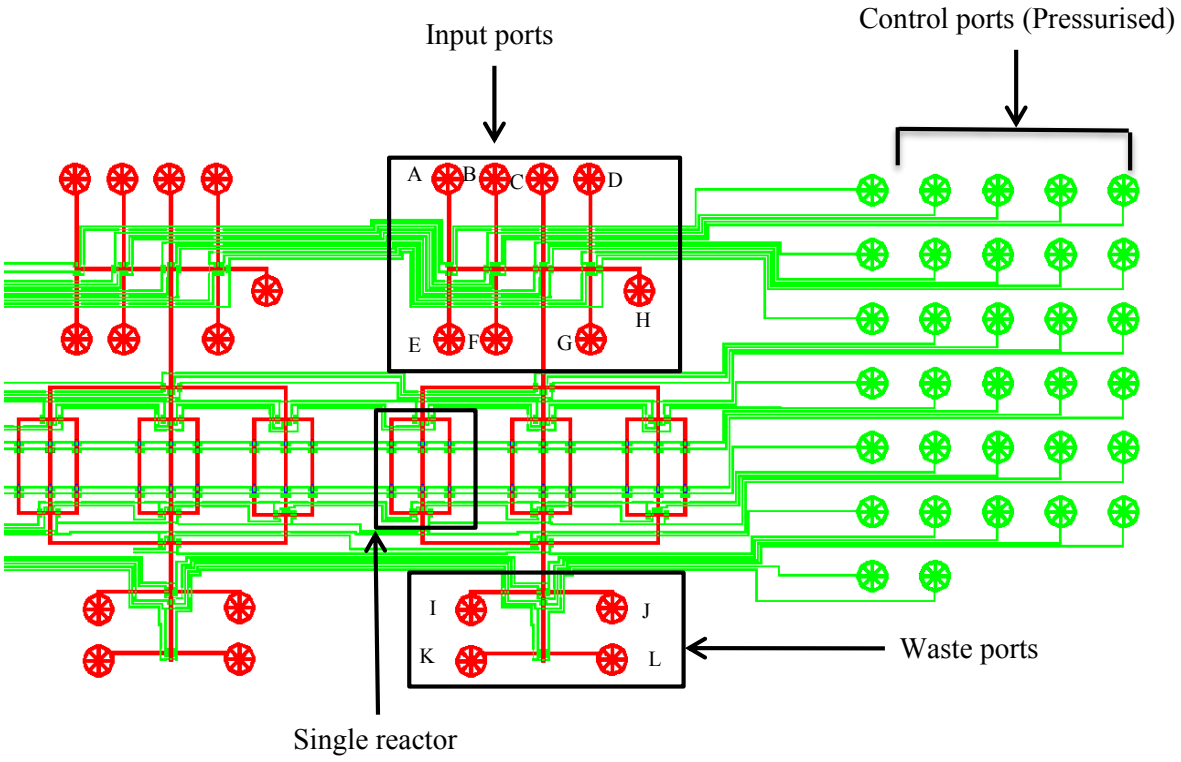

Figure 23: AutoCAD diagram of the microfluidic chip layout, highlighting the inputs (A-H), outputs (I-L), control ports (green circular structures), flow layer (red), control layer (green) and a single reactor. The device has a push-down geometry, meaning the valves are above the flow layer, pushing down onto the flow layer. The control layer channels are 25 µm tall and 50 µm wide with a rectangular geometry. The control lines control the opening and closing of valves and sieve valves throughout the device. The bottom layer, flow layer (red) is a hybrid layer containing two different heights within the same layer. There is the height for the flow layer and a height for sections of the flow layer where the sieve valves

are situated. The flow layer contains channels that are 12 µm high and 100 µm wide with a parabolic cross section (rounded geometry), while the sieve valves (blue) are situated in sections of the flow layer with a rectangular geometry, height of 8 µm and width of 100 µm.

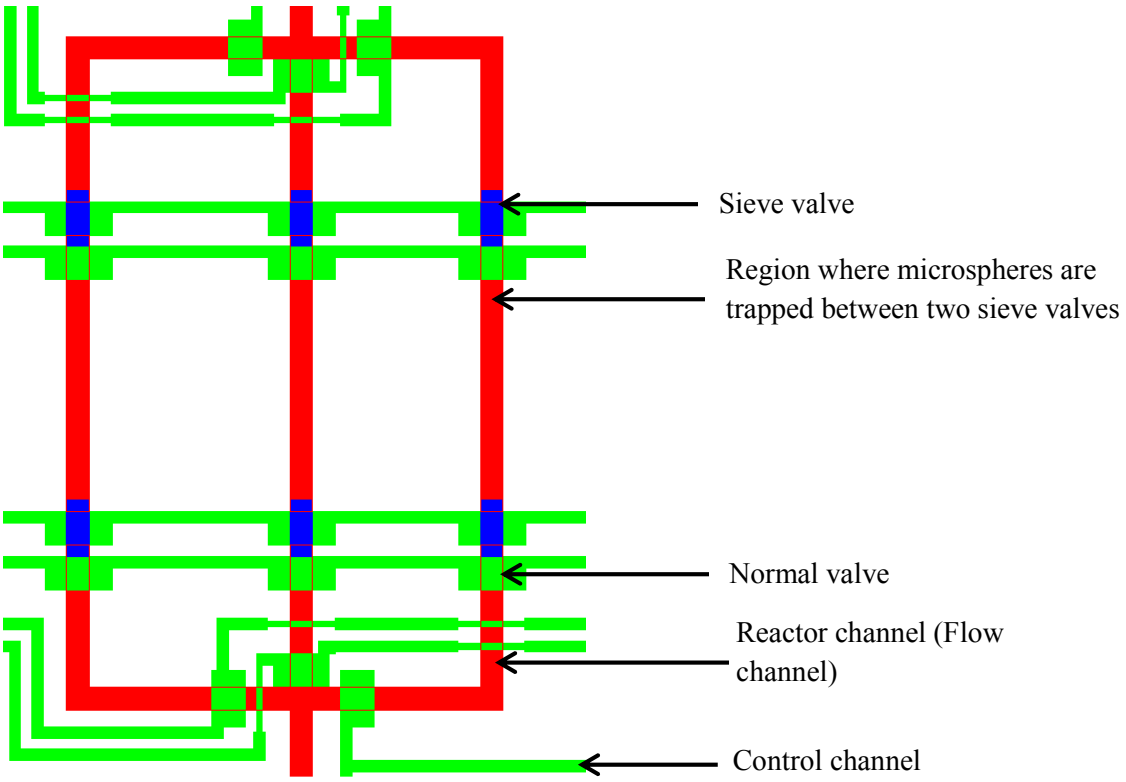

Figure 24: An enlarged view of a single reactor containing sieve valves (blue rectangles), normal valves (green rectangles) and three individual flow channels where experiments were conducted. Sieve valves are actuated separately compared to normal valves, due to requiring a different threshold pressure in order for the sieve valves to create a narrow passage for fluids to pass through, but retaining any object that is larger than 5 µm in diameter. Each reactor contains three channels that can be supplied by any of the input ports (~1000 µm in diameter) with desired reagents.

Figure 25: Illustration of the microfluidic layers that assemble to make the microfluidic chip device. The first layer (blue) is the glass cover slip, middle layer is the flow layer (red) and the top layer is the control layer (green).

3.2. Chip Fabrication by MSL

From the master mold generated from the AutoCAD design, the microfluidic device was built using the MSL chip fabrication method (Appendix A). A photograph of the built microfluidic device can be seen in Figure 26. This figure can be identically compared to the AutoCAD design as shown in Figure 23. Within the device, the designed valves and sieve valves can also be seen (Figure 27).

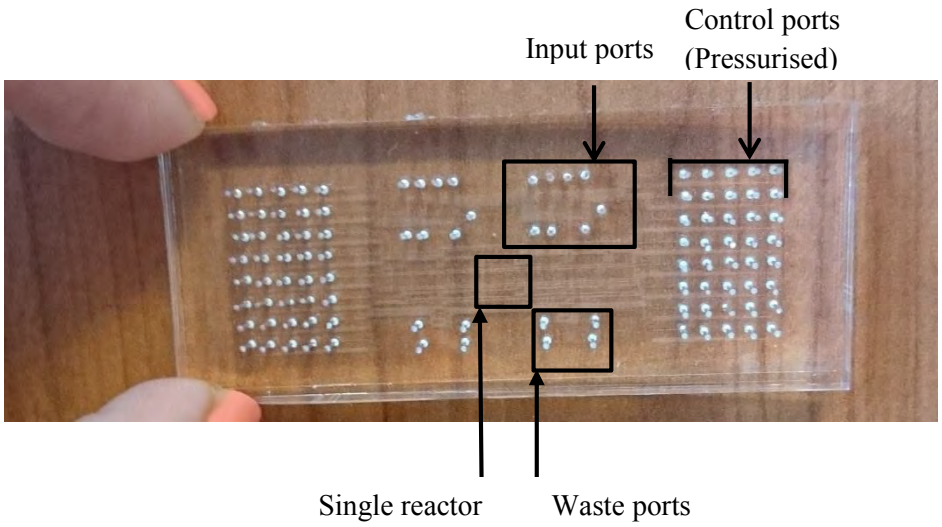

Figure 26: Image of the HIV-1 microfluidic chip after chip fabrication by MSL. Inputs, waste ports, control ports and a single reactor have been highlighted for comparison with the AutoCAD design as previously shown in Figure 23. The length of the chip is 7cm, width is 3cm and height is 5mm.

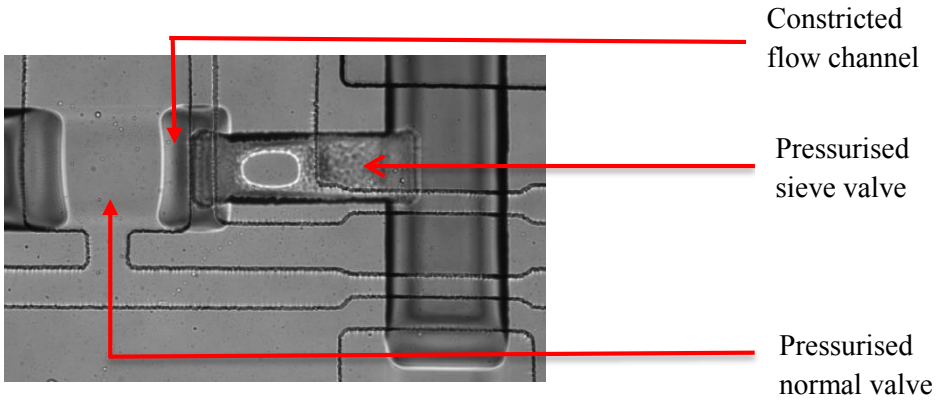

Figure 27: Micrograph of a normal pressurised microfluidic valve versus a pressurised sieve valve. When a normal sieve valve has been pressurised, no flow can occur at all due to the complete

constriction of the flow channel. When a sieve valve is pressurised, any item or reagent below 5 µm can pass through.

3.3. Automation of Microfluidic Device

The graphical user interface was created, (Figure 28) allowing for the control of the microfluidic chip and microscope. The primary function of the LabVIEW script was to relay a recipe provided to the graphical user interface, prior to the start of an assay that controlled the assay. This recipe controlled valves, inputs, outputs, UV light, bright light and the camera. These were all controlled without human intervention once the assay commenced.

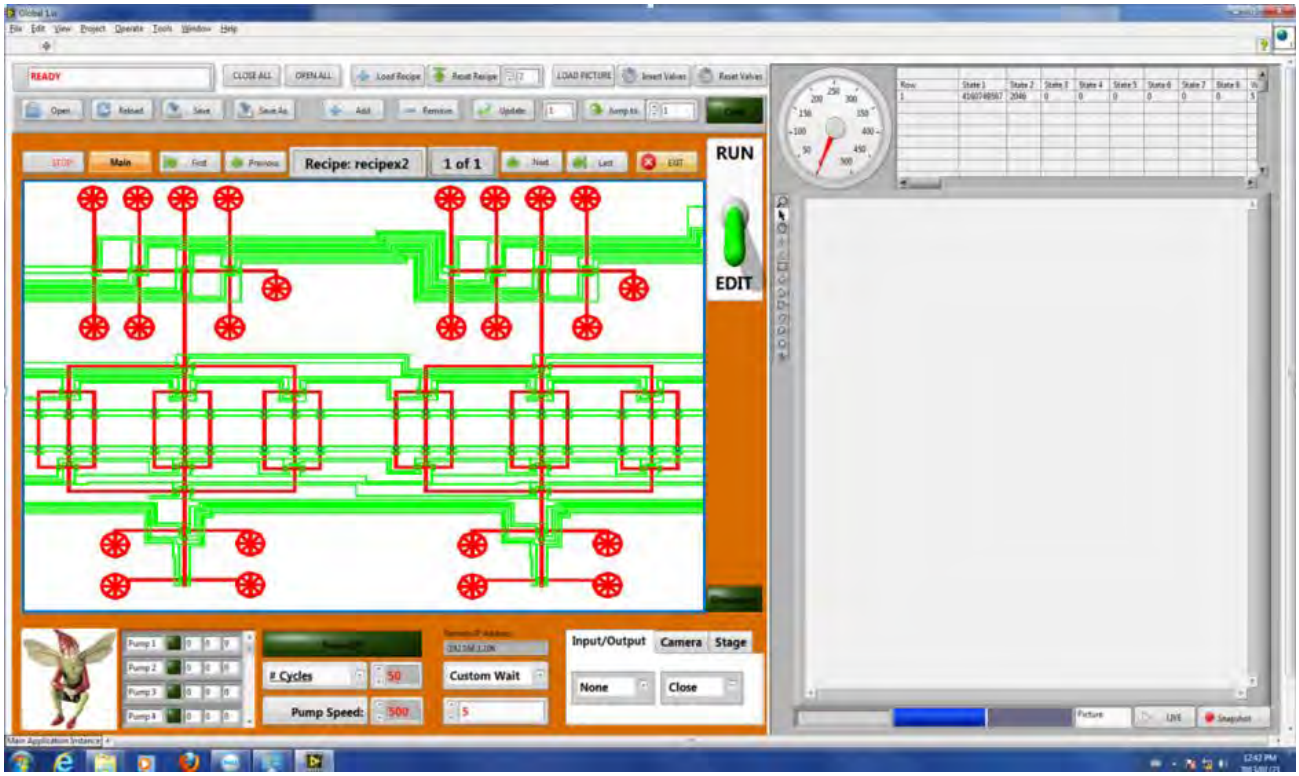

Figure 28: Graphical user interface for the LabVIEW program, which enabled the experiment to be controlled via automation, once recipes were set up.

3.4. Testing Experimental Setup and Reagents

The importance of testing the biological components with the microscope components, was to verify that the biological components could be detected. The results were as follows.

Microspheres were illuminated by bright light (microscopes light bulb), (Figure 29). Next, the microspheres were viewed under excitation light originating from the UV mercury lamp, through the appropriate filter set, (Figure 30). Lastly, to determine if the FITC detection antibody could be detected by the microscope filter cube set, the channel with the microspheres was filled with FITC detection antibody and imaged, (Figure 31).

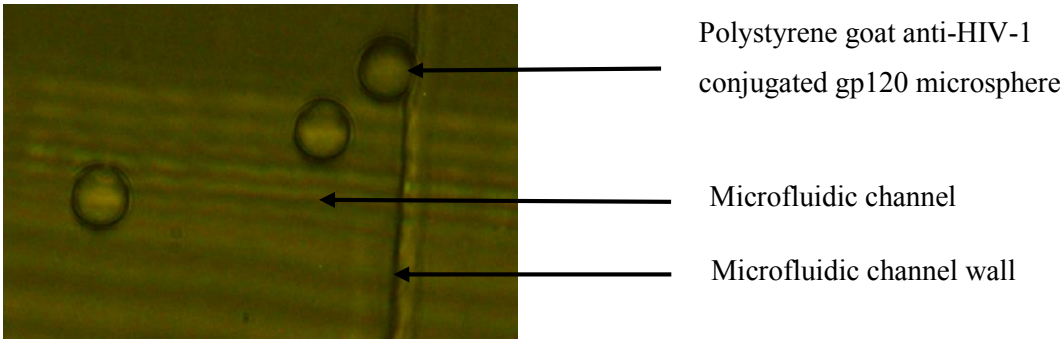

Figure 29: Bright field image of three polystyrene goat anti-HIV-1 conjugated microspheres trapped within the microfluidics channel.

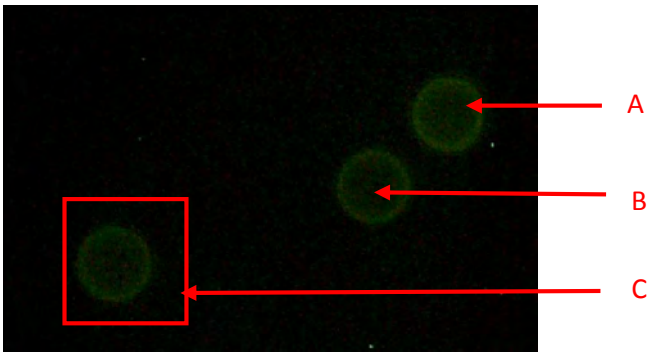

Figure 30: Fluorescent image of the three polystyrene goat anti-HIV-1 conjugated microspheres (A-C) in the microfluidics channel. This image highlighted that the microspheres had their own natural auto fluorescence.

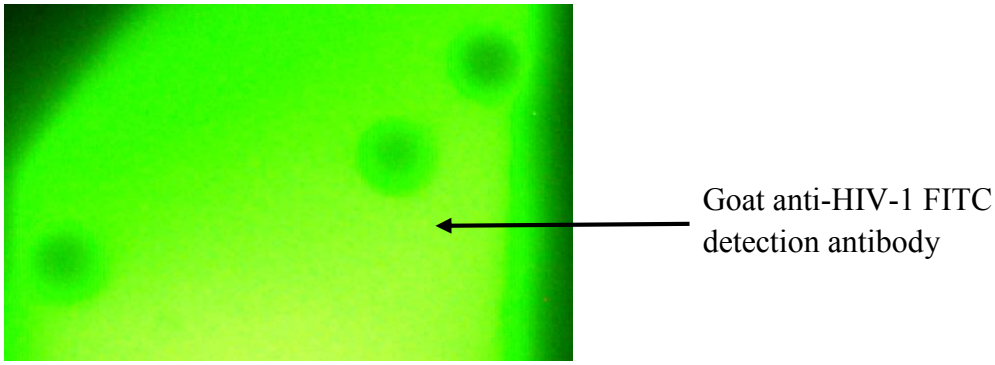

Figure 31: Fluorescent image of the microfluidic channel completely filled with goat anti-HIV-1 FITC detection antibody.

3.5. Photobleaching Autofluorescence

A microsphere was exposed to UV light for an extended period of time and as a result, the microsphere's autofluorescence could be seen decreasing every 30 seconds (Figure 32). An image of the microsphere before and after UV exposure can be seen in Figure 33.

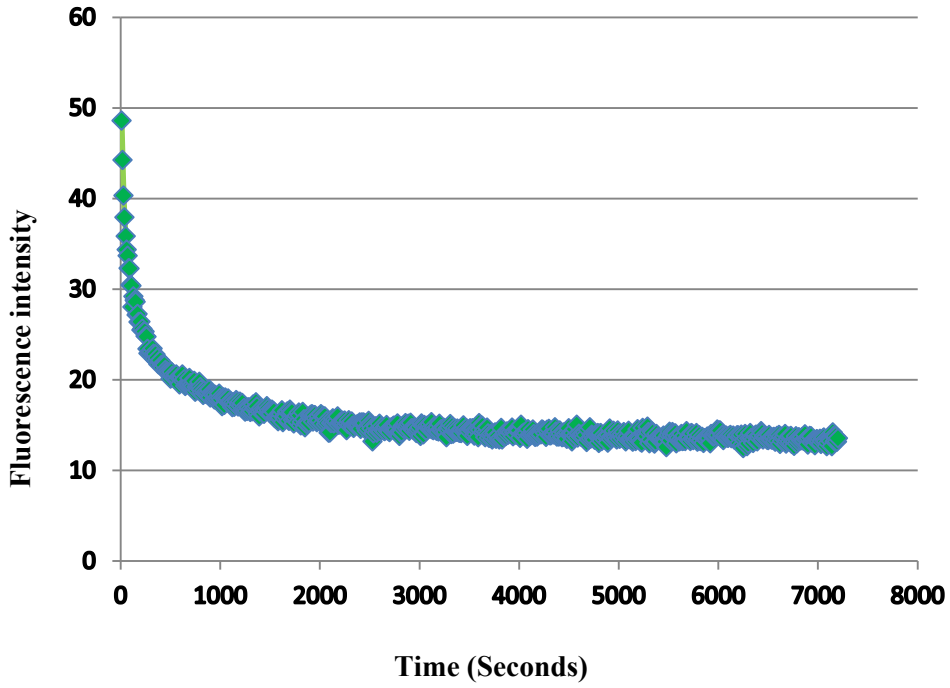

Figure 32: A polystyrene goat anti-HIV-1 conjugated gp120 microsphere previously incubated with goat anti HIV-1 FITC, being subjected to UV light over time. As it can be seen the microsphere's fluorescence intensity slowly decreased over time. The fluorescence intensity eventually reaches a

fluorescence plateau around 5500 seconds corresponding to a fluorescence intensity of 13. Each point seen in the graph is the fluorescence intensity at a specific time point every 30 seconds. This experiment was repeated 3 times and the overall trend observed was a fluorescence decrease over time, until a plateau was reached. This figure is the raw data points obtained for 1 of the 3 experiments.

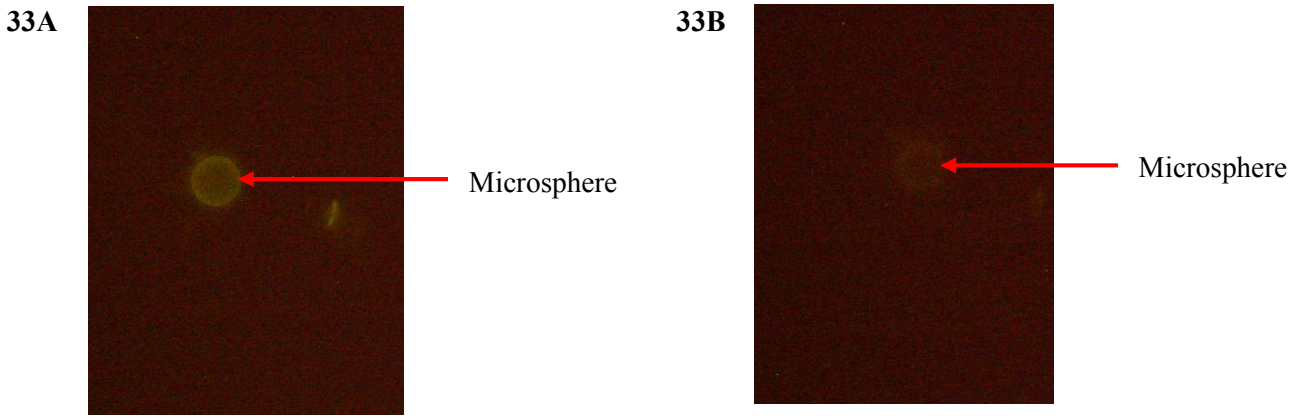

Figure 33: Images taken of a polystyrene goat anti-HIV-1 conjugated gp120 microsphere at its natural auto fluorescence before (A) and after UV light exposure (B). As it can be seen, the microsphere in A is brighter compared to B, conferring photobleaching had occurred. Within this figure, only 1 microsphere has been shown, this experiment was carried out 3 times and all resulted in the microsphere losing fluorescence over time.

3.6. FITC Detection Antibody and gp120 Conjugated Microsphere Non-Specific Binding

The result of the microsphere previously photobleached (Figure 32) and then being exposed to multiple 4mg/mL FITC detection antibody incubations was that at each incubation with FITC detection antibody, the fluorescence intensity increased, (Figure 34). This occurred despite removal of the microsphere's auto fluorescence through UV photo bleaching prior and the removal of excess FITC detection antibody via PBS washing before each incubation cycle. Therefore, non-specific binding was occurring between the microsphere and the FITC detection antibody. The image of the microsphere gaining fluorescence after 1 FITC detection antibody incubation can be seen in Figure 35.

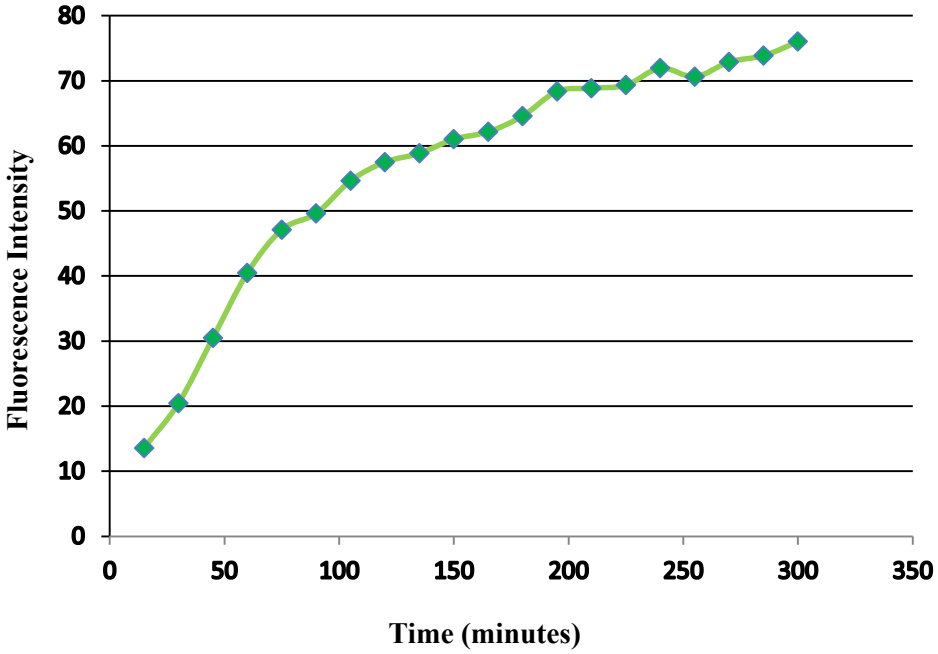

Figure 34: A graph depicting a previously photobleached microsphere having gone through multiple FITC detection antibody incubations. The fluorescence intensity can be seen increasing over time, per each incubation point, indicating that non-specific binding was occurring between the microsphere and the FITC detection antibodies. Here only 1 microsphere was analysed. This experiment was repeated 4 times and all experiments showed an increase in fluorescence after each new incubations with FITC detection antibody. The diamond points on the graph, represent the raw fluorescence intensity values each 15 minutes during 1 of the 4 experiments and not an average value of all the repetitions.

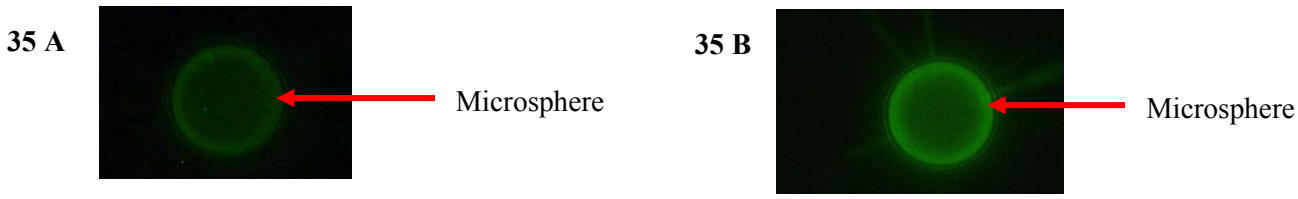

Figure 35: (A) Image highlighting the microspheres natural auto fluorescence and (B) the increase in fluorescence intensity after one incubation round with FITC detection antibody.

3.7. Evaluation of Efficient Blocking Agents

The first experiment conducted involved the recording of the microsphere's natural auto fluorescence and subsequently, the fluorescence signal when the microsphere had been incubated with FITC detection antibody, (Figure 36). Thereafter, the following experiment was conducted for every blocking agent. A microsphere was incubated with a specific blocking agent, rinsed and incubated with FITC detection antibody. The fluorescent signal was then analysed, (Figure 36). The methodology for different blocking agents can be seen in Appendix I. Figure 36 shows a highly significant decrease ($p \leq 0.0001$) in FITC detection antibody binding to the microsphere by the blocking agent BSA. There was a decrease from 29.8±2.387 fluorescence intensity in the control (Microsphere + FITC) to 5.941±1.968 fluorescence intensity in the Microsphere + BSA (10mg/mL) + FITC (4mg/mL). There was another significant decrease ($p \leq 0.01$) in FITC detection antibody binding for the blocking agent rabbit IgG. A decrease from 29.8±2.387 fluorescence intensity in the control (Microsphere + FITC) to 19.12±1.876 fluorescence intensity in the Microsphere + Rabbit IgG (1mg/mL) + FITC (4mg/mL). No significant change ($p > 0.05$) was observed for the goat IgG (25.09±9.523 fluorescence intensity) and the casein (23.39±2.563) blocking agents. As shown in Figure 36, BSA effectively prevented non-specific binding the most and is the better blocking agent compared to the others. Therefore, BSA was chosen as the blocking agent for the on-chip HIV-1 assay.

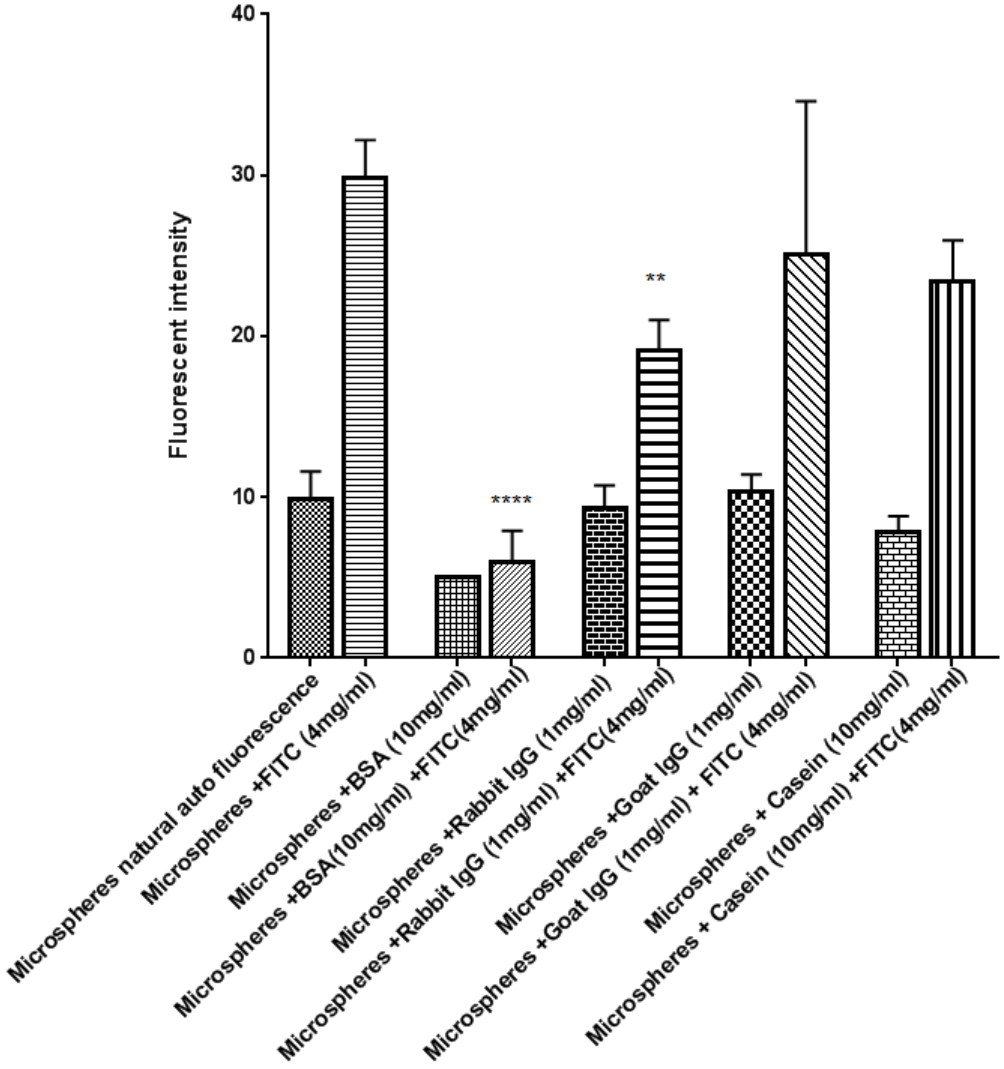

Figure 36: Graph depicting the comparison between blocking agents; 10mg/mL BSA, 1mg/mL rabbit IgG, 1mg/mL Goat IgG antibody and 10mg/mL casein and their effectiveness in preventing 4mg/mL FITC detection antibody binding to the microsphere. Only BSA and rabbit IgG resulted in a significant decrease in FITC detection antibody binding to the microsphere ****$p \leq 0.0001$, and **$p \leq 0.01$ respectively in comparison to the control (Microsphere + FITC). This assay was performed three times to validate data. The data represented in this figure, are the averages from all three repeated experiments.

3.8. Removing Debris in the System

During the running of assays within the microfluidic device, debris would come through and bind onto the microspheres. This caused the FITC detection antibodies to bind to the debris, leading to a false fluorescence increase as it created bright spots. Despite the filtering and washing of the reagents, debris

was still present in the microfluidic system (Figure 37). However the presence of debris did reduce slightly as a result of filtering and washing reagents.

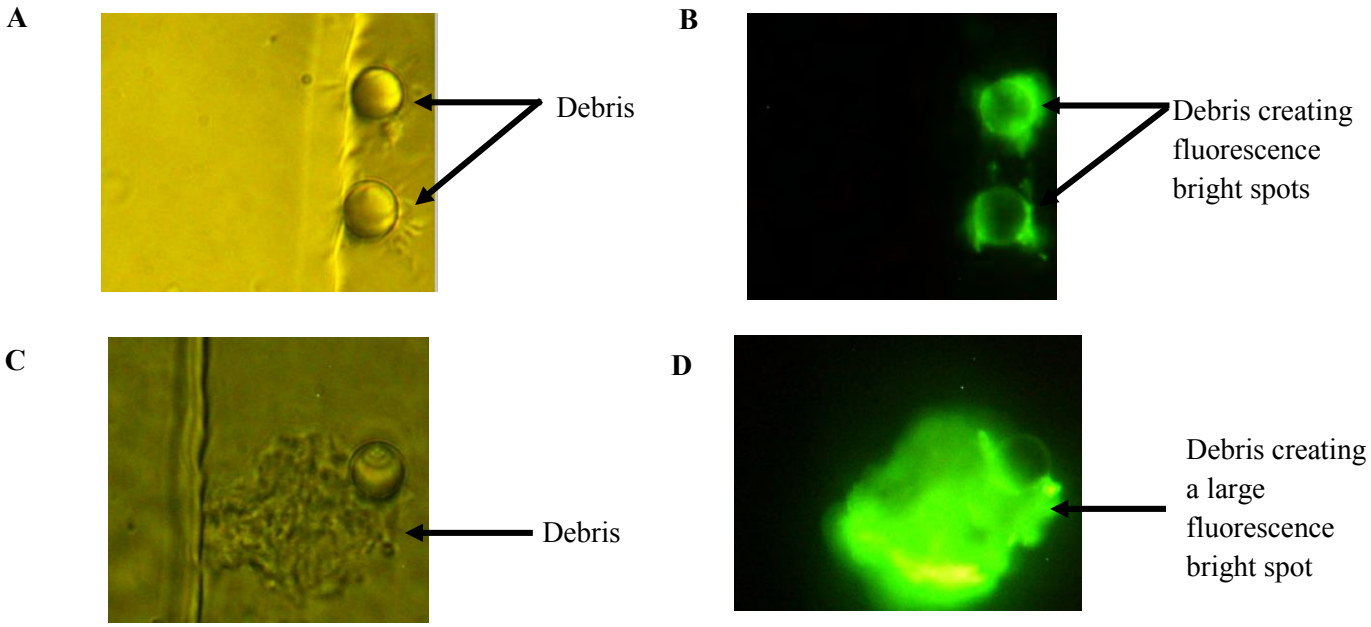

Figure 37: (A and C) Images showing the bright field image of two microspheres with debris around them. (B and D) Images of the fluorescent images of (A and C) where FITC detection antibody has bound to the debris and caused additional fluorescence on the microsphere.

3.9. Off-Chip Viral Stock Validation

Through FACS, the viral stock of $1x10^6$ was validated as being at that concentration. The FACS data showing percentage infection of REV-CEM cells at certain dilutions can be seen in Figure 38. A table was made highlighting the percentage infection and the REV-CEM dilution, see Table 3.

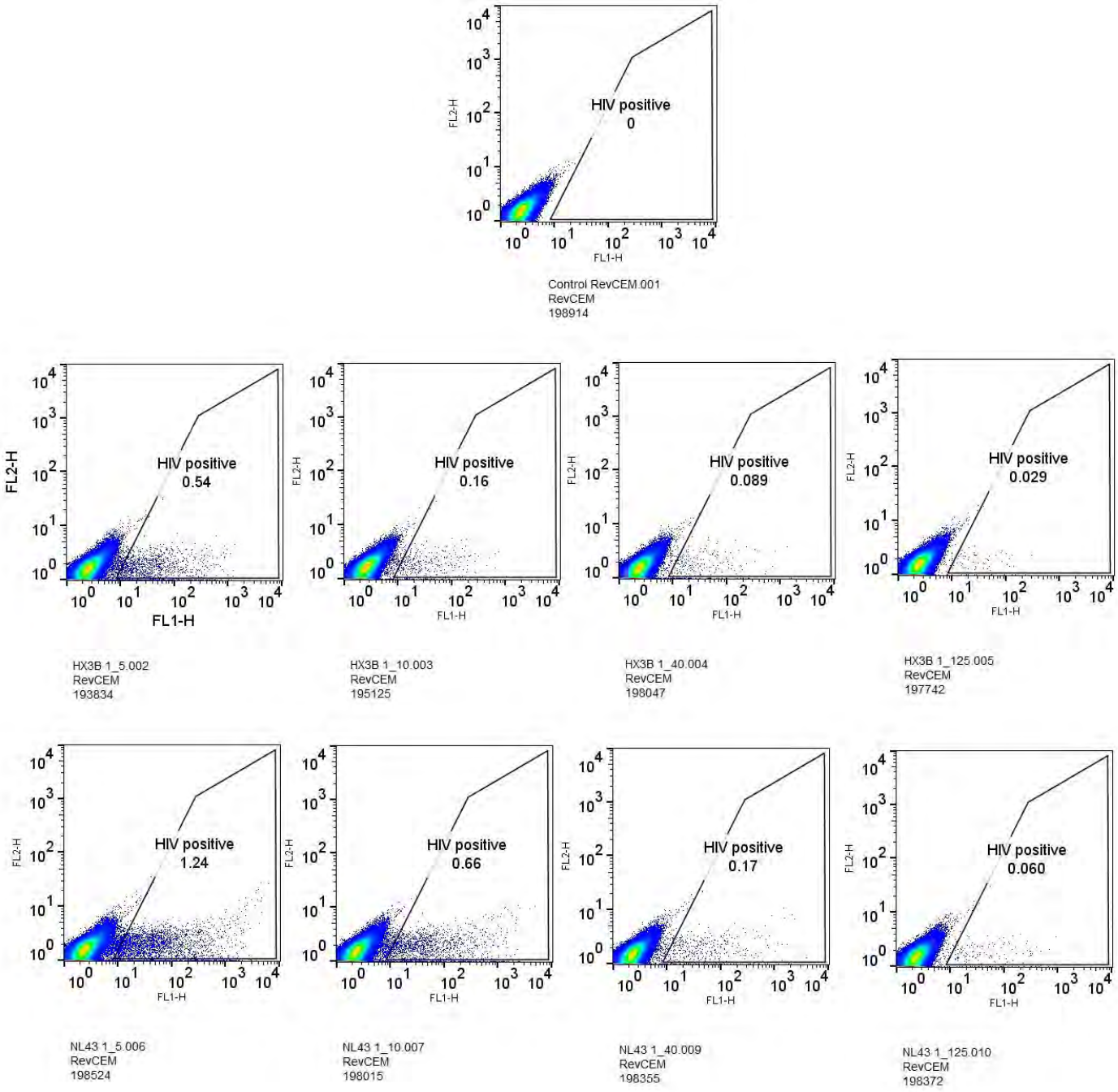

Figure 38: FACS plots showing percentage infection for NL-43 and HIV-1 IIIB

Dilution of REV-CEM	% Infection Generated by the FACS machine	
	HIV-1 (IIIB)	NL4-3
1:5	0.54	1.24
1:10	0.16	0.66
1:40	0.089	0.44
1:125	0.029	0.17

Table 3: Percentage virus infection at certain REV-CEM dilutions for viruses HIV-1 (III B) and NL4-3.

From the percentage infection, the number of HIV-1 virus particles could be calculated:

At a 1:5 dilution with a 0.54% infection
= 0.54 x 0.25
= 0.1325 ng/p24
=132.5pg p24
132.5pg p24 /µl is equal to $1x10^6$ virus copies/µl

Here it was shown that there was 1 million vpc/µl present in the 1 million vpc/µl stock made. See Appendix F for further information on the calculation and the protocol.

3.10. On-Chip Detection of Virus Particle Concentrations

The results of testing different HIV-1 virus concentrations through the microfluidic device using the ELISA approach was as follows; there was a significant increase ($p \leq 0.01$) in fluorescence intensity for the 1 million vpc/µl concentration (19.27±2.269) compared to 0 vpc/µl (8.226±0.599) fluorescence intensity. However, there was no significant increase ($p > 0.05$) for the 500 000 vpc/µl (10.06±4.252) concentration compared to 0 vpc/µl. As seen in Figure 39.

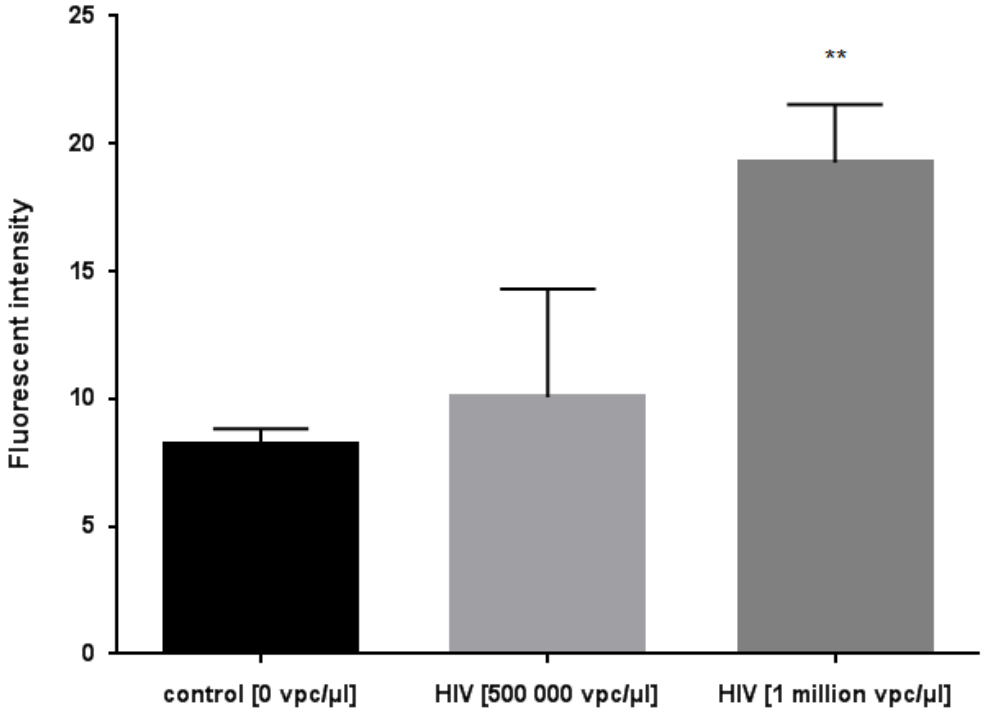

Figure 39: Depiction of the final average fluorescence intensity for 3 repetitions of each viral concentration, (0 vpc/µl, 500 000 vpc/µl and 1million vpc/µl). Only 1 million vpc/µl resulted in a significant increase in fluorescence intensity **$p \leq 0.01$ by comparison to the control. The average fluorescence intensity for each of the concentrations were derived as follows; for each concentration the experiment was replicated a total of 3 times. Within each experiment, 3-7 microspheres were trapped within the channel. These individual microspheres were averaged for each of the 3 experiments. Lastly, the average value of each of the 3 experiments were averaged together resulting in an overall average for each of three experiments for each viral concentration. Please refer to Appendix H.

CHAPTER 4

4. Discussion and Conclusion

HIV-1 has caused a global pandemic over the last couple of decades. There is an unmet need to develop POC viral load diagnostics to initiate and monitor ART in resource-constrained settings[1]. The need for monitoring of HIV-1 and the treatment of AIDS involves consistent and regular monitoring of patients. This has led to the need to develop convenient and accurate methods for monitoring HIV-1 infected individuals efficiently in resource-poor countries[49]. Viral load tests are often performed in specialized laboratories, as they require specifically dedicated materials and are mainly based on extremely specific RT-qPCR reactions, in order to determine viral serum concentration[49]. These tests can be difficult to conduct in developing countries where there is often a lack of resources such as funds and proper infrastructure to conduct specific RT-qPCR assays. However, microfluidics has recently emerged as an interesting field that may provide POC monitoring solutions. The goal of this dissertation was to design, develop and investigate the utility of an automated microfluidic device in its ability to capture, concentrate and quantify whole HIV-1 particles through an ELISA based approach.

As seen within the results, the journey to the actual HIV-1 testing assay contained many hurdles. It was initially observed that the microspheres contained their own natural auto fluorescence, (Figure 30)**.** This auto fluorescence was removed by photobleaching the microsphere with UV light for a prolonged period of time (Figure 32 and 33) as shown by Osborne *et al. 2000* [109]. Next it was discovered that the FITC detection antibody was binding non-specifically to the microsphere itself (Figure 34 and 35). This was overcome by testing different blocking agents' effectiveness in preventing FITC detection antibody binding to the microsphere. It was found that BSA was the most significant ($p \leq 0.0001$) in preventing the FITC detection antibody binding to the microsphere (Figure 36). A similar situation was seen in a study conducted by ShuQi Wang *et al.,* 2012, whereby the capability of BSA to reduce non-specific binding of quantum dots to the microfluidic channel wall was investigated. It was found that by the use of BSA, the fluorescent intensity was significantly reduced, indicating that the BSA blocked binding sites of the channel surface, preventing the quantum dots from binding. The next thing observed was debris within the microfluidic system, (Figure 37). The debris resulted in the FITC detection antibody binding to the microsphere. Despite efforts to filter all reagents that were placed in the microfluidic device, the debris presence was only slightly alleviated. The remaining presence of debris could suggest that the pore size of the syringe was not small enough to capture the debris. It also could suggest that the debris did not come from the solutions being passed through, but rather are pieces of PDMS that get

trapped within channels during the MSL process of chip making. The debris could possibly prevent the sample (HIV-1 virus particles) binding to or interacting with the microspheres anti-gp120 antibodies, due to being blocked by the debris. Next, before on-chip virus particle counting could be performed, the $1x10^6$ vpc/µl stock solution needed to be validated so the concentrations being flowing through the chip were known in order to be able produce a standard curve of concentration vs fluorescence intensity. The $1x10^6$ vpc/µl stock solution was validated by FACS and the on-chip viral load was able to be done. The microfluidic device was setup and different concentrations of HIV-1 virus particles were flown through. It was detected that the ELISA based microfluidics approach could significantly ($p \leq 0.01$) capture and detect 1 $x10^6$ vpc/µl ($1x10^9$ vpc/mL) in 3.8 hours.

Over the years there have been many microfluidic systems made to quantify HIV-1 particles.

In 2009, *Byoung Chan Kim et al.,* reported direct detection of HIV-1 particles. Within the assay, broadly neutralizing HIV-1 gp120 monoclonal antibodies (gp120-Mabs) - conjugated magnetic beads (MBs) and fluorescent nanosized polymeric beads (FNBs) were used to capture HIV-1 particles within 1.5 hours[110]. The HIV-1 virus particles were mixed with the conjugated magnetic beads off chip on a shaker for 30 minutes at room temperature. However, despite being able to detect $2x10^4$ vpc/mL, this was detected off-chip via FACS. In 2010, *Grace D.Chen et al.,* reported the concentration and purification of HIV-1 by microfluidic separation of superparamagnetic nanoparticles[111]. Firstly, HIV-1 virions were incubated on a rocker for 1 hour with superparamagnetic beads. Once the incubation period had been completed, the superparamagnetic beads were injected into the microchip. Secondly, once in the device, the solution was lysed and the lysate was collected for analysis via p24 ELISA off-chip. The assay had an 80 –fold concentration detection of HIV-1 from the initial plasma sample containing 10^6 vpc/mL[111]. In 2012, *ShuQi* Wang *et al.,* achieved efficient on-chip isolation of HIV-subtypes. HIV-1 particles were captured by protein G on the microfluidic channel surface whereby anti-gp120 antibodies were immobilized onto[112]. The use of protein G made the antibody orientation perfect for HIV-1 capture. The captured HIV-1 particles were lysed and the lysate used for HIV-1 RNA extraction. The RNA was quantified using RT-qPCR. The assay as a whole could detect between 1000-10 000 vpc/mL[111]. Lastly, *Hadi Shafiee et al.,* in 2015 fabricated flexible plastic microchips for viral load measurements in both plasma and saliva. They utilised electrical sensing to detect a range of 10^2 vpc/mL - 10^7 vpc/mL[113]. Through the use of biotinylated polyclonal anti-gp120 antibodies anchored to streptavidin-coated magnetic beads, they were able to capture HIV-1 particles. The captured particles were then lysed and via capacitance spectroscopy quantified on chip[113]. The only disadvantage within this application was that there were a lot of off-chip pre sample preparation. For example, the HIV-1 particles were introduced to the biotinylated polyclonal anti-gp120 antibodies off-chip. As it can be seen all of these studies employ the basic principle of ELISA to capture the whole HIV-1 virus particles, in the pursuit of microfluidics as an HIV-1 detection assay. However, these assays are not fully on-chip, but have a

lot of off-chip sample preparation, antigen capturing or detection. Such events, prevent these assays from being accepted as POC technology.

Despite there being many POC tests available that utilise the core principle of microfluidics, there are none that employ the simple ELISA approach to quantify HIV-1 cost effectively, without human involvement and at high sample throughput. There are some devices such as the Liat HIV quant Assay, which utilises whole blood and can be completed within 88 minutes with a detection limit of 57 vpc/mL[10]. This assay uses a similar approach to the GeneXpert MTB/RIF cartridge whereby the PCR reagents are within microspheres. The disadvantage of this assay however is that only one specimen can be sampled at a time and a technician needs to move the sample tube from the Liat tube into the Liat analyser[114].

In summary, the results provide a basic proof-of-concept that the utility of the created microfluidic device in conjunction with HIV-1 neutralizing antibody gp120-conjugated microspheres captured and concentrated 1 $x10^6$ vpc/µl ($1x10^9$ vpc/mL). This was done within 3.8 hours via complete automation and no pre- or post-sample treatment was done. Within this dissertation, objectives (A) design and create a microfluidic device in-house, (B) automate operations of the microfluidic device, whereby the experiment would run with minimal human involvement and (C) to test the ability of the microfluidic system to quantify whole HIV-1 virus particles were completed. However, due to the low limit of detection of the system, the final objective (D) create a standard curve of known HIV-1 viral particle concentrations was not able to be completed very well. The only concentrations that were able to be detected were, 0, 500,000 and 1,000 000 vpc/µl. The reason why objective (D) was unable to be completed well is due to the limit of detection of the microfluidic system. The limit of detection of this system was 1 $x10^6$ vpc/µl, which compared to the detection limit required for potential POC assays being1000-5000 vpc/mL[1], this assay was not as sensitive. Comparing our microfluidics device to current POC applications such as Amplicor HIV-1 Monitor Test 1.5, (PCR based machine), which is able to detect 50 vpc/mL and above[74] the microfluidic device needs to be greatly improved on.

From studies cited in the literature review and discussion[110,111], most current microfluidic HIV-1 detection approaches are more sensitive and capable of detecting much lower HIV-1 particle numbers[2]. Interestingly, successful virus capture approaches have incorporated neutravidin, streptavidin and biotin components [113,107]. There is a strong interaction between biotin and streptavidin, with a dissociation rate of $2.4x10^6$ $s\text{-}1$[115] compared to a normal rabbit monoclonal antibody which is $7x10^{-11}$ $s\text{-}1$[116]. A very similar approach to this dissertation's assay was done by Kim *et al*., 2009; however, biotinylated anti-gp120 capture antibodies were coated onto the neutravidin coated microfluidic channel, creating a strong bond. Thereafter, captured HIV-1 was co-detected by biotinylated anti-gp120 antibodies binding

with streptavidin conjugated green quantum dot antibodies and biotinylated ConA lectin antibodies with streptavidin conjugated red quantum dot antibodies. Not only does dual labelling provide a more reliable countable virus image, but the use of quantum dots, which provide greater photostability and brightness[107] are said to provide a promising alternative to organic fluorophores for fluorescence-based biological imaging.

In conclusion, the use of this in-house microfluidic device and assay approach was not sensitive enough. A possible hypothesis that could have occurred in this dissertations' microfluidic system was that there was not a strong enough interaction between the anti-gp120 capture antibodies on the microsphere and the HIV-1 virus particles themselves. Thus when rinsing occurred, the HIV-1 particles were removed from the anti-gp120 capture antibodies. To possibly provide more sensitivity for this dissertations microfluidic device and future microfluidic devices, it is recommended to employ a different biological assay approach. For future research using an ELISA based approach for HIV-1 whole virus or particles, detection within a microfluidics device, researchers could look at incorporating the reaction between streptavidin and biotin with quantum dots for detection and also the use of broadly neutralizing antibodies (Bnabs). Bnabs have been said to be able to more efficiently neutralize many HIV-1 subtypes and epitopes with more efficiency[117]. Both these approaches could increase the sensitivity of the reaction.

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APPENDICES

APPENDIX A

Multilayer Soft Lithography Fabrication

1- Priming: all molds
   TMCS vapour 3 minutes in a sealed Tupperware container.
2- Cast Thick layer: Combine 5:1 Part A: Part B and mix in a centrifugal mixer for 10 minutes. Place mixture into petri dish lined with aluminium.
3- Cast flow layer: Combine 20:1 Part A: Part B and mix in a centrifugal mixer for 10 minutes.
4- Place mixture onto flow layer and spin at 1800 g for 1 minute.
5- 1st Cure Thick layer: convection oven
   80 °C for 40 minutes.
6- 1st Cure Flow layer: convection oven
   80 °C for 40 minutes.
7- Punching push up control input holes: peel thick layer from mold.
8- Punch control input holes.
9- Align the thick layer onto the spin coated flow layer mold.
10- Bake in convection oven
   80 °C for 40 minutes.
11- Thick/flow control bonding: peel thick/flow bonded layer from mold.
12- Punch flow input and output port holes.
13- Cast blank glass coverslip layer: Combine 30:1 Part A: Part B and mix in a centrifugal mixer for 10 minutes.
14- Place mixture on glass coverslip and spin at 1800 g for 1 minute.
15- 1st Cure blank glass coverslip layer: convection oven
   80 °C for 40 minutes.
16- Place thick/flow bonded layer onto cured blank glass coverslip.
17- Ensure no air bubbles.
18- Bake in convection oven
   80 °C for 6 hours.

APPENDIX B

Background Information on Specific Materials

Polystyrene goat anti_HIV-1 conjugated gp120 microsphere

The anti_HIV-1 conjugated gp120 antibodies bound to the polystyrene microsphere were raised against recombinant gp120 from HIV-1 IIIB (Subtype B, Group M).

Goat anti-HIV-1 (Human Immunodeficiency Virus-1) FITC (Fluorescein isothiocyanate)

These antibodies were generated in goats using a mixture of two immunogens, two purified virus strains, IIIB (Subtype B, Group M) and the Murine Norovirus.

HIV-1 (IIIB strain) Purified Virus

This virus was propagated in H9 cells and purified from culture supernatant when cells show cytopathic effects (cell death). The virus was then purified via the multi-step proprietary method and viral concentration was then determined by transmission electron microscopy (TEM), which resulted in a count of virus particles per mL (vpc/mL). This strain of virus corresponds to subtype B, group M. The concentration of the Purified Virus that was ordered was $6.7x10^{10}$ vpc/mL.

APPENDIX C

Polystyrene Goat Anti-HIV-1 Conjugated gp120 Microsphere Preparation

1- 10 µl of the microsphere solution was placed into 90 µl of PBS.

2- The solution was centrifuged for 5 minutes at 2500 g.

3- The supernatant was removed and the pellet was re-suspended in 90 µl PBS.

4- Repeat number 1- 3, 3 times.

APPENDIX D

10mg/mL BSA and Casein Preparation

1- 0.1g of BSA/casein was weighed out.

2- The weighed out BSA/casein was then placed in 10 mL of PBS.

3- The solution was vortexed.

APPENDIX E

Preparation of Viral Stock Concentrations

Initial product information: HIV-1 (IIIB strain) Purified Virus
Concentration: $6.7x10^{10}$ vpc/mL or $6.7x10^{7}$ vpc/µl
Volume: 800 µl (0.8mL)

Firstly, I made a working stock of 1 million vpc/µl. From this stock I was able to use it to obtain the 500 000 vpc/µl concentration.

Volume of initial (V1) product needed =?
Concentration of initial (C1) product = $6.7x10^{10}$ vpc/mL or $6.7x10^{7}$ vpc/µl
Volume you want to make (V2) = 1mL (1000 µl)
Concentration you want to have (C2) = $1x10^{6}$ vpc/µl

Equation: $V_1 C_1 = V_2 C_2$

$V_1C_1=V_2C_2$
V_1 =?
$C_1 = 6.7x10^{7}$ vpc/µl
V_2 = 1 mL (1000 µl)
$C_2 = 1x10^{6}$ vpc/µl

$$V_1 = \frac{V_2(C_2)}{C_1}$$

$$V_1 = \frac{1000\mu l(1x10^{6} vpc/\mu l)}{6.7x10^{7} vpc/\mu l}$$

$$V_1 = \frac{1x10^{9}\mu l}{6.7x10^{7}}$$

$$V_1 = 15\mu l$$

Therefore, 15 µl of $6.7x10^{7}$ vpc/µl must be taken and placed into 985 µl of PBS to make a new stock concentration of $1x10^{6}$ vpc/µl.

To make a Stock with a concentration of 500 000vpc/ µl

V_1C_1=V2C2

V_1 =?

C_1 = 1x10^6 vpc/ µl

V_2 = 1 mL (1000 µl)

C_2 = 500 000 vpc/µl

$$V_1 = \frac{V_2(C_2)}{C_1}$$

$$V_1 = \frac{1000\mu l(500\ 000\ vpc/\mu l)}{1x10^6 vpc/\mu l}$$

$$V_1 = \frac{5x10^8 \mu l}{1x10^6}$$

$$V_1 = 500\ \mu L$$

Therefore, 500 µl of 10x10^6 vpc/µl must be taken and placed into 500 µl of PBS to make a new stock concentration of 500 000 vpc/µl.

APPENDIX F

Information and Protocol to Validate 1 million vpc/µl Viral Stock

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Information

1- The % infection values were provided by the FACS machine.
2- HX3B is the HIV-1(IIIB) strain and NL4-3 is a well-known and characterised lab strain, therefore was the control for the FACS machine.

Known information to work out the number of virus copies present:

1- 1pg p24 = $1x10^4$ virus copies[118].
2- 1ng p24=$1x10^7$ virus copies[119].
3- 100pg p24 = $1x10^6$ virus copies.
4- For a 3-4% REV-CEM infection from a 1:5 dilutions is around 1ng p24.

Therefore, HIV-1(IIIB) at 0.53 % infection;
If 4 % REV-CEM infection at 1:5 dilution represents 1 ng of p24
Than a 1% REV-CEM infection would represent 0.25 ng p24 (1÷ 4 =0.25)

Protocol

1- Two well plates each had Rev-CEM cells placed into the wells at the following dilutions; 1:5. 1:10, 1:20, 1:40 and 1:125.

2- 200 µl of the virus NL4-3 was placed into the different dilutions in plate 1.

3- 200 µl of the virus HIV-1 III B was placed into the different dilutions in plate 2.

4- 4 day incubation occurred at 37 ºC.

5- Cells were collected in an Eppendorf tube and spun at 3500 g for 5 minutes.

6- Supernatant was removed.

7- Cells were re-suspended in 900 µl PBS and spun as in step 5.

8- Supernatant was removed and re-suspended in FACS buffer.

9- Each sample was flown through the FACSCalibur and % infection was generated.

This assay was performed in Dr Alex Sigal's laboratory, where they had Rev-CEM cells and Nl-43 virus already set up, so both the Rev-CEM cells and NL-43 control virus were gifts from the Sigal laboratory. Rev-CEM cells are cells that automatically express GFP (green fluorescent protein) in 3-5 days of HIV infection.

APPENDIX G

Experimental Assay Procedure

Experimental Procedure for the Control (0 vpc/µl), 500 000 vpc/µl and 1 million vpc/µl.

1- 10mg/mL BSA was flowed through the desired reactor and channel and incubated for a few minutes to reduce nonspecific binding.

2- 5-10 polystyrene goat anti-HIV-1 conjugated gp120 microsphere were flowed into the desired experiment channel and captured by the sieve valve.

3- PBS (control)/500 000 vpc/µl or 1 million vpc/µl were flown into the channel with the microsphere and incubated for 15 minutes.

4- Repeat Step 3 a total of 6 times.

5- 10mg/mL BSA was introduced to the microsphere and incubated for 10 minutes.

6- PBS was then flowed through the channel for 3 minutes.

7- 4mg/mL goat anti-HIV-1 FITC was introduced and incubated for 10 minutes.

8- PBS was flowed through for 3 minutes.

9- Steps 7-8 was repeated a total of 10 times.

10- Fluorescent image was taken.

APPENDIX H

Raw Graphs and Calculations for Data Shown in Figure 39

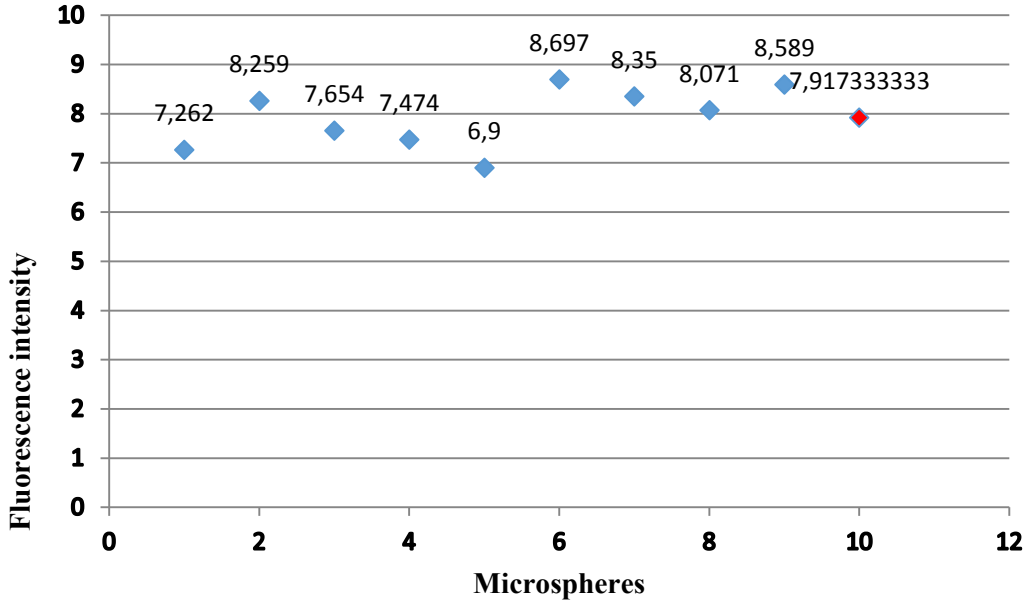
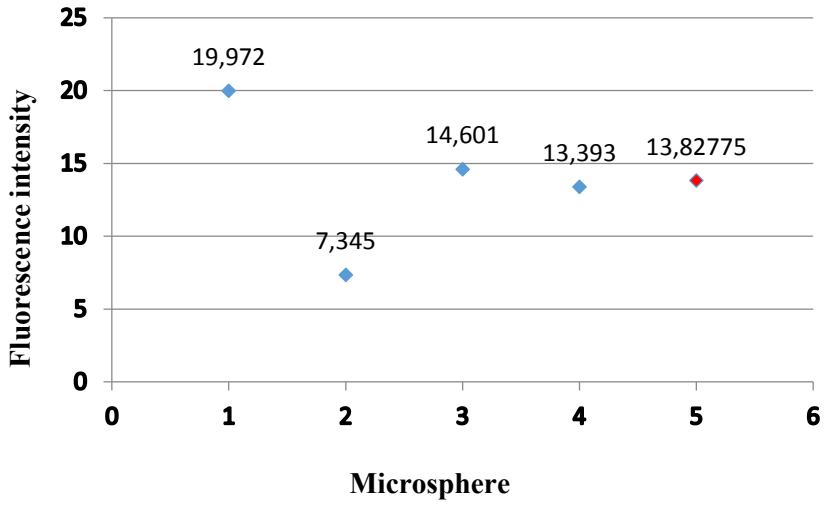


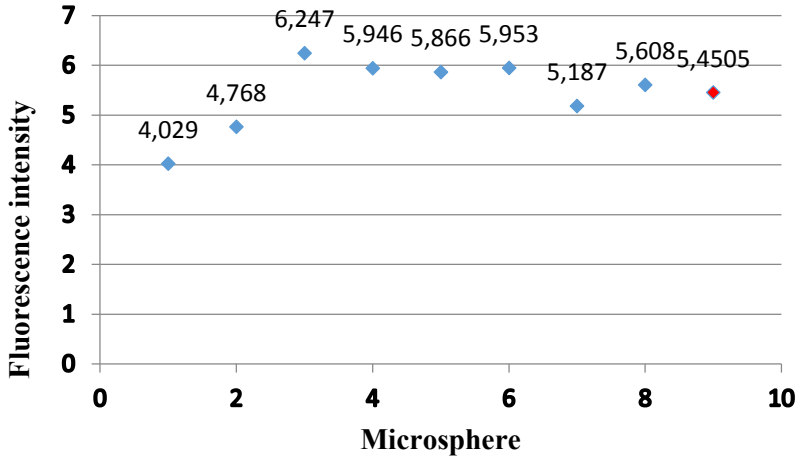
Figure 40: Graph showing the individual microsphere values (Blue diamond) and the overall average of all microspheres (Red diamond) for the control experiment.

As a result, the average fluorescence intensity for the control was 8.

A



B



C

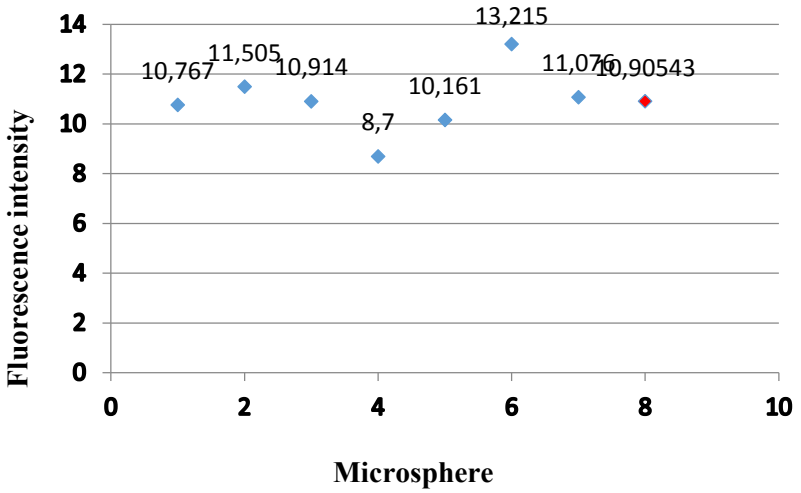


Figure 41 A-C: Graph showing the individual microsphere values (Blue diamond) and the overall average of all the microsphere (Red diamond) for the each of the 3 experiment testing 500 000 vpc/µl.

Thus, the average value for 500 000 vpc/µl is the average of the averages for the 3 experiments.

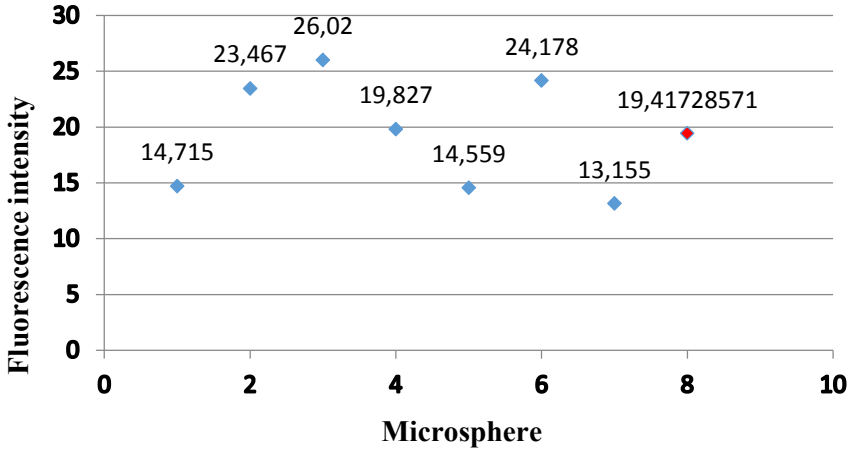
A Fluorescence intensity (14).

B Fluorescence intensity (11).

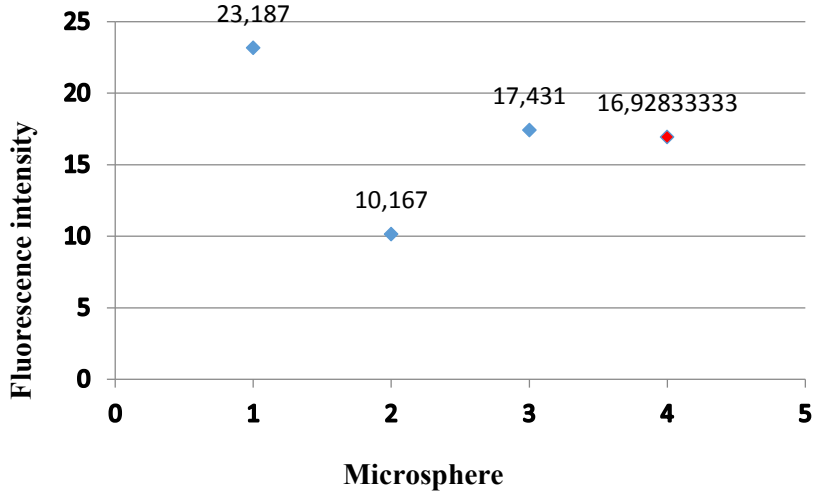
C Fluorescence intensity (5).

The overall average fluorescence intensity was 10.

A



B



C

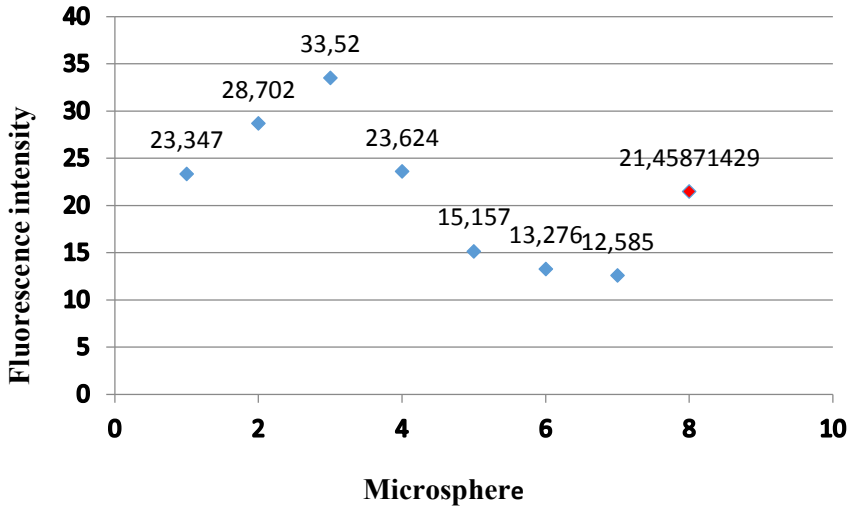


Figure 42A-C: Graph showing the individual microsphere values (Blue diamond) and the overall average of all microspheres (Red diamond) for the each of the 3 experiment testing 1 million vpc/µl.

Therefore, the average value for 1 million vpc/µl is the average of the averages for the 3 experiments.

A Fluorescence intensity (19).

B Fluorescence intensity (17).

C Fluorescence intensity (21).

The overall average fluorescence intensity was 19.

*Note: The microspheres analysed for these experiments conducted were all microspheres that did not have debris present.

APPENDIX I

Methodology for Assessing Different Blocking Agents

1- A microsphere was incubated in either 10mg/mL BSA / 1mg/mL rabbit IgG antibody / 1mg/mL Goat IgG antibody or 10mg/mL casein for 15 minutes.
2- Microsphere were washed with PBS.
3- Thereafter the microspheres were subsequently incubated with Goat anti HIV-1 FITC detection antibody for 15 minutes.
4- Microspheres were washed as in step 2.
5- Fluorescence intensity was recorded.