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Anti-gp120 and anti-p24 aptamers: potential for use as flow cytometry reagents

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A Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine by Research.

Johannesburg, 2018

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Dr. Kabamba Alexandre

DECLARATION

I Kanyane Bridgett Malatji declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine by research at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

_____

(Signature of candidate)

_____ day of _____ 20 _____ at _____

DEDICATION

I dedicate this Dissertation to my mother Grace Matobole Malatji, my late father Mochai Judah Malatji and my siblings. The support you gave me throughout my studies was amazingly inspiring. This whole journey wouldn't have been possible without the Lord almighty. Thank you God.

PRESENTATIONS ARISING FROM THIS STUDY

1. This study, which was titled; Isolation of HIV-1 anti-gp120 and anti-p24 aptamers for use as reagents in flow cytometry, has been presented as a poster presentation at an HIV Research for Prevention Conference held in Madrid, Spain, October 2018. The conference organising committee awarded me a partial scholarship to attend the meeting.

ABSTRACT

Introduction: Aptamers are nucleic acids selected by systematic evolution of ligands by exponential enrichment (SELEX). They have potential as alternatives to antibodies in research and diagnosis. Their main advantages over antibodies are that they are non-immunogenic and relatively inexpensive to produce. The aim of this study was to generate fluorescein isothiocyanate (FITC) conjugated gp120 aptamers as well as synthesize p24 aptamers and to potentially use them for detecting human immunodeficiency virus (HIV-1) infected cells.

Methods: The gp120 aptamer was conjugated with FITC by incubation with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and imidazole. The conjugation and binding to the glycoprotein was confirmed using flow cytometry and capture assay. Aptamers against p24 were selected using SELEX and their sequences elucidated by next generation sequencing.

Results: The conjugation of the gp120 aptamer with FITC increased fluorescence emission 24 -fold from baseline. Similar data were obtained when beads coupled with HIV-1 gp120 or whole viruses were detected with the FITC-conjugated aptamer by flow cytometry and capture assay, respectively. When compared to a commercially available antibody (biotinylated anti-gp120 polyclonal antibody), the FITC-conjugated aptamer showed better emission of fluorescence. During the synthesis of p24 aptamers an enrichment of binding sequences was observed. Furthermore, 90,500 sequences were identified by deep sequencing out of the initial 10^{14} ss-DNA library.

Conclusion: A FITC-conjugated gp120 aptamer that can bind the glycoprotein on coated beads or on a whole viral particle was generated. The aptamer is a potential low cost reagent for use in HIV/AIDS research or diagnosis. Furthermore, the selection of p24 aptamers was performed and sequences that bind the target were identified.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr Hazel Mufhandu, Dr Kabamba Alexandre and Dr Pascaline Fonteh for this learning opportunity. I would like to show my appreciation for the support and guidance they gave me throughout the study. A special thanks to Ms Nompumelelo Mthimkhulu for assisting with the use of the flow cytometer instrument, and data analysis with FlowJo. Lastly I thank Dr Arshad Ismail and Dr Mushal Ali from the National Institute for Communicable Diseases (NICD) sequencing laboratory for assisting with the deep sequencing of the aptamer library.

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NOMENCLATURE

AIDS	Acquired Immunodeficiency Syndrome
APS	Ammonium Persulfate
ARV	Antiretroviral therapy
Bp	Base pair
BSA	Bovine Serum Albumin
ConC	Consensus C
CSIR1.1 aptamer	Council for Science and Industrial Research 1.1 aptamer
DAPI	4',6-diamidino-2-pheylidole
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ds-DNA	Double stranded deoxyribonucleic acid
EDAC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetra acetic acid
ELONA	Enzyme Linked Oligonucleotide Assay
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
gp120	Glycoprotein120
HIV	Human Immunodeficiency Virus
IC_{50}	50% Inhibitory Concentration
LTR	Long Terminal Repeat
M	Molar
mAb	Monoclonal antibody
µm	Micrometer
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institute of Health
NICD	National Institute for Communicable Diseases
nM	Nanomolar

p24	Protein 24
pAb	Polyclonal antibody
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
RLU	Relative Light Unit
RNA	Ribonucleic acid
SELEX	Systemic Evolution of Ligands by Exponential Enrichment
SPR	Surface Plasmon Resonance
ss-DNA	Single stranded deoxyribonucleic acid
TBE	Tris-borate-EDTA
TCID	Tissue Culture Infectious Dose
TEMED	Tetramethylethylenediamine
UCLA1 aptamer	University of California, Los Angeles1 aptamer
WHO	World Health Organisation

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Chapter 1 : INTRODUCTION

This chapter will cover the study rationale, a background on human immunodeficiency virus (HIV) including statistics, life cycle, structure, compounds that target the viral envelope, background on aptamers, and the systemic evolution of ligands by exponential enrichment (SELEX) technology used to synthesise aptamers.

1.1. Study Rationale

Antibodies are used in flow cytometry for the detection of cells infected with HIV-1, these include antibodies specific to gp120 and those against p24 (Waldrop *et al*., 1997, Sattentau and Moore, 1991). However, these reagents have a number of disadvantages. For example they are produced by the immunisation of animals with the target of interest, as a result, antibodies are expensive to produce and the production is time consuming (Guo *et al*., 2008). In addition, target molecules that do not elicit an immune response cannot be detected using antibodies (Beltramello *et al*., 2010). Moreover, they can be unstable and can also lose their native structure and function (Yang *et al*., 2012).

These limitations associated with antibodies highlight the need for better and cheaper reagents for use to detect HIV infected cells. One such approach would be to use more stable and less expensive molecules such as aptamers that target the viral gp120 or p24 proteins (Zeng *et al*., 2017; London *et al*., 2015, Mufhandu *et al*., 2012). Although anti-gp120 and anti-p24 aptamers have been isolated (Zeng *et al*., 2017; London *et a*l., 2015), their conjugation to Fluorescein isothiocyanate (FITC) for the detection of HIV-1 infected cells by flow cytometry have not been explored extensively. FITC is an inexpensive and commonly used fluorophore that contains an isothiocyanate reactive group, which reacts with nucleophiles.

The study starts with giving the background information which includes the statistics of HIV-1, pathogenesis and structure, background information on HIV-1 gp120 and p24,

compounds that target the viral envelope and detection of HIV-1 by flow cytometry. The introduction concludes with information on aptamers, the method used for isolation of aptamers, SELEX, and finally the study objectives. This chapter will be followed by chapter two which covers the materials and methods used to conduct the study. The materials and methods chapter covers the conjugation of anti-gp120 aptamers to FITC followed by the confirmation of conjugation with fluorescence imaging, flow cytometry, and capture assay. The integrity of the aptamer after conjugation with FITC was determined using the neutralisation assay in TZM-bl cells. The materials and methods are followed by chapter 3, which covers the results and discussion. Finally the conclusion and future work are covered in chapter 4.

1.2. HIV-1

1.2.1. The HIV Pandemic

Since the start of the HIV epidemic, 70 million people have been infected with the virus and 35 million have died of the associated acquired immunodeficiency syndrome (AIDS) related illnesses worldwide (WHO, 2017). Currently there are 38.7 million people living with HIV. Sub-Saharan Africa is the most affected region in the world (Figure 1.1); while South Africa is the country with the highest number of infected individuals standing at about 7.2 million in 2017 (WHO, 2017). These statistics suggests that South Africa is facing the greatest challenge in HIV/AIDS cases compared to the rest of the world, thus, it remains critical to study the virus further with the aim of reducing these challenges at the country level and worldwide. Figure 1.1 shows the prevalence of HIV among individuals aged 15-49 years in 2017 (WHO).

1.2.2. HIV-1 pathogenesis and structure

HIV is a lentivirus from the Retroviridae family (Fanales-Belasio *et al*., 2010, Freed, 1998) (Figure 1.2). Retroviruses are viruses that reverse transcribe their RNA genome into core viral DNA that integrates the host genome (Sundquist and Krausslich, 2012; Temin, 1993). Lentiviruses are retroviruses that infect non-dividing cells and depend on

the use of nuclear import pathway to enable their DNA to cross the host nuclear cell membrane and integrate into its genome (Zennou *et al*., 2000).

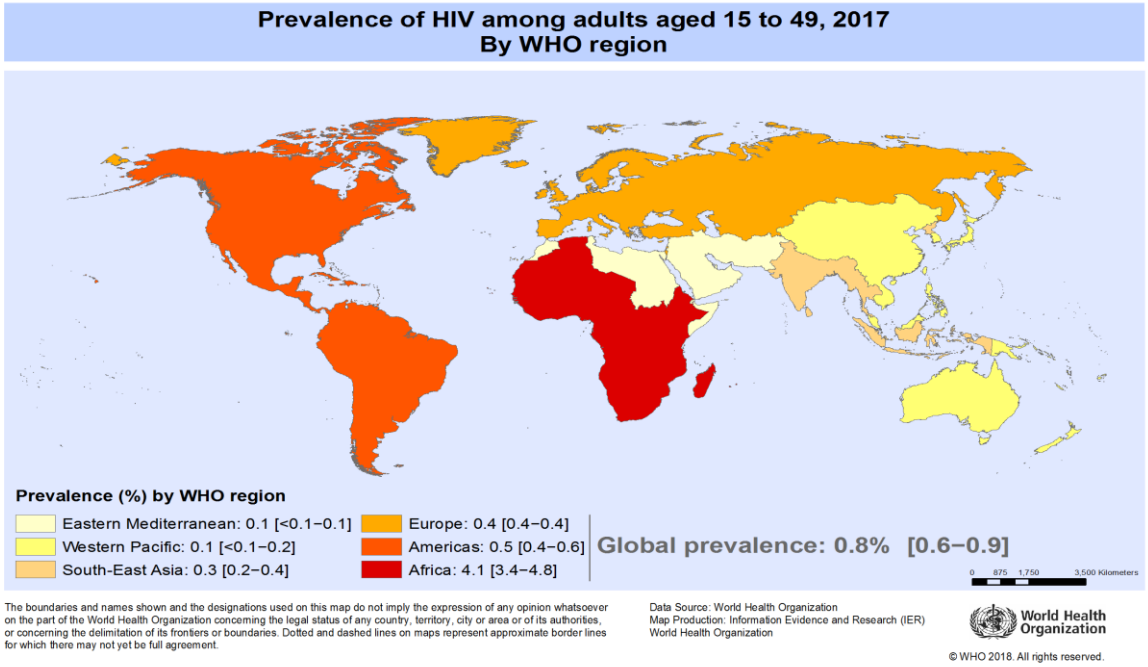


Figure 1.1: The number of people living with HIV around the world in 2017 (WHO, 2017).

The warmer the colour in the figure the higher the prevalence.

HIV has two different types which are HIV-1 and HIV-2. HIV-1 is divided into groups; group M, O, and N. The group M, which is the largest, is further divided into subtypes, namely subtype A, B, C and D that account for the majority of infections worldwide (McCutchan, 2006). In sub-Saharan Africa, subtypes A and C viruses are the most prevalent. The type 2 (HIV-2) is largely restricted to West Africa and has a low prevalence (McCutchan, 2006).

The virus has an RNA genome that is surrounded by p24 capsid proteins detected in blood during infection (Zeng *et al*., 2017; Freed, 1998). The virus is also made up of a p17 matrix and has the following enzymes used during viral replication in host cells: Protease, reverse transcriptase and integrase (Freed, 1998) (Figure 1.2). The viral

envelope is made up of a trimeric complex composed of the heterodimer proteins gp120 and gp41 derived from gp160 (Blumenthal *et al.*, 2012; Willey *et a*l., 1988). These proteins are essential for the recognition and entry into the target cell (Wyatt *et al*., 1998, Chan and Kim, 1998). The gp41 protein is responsible for the fusion of the virus and cellular membranes whereas the gp120 binds to CD4 receptors expressed on host cell surface (Chan and Kim, 1998).

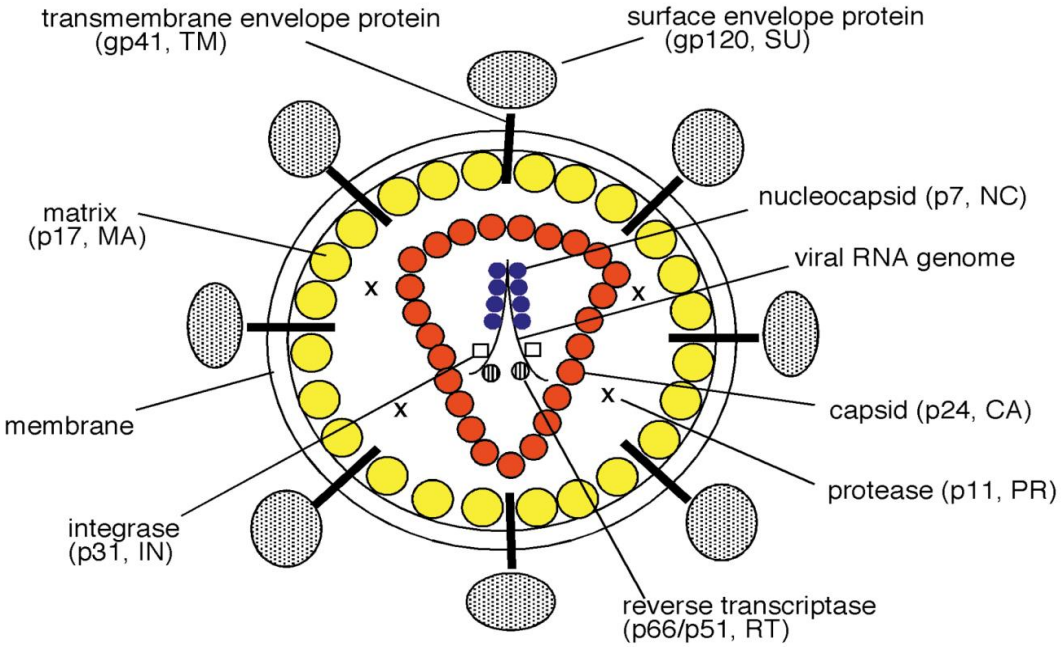


Figure 1.2: The structure of HIV-1 virus.

The image shows the envelope proteins and the nucleic acid along with the enzymes involved in viral replication during infection. The spikes represent the envelope proteins, and the viral matrix is represented by the yellow circles. The viral capsid, which houses the viral integrase, nucleocapsid and the viral RNA genome, is indicated in red (Acquired from Freed, 1998 and used with permission from American Society for microbiology).

HIV-1 life cycle begins with the binding of viral envelope gp120 (Figure 1.3) to the target cell CD4 receptor and the chemokine co-receptor CXCR4 or CCR5 (Brenchley *et al*., 2004; Geijtenbeek *et al*., 2000). This is followed by the entry of the virus into the host cell cytoplasm. Then uncoating; which happens when there is a release of the two

copies of viral RNA (containing *gag*, *pol* and *env* genes) from the capsid into the cytoplasm of the target cell. This uncoating is then followed by reverse transcription of HIV-1 ss-RNA into a ds-DNA by the viral reverse transcriptase. This ds-DNA then migrates to the nucleus to integrate the host cell genome as a pro-virus. This integration is mediated by HIV-1integrase (Fanales-Belasio *et al.*, 2010). The final steps of the viral life cycle include the synthesis of viral proteins, such as the envelope gp160 and p24, followed by assembly and budding of virions from then infected cell as depicted in Figure 1.3 (Hu and Hughes, 2012; Fanales-Belasio *et al*., 2010; Arhel, 2010).

HIV infection begins with an acute period of infection that is followed by the chronic phase of the disease that can be characterized by a period of clinical latency (Fanales-Belasio *et al*., 2010). The systemic spread of the virus requires its circulation from the sites of infection (usually in mucosal surfaces) to zones where T-cells are located such as secondary lymphoid organs where viral replication occurs in CD4+ T-helper cells (Geijtenbeek *et al*., 2000). The viral infection of CD4+ T-helper cells is achieved via its interaction with the CD4 receptor and CCR5 or CXCR4 co-receptor (Brenchley *et al*., 2004; Geijtenbeek *et al*., 2000).

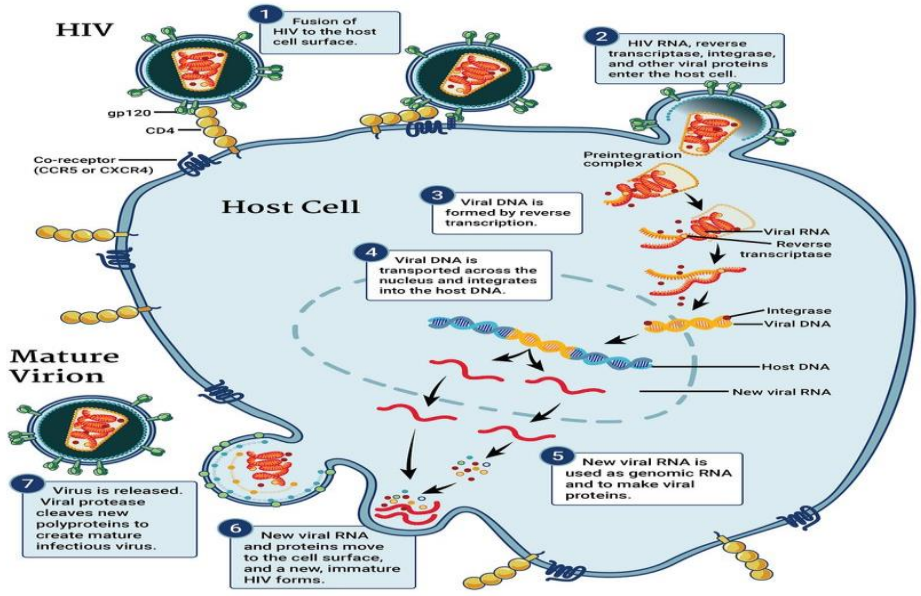


Figure 1.3: The HIV replication cycle.

The image shows the events from when the virus attaches to the host cell to when it buds after replication and assembly within the cell. The attachment of the virus is mediated by the fusion of

the viral proteins with those on the host cell. Attachment is followed by the release of viral genome (3), reverse transcription of RNA into DNA and integration (4) into the host genome. Mature virions are produced after protein synthesis, assembly and budding (5, 6 and 7) (Acquired and used with permission from NIAID, 2010).

1.2.3. The HIV gp120 structure

The HIV-1 gp120 contains five variable regions named from V1-V5 (Figure 1.4) inserted between conserved regions. These conserved regions are targets for neutralising antibodies (Walker *et al*., 2010, Zhou *et al*., 2007). The gp120 core consists of an inner domain, an outer domain and a bridging sheet that contributes either directly or indirectly to the cellular CD4 and chemokine-receptor binding, which results in viral entry into the host cell (McCaffrey *et al*., 2004; Wyatt *et al*., 1998).

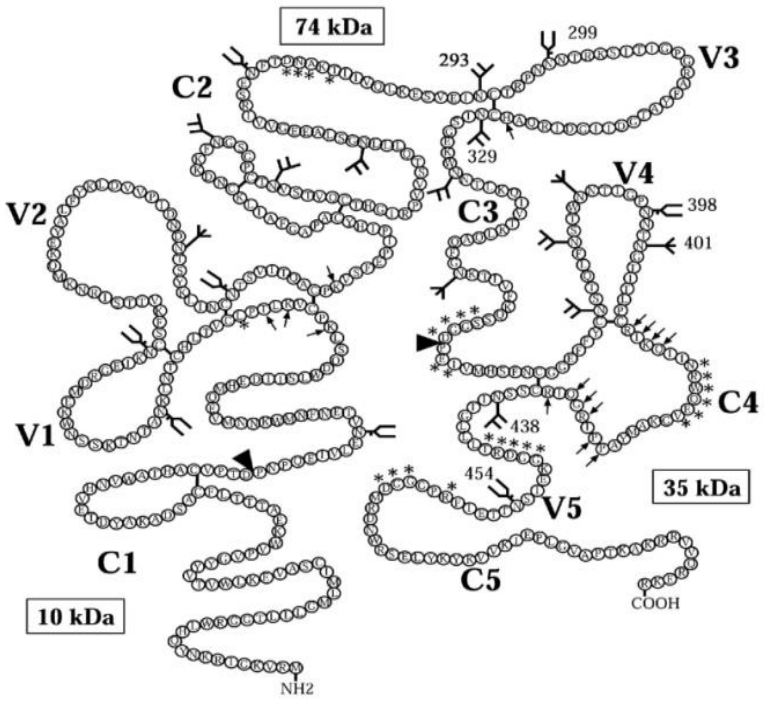


Figure 1.4: The structure of the HIV-1 gp120 glycoprotein.

V1-V5 indicate the variable regions and C1-C5 indicate the conserved regions (Acquired from Mccaffrey *et al*., 2004 and used with permission from American Society for microbiology).

HIV-1 gp120 is the most studied viral protein or antigen due to the fact that it is expressed on the HIV-1 surface and is vital for entry into target cells. For example, HIV entry inhibitors have been produced for use as therapeutics to prevent gp120 binding to host cell receptors (Liu *et al*., 2005). To be precise, Zhao *et al*., identified two N-phenyl-N V-(2, 2, 6, 6-tetramethyl-piperidin-4-yl)-oxalamide small organic compounds that inhibit HIV-1 entry by interacting with gp120 (Zhao *et al*., 2005). Also a tetrameric CD4-IgG_2 fusion protein that binds to gp120 with an affinity in the nanomolar range was expressed by Allaway *et al*., and reported to have neutralising effects of the cell-to-cell HIV-1 transmissions (Allaway *et al.*, 1995). Compounds such as the water-soluble polymer carbohydrate binding agents (CBAs) and BMS-378806 which binds to gp120 were also developed for use as therapeutics (Jay *et al*., 2010, Lin *et al*., 2003).

1.2.4. HIV p24 structure

HIV p24 is the major capsid protein that surrounds the viral nucleic acid. It is initially expressed as part of the gag polyprotein and when processed, is made up of 231 amino acid residues (Berthet-Colominas *et al*., 1999). This protein is produced after the cleavage of gag into its individual components by the viral protease. HIV p24 then condenses into the mature capsid that surrounds the viral RNA and plays important roles during processes of viral assembly and maturation (Berthet-Colominas *et al*., 1999, Freed, 1998).

HIV p24 has a molecular weight of 24 KDa (Zeng *et al*., 2017). This protein is made of two α-helical domains; each of which comprises seven α-helices at the N-terminal and four at the C-terminal interconnected by a flexible linker (Gillespie *et al*., 2002; Berthet-Colominas *et al*., 1999) (Figure 1.5). The protein's α-helices H1 and H2 play a significant role in p24 dimer interface formation and in stabilizing the HIV capsid assembly and maturation (Gillespie *et al*., 2002). The p24 protein is a significant marker in early HIV-1 detection as it is expressed in blood during infection (Zeng *et al*., 2017; Zhang *et al*., 2002).

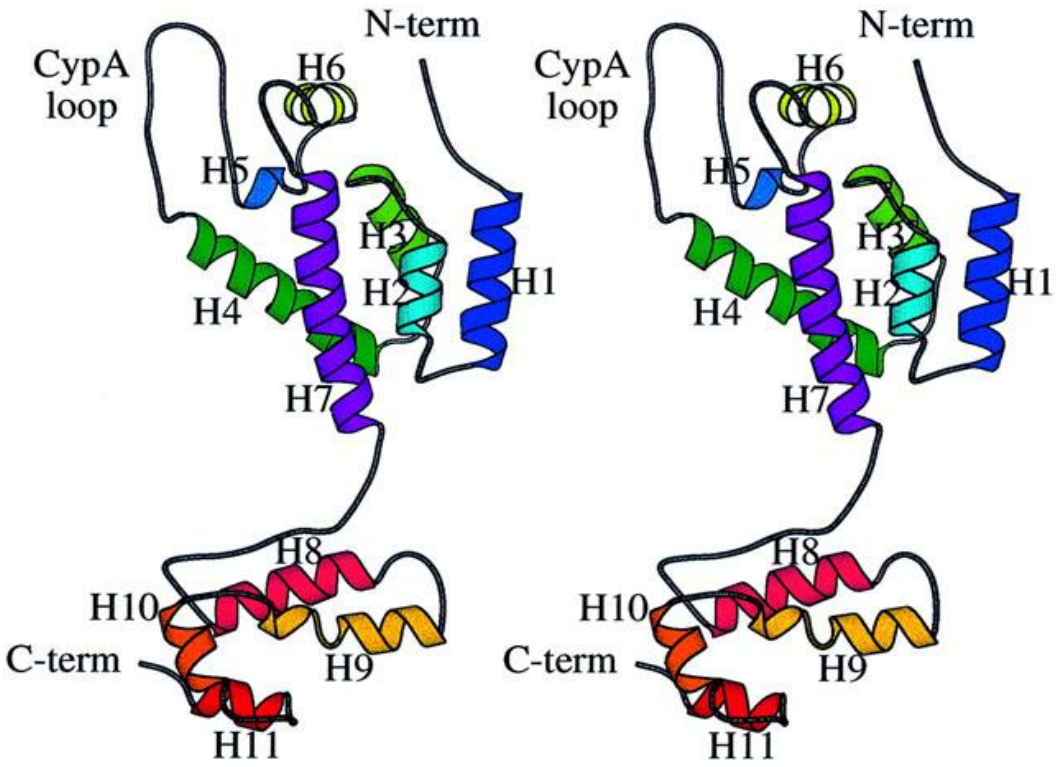


Figure 1.5: The structure of HIV-1 p24.

The molecule shows eleven colour-coded α-helices that constitute the protein (Acquired from Berthet-Colominas & Cusack, 1999 and used with permission from Journal of European Molecular Biology Organisation (EMBO).

1.2.5. Antibodies that target HIV-1 envelope

Among the compounds targeting the HIV-1 envelop, antibodies are the most used (Ulrich *et al*., 2004; Brody and Gold, 2000). They target different sites on the viral envelope and include antibodies such as VRC01, PG9, 2G12, b12, 4E10, 2F5 and PG16 (Zhou *et al*., 2010; Walker *et al*., 2010; Ofek *et al*., 2004). VRCO1 and b12 are broadly neutralising antibodies that bind to the viral CD4 binding site (Zhou *et al*., 2010, Douek *et al*., 2006). The 2F5 and 4E10 antibodies are also broadly neutralising to the virus and recognise the membrane proximal region of gp41 (Douek *et al*., 2006; Cardoso *et al*., 2005; Ofek *et al*., 2004); while 2G12 binds carbohydrate chains of HIV-1 envelope spikes (Botos and Wlodawer, 2005; Calarese *et al*., 2003). Lastly, PG9 and

PG16 are somatically related neutralising antibodies that target an epitope expressed on trimeric HIV Env (Walker *et al*., 2010).

HIV-1 antibodies have been conjugated to different fluorescent dyes for detection of infections. An anti-Rev IgG2b and anti-Tat monoclonal antibodies have been conjugated to FITC and phycoerythrin (PE) for the differentiation of HIV-1 infected from uninfected cells (Rigg *et al*., 1995). In addition, HIV-1 anti-p24 antibody conjugated to FITC was also used to detect intracellular p24 expression after infection (Lee *et al*., 1994).

1.2.6. Detection of HIV-1 by flow cytometry

Flow cytometry is a technique utilized to analyse the physical and chemical characteristics of particles in a fluid that passes through at least one laser. In this technology a number of measurements are made on cells, cell organelles, and other objects such as beads suspended in a liquid and flowing at a rate of several thousand per second through a flow chamber (Sladek and Jacobberger, 1990; Clevenger *et al*., 1985) (Figure 1.6).

Briefly, in flow cytometry cells or beads are suspended in a liquid and after obtaining a single cell suspension, they are stained with antibodies conjugated to fluorescent dyes that bind to the specific features being measured. The stained cells or beads are then allowed to flow through a flow cell and interrogated by the laser beam one at a time. Measurements are made as the cells pass through the beam by light detectors such as photomultiplier and photodiodes (Jaye *et al*., 2012, Brown and Wittwer, 2000).

Flow cytometry has been used for decades for the diagnosis of different infections using antibodies conjugated to dyes. The technique is used to detect HIV-1 infection from measuring p24 antigens using reagents such as FITC-conjugated anti-p24 antibodies. It is also used to detect infections from HIV-1 infected cells expressing viral Rev and Tat using anti-Rev and anti-Tat antibodies conjugated to fluorescent dyes (Rigg *et al*., 1995, Lee *et al*., 1994). Although antibodies are mostly used in flow cytometry, they have various limitations; for example, they are often expensive to generate as they require the use of animals, and some target molecules may have limited immunogenicity (Guo

et al., 2008). Aptamers are alternatives which amongst other advantages are cheaper. They are discussed in the next section.

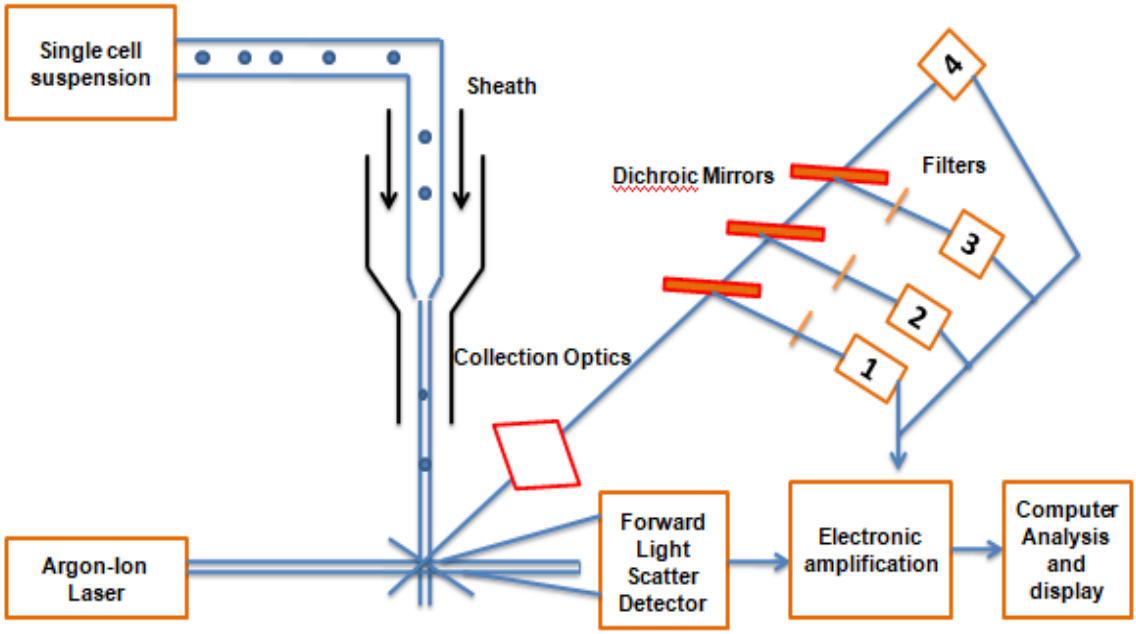


Figure 1.6: An outline of the flow cytometry technique.

The single cell suspension in a sheath and the light detectors are shown. Side scatter (1) and emission detectors (2-4) are also shown in the image. Electronic amplification and conversion to digital form occurs and analysis is performed on a computer screen as indicated (Adapted from Brown and Wittwer, 2000).

1.3. Aptamers

Nucleic acids are very attractive for combinatorial chemistry since they have the ability to fold into defined secondary and tertiary structures and can be easily amplified by polymerase chain reaction (PCR) or *in vitro* transcription (McKeague and Derosa, 2012; Stoltenburg *et al*., 2007). Aptamers are short, synthetic single stranded nucleic acid

(DNA or RNA) oligomers that fold by intramolecular interactions into unique 3-D structures characterized by stems, loops, bulges, hairpins, pseudoknots, triplexes or quadruplexes that allow them to bind with high affinity and specificity to different target molecules (London *et al*., 2015; Zhou *et al*., 2011; Fan *et al*., 2008; Stoltenburg *et al*., 2007; Proske *et al*., 2005) (Figure 1.7).

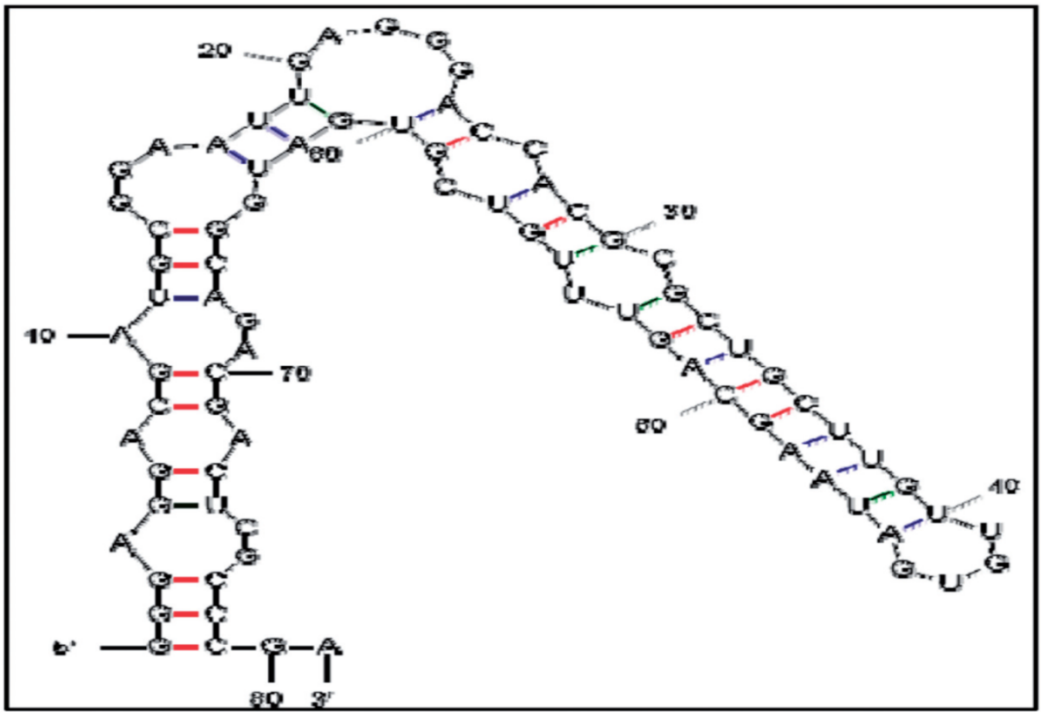


Figure 1.7: A two dimensional structure of an HIV-1 anti-gp120 aptamer.

Acquired from Zhou *et al*., 2009 and used with permission from Oxford University Press.

Aptamers are known to be equivalent to antibodies in their potential as research, diagnosis, and therapeutic reagents (Burnett and Rossi, 2012; Ishida *et al*., 2003, Brody and Gold, 2000). However, aptamers have several advantages over antibodies that include their lack of immunogenicity, they are easy to modify with chemical substitutions and fluorescent dyes, and can be used in the delivery of drugs into target cells (Zhou *et al*., 2011; Ulrich *et al*., 2004). In addition, they are small in size, can retain their function

after truncation and are easy to produce (Mufhandu *et al*., 2012; Burnett and Rossi, 2012; Farokhzad *et al*., 2004). Aptamers have been produced against a variety of targets such as small molecules, peptides, amino acids and proteins (Blind and Blank, 2015, Proske *et al*., 2005, Farokhzad *et al*., 2004). Rong *et al*., isolated RNA aptamers which specifically targets HIV-1 susceptible cells (Rong *et al*., 2016).

Aptamers are isolated from a random ss-library using a technique called SELEX in as little as five to nine rounds of selection (Zeng *et al*., 2017; London *et al*., 2015). Previously, they have been selected against HIV-1 gp120 for the inhibition of its interaction with the CD4 receptor. Some of these aptamers have been reported to inhibit or neutralize HIV-1 infection with 50% inhibition concentration (IC_{50}) values in the nanomolar range (London *et al*., 2015, Mufhandu *et al*., 2012). For example, Zhou *et al*. combined an anti-gp120 aptamer with a si(RNA) to inhibitHIV-1 infection. Similarly, these researchers isolated ss-DNA aptamers against the CD4 receptor that were inhibitory to the viral infection (Zhou *et al*., 2011). Lastly, aptamers specific to p24 have also been produced (Zeng *et al*., 2017) for use in HIV treatment and diagnosis.

1.4. Systematic evolution of ligands by exponential enrichment

The conventional SELEX method that is most commonly used for the selection of aptamers is discussed in section 1.4.1. Other methods that were derived from SELEX are outlined in the sections that follow.

1.4.1. Conventional SELEX method

Here DNA or RNA aptamers are selected from a random nucleic acid library exposed to the target molecule (Guo *et al*., 2008; Stoltenburg *et al*., 2007). This procedure employs a repetitive process of selection and amplification of the high binding oligonucleotides which results in the exponential increase of the best binding aptamers (Walker *et al*., 2009; Wen and Gray, 2004; Tuerk and Gold, 1990).

The amount of selection rounds in this technique depends on the diversity of the library, target features and concentration, ratio of target molecules to oligonucleotides, stringency of selection, and bias of amplification (Glokler *et al*., 2010; Stoltenburg *et al*., 2007). A random ss-DNA library consists of a central randomized region of 20-80 nucleotides, flanked by constant regions used as primer annealing sites for amplification during selection (Khati *et al*., 2003). The first step of selection of DNA aptamers includes amplification of ss-DNA library of about 10^{14} random sequences. The amplification is followed by the digestion of the PCR product to ss-DNA for exposure to the target to allow specific sequences to bind. Bound ss-DNA molecules are then separated from the unbound sequences and eluted from the target molecule. This is followed by PCR and generation of ss-DNA for the next round of SELEX. After every round of selection the percentage of bound aptamers is determined by measuring the concentration of the eluted aptamers that were exposed to the target in that round. The selection is stopped when this percentage reaches a plateau (Figure 1.8) (London *et al*., 2015; Proske *et al*., 2005; Khati *et al*., 2003). Note that the isolation of RNA aptamers is similar to that of DNA aptamers, the difference being that in every round ss-DNA library is converted into RNA before exposure to the target.

1.4.2. Other SELEX techniques

There are different types of SELEX derived from the conventional method laid out by Tuerk and Gold (Tuerk and Gold, 1990). One of them is cell-SELEX that specifically produces aptamers able to recognise and differentiate molecular signatures found on a number of abnormal cells such as cancer cells (Rong *et al*., 2016). This technique can be used to identify new biomarkers. This by employing affinity separation to purify and isolate the aptamer targets which have the potential to be used as biomarkers (Ray and White, 2017, Rong *et al*., 2016). Capillary electrophoresis SELEX is another type of SELEX which selects binding sequences based on a mobility shift induced by the formation of complexes with the target of interest. Capillary electrophoresis SELEX produces aptamers with high affinity and specificity for their target in significantly fewer rounds than the conventional method (Mendonsa and Bowser, 2004).

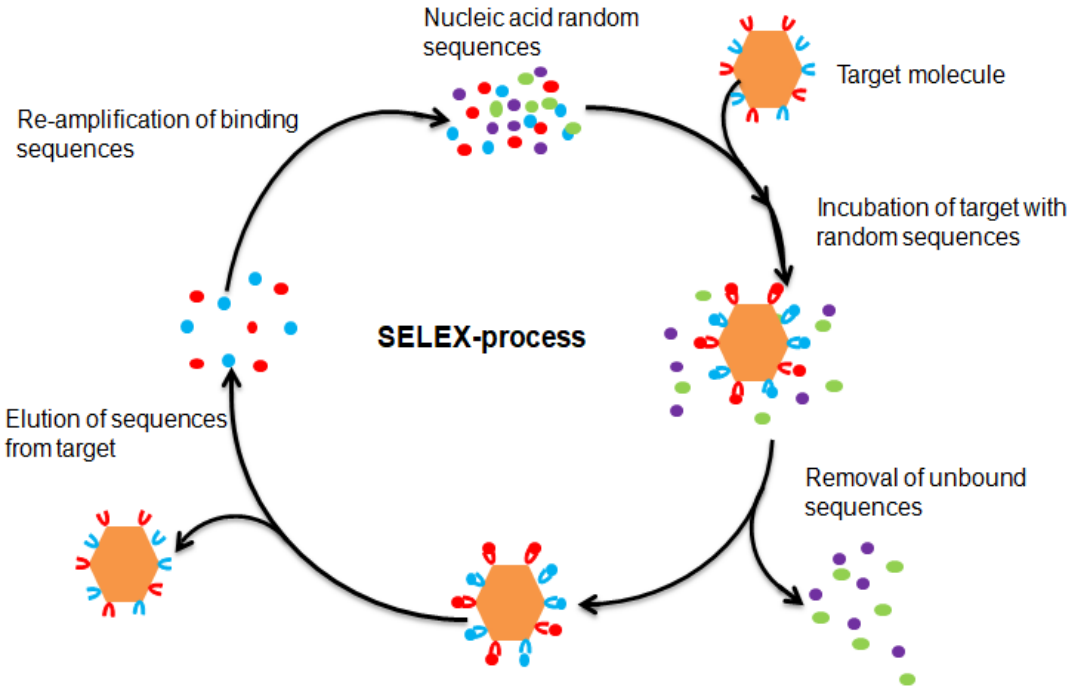


Figure 1.8: A schematic diagram of the SELEX process during aptamer isolation.

The nucleic acids are represented by the mixed coloured circles and the target is represented by the orange circle with binding sides on it. The process starts with the incubation of target with nucleic acid library, then the removal of unbound nucleic acid molecules and ends with the elution of bound species from target and amplification of the library for the next round (adapted from Proske *et al*., 2005).

Other SELEX methods include counter SELEX which was developed by Jenison *et al* (1994). This method improves aptamer selection by excluding RNA molecules which have the same affinity for molecules that are similar to the target (Jenison *et al*., 1994). Photo SELEX, developed by Jenison *et al* (1994), uses photosensitive nucleotides to promote covalent cross link with other molecules under particular wavelengths (Aquino-Jarquin and Toscano-Garibay, 2011, Jenison *et al*., 1994).

1.5. Study aim

Aptamers have been isolated for a number of cell and viral proteins (Mufhandu *et al*., 2012, London *et al*., 2015). However, their conjugation to fluorescent dyes for the purpose of generating low cost reagents that can be used in HIV research or even in diagnosis kits is still largely unexplored. Therefore, this study aimed to conjugate a council for scientific and industrial research (CSIR) owned anti-gp120 RNA aptamers called CSIR1.1 (London *et al*., 2015) and University of California Los Angeles 1 (UCLA1) to FITC for use to detect HIV infected cells by flow cytometry. In addition, the study sought to isolate aptamers against HIV-1 p24 for future conjugation with fluorescent dyes. Since aptamers isolation is inexpensive we expect these reagents to be cheap.

1.6. Study Objectives

- The CSIR aptamer lab holds a patent for HIV-1 parental/original anti-gp120 aptamers. UCLA1 is a derivative of one of the original aptamers called B40 and CSIR1.1 was isolated with the use of the patented method. The first objective of the study was to label these aptamers with FITC for detection of HIV-1 infected cells that express gp120 on their surface by flow cytometry.
- The second objective was to generate anti-p24 aptamers by conventional SELEX for future labelling with fluorescent dyes to detect HIV-1 by flow cytometry.

Chapter 2 : MATERIALS AND METHODS

2.1. Summary

In this chapter detailed descriptions of the methods used in the conjugation of FITC to anti-gp120 RNA aptamers (CSIR1.1 and UCLA1) and the isolation of anti-p24 aptamers are provided. We start off with the methods for the first objective which involves the conjugation of the anti-gp120 aptamers to FITC, followed by fluorescence imaging, flow cytometry and capture assay to confirm conjugation. This is followed by a description of the neutralisation assay method used for comparing the neutralising effects of the FITC-conjugated anti-gp120 aptamer to the parent aptamer (CSIR1.1). The chapter ends by providing methods employed to isolate aptamers targeting HIV-1 p24 (objective two methods). Figure 2.1 is a schematic diagram showing the flow of the methods and assays performed.

In Figure 2.1, different confirmatory assays were performed for anti-gp120 CSIR 1.1 and UCLA1 in order to confirm conjugation to FITC. While this was not ideal, costs and methodological constraints in some instances and the fact that this was an exploratory phase with potential for future studies meant this was acceptable.

2.2. Reagents, cell lines and pseudoviruses

2.2.1. Reagents

The UCLA1 anti-gp120 aptamer was obtained from the University of California, Los Angeles. The CSIR1.1 aptamer was isolated at the CSIR (Pretoria, South Africa) by London *et al*., and is made up of 112 nucleotides in length (London *et al*., 2015). The plasmids to make the ZM53M.PB12, CAP239.2.00.G3J and DU156.12 pseudoviruses were obtained from the National Institute for Communicable Diseases (NICD) (Modderfontein, JHB, South Africa). The ss-DNA library was designed and synthesized by Integrated DNA Technology Inc. (Coralville, IA, USA).

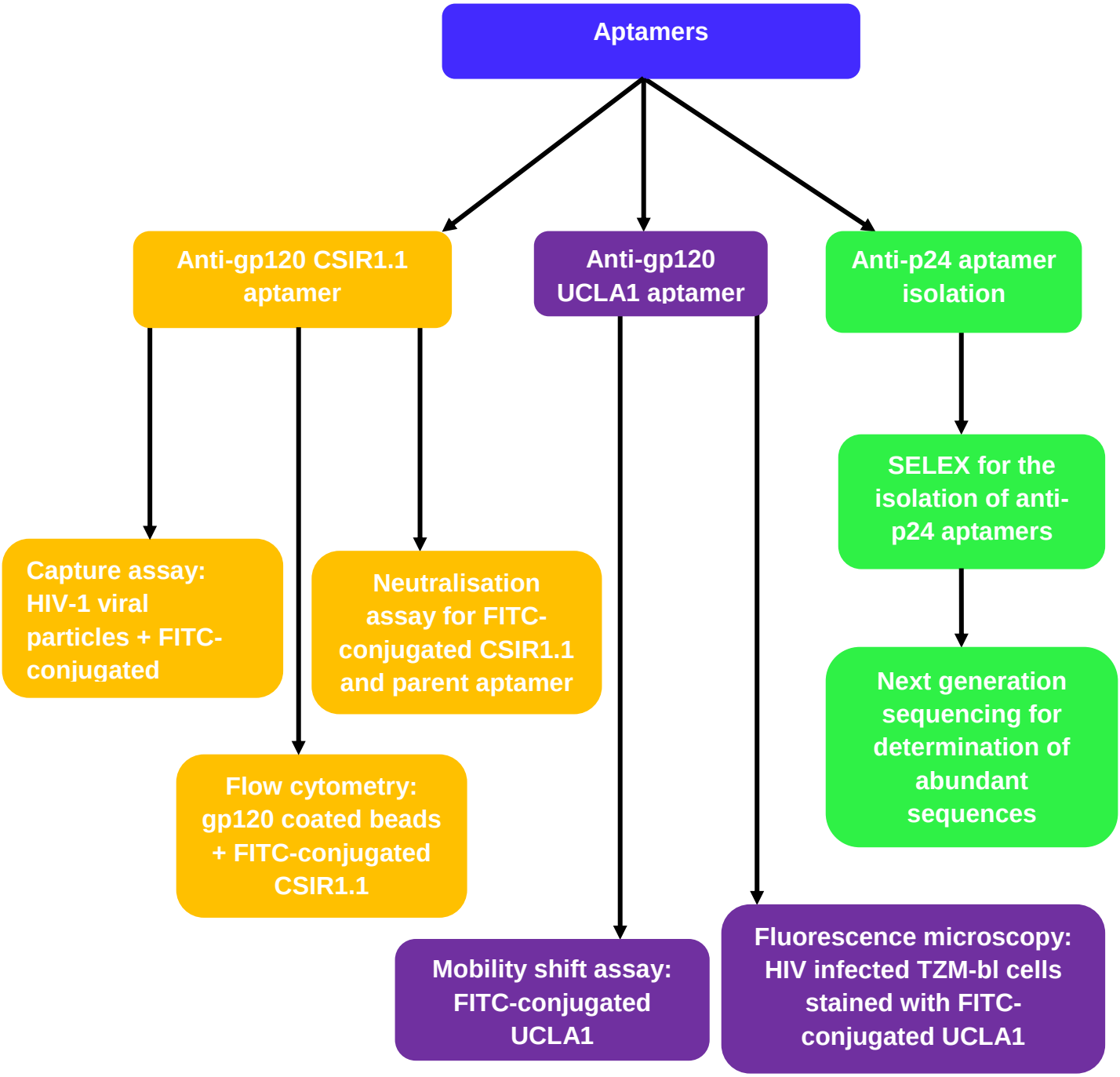


Figure 2.1: Flow diagram of the methods used in this study.

The first objectives were pursued by conjugating anti-gp120 aptamers (CSIR1.1 and UCLA1) to FITC and confirming the conjugation by fluorescence imaging, flow cytometry and capture assay (colour shaded yellow and purple). To investigate the second objective of isolating anti-p24 aptamers, the SELEX method for aptamer isolation was used (illustrated with the green shaded boxes).

The nitrocellulose membrane used for counter selection was purchased from Merck Millipore (Burlington, MA, USA). The PCR reagents for isolating the p24 aptamers were purchased from Promega (Madison, WI, USA).

2.2.2. Cell lines

Two types of cell lines were used in the study namely; TZM-bl and 293T cells obtained from National Institutes of Health (NIH) (Bethesda, MD, USA). TZM-bl cells are HeLa cell clones engineered to express CD4 receptor, as well as CCR5 and CXCR4 co-receptors. These cells also contain integrated reporter genes for firefly luciferase and *E.coli* β-galactosidase regulated by HIV-1 long terminal repeat (Sarzotti-kelsoe *et al*., 2015). The 293T cells are adherent human embryonic kidney cells. Both cell lines were sub-cultured at a concentration of 1×10^6 cells/ml every two days using DMEM supplemented with 10% FBS and gentamycin at 37ºC, 5% CO_2 and 95% humidity.

2.2.3. Pseudoviruses

2.2.3.1. Transfection of Env-pseudotype viruses using 293-T cells

To transfect 293-T cells, 2.5×10^6 cells were added to a 6 cm culture dish, Corning (Riverfront, NY, USA) containing 10 ml DMEM, Sigma Aldrich (St Louis, MI, USA), and incubated overnight until cell monolayer was 80% confluent. To an Eppendorf tube, 2 µg of HIV envelope plasmid DNA and 4 µg envelope backbone plasmid DNA, NICD (Modderfontein, JHB, South Africa) were mixed. The final volume was adjusted to 50 µl with serum free DMEM. To the tube, 326 µl of DMEM and 24 µl of X-tremegene transfection reagent, Sigma Aldrich (St Louis, MI, USA) were added and the reaction was briefly vortexed and incubated at room temperature for 45 minutes. The contents of the reaction were added drop wise to the 293-T cells and mixed gently to ensure uniform distribution across the cell monolayer. The cells were incubated for 48 hours at 37ºC, 5% CO_2 and 95% humidity. The pseudovirion-containing supernatants were harvested and the concentration of FBS was adjusted to 20%. The supernatant containing the pseudovirus was filtered using a 0.45 micron nitrocellulose membrane filter, Merck Millipore (Burlington, MA, USA), aliquoted, and stored at -80°C.

2.2.3.2. Titration of infectious pseudoviruses (TCID assay)

To test the infectivity of the pseudoviruses (ZM53M.PB12, CAP239.2.00.G3J and DU156.12), 150 µl of DMEM media was added to all wells of a 96 well plate, Corning Incorporated (Corning, NY, USA), followed by the addition of 50 µl of pseudoviruses to the first wells. A 4-fold dilution series of the viruses was performed. To the different concentrations of pseudoviruses, 10,000 TZM-bl cells/100 µl/ well were added and incubated at 37 ºC, 5% CO_2 and 95% humidity for 48 hours. After 48 hours incubation, 150 µl of spent media was discarded from the wells and 100 µl Bright-Glo substrate, Promega (Mandison, WI, USA) was added. The cells were incubated at room temperature for 2 minutes to allow complete cell lysis, followed by mixing and the transfer of 150 µl to a corresponding 96 well black plate. The plate was read immediately at 510 nm using the TECAN Infinite F500 Luminometer and i-control 1.5 software, Tecan Group Ltd. (Männedorf, Switzerland).

2.3. Conjugation of anti-gp120 RNA aptamers to FITC for the detection of HIV-1 infected cells expressing gp120 on their surface by flow cytometry.

2.3.1. Interaction between FITC and gp120 aptamers

2.3.1.1. Structures of FITC, UCLA1 and CSIR1.1

FITC is a fluorophore that contains reactive isothiocyanate and carboxylic acid groups (Figure 2.2) that reacts with nucleophiles such as amines. The fluorophore has a molecular weight of 389.38 Da.

UCLA1 is an anti-gp120 RNA aptamer that is 54 nucleotides in length and has a molecular weight of 18,534 Da. The aptamer was derived and truncated from a longer aptamer, B40t77 with a length of 77 nucleotides, which was also a truncated derivative of an RNA aptamer called B40 (117 nucleotides) (Mufhandu *et al*., 2012, Cohen *et al*., 2008).

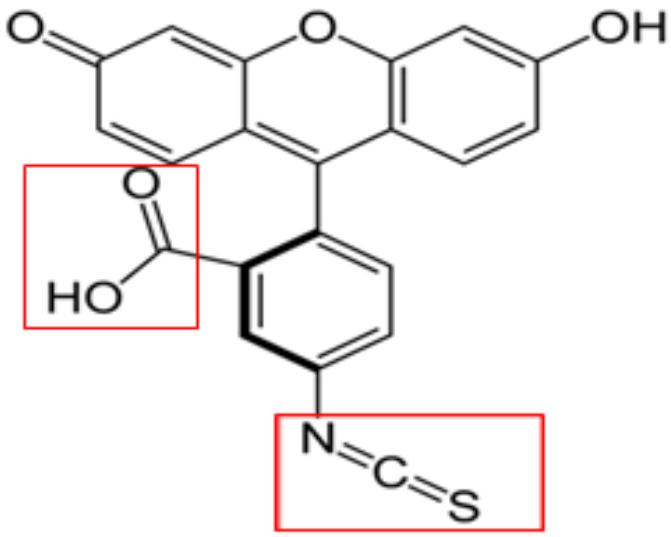


Figure 2.2: The structure of FITC fluorophore.

The reactive isothiocyanate and carboxyl groups are highlighted by the red box.

UCLA1 contains modifications at the 3' and 5' ends (Figure 2.3). These modifications are necessary for the stability of the aptamer and for protection against degradation by exonucleases (London *et al*., 2015; Wang *et al*., 2011). In addition, these modifications enable the restoration of the neutralisation potency of the aptamer relative to the B40 aptamer from which it was derived (Mufhandu *et al*., 2012; Cohen *et al*., 2008).

The modifications include a dimethoxyltrityloxy-(CH2)6-SS-(CH2)6-phospho linker at the 5' end and an inverted deoxy-thymidine (Dt) at the 3' end. UCLA1 interacts with eight amino acid residues on gp120 namely K121, L125, K305, I307, R308, H330, L369 and R419 (Mufhandu *et al*., 2012) and in so doing, reduces and neutralises virus infectivity (Cohen *et al*., 2008). The aptamer was reported to reduce virus infectivity by 82% and neutralise HIV infection by 84% (Cohen *et al*., 2008).

The CSIR1.1, synthesised by London *et al* (2015), is an anti-gp120 RNA aptamer that binds with high affinity and specificity to HIV-1 gp120 (London *et al*., 2015). The aptamer has a molecular weight of 54,672 Da and was selected from a ss-DNA library containing 10^{14} random sequences against whole subtype C HIV-1 CAP_{45} (Figure 2.4).

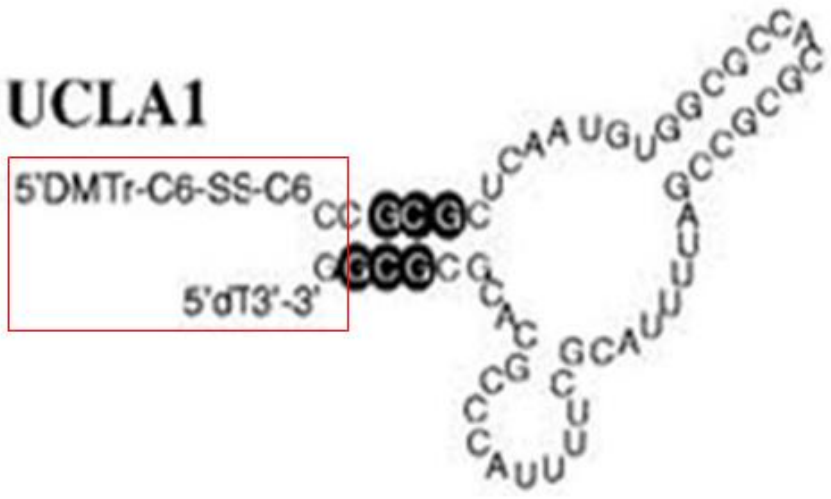


Figure 2.3: The structure and sequence of the anti-gp120 UCLA1 aptamer.

The modifications of the aptamer at the 5' and 3' end are highlighted by the red box (acquired from Cohen *et al*., 2008 and used with permission from American Society for microbiology).

Nine rounds of conventional SELEX were performed to obtain this aptamer, where transcription using the T7 promoter sequence present in the reverse primer sequence and a T7 RNA polymerase was employed to convert the DNA library to RNA. Modifications for its stability involved the use of 2'Flouropyrimidines during synthesis. Compared to the UCLA1 sequence above (Figure 2.3), CSIR1.1 aptamer has fewer modifications, that is, with the 2'Flouropyrimidines only.

2.3.1.2. Expected reactions

The FITC isothiocyanate and carboxyl groups are reactive groups that react with nucleophiles such as amines and hydroxyls (Figure 2.5) (Koniev and Wagner, 2015). A free hydroxyl group is found on the 3' end of nucleic acids when the sequence terminates with unmodified nucleotide; whereas a free amine is found at the 5' end when it has an unmodified purine or pyrimidine. Of note is the fact that within a nucleic acid molecule there are also amine groups attached to different bases (Shapiro *et al.*, 1998). These, however, are unlikely to react with FITC due to steric hindrance caused by their location within the molecule (see Figures 2.3. and 2.4 showing interaction between nucleotides within aptamer molecules).

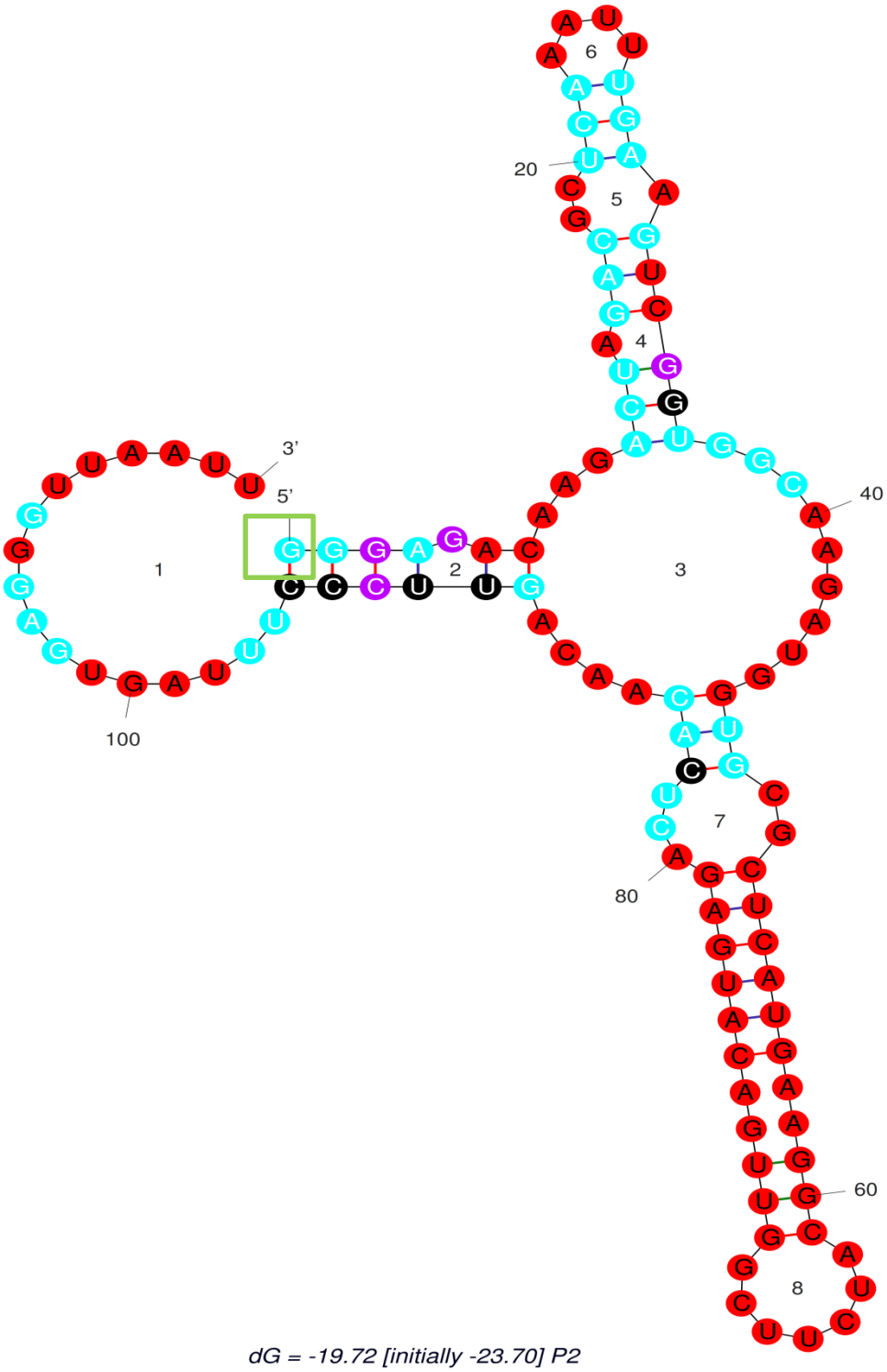


Figure 2.4: The structure and sequence of the anti-gp120 CSIR1.1 aptamer.

The nucleotide containing a nucleophile expected to react with FITC is highlighted by the green box (Acquired from London *et al*., 2015).

Thus, the expectation is that the carboxyl group on the FITC will react with the hydroxyl group at the 3' end or the amine group at the 5' end of the UCLA1 and CSIR1.1 aptamers.

Although nucleic acid molecules do contain amine groups, many researchers modify the aptamer with an amine group or 5'Fluoresceinphosphoramidite at the 5' end to facilitate interaction with fluorophores such as FITC (Nutiu and Li, 2005, Potyrailo *et al*., 1998). One of the main reasons is that free amine is less hindered than the one within the molecule, thus, would react better with the FITC molecule. In this study, the amine groups at the 5' end of the aptamers were not modified with an additional amine group during conjugation (Figure 2.5).

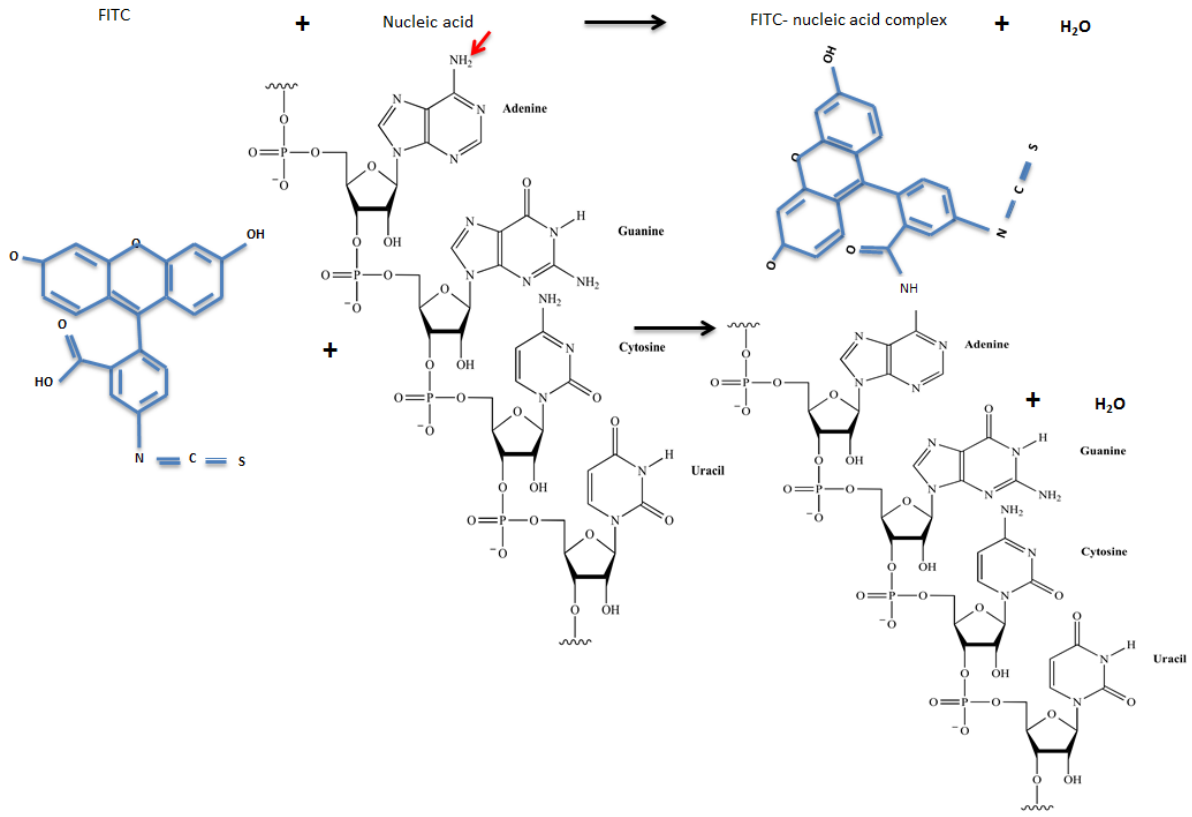


Figure 2.5: The possible reaction between FITC and a nucleic acid (RNA) at the 5' amine group.

The FITC molecule is shown in blue and the reactive amine is also indicated.

2.3.2. FITC-UCLA1 aptamer conjugation to FITC using the FluoroTag™ FITC conjugation kit

To conjugate the anti-gp120 UCLA1 aptamer to FITC, the FluoroTag™ FITC conjugation kit was used Sigma Aldrich (St Louis, MI, USA). Here 0.1 M sodium carbonate–bicarbonate buffer pH 9.4 (0.0125 M of sodium bicarbonate, 0.0875 M sodium carbonate) was made by dissolving the contents of one sodium carbonate–bicarbonate capsule Sigma Aldrich (St Louis, MI, USA) into 50 ml of distilled water. FITC was reconstituted in 2 ml of the buffer. Then 89 µl of anti-gp120 UCLA1 aptamer dissolved in the same buffer was added to a 1.5 ml Eppendorf tube and the volume was made up to 200 µl with the sodium carbonate-bicarbonate buffer. This was followed by the drop wise addition of 50 µl of FITC while stirring for two hours in the dark at room temperature.

2.3.3. Conjugation of FITC to anti-gp120 UCLA1 and CSIR1.1 RNA aptamers using an in-house method

To conjugate UCLA1 and CSIR1.1 aptamers to FITC, 7.5 µl of the aptamers (1750 ng/µl) were mixed with 1.25 mg of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) Sigma Aldrich (St Louis, MI, USA) and 5 µl of FITC (0.25 M) in imidazole (0.1 M, pH 6) Sigma Aldrich (St Louis, MI, USA). The mixture was thoroughly vortexed and centrifuged at 17000 ×*g* for 5 minutes. To the reaction, 20 µl of imidazole (0.1 M, pH 6) was added and mixed using a shaker at room temperature for 48 hours. This was followed by the centrifugation of each conjugated aptamer in the 3,000 and 10,000 molecular weight cut off (MWCO) vivaspin columns Sartorius (Goettingen, Germany) (Figure 2.6) to remove unconjugated FITC as the filters were designed to retain compounds with a mass above 3,000 Da and 10,000 Da respectively.

The FITC-conjugated aptamer was expected to be retained on the filter and the free FITC molecules to pass through. Afterwards, the retained aptamer was washed three times with 0.1 M imidazole by centrifuging for 20 minutes at 3,901 ×*g*. The washed solution was collected and stored at -20°C.The two aptamer solutions were transferred

to different wells of a 96 well black plate and fluorescence was measured using the DTX 880 multimode detector at the wavelength of 485 nm. Unconjugated FITC was used as the positive control.

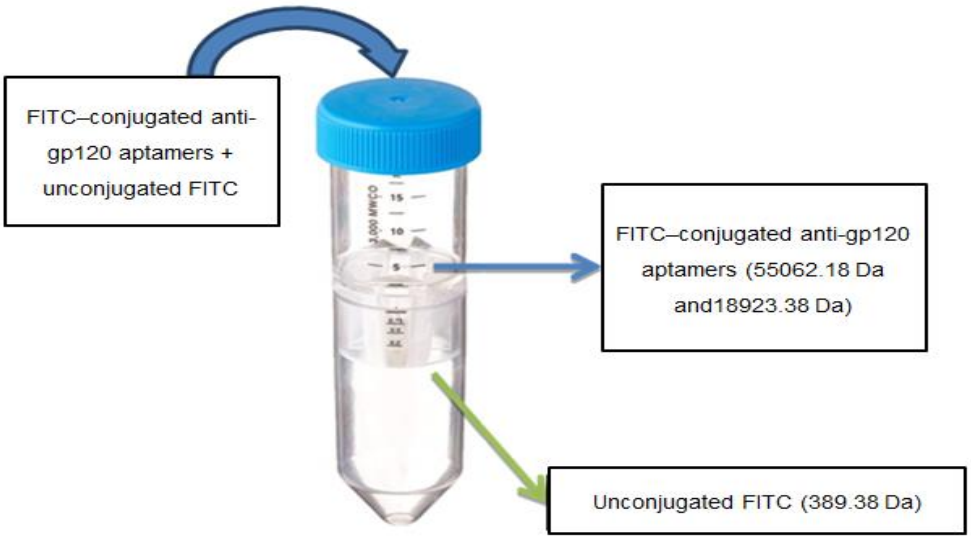


Figure 2.6: A 3000 MWCO vivaspin column for purification of FITC-conjugated anti-gp120 aptamers.

The large blue arrow represents the addition of conjugated aptamers solution, the small one shows where the conjugated FITC-aptamer was retained; while the green arrow indicates where the unconjugated FITC was collected and discarded.

2.3.3.1. Confirmation of conjugation of FITC to UCLA1 and CSIR1.1

2.3.3.1.1. Mobility shift assay to confirm conjugation of UCLA1 to FITC

The assay was performed to detect mobility shift of the conjugated aptamer (UCLA1), which was expected to migrate slower than the unconjugated one due to it having a bigger size. For this assay a urea gel and an agarose gel were used. The urea gel was prepared by mixing 8 M urea Sigma Aldrich (St Louis, MI, USA) with 40% (w/v) bis acrylamide Sigma Aldrich (St Louis, MI, USA), 10× Tris-Borate-EDTA (TBE) buffer Sigma Aldrich (St Louis, MI, USA), and distilled water. The gel was then solidified by adding 10% ammonium persulfate (APS) and tetramethylethylenediamine (TEMED)

Sigma Aldrich (St Louis, MI, USA). The gel was set between two casting plates with a 10 well comb Bio-Rad (Hercules, CA, USA) inserted between them to create the wells to which 15 µl of sample, containing 5 µl 2× RNA gel loading dye Thermo Scientific (Waltham, MA, USA) and 10 µl of FITC-conjugated UCLA1 aptamer and free aptamer, were loaded. The gel was placed in a vertical electrophoresis gel tank Bio-Rad (Hercules, CA, USA) containing 1× TBE buffer and ran at 200V for 30 minutes prior to loading the samples. Then the samples were loaded to the wells; and 15 µl RiboRuler Low Range RNA ladder Thermo Scientific (Waltham, MA, USA) was also loaded, in the first well of the gel. This was followed by running the gel at 200 V for 30 minutes using a PowerPac High-Current supply Bio-Rad (Hercules, CA, USA).

The agarose gel was prepared by weighing 4 g of agarose powder Sigma Aldrich (St Louis, MI, USA) followed by dissolving it in 100 ml of TBE buffer. Afterwards, 10 µl of ethidium bromide was added to the gel for visualisation. The gel was then solidified in a gel casting tray at room temperature before loading the samples containing 5 µl of 2× RNA gel loading dye and 5 µl of the FITC-conjugated UCLA1 aptamer and unconjugated aptamer. Also 10 µl RiboRuler Low Range RNA ladder Thermo Scientific (Waltham, MA USA) was loaded in the first well of the gel, which was run at 200 V for 30 minutes at room temperature in TBE buffer. Both the urea and agarose gels were viewed on a gel Doc™ XR+ System using the Image Lab™ Software Bio-Rad (Hercules, CA, USA).

2.3.3.1.2. Detection of fluorescence emitted by FITC-conjugated aptamer (UCLA1) using HIV-1 infected TZM-bl cells

To check if the conjugation was successful, fluorescence microscopy imaging was performed using TZM-bl cells infected with HIV-1 ZM53M.PB12. Here cells were permeabilised with DEAE-dextran Sigma Aldrich (St Louis, MI, USA) and cultured in a flat bottom 96 well plate at the concentration of 10 000 cells/well/100 µl of growth medium i.e. Dulbecco's modified eagle's medium (DMEM) Thermo Scientific (Waltham, MA, USA) supplemented with 10% FBS Thermo Scientific (Waltham, MA, USA) and gentamicin Sigma Aldrich (St Louis, MI, USA) as the antibiotic. This was followed by infection with 200 tissue culture infectious dose that causes 50% infection ($TCID_{50}$) of

ZM53M.PB12 pseudovirus; similar to what has been done before in neutralisation assays (Alexandre *et al*., 2013; Alexandre *et al*., 2012). The reaction was incubated for 48 hours at 37°C, 5% CO_2 and 95% humidity.

The infected cells were stained with the FITC-conjugated anti-gp120 UCLA1 aptamer before imaging using a Cytation 3 Cell Imaging Multi-mode reader BioTek (Winooski, VT, USA). The staining procedure was performed by first removing the growth medium and washing the plate three times for 10 minutes with 100 µl PBS BioUltra pH 7.4 Sigma Aldrich (St Louis, MI, USA). The cells were then incubated with 100 µl of 4% paraformaldehyde (PFA) Sigma Aldrich (St Louis, MI, USA) for 10 minutes. Afterwards, the PFA was discarded and fixed cells were washed three times with PBS BioUltra pH 7.4 for 10 minutes with shaking. Then 100 µl/well of a 3-fold serial dilution of the conjugated aptamer starting with the concentration of 8.96×10^{-7} M in 10% goat serum Gibco (Waltham, MA, USA) was added to the cells.

This was followed by 1 hour incubation in the dark at room temperature. The cells were washed three times with PBS BioUltra pH 7.4 for 10 minutes followed by cell nuclei staining with DAPI at a concentration of 10 mg/ml Sigma Aldrich (St Louis, MI, USA). To remove excess DAPI, cells were washed three times with PBS BioUltra pH 7.4 before imaging at 488 nm using the Cytation 3 Cell Imaging Multi-mode reader Biotek (Winooski, VT, USA) with Gen5 2.08 software.

2.3.3.1.3. Detection of fluorescence emitted by FITC-conjugated aptamers (UCLA1 and CSIR1.1) using 293-T cells transfected with HIV-1 genome

The 293-T cells express HIV-1 gp120 on their cell membrane upon transfection with a plasmid encoding the glycoprotein. Thus, 2.5×10^6 cells were added into a 6 cm culture dish Corning (Riverfront, NY, USA) containing 10 ml of DMEM. The cells were allowed to grow overnight until the monolayer was between 50-80% confluent. To a sterile Eppendorf tube, 2 µg of ZM53M.PB12 virus plasmid, 4 µg of backbone plasmid, 24 µl of Xtremegene transfection reagent Sigma Aldrich (St Louis, MI, USA) and 650 µl of plain DMEM were mixed and incubated for 45 minutes. The contents of the tube were added drop wise to the cell monolayer and the cells were then incubated for 48 hours at 37°C

and 5% CO_2. After the incubation, the supernatant was discarded to remove gp120 associated with budded pseudoviruses, leaving only those on the cell membrane. This was followed by the transfer of 10,000 infected 293-T cells/100 µl of growth medium per well of a 96 well flat bottom plate. The cells were allowed to grow overnight in a 37°C and 5% CO_2 incubator. Again the supernatant was removed and cells were stained with FITC-conjugated UCLA1 and CSIR1.1 aptamers as described above in section 2.3.3.1.2 and fluorescence of the samples was analysed by DTX 880 multimode detector Beckman Coulter (Brea, CA, USA).

2.3.3.1.4. TZM-bl neutralisation assay

This assay measures HIV-1 neutralisation in TZM-bl cells as a function of reductions in luciferase reporter gene expression after a single round of pseudovirus infection (Montefiori, 2005). TZM-bl cells are engineered to highly express the CD4, CCR5 and CXCR4 receptors making them very susceptible to HIV-1 (Montefiori, 2005). These cells also contain integrated reporter genes for luciferase expression controlled by HIV-1 long terminal repeat (LTR) (Montefiori, 2005). In this assay, a 3-fold dilution series of the inhibitor (CSIR1.1 FITC-conjugated aptamer or the parent unconjugated aptamer) was prepared in 100 µl of DMEM containing 10% FBS. The dilution series started with 11.9 nM concentration of each aptamer. This was followed by the addition of 50 µl of HIV-1 CAP239.G3J pseudovirus that is known to be sensitive to these aptamers. The negative control wells included uninfected and untreated infected TZM-bl cells.

The contents of the plate were incubated for an hour at 37°C, 5% CO_2 and 95% humidity. After an hour, 1×10^5 TZM-bl cells in 100 µl of growth medium were added per well and incubated at 37°C, 5% CO_2 and 95% humidity for 48 hours. This was followed by the removal of 150 µl of the growth media and the addition of 100 µl of Bright Glo substrate solution Promega (Mandison, WI, USA). The contents of the plate were then incubated in the dark for 2 minutes at room temperature to allow for cell lysis. This was followed by the transfer of 150 µl of each well to the corresponding wells of a black 96 well plate for luminescence readings at 510 nm using the TECAN Infinite F500 Luminometer and i-control 1.5 software Tecan Group Ltd. (Männedorf, Switzerland).

The IC_{50} value of each aptamer was calculated as the inhibitory concentration that causes 50% reduction of relative light unit (RLU) compared to the virus control (wells with no inhibitor) after subtraction of the background (wells without both the virus and the inhibitor).

2.3.3.1.5. Detection of gp120 coated latex beads with the FITC-conjugated aptamer using flow cytometry

The conjugation of the anti-gp120 CSIR1.1 aptamer to FITC was confirmed by flow cytometry. Initially, neutravidin beads were used for this confirmation but due to significant auto-fluorescence, the 0.2 µm neutravidin beads were replaced with the 0.3 µm latex beads Sigma Aldrich (St Louis, MI, USA). This work was performed to further confirm the ability of FITC-conjugated aptamer to bind gp120. Here, the latex beads were diluted to a 1% working stock solution in double distilled water (ddH_2O). Then 10 µl of gp120 at a concentration of 1.29 mg/ ml was added to 4 µl of the 1% latex beads, and the solution was incubated overnight at 4°C. This was followed by washing the coupled beads and blocking twice with 500 µl of 0.1% BSA-PBS Sigma Aldrich (St Louis, MI, USA) through centrifugation at 17,000 ×g. The beads were then re-suspended in 700 µl of the 0.1% BSA-PBS.

The beads were then transferred to flow cytometry tubes and centrifuged at 3,901 ×g for 5 minutes and then re-suspended in 100 µl of 1× BD binding buffer Becton Dickinson (Franklin Lakes, NJ, USA). The four test tubes contained gp120 coated (or coupled) beads stained with a three-fold dilution series of FITC-conjugated CSIR1.1 aptamer starting at 869 nM. The control tubes were made of gp120 coated beads stained with a three-fold dilution series of FITC also starting at 869 nM, uncoated beads stained with the dilution series of FITC-conjugated CSIR1.1 aptamer, and uncoated beads stained with the dilution series of FITC. Fluorescence was subsequently measured using BD LSRFortessa cell analyser Becton Dickinson (Flanklin Lakes, NJ, USA) across the FITC channel with an excitation of 485 nm and 10,000 events recorded for each sample. The flow data was analysed using FlowJo version 10 FlowJo LLC (Ashland, OR, USA).

2.3.3.1.6. Detection of whole HIV-1 particle with FITC-conjugated aptamer using the capture assay

The ability of the CSIR1.1 FITC-conjugated anti-gp120 aptamer to bind gp120 on HIV-1 particle was determined using the capture assay. This was performed as previously described with some modification (Alexandre *et al.*, 2010). A high binding black 96 well plate Corning (Riverfront, NY, USA) was coated overnight at 4°C with 1 µg/ml of the anti-gp41 monoclonal antibody 10E8. Because the FITC-conjugated CSIR1.1 aptamer binds to gp120 (London *et al*., 2015), the gp41 10E8 monoclonal antibody was used to avoid competitive binding.

After coating with the antibody, the plate was washed three times with PBS at room temperature to remove unbound antibodies. This wash was followed by blocking with 3% BSA for 2 hours at 37°C. The blocking reagent was discarded; then HIV-1 pseudovirus was added and incubated for 2 hours at 37°C to allow binding to 10E8. HIV-1 ZM53.PB12, CAP210.G3J, and Du156.12 were used as they are known to strongly bind the CSIR1.1 aptamer (Grace London PhD thesis, 2013). After 2 hours, unbound viruses were washed with PBS three times at room temperature. This was followed by the addition of a three-fold dilution series of FITC-conjugated CSIR1.1 aptamer, starting at 50 µg/ml, and one hour incubation at 37°C.

Biotinylated gp120 antibody and FITC-conjugated streptavidin were used in this study as the positive controls while the negative control was the well containing everything minus the fluorescent stain. Then the plate was washed with PBS three times and fluorescence was measured at 480 nm using the DTX 880 multimode detector Beckman Coulter (Brea, CA, USA). The excitation wavelength was 485 nm while the emission occurred at 535 nm. SoftMaxPro version 6.4 was used for data acquisition and analysis.

2.4. Isolation of anti-p24 aptamers by SELEX

The SELEX procedure was performed for 8 rounds in order to obtain aptamers that can bind to HIV-1 p24 antigen i.e. the assays described below from paragraph two of section 2.4.1 to section 2.4.6 were repeated 8 times.

Table 2.1 is a summary of the assays done for the different aptamers (also reflected in Figure 2.1).

Table 2.1: Summary of experiments performed for UCLA1 and CSIR 1.1 aptamers

Assay Type	UCLA1	CSIR1.1
Mobility shift assay	√	
Flow cytometry (beads)		√
Fluorescent microscopy (imaging with TZM-bl cells)	√	
Fluorescence (DTX 880 multimode reader using 293T cells)	√	√
Capture assay		√
Neutralisation assay (TZM-bl cells)		√

2.4.1. Amplification of the aptamer library by Polymerase chain reaction

A random DNA library consisting of 10^{14} oligonucleotide sequences, each containing a 49 nucleotides random region flanked by identical primer binding sites on the 5' and 3' ends was used during SELEX. The total length of each sequence in the library was 134 bases. The polymerase chain reaction (PCR) was performed using the DNA Engine Peltier Thermal cycler Bio-Rad (Hercules, CA, USA).

The following primers in the 5’ to 3’ direction were used for amplification of the library; (Forward) AAT TAA CCC TCA CTA AAG GGA ACT GTT GTG AGT CTC ATG TCG AA and (Reverse) TAA TAC GAC TCA CTA TAG GG AGA CAA GAC TAG ACG CTC AA. For SELEX or selection of aptamers a different primer set from the one described above was used: (Forward) GCCTGTTGTGAGCCTCCTAAC and (reverse) 5’-phosphate (PO_4)-GGGAGACAAGAATAAGCATG, Inqaba Biotech (Muckleneuk, PTA, South Africa). The reverse primer contained a 5’ phosphate group needed for exonuclease digestion.

The PCR reactions consisted of 1 × Taq Polymerase buffer Promega (Mandison, WI, USA), 25 mM $MgCl_2$, 10 mM dNTP Thermo Scientific (Waltham, MA USA), 100 µM Forward primer Inqaba Biotech (Muckleneuk, PTA, South Africa), 100 µM reverse primer Inqaba Biotech (Muckleneuk, PTA, South Africa), 5 U/µl Taq Polymerase enzyme and 12 ng/µl DNA aptamer library. For master mix preparation see Appendix B, Table B1. The template was denatured at 94°C for 5 minutes followed by 8 rounds of PCR with a denaturing step at 95°C for 45 seconds, an annealing step at 55°C for 45 seconds and an elongation step at 72°C for 45 seconds. A final extension step was performed at 72°C for 5 minutes.

To verify whether the PCR amplification of the library gave products of correct size we ran on a 2% agarose gel containing 10 µl ethidium bromide Sigma Aldrich (St Louis, Missouri, USA). The gel was viewed on a molecular imager ChemiDoc XRS imaging system Bio-Rad (Hercules, CA, USA). To determine the size of the sequences in base pairs (bp) a low range DNA ladder (O'GeneRuler Low Range DNA Ladder, Ready-to-Use 25-700 bp) Thermo Scientific (Waltham, MA, USA) was run on the same gel. The PCR product was washed using a Promega Wizard PCR clean-up Promega (Mandison, WI, USA) followed by quantification on Nanodrop 1000 spectrophotometer Thermo Scientific (Waltham, MA, USA) using the 3.71 software.

2.4.2. ss-DNA generation by exonuclease digestion

Lambda exonuclease is a 5' to 3' exodeoxyribonuclease that specifically digests the phosphate at the 5' end of the ds-DNA incorporated in one of the primers during PCR as described above in Section 2.4.1. Figure 2.7 below shows the process that occurs during exonuclease digestion of a ds-DNA. Thus, after the PCR amplification with the phosphate labelled reverse primer, 15.6 µg of ds-DNA was incubated with, 50 U of lambda exonuclease and 10 µl of lambda exonuclease buffer Biolabs (Ipswich, MA, USA). The reaction was made up to 100 µl with double distilled water and incubated for 6 hours in a 37°C water bath. The reaction was terminated by incubation at 75°C for 10 minutes. The ss-DNA was then extracted by phenol: chloroform extraction. For partially digested ds-DNA, a Promega Wizard gel and PCR clean-up kit Promega (Mandison, WI, USA) was used to extract the ss-DNA from the gel according to the manufacturer's protocol.

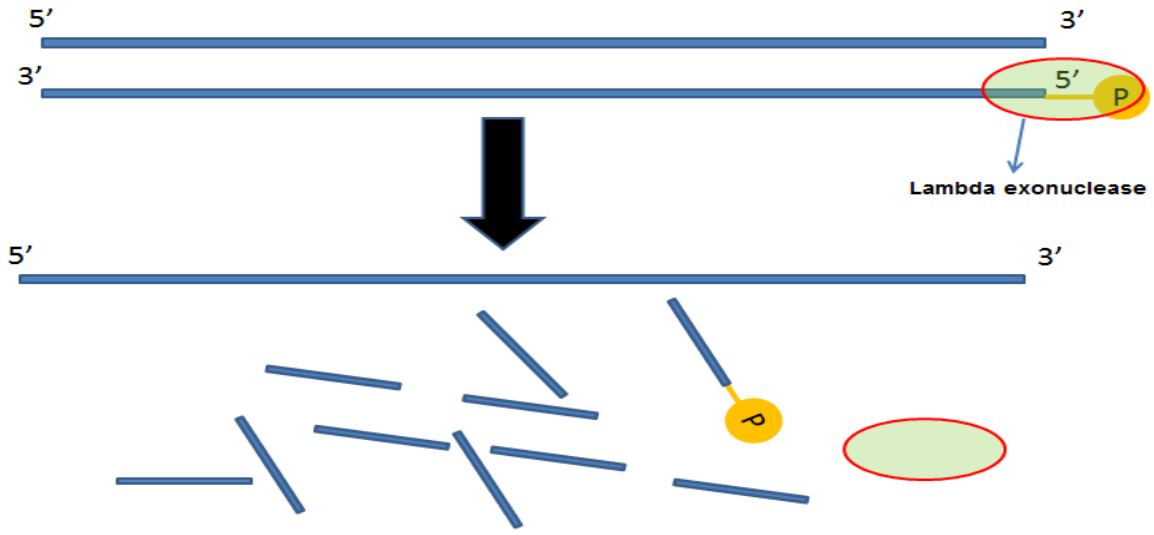


Figure 2.7: Lambda exonuclease digestion

The DNA strand to be digested contains a phosphate group at the 5' end. The exonuclease enzyme is represented by the red oval, whereas the chopped lines represent the digested DNA strand. The digestion by the exonuclease starts at the 5' end containing a phosphate group and ends at the 3' end of the DNA molecule.

2.4.3. Phenol: chloroform extraction

Phenol: chloroform is an extraction method that separates nucleic acids from proteins. During SELEX, the method is used to separate the ss-DNA sequences from the lambda exonuclease enzyme used in the digestion (Avci-Adali *et al*., 2010). To extract the single stranded DNA, 600 µl of phenol: chloroform: isoamyl alcohol (25:24:1) (saturated with 10 mM Tris, 1 mM EDTA, pH 8) Sigma Aldrich (St Louis, MI, USA) was added to the digestion reaction, briefly vortexed and incubated for 30 minutes at room temperature. After the incubation, 200 µl of double distilled water was added followed by vortexing and centrifuging at 13,300 rpm for 5 minutes. The aqueous layer containing the ss-DNA was extracted. The extraction process was repeated followed by combining the aqueous layers.

To the final aqueous solution, a 1:1 volume of phenol: chloroform was added and the solution was centrifuged as above. To precipitate the DNA, the aqueous layer was extracted by adding 0.1 × volume of sodium acetate (3 M, pH 5.2) Sigma Aldrich (St Louis, MI, USA), and 2.5 × volume of 99% (v/v) ethanol Lasec (Midrand, JHB, South Africa). The solution was then left overnight at -80°C. After the incubation it was centrifuged at 13,300 rpm for 30 minutes at 4°C, followed by ethanol precipitation for 10 minutes using 500 µl of ice cold ethanol (70%). The supernatant was discarded and the pellet left to dry at room temperature. The ss-DNA was then re-suspended with 30 µl double distilled water and quantified on the Nanodrop spectrophotometer. To verify the product, samples were run on a 4% agarose gel at 200 V.

2.4.4. Negative selection

Negative selection was performed to eliminate sequences that did not bind to the p24 target protein. The nitrocellulose membrane was employed in negative selection to eliminate sequences that bind to it and not to the target protein. This was performed at round 1 and 4 using a 0.45 µm nitrocellulose membrane Merck Millipore (Burlington, MA, USA). Also gp120 was used for negative selection at round 5 given that it is the most prominent protein on the virus (Wyatt *et al*., 1998). To the extracted ss-DNA after

negative selection, 40 µl of binding buffer (20 Mm Hepes, 150 mM NaCl, 2 mM KCL, 2 mM $MgCl_2$ and 2 mM $CaCl_2$, pH 7.4) and 130 µl of double distilled water was added. The DNA was then heat denatured for 10 minutes at 95°C and immediately cooled on ice for 5 minutes, followed by a further incubation at room temperature for an additional 5 minutes.

2.4.5. Positive selection

For positive selection the target protein p24 was exposed to the ss-DNA library derived from the negative selection. Here, to allow sequences that are specific to p24 to bind, 20 µl of the protein (10 µg/ml) was added to the supernatant from the negative selection and incubated at room temperature for an hour on a shaking incubator. Afterwards, the reaction was loaded on a nitrocellulose membrane moistened with a 1× HMCKN buffer (20 mM Hepes, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM KCl, 150 mM NaCl; pH 7.4) and locked in a 10 ml syringe. This was followed by washing the membrane with 300 µl of 1× HMCKN buffer to allow only those sequences that bind p24 to remain attached to the membrane and the unbound ones to wash through.

2.4.6. DNA elution

The membrane that had the p24-ss-DNA complex from the positive selection was placed on a clean glass plate, cut into eight pieces and transferred into an Eppendorf tube. To allow the bound DNA sequences to elute from the p24 protein, 200 µl of 8 M urea was added to the membrane and the reaction was heated at 100°C for 5 minutes. Then the phenol: chloroform was performed as described in Section 2.4.3. The bound DNA was quantified by measuring the concentration using a Nanodrop.

To confirm the size of the eluted ss-DNA, a 4% agarose gel (in TAE buffer containing 108 g Tris, 55 g Boric acid and 0.5 M Na_2 EDTA, pH 8.0) was run. Ethidium bromide (10 µl) Sigma Aldrich (St Louis, MI, USA) was added to the gel to allow visualisation of the DNA. The gel was run for 30 minutes at 200 V. The visualisation was achieved using

the molecular imager ChemiDoc XRS+ imaging system and the quantity one 4.6.9 analysis software Bio-Rad (Hercules, CA, USA).

2.5. Next generation sequencing of anti-p24 aptamers

To determine the evolution of p24 specific aptamer sequences, ss-DNA from round 2, 3, 4, 5, and 8 were first amplified by PCR and their concentrations measured using Qubit Fluorometer at 485 nm Invitrogen (Carlsbad, CA, USA), which measures RNA and DNA concentrations with high accuracy and sensitivity. The Qubit Fluorometer results are shown in Table 2.1 below. This was followed by library preparation where the DNA was tagged and fragmented with a Nextera XT transposome from a Nextera XT Sample Preparation Kit Illumina (San Diego, CA, USA). The transposome fragmented the input DNA and added adapter sequences to allow amplification. PCR was performed under the following conditions; 72°C for 3 minutes, 95°C for 30 seconds and 15 cycles of denaturing at 95°C for 10 seconds, followed by an annealing step at 55°C for 30 seconds and an elongation step at 72°C for 30 seconds.

A final extension step was performed at 72°C for 5 minutes. The PCR product was purified using AMPure XP PCR clean-up kit Whitehead Scientific (Modderfontein, JHB, South Africa) followed by gel electrophoresis for the verification of the size of the DNA fragments against a DNA ladder. The fragments were visualized under ultraviolet light using a Gel Doc XR+ Molecular Imager System Bio-Rad (Hercules, CA, USA). The concentration of the DNA was measure using Qubit Fluorometer (Table 2.2) and the size of library preparation was verified using Agilent 2100 Bioanalyser Agilent Technologies (Santa Clara, CA, USA). A sample sheet for setting up a sequencing run on the Miseq instrument Illumina (San Diego, CA, USA) was created using IEM software. The library samples were normalized to 2 nM pooled and denatured to create a sequencing library followed by a MiSeq sequencing run using a MiSeq Reagent Kit Illumina (San Diego, CA, USA) and a MiSeq Control Software.

2.6. Statistical analysis

Unless stated otherwise, three independent experiments were performed for each assay. The graphs were obtained using GraphPad Prism 5, Microsoft excel 2010 and FlowJo software. The data represent the mean value for all experiments and error bars represent the standard deviation of the mean.

Table 2.2: The Qubit concentrations (initial and after library preparation) for the 5 selected rounds of SELEX.

Sample ID	Initial concentration (ng/µl)	Concentration after library Prep (ng/µl)
R4	17.1	45.0
R5	18.7	46.9
R2	10.6	42.6
R3	6.83	33.3
R8	9.92	50.0

Chapter 3 : RESULTS AND DISCUSSION

3.1. Summary

The first part of this chapter reports on the results obtained for objective one of the study, which involved the conjugation of anti-gp120 RNA aptamers (UCLA1 and CSIR1.1) to FITC. To confirm the success of the conjugation between FITC and UCLA1, a mobility shift assay was performed using urea and agarose gels. The assay gives information as to whether the conjugation was successful based on the increased size and slow mobility of the conjugated nucleic acid. It was expected that the molecular weight of the FITC-conjugated UCLA1 aptamer will be much higher compared to the parent aptamer, and thus will migrate slower.

Fluorescence imaging of HIV-1 infected cells was further used as another technique of confirming UCLA1 conjugation to FITC, while the conjugation of FITC to CSIR1.1 was confirmed using flow cytometry, and virus capture assay. In addition, the TZM-bl neutralisation assay was performed to determine the structural integrity of FITC-CSIR1.1 aptamer conjugation.

The second part of this chapter covers the results obtained for the last objective of the study which involved the isolation of anti-p24 aptamers. The chapter ends with data on deep sequencing that was performed to determine the most abundant sequences, especially in the last round of selection.

3.2. Confirmation of anti-gp120 UCLA1 aptamer conjugation to FITC

3.2.1 Mobility shift assay for FITC-conjugated UCLA1 aptamer using urea and agarose gels

Following the conjugation procedure, mobility shift assays using a urea gel (Figure 3.1A) and an agarose gel (Figure 3.1B) were performed to confirm the conjugation of the aptamer with FITC. With this assay the success of the conjugation can be

determined by the difference in mobility of the conjugated and unconjugated aptamers through the gel.

The FITC-conjugated aptamer should have had a higher molecular weight and therefore should migrate slower compared to the parent aptamer. However, both urea (Figure 3.1A) and agarose (Figure 3.1B) gels indicated no mobility shift between the unconjugated and the FITC-conjugated UCLA1 aptamer. Different FITC to UCLA1 ratios (20:1, 10:1 and 5:1) were tried and the outcome was the same i.e. the mobility of the conjugated and unconjugated aptamers were similar. This meant two things: one that the conjugation of FITC with UCLA1 was unsuccessful or that the mass of FITC was too small to make a noticeable difference in the aptamer migration through the gels.

The mobility shift assay is not without limitations. Some of the limitations include the fact that multiple bands or smears on the gel due to co-migration of complexes may complicate analysis of results. In addition, dissociation during electrophoresis could occur since samples are not at equilibrium during the run, hence preventing detection (Holden and Tacon, 2011).

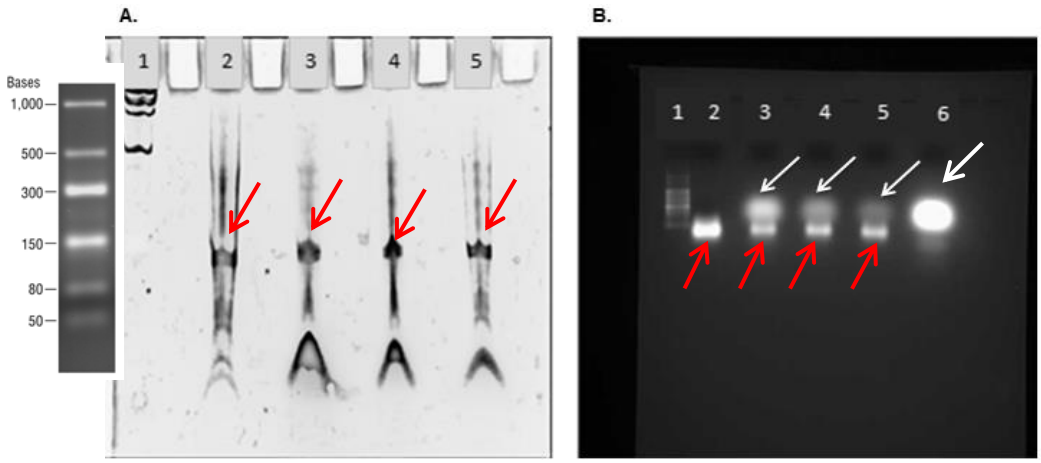


Figure 3.1: The confirmation of FITC-UCLA1 conjugation by mobility shift using urea and agarose gels.

An 8 M urea gel (A) and 4% agarose gel (B) were run. For both gels, lane 1 contains RNA molecular weight markers, lane 2 the parent aptamer, lane 3 has FITC: UCLA1 at a ratio of

20:1, lane 4 at a ratio of 10:1 and lane 5 at a ratio of 5:1. Lane 6 in the agarose gel is FITC only. The red arrows show the UCLA1 aptamer, whereas the white arrows show the FITC only.

The unconjugated FITC in lane 6 of the agarose gel (Figure 3.1B) migrated the same distance as the bands indicated by white arrows in lanes 3, 4 and 5. This shows that some of UCLA1 aptamer and FITC are in solution as separate entities.

3.2.2. Confirmation of conjugation of anti-gp120 UCLA1 aptamer to FITC by fluorescence microscopy

The conjugation of FITC with the aptamer was also investigated using fluorescence microscopy to detect stained TZM-bl cells after their infection with HIV-1 ZM53M.PB12. The idea here was that infected cells will have viral gp120 on their surface resulting from the fusion of HIV-1 envelope with the cell membrane. The fluorescence emitted by cells stained with the conjugated aptamer was compared to cells incubated with the parent aptamer and with FITC only.

When the cells were imaged there was a lot of background. To be precise, the fluorescence emitted by uninfected and infected cells stained with FITC-conjugated UCLA1 was the same. It is possible that the aptamer bound these cells without specificity or that the emission was due to auto-fluorescence from the cells (see Appendix A, Figure A1.1). This is one of the major drawbacks of fluorescence imaging i.e. the negative effects of background fluorescence on the quality of the image (Peng *et al*., 2017; Poland *et al*., 2016). This negativity may include non-specific auto-fluorescence which masks the specific fluorescent label (Cowen *et al*., 1985).

The data for confirming UCLA1 conjugation using the mobility shift assay and fluorescent microscopy suggested that there was no conjugation. As indicated above, these techniques are not without limitations and the assays probably required additional optimization or the use of alternative assays, results for some of which are provided below for CSIR1.1.

3.3. Comparison of fluorescence emitted by UCLA1 and CSIR1.1 aptamers

The UCLA1 aptamer was the first aptamer for which conjugation with FITC was attempted. Due to the absence of efficient conjugation, a different method of conjugation for both UCLA1 and CSIR1.1 was employed for comparison purposes.

Different concentrations of UCLA1 and CSIR1.1 aptamers were conjugated with FITC as explained in section 2.3.3. Each conjugated aptamer was centrifuged in the 3,000 MWCO (Figure 3.2A) and 10,000 MWCO (Figure 3.2B) vivaspin columns to remove unconjugated FITC. Fluorescence of the FITC-conjugated aptamers was measured using the DTX 880 multimode detector at the wavelength of 485 nm. Unconjugated FITC was used as the positive control. As expected FITC gave the highest fluorescence reading, which decreased with the decreasing concentration of the fluorophore (see Figure 3.2).

The second highest fluorescence was observed with FITC-conjugated CSIR1.1 aptamer for both the 3,000 and 10,000 MWCO filters and this was also dose dependent i.e. increased with the increasing concentration of the aptamer. The lowest fluorescence detection was obtained with FITC-conjugated UCLA1. Therefore, the samples that were centrifuged through the 3,000 and 10,000 MWCO filters exhibited similar results (Figure 3.2). So from this we concluded that the interaction or conjugation between CSIR1.1 aptamer and FITC was more efficient than the UCLA1-FITC conjugation.

The conjugation of the UCLA1 aptamer to FITC was less efficient than that of CSIR1.1 aptamer (Figure 3.2). One possible reason is the presence of extensive modifications of UCLA1at the 5’ and 3’ ends that could have prevented effective conjugation with FITC (Figure 3.2). Notably, the 5’ end of UCLA1 is modified with a dimethoxyltrityloxy-(CH2)6-SS-(CH2)6-phospho linker whereas the 3’ end of its sequence is modified with an inverted dT probably resulting in a less efficient reaction between FITC and UCLA1. Based on the results obtained for the UCLA1 aptamer, we came to the conclusion that the conjugation between UCLA1 and FITC was inefficient; and as a result, all

subsequent studies were performed with the CSIR1.1 aptamer which was found to perform better in a head to head comparison.

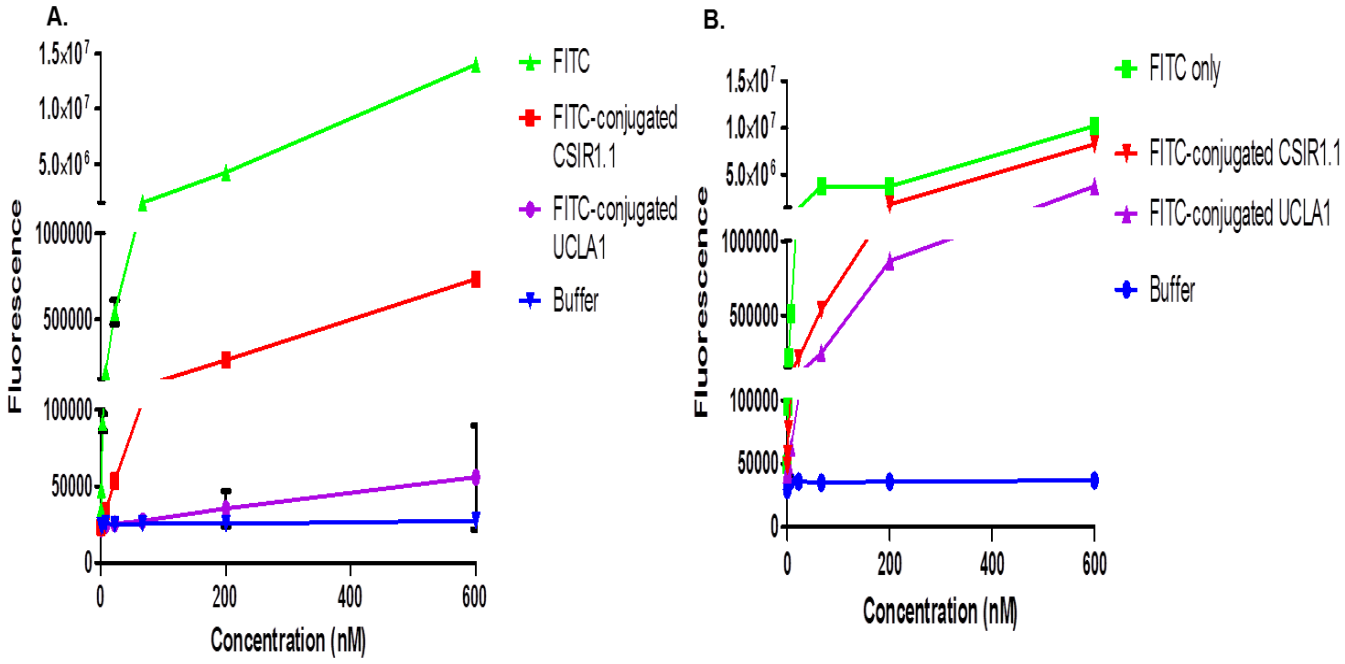


Figure 3.2: Graph of fluorescence emission against concentrations for FITC-conjugated UCLA1 and CSIR1.

The green line represents FITC only in solution; the red line is for FITC-conjugated CSIR1.1 aptamer. The conjugated UCLA1.1 and buffer only are represented by the purple and blue lines, respectively. The conjugated aptamers were filtered through a 3,000 MWCO (A) and a 10,000 MWCO (B) vivaspin columns.

The same experiment was repeated with higher concentrations of the conjugated CSIR1.1 aptamer and the fluorescence compared to FITC. At this stage UCLA1 was not used given that its conjugation to FITC was not as efficient. The increased concentrations of conjugated CSIR1.1 aptamer exhibited a dose response (Figure 3.3) and the fluorescence emitted was higher than that obtained in Figure 3.2. However, compared to FITC it was still lower.

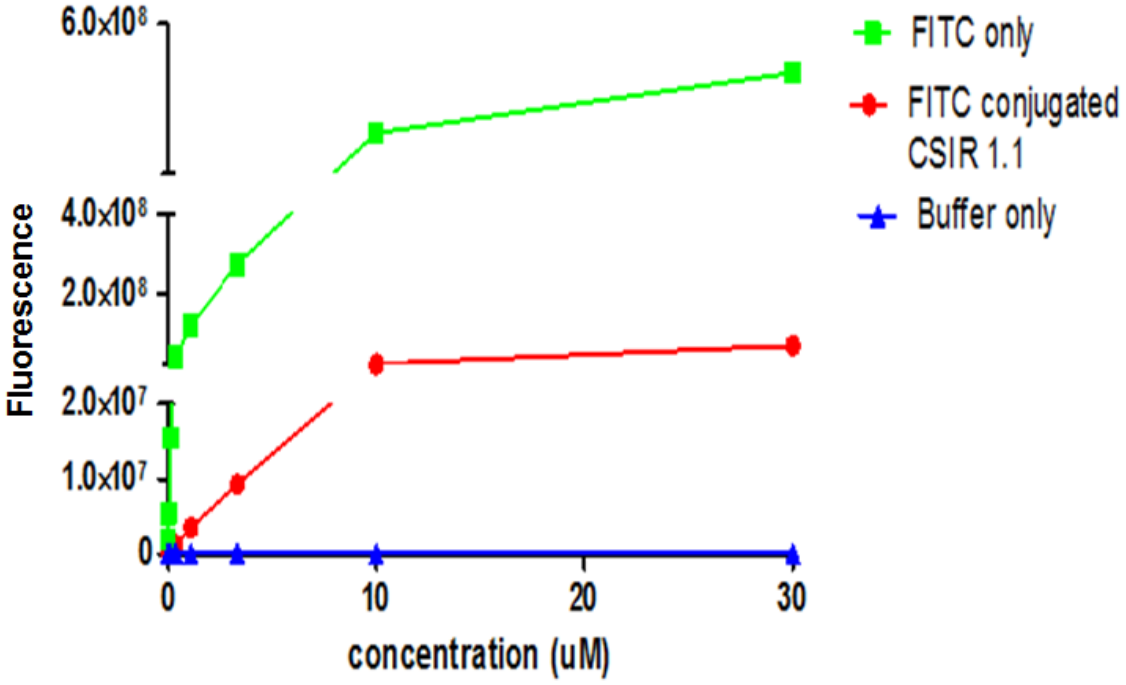


Figure 3.3: Graph of fluorescence against concentrations of FITC-conjugated CSIR1.1.

FITC only used as positive control is shown with the green line and CSIR1.1 aptamer is represented by the red line. The blue line represents the buffer only used as negative control**.**

3.4. Comparison of HIV-1 neutralisation ability between FITC-conjugated CSIR1.1 and the parent aptamer

To determine whether the conjugation of CSIR1.1 aptamer with FITC did not alter the aptamer's structure, its neutralisation of HIV-1 was compared with the parent aptamer. The subtype C virus CAP239.G3J that was previously shown to be sensitive to CSIR1.1 aptamer (Appendix A, Table A2.1, Grace London PhD thesis, 2013) was used. The idea here was that if the FITC-conjugation did not alter the aptamer's structure, it's neutralisation of the virus will be similar to the parent molecule.

As shown in Table 3.1, both parent and conjugated-aptamer neutralised the virus with similar IC_{50} values (see also Appendix A, Figure A3.1 and A3.2). To be precise the

parent aptamer had an IC_{50} of 19.2 nM while for the conjugated derivative the IC_{50} was 21.3 nM. The data was not statistically significant as the p-value was 0.2148 (P>0.05). These data indicates that conjugation with FITC probably had no effect on the structure of the CSIR1.1 aptamer.

Table 3.1: IC_{50} values for HIV-1 neutralisation with parent and conjugated CSIR1.1

Compound name	IC_{50} (nM)
Parent aptamer	19.2
FITC-conjugated CSIR1.1	21.3

3.5. Investigation of FITC-conjugated CSIR1.1 aptamer binding to HIV-1 gp120 by Flow cytometry

First optimisation assays with different beads (neutravidin and latex), blocking reagents (BSA and skim milk) and times of blocking, concentrations and volumes of FITC were conducted (see Appendix A, Figures A4.1-A4.3). From these; latex beads, BSA, 30 minutes blocking time, 4 µl of 896 nM FITC and conjugated CSIR1.1 aptamer were found to be optimal. Next, the beads were coupled with recombinant gp120 from the virus Consensus C (ConC). This virus is representative of all circulating HIV-1 subtype C viruses (Gray *et al*., 2011) . Note that the ConC was chosen because CSIR1.1 aptamer was selected against a subtype C virus (London *et al*., 2015). The gp120 coupled beads were incubated with the FITC-conjugated CSIR1.1 aptamer. As a control the beads were also stained with FITC. Other controls involved unstained gp120 coupled beads, and uncoupled beads (those that do not bear the gp120 on their surface) incubated with the conjugated aptamer and with FITC alone.

After the acquisition of 10,000 events there was no fluorescence observed with the coupled beads only i.e. the fluorescence was at 0.8% as shown in the representative

histogram in Figure 3.4. However, when these beads were stained with the FITC-conjugated aptamer the fluorescence increased to an average of 16%. The staining of the coupled beads with FITC alone resulted in an increase of only 3% from baseline, indicating that the fluorophore interaction with the glycoprotein was negligible. With uncoupled beads stained with FITC and with the FITC-conjugated CSIR1.1 aptamer, the percentage fluorescence was 3.5 and 4%, respectively (Figure 3.4B), confirming that the conjugated aptamer interacted only with gp120 coupled to the beads.

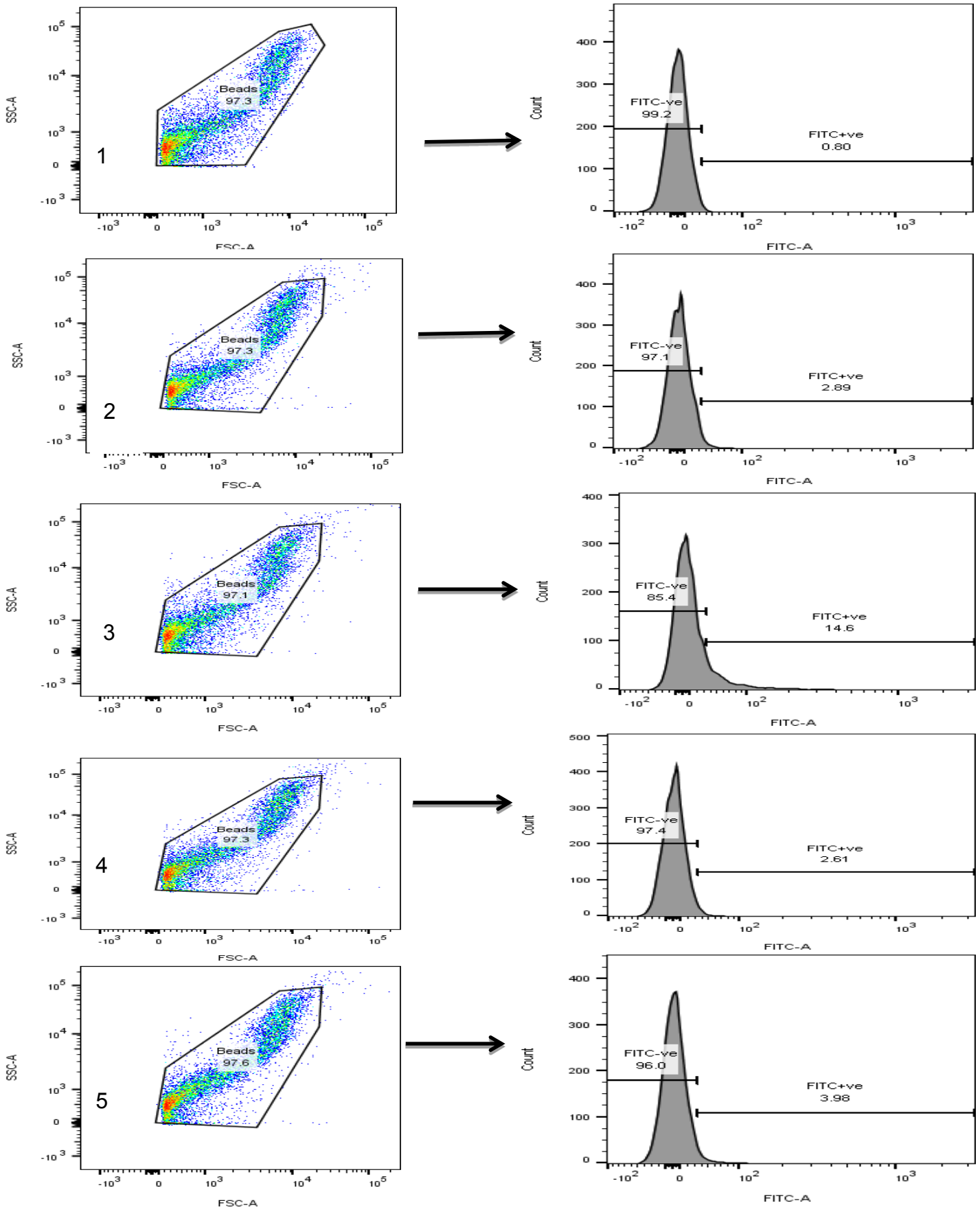
The data obtained for coupled beads stained with FITC-conjugated CSIR1.1 aptamer, coupled beads stained with FITC only and uncoupled beads stained with FITC-conjugated CSIR1.1 aptamer was significantly different with a p-value of 0.0016 ($P<0.05$). In conclusion, these data showed that the FITC-conjugated CSIR1.1 aptamer binds to HIV-1 gp120. Because of its ability to recognise the ConC gp120, it is likely that the conjugated aptamer will also be able to bind gp120 from other subtype type C viruses.

Similar to what was reported by Rong *et al*. and Song *et al.*, who showed that FITC-conjugated aptamers could be used as a reagent to differentiate cancerous and non-cancerous cells by flow cytometry, the FITC-conjugated CSIR1.1 aptamer has potential for use to differentiate HIV infected and uninfected cells (Rong *et al*., 2016, Song *et al*., 2012). Therefore, it could be used as an inexpensive research or diagnosis reagent. While saturation levels of fluorescence were not reported, further optimization of these initial findings is required.

3.6. Investigation of the ability of FITC-conjugated CSIR1.1 aptamer to bind gp120 on intact HIV-1 particles

HIV-1 capture assay was performed to determine whether the FITC-conjugated CSIR1.1 aptamer was able to bind gp120 expressed on intact viruses.

A.


1
Beads
97.3
SSC-A
FSC-A
Count
FITC-ve
99.2
FITC+ve
0.80
FITC-A
2
Beads
97.3
FITC-ve
97.1
FITC+ve
2.89
3
Beads
97.1
FITC-ve
85.4
FITC+ve
14.6
4
Beads
97.3
FITC-ve
97.4
FITC+ve
2.61
5
Beads
97.6
FITC-ve
96.0
FITC+ve
3.98

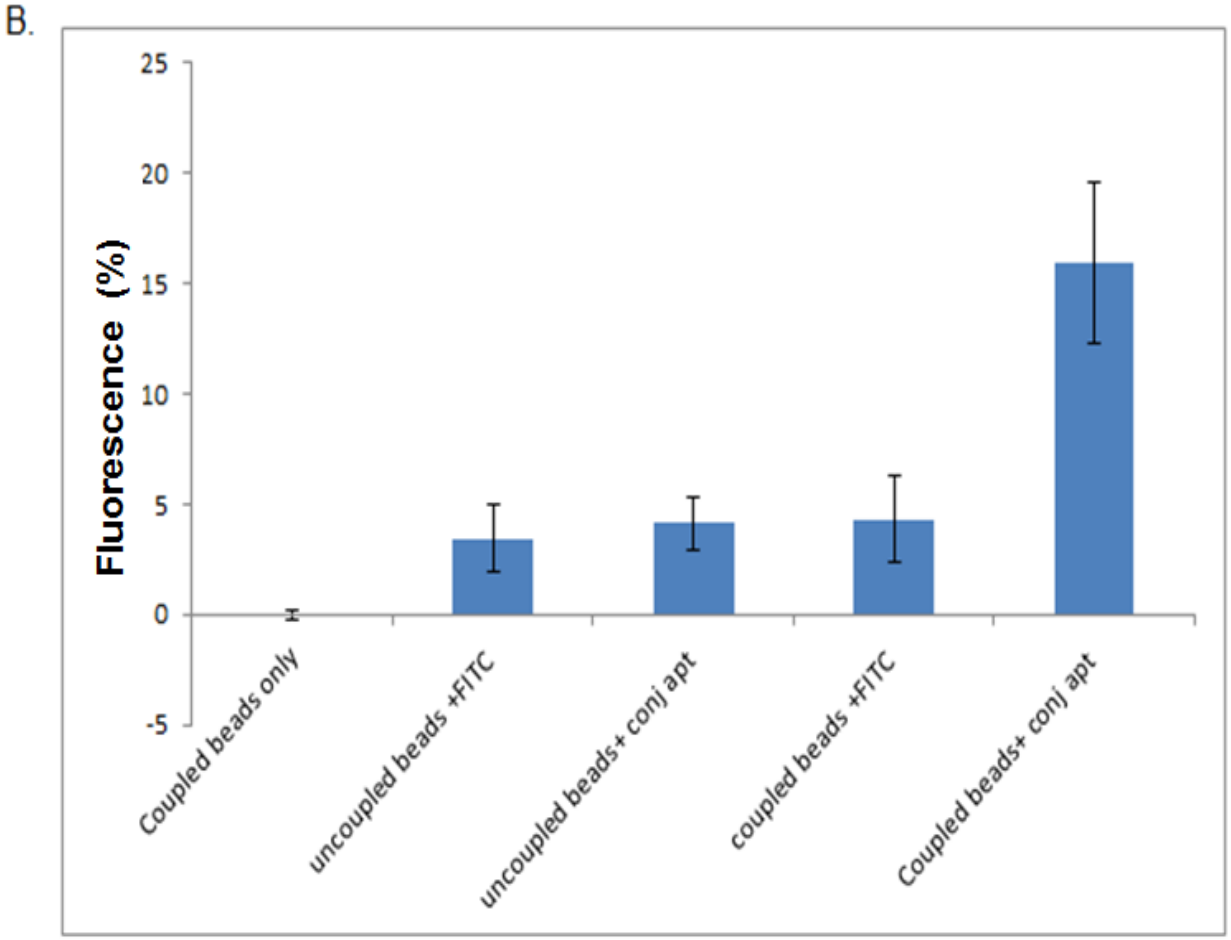


Figure 3.4: The binding of FITC-conjugated CSIR1.1 aptamer to gp120 on latex beads.

The dot plot and histograms of flow cytometry data (A); (A1) coupled beads alone, (A2) coupled beads stained with FITC only, (A3) coupled beads stained with conjugated aptamer, (A4) uncoupled stained with FITC only, (A5) uncoupled bead stained with conjugated aptamer. (B) The results obtained in flow cytometry converted into a bar graph; data represent the mean fluorescence and error bars the standard deviation. The experiment was performed 3 times in duplicates.

Here the virus was captured with the monoclonal antibody 10E8 that binds gp41 (Huang *et al*., 2014), and detected using different concentrations of FITC-conjugated CSIR1.1 aptamer.

We used three HIV-1 subtype C pseudoviruses; ZM53M.PB12, DU156.12, CAP210.E8 selected from the panel of viruses that are known to be sensitive to CSIR1.1 aptamer (Grace London PhD thesis, 2013).

All three viruses could be detected by FITC-conjugated CSIR 1.1 and this was consistent with the fact that they are sensitive to this aptamer (Grace London PhD thesis, 2013). The CAP210.E8 pseudovirus resulted in the highest fluorescence reading as compared to the other two viruses (Figure 3.5). This is probably due to the fact that of all three viruses it is the most sensitive to the aptamer (Appendix A, Table A1, Grace London PhD thesis, 2013). The second highest fluorescence was observed with DU156.12 while the lowest was from ZM53M.PB12, consistent with the latter being previously shown to be the least sensitive of the viruses used (Figure 3.5) (Grace London PhD thesis, 2013).

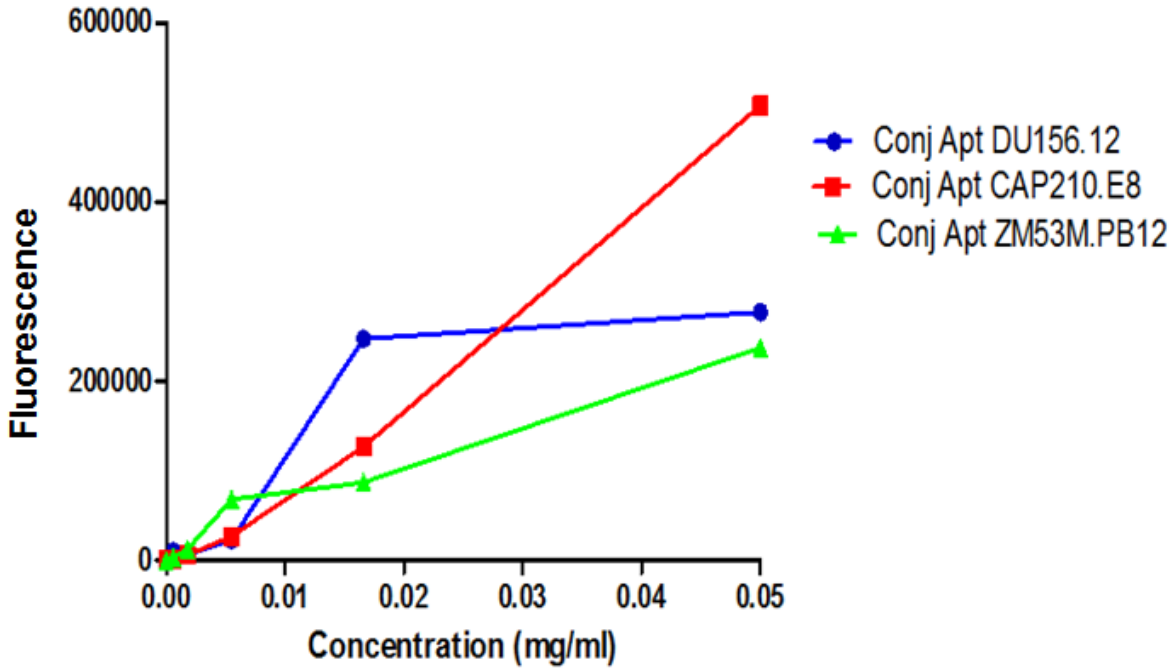


Figure 3.5: The binding of FITC-conjugated CSIR1.1 aptamer to HIV-1 pseudoviruses.

The pseudoviruses used were DU156.12 represented by the blue line; CAP 210.E8 and ZM53M.PB12 represented by the red and green lines, respectively. The fluorescence readings were subtracted from negative control.

After testing FITC-conjugated CSIR1.1 aptamer binding to pseudoviruses, the aptamer was compared to a commercially available polyclonal biotin-conjugated anti-gp120 antibody for binding to intact HIV-1 particle. This antibody is detected using a FITC-conjugated streptavidin. The biotin interaction with streptavidin was optimised and the strongest signal was obtained at 1/2000 biotin-conjugated antibody to streptavidin ratio (see Appendix A, Figure A5.1). This was followed by a comparison of the fluorescence emission after HIV-1 binding between the same concentrations of the FITC-conjugated CSIR1.1 aptamer and biotin-conjugated gp120 antibody.

At almost all concentrations used, the FITC-conjugated aptamer emitted stronger fluorescence than the antibody (Figure 3.6). This difference became increasingly larger as the concentrations of the aptamer and antibody increased. At the highest concentration, the difference in fluorescence between the FITC-conjugated aptamer and antibody was 8-fold. One can speculate that the higher signal obtained with the aptamer is due to its stronger binding to gp120 compared to the biotinylated antibody. In addition, it is also possible that this higher fluorescence was due to a more efficient conjugation between the aptamer and FITC compared to the fluorophore and streptavidin.

The observed better binding of the FITC-conjugated CSIR1.1 aptamer to gp120 may have been due to the fact that antibodies are unstable and larger in size which causes interference with target binding (Xiang *et al*., 2015; Song *et al*., 2012). Aptamers on the other hand, are smaller and bind to the target molecules with high specificity and affinity (Ying *et al*., 2015). Based on these data it can be argued that the FITC-conjugated CSIR1.1 aptamer has potential as an alternative reagent to antibodies in HIV-1 research.

3.7. Anti-p24 aptamer Isolation using SELEX

To perform the SELEX for the isolation of anti-p24 aptamers, the number of cycles for the PCR amplification of the ss-DNA library was optimised. The number of PCR cycles

was determined to be eight (see Appendix B, Figure B1.1). Then the amplified library was exposed to HIV-1 p24 target protein for eight rounds of selection.

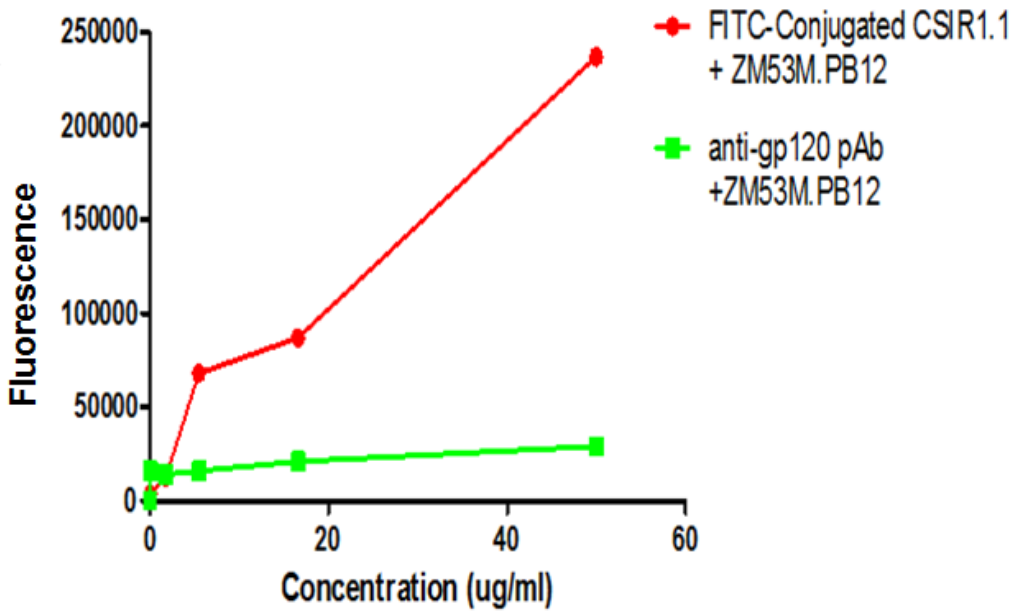


Figure 3.6: Comparison of the binding ability of FITC-conjugated CSIR1.1 aptamer and a biotinylated gp120 polyclonal antibody, detected with FITC-conjugated streptavidin.

The FITC-conjugated CSIR1.1 aptamer bound better to HIV-1 ZM53M.PB12 as compared to the commercially available gp120 antibody.

A nitrocellulose membrane and HIV-1 gp120 were used for negative selections, respectively. After exposing the library to the p24 target the bound ss-DNA were eluted, amplified and digested by lambda exonuclease. Shown below are 4% agarose gels for all eight rounds of SELEX after PCR, exonuclease digestion, and phenol: chloroform extraction (Figure 3.7). Gel electrophoresis was performed to determine the size of the ss-DNA, expected to be 100 base pairs, before exposure to the target.

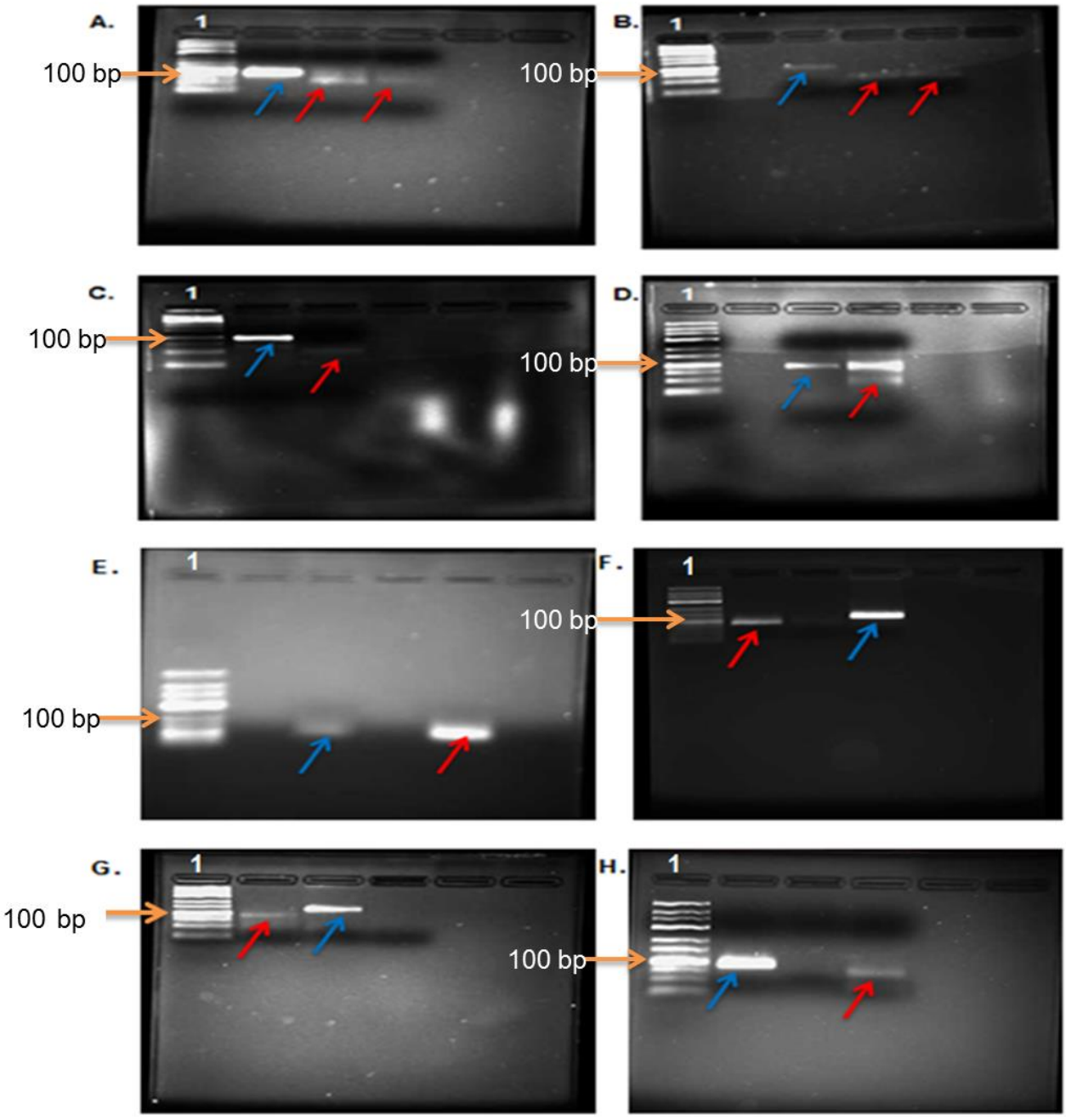


Figure 3.7: Gel electrophoresis of the PCR products and ss-DNA after lambda exonuclease digestion.

Round 1 of SELEX (A), round 2 (B), round 3 (C), round 4 (D), round 5 (E), round 6 (F), round 7 (G) and round 8 (H). Lane 1 in all the gels is the DNA ladder with a weight range of 25-500 base pairs (bp), similar to that shown in Figure 3.9 below. The ds-DNA is shown by the blue arrows, the ss-DNA by the red arrows and the position of the100 bp band is indicated by the orange arrow.

During gel electrophoresis it was expected that the ss-DNA will migrate faster than the ds-DNA given that the latter was double in size i.e., 100 base pairs. The difference in migration was observed in all the gels that were run (Figure 3.7). However, there was also a case of partial digestion in round 4 i.e. after incubation with lambda exonuclease there were still some ds-DNA present in solution (Figure 3.7D). To solve this problem gel extraction of the single ss-DNA was performed. The concentration of ss-DNA before extraction was 1,821.1 ng/µl and after extraction was 102.5 ng/µl respectively.

The concentrations of the ss-DNA after the phenol: chloroform extractions were measured using the Nanodrop spectrophotometer (Figure 3.8; and see also Appendix B, Figure B2.1-B2.2). As shown in the Figure 3.8, the concentration of ss-DNA collected increased with increasing rounds of selection. To be precise, the concentration of the recovered ss-DNA after the first round of selection was 64.3 ng/µl which increased to 88.7 ng/µl after round 3 of selection. It reached 135.6 ng/µl in round 5 and 225.7 ng/µl in round 8. What this means is that earlier, i.e., in round 1 and 2 of selection there was low recovery of target specific ss-DNA levels shown by the low concentration.

As the amount of these ss-DNA increased the concentrations also increased accordingly. These results were similar to those obtained by previous investigators where they showed that the early rounds of selection for the isolation of aptamers corresponded to low amount of ss-DNA recovered (London *et al*., 2015, Song *et al*., 2012). This amount increased with the increasing enrichment indicating that there was enrichment of ss-DNA sequences that were specific to HIV-1 p24 protein.

Previously it was found that the use of a different molecule for counter selection increased the stringency of the selection process, reflected by increased amount of collected ss-DNA (Zeng *et al*., 2017; London *et al*., 2015). In this study similar results were also observed, i.e., after counter selection with HIV-1 gp120 protein in round 5 the concentration of the recovered DNA was more than double in the following round (Figure 3.8).

A decrease in concentration of the recovered ss-DNA was observed from round 6 to round 7. This might have been due to target specific sequences being lost during

amplification. For example, one can speculate that the introduction of errors in the sequences during PCR can result in their loss of specificity for the target.

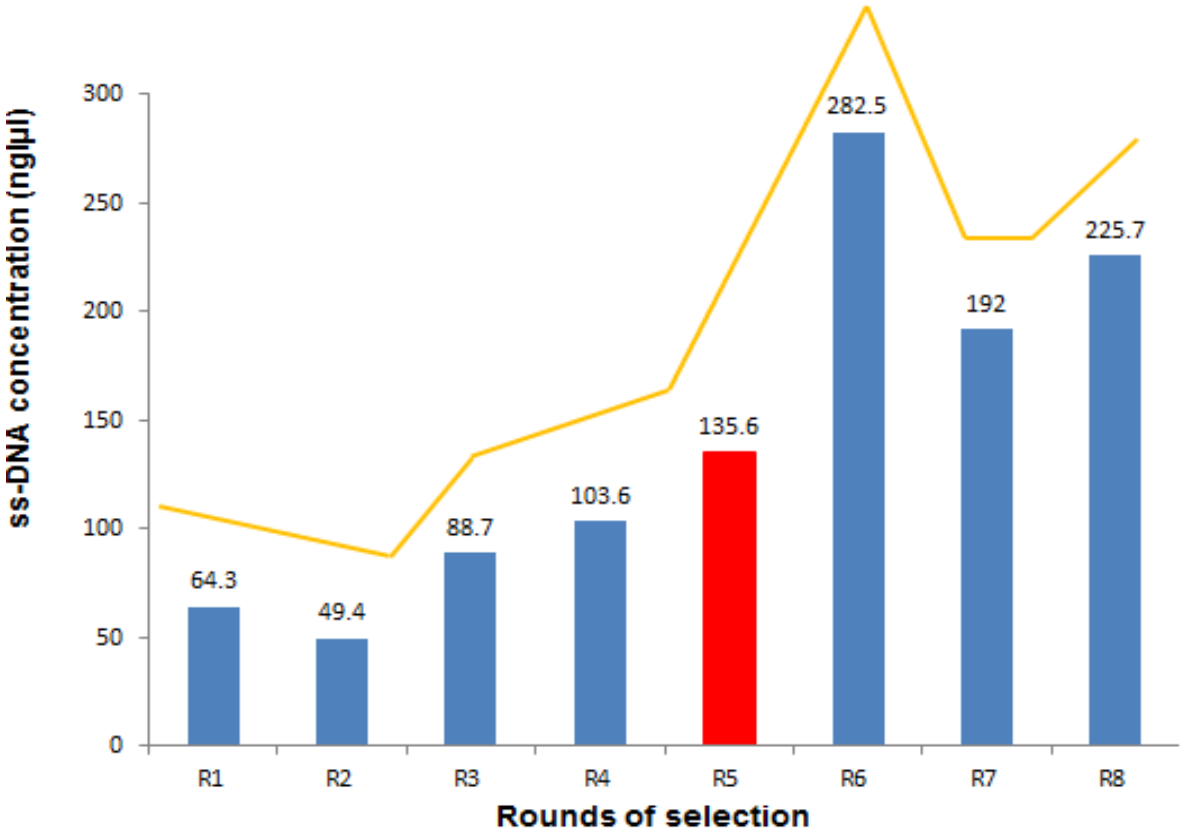


Figure 3.8: The binding of the ss-DNA library sequences to p24 target protein.

Shown are concentrations of ss-DNA after each round of SELEX i.e. from round 1 to round 8. The red bar represents the ss-DNA concentration after counter selection with gp120, which happened at round 5. Note that R stands for round of selection.

Round 8 was the last round of anti-p24 aptamers selection. After the extraction of ss-DNA using phenol: chloroform extraction, the DNA was amplified by PCR before performing next generation sequencing (see Figure 3.9).

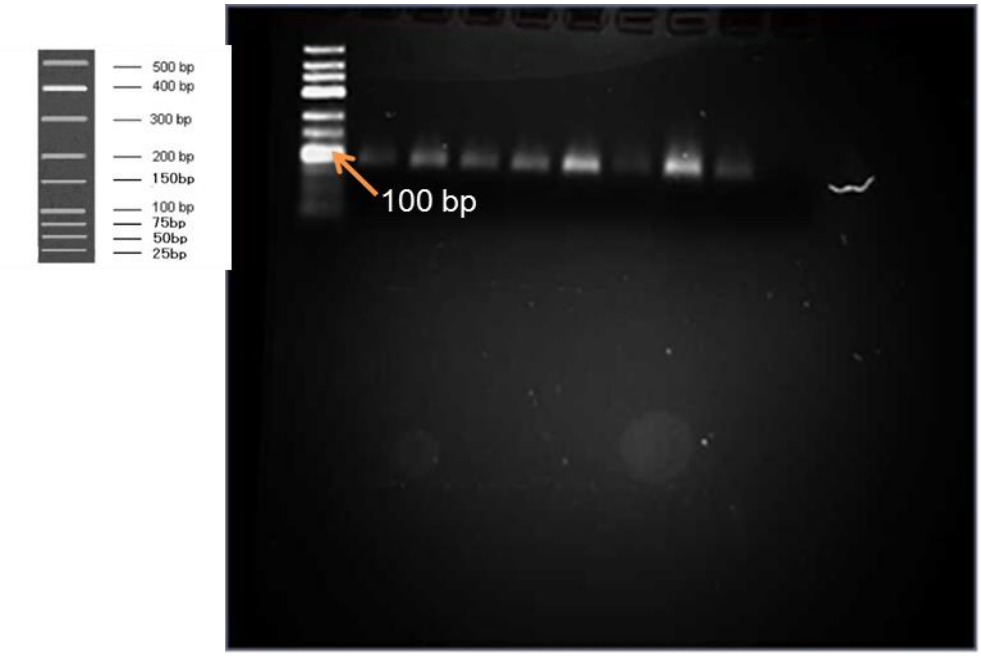


Figure 3.9: The ds-DNA PCR product after the 8th round of selection.

The first lane is the DNA ladder with a weight range of 25-500 bp. Note that all the remaining wells were loaded with exactly the same sample to produce bulk amounts of the DNA for the next steps of selection.

The next generation sequencing was performed with the PCR products to determine the most abundant sequences, i.e., to identify the aptamer sequences that were specific to HIV-1 p24. Sequencing was performed for rounds 2, 3, 5 and 8 (see the PCR products in Figure 3.7). The general structure of all oligonucleotide sequences in the library is shown in Figure 3.10. The section that was analysed with next generation sequencing to identify the enriched sequences is the N49 region; since this is where the oligonucleotides differed.

5'-GCCTGTTGTGAGCCTCCTAAC(**N49**)CATGCTTATTCTTGTCTCCC-3'

Forward primer | Reverse primer

Figure 3.10: The general structure of oligonucleotide sequences in the ss-DNA library.

From the figure, the variable region is shown in purple. The forward and reverse primer sequences are also indicated.

The next generation sequencing data analysis showed that a total of 50,932 to 90,464 sequences were identified, out of the initial amount of 10^{14} in the 4 rounds that were sequenced (see Table 3.2). However, among these identified sequences only few had more than two copies (Figure 3.15, red highlight), while the large majority had only one copy each. Figure 3.11 shows a representative number of sequences obtained in each round (for more sequences see Appendix B3). Note that the sequences that had two copies differed from all the rounds that were sequenced. As speculated earlier, one explanation is that they were lost during amplification that is why they couldn't be carried over in the following rounds. However, it was also observed that the number of sequences with two copies increased from 2 in round 2 to 8 in round 8. The highest number of duplicate sequences was seen in round 5 that had 10 duplicates.

Table 3.2: The number of sequences obtained after next generation sequencing

Rounds	Number of sequences
R2	84,084
R3	50,932
R4	75,665
R5	90,464
R8	76,054

In all the rounds that were sequenced, enrichment was not observed as expected, i.e., there was no single sequence that was the most abundant among all the sequences, particularly in round 8 of selection. One possible reason is that the selection needed to be carried out beyond the 8^{th} round. However, it is also a possibility that all of the identified sequences (Figure 3.11 and Appendix B3), especially those in round 8, are p24 binders given that they have been selected from a pool of 10^{14} sequences.

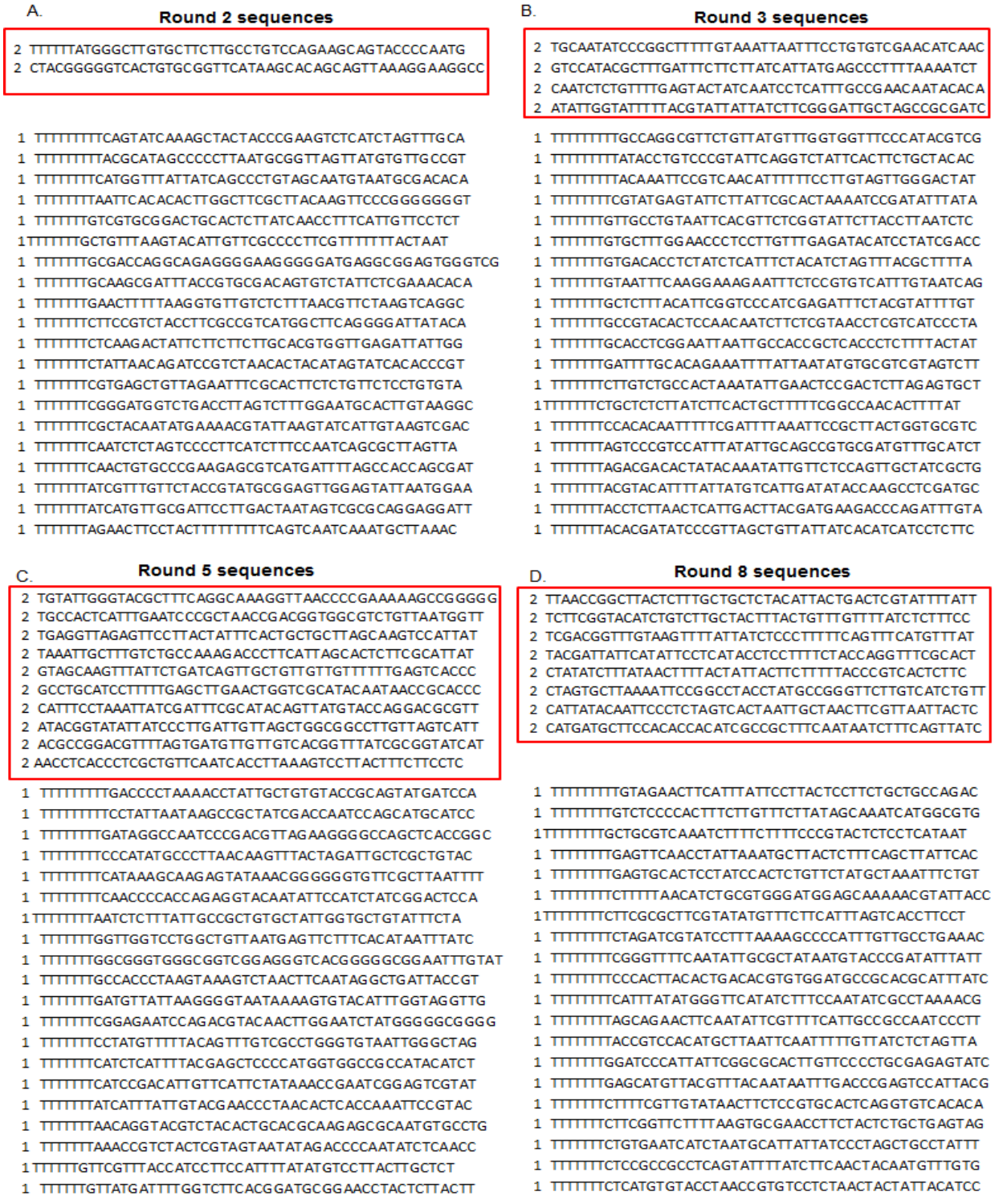


Figure 3.11: The DNA sequences obtained from next generation sequencing of round 2, 3, 5, and 8.

The figure shows some of the sequences obtained after p24 aptamer isolation by SELEX. (A) Sequences obtained in round 2; (B) round 3; (C) round 5 and (D) round 8. The sequences that had more than one copy are highlighted in the red box.

Chapter 4： CONCLUSION AND FUTURE WORK

4.1. Conclusion

The use of antibodies in HIV-1 research can be expensive partly due to the high cost of producing these reagents in animals (Guo *et al*., 2008). Also antibodies cannot be synthesized for targets that are not immunogenic (Jayasena, 1999). These disadvantages suggest that new strategies or molecules that can be employed as alternatives are required. This study was aimed towards that goal, i.e., the synthesis of reagents that can be used as alternatives to antibodies in flow cytometry. For this, anti-gp120 aptamers called CSIR1.1 previously isolated at the CSIR aptamer laboratory (London *et al*., 2015) and UCLA1 obtained from the University of California were conjugated to FITC. In addition aptamers against HIV-1 p24 were selected for possible conjugation with fluorophores in the future.

Unlike UCLA1 which showed inefficient conjugation to FITC, the conjugation of CSIR1.1 aptamer with FITC was successful and was shown by analysis of fluorescence emitted by the FITC conjugated aptamer. In addition, the ability of this aptamer to interact with gp120 and whole HIV-1 particles was demonstrated by flow cytometry and capture assay, respectively. This aptamer was also compared to a commercially available antibody (biotinylated anti-gp120 polyclonal antibody) in HIV-1 capture assay and found to perform better. Therefore, the FITC-conjugated CSIR1.1 aptamer can be developed into a reagent for HIV-1 research in techniques such as flow cytometry and enzyme-linked oligonucleotide assay (ELONA), capture assays. Since the cost of producing aptamers is very low, the reagents are likely to be inexpensive.

The second part of the study focused on the isolation of HIV-1 anti-p24 aptamers using SELEX. However, some challenges were encountered in the isolation of these aptamers, such as the partial digestion of ds-DNA (Figure 3.7D) as well as the enrichment observed with the increasing concentrations of eluted ss-DNA not matching the sequencing results as expected (Figures 3.8 and 3.11). The partial digestion was dealt with by performing gel extraction of the ss-DNA band and this remedied the

problem. With the sequencing results, there are two possibilities; the first is to carry on with the selection of aptamers beyond the 8th round to get more enriched sequences. The second is that the 76,000 plus sequences obtained are the actual enrichment; but this can only be confirmed by synthesizing the aptamers and testing their binding to HIV-1 p24 protein.

4.2. Future work

For objective one of the study, future work should involve testing the FITC-conjugated CSIR1.1 aptamer for detection of HIV-1 infected cells by flow cytometry. Here, uninfected cells will have to be used as controls to eliminate the possibility of non-specific binding to the cells. Such data will complement what was observed with the latex beads (Figure 3.4). In addition, the flow cytometry assay conducted in this study may also require more optimisation to see if fluorescence emission beyond the average 16% could be obtained. This optimisation can involve increasing the ratio between the FITC-conjugated CSIR1.1 aptamer and gp120 on the beads. Lastly, it would be interesting to also conjugate CSIR1.1 aptamer with other fluorophores such as PE that is known not to bleach (lose fluorescence) as quick as FITC (Song *et al*., 1995).

In this study the structural integrity of CSIR1.1 aptamer conjugated to FITC was determined using the TZM-bl neutralisation assay and comparing its IC_{50} to the parent aptamer. However, only one virus was used. Thus, in future this investigation should involve comparing these aptamers against more pseudoviruses chosen among those that are known to be sensitive to the parent aptamer (Grace London PhD thesis, 2013).

For studies of FITC-conjugated aptamer affinity to HIV-1 gp120, surface plasmon resonance (SPR) can be performed to compare its binding to the glycoprotein with the parent aptamer. This technique measures the refractive index, in real time, of materials adsorbed on planar metal surfaces such as gold and silver (Pattnaik, 2005; van der Merwe and Barclay, 1996), and it is widely used to investigate interactions between molecules. SPR is, therefore, the ideal method for the determination of whether the conjugation to FITC did not affect CSIR1.1 aptamer affinity to HIV-1 gp120.

The future work for objective two, which involved the isolation of anti-p24 aptamers by SELEX, would include further isolation rounds to see if more enrichment of sequences could be obtained. This can also be followed by the determination of the three dimensional structure of the aptamer that will show the strongest binding affinity to p24. Also this aptamer will have to be conjugated to FITC and its interaction with the protein studied by flow cytometry, SPR, or electrophoretic mobility shift assay (Zeng *et al*., 2017). Lastly, the FITC-conjugated p24 aptamer will have to be tested for detection of HIV-1 infected cells.

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APPENDIX A

- The conjugation of FITC to anti-gp120 aptamers for detection of HIV-1 infected cells that express gp120 on their surface by flow cytometry.

A1. Fluorescence imaging

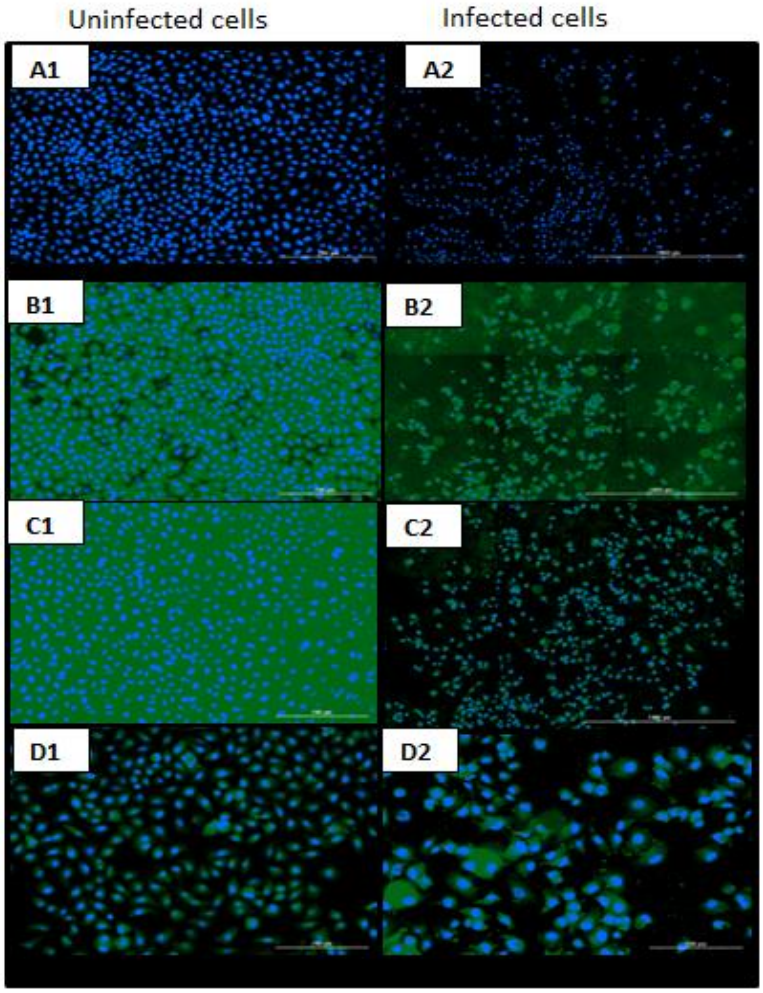


Figure A1.1: Fluorescence microscopy of TZM-bl cells treated with FITC-conjugated anti-gp120 UCLA1 aptamer. The cells were infected with ZM53M.PB12 pseudovirus. A1 is the uninfected cells only. A2 is the infected cells treated with anti-gp120 UCLA1 parent aptamer. The three images on the left from top to bottom (B1-D1) are the uninfected cells treated with 20:1, 10: 1, and 5:1 FITC: anti-gp120 UCLA1 ratios respectively. The three on the right (B2-D2) are the infected cells treated with the above mentioned FITC: anti-gp120 UCLA1 ratios from top to bottom respectively. The images show a high proportion of green fluorescence in the uninfected cells as compared to the infected cells.

A2. CSIR1.1 aptamer IC_{50} values obtained from Grace London PhD Thesis, 2013.

Table A2.1. The IC_{50} values obtained for CSIR1.1 aptamer with different pseudoviruses. The aptamer and IC_{50} values are highlighted in blue while the pseudoviruses used in this project are highlighted in red.

	Aptamer IC_{50} (nM)			
Env clone	**CSIR1.1**	**CSIR1.4**	**CSIR1.5**	**CSIR1.6**
CAP45.200.G3	2.6	2.2	1.1	1.1
ZM233M.PB6	4.9	4.2	4.2	2.4
ZM249M.PL1	>50	>50	>50	>50
ZM53M.PB12	20.1	6.2	7.1	23.4
ZM109F.PB4	14.2	2.9	25.1	5.6
ZM197M.PB7	19.2	7.1	7.1	15.1
CAP210.200.E8	2.2	2.9	1.5	1.7
ZM135M.PL10a	2.8	1.1	3.2	2.1
ZM214M.PL5	17.6	15.5	>50	7.2
DU172.17	12.1	46.1	9.1	>50
DU156.12	3.3	4.9	2.1	2.8
DU422.1	>50	>50	>50	>50
CAP08.2.00.F6	1.2	2.9	2	1.9
CAP61.2.00.F10	0.3	1.2	2.3	0.8
CAP63.2.00.A9J	1.7	7	2.5	4.3
CAP84.2.00.32J	29.1	3.3	10.1	15.1
CAP85.2.00.09J	16.1	9.7	10.7	20.1
CAP88.2.00.D3	1.6	3.2	>50	3.6
CAP239.2.00.G3J	7.1	4.9	9.5	6.2
RP1.12	4.7	49	20.7	8.9
RP4.3	>50	10.5	1.5	1.5
RP6.6	3.7	5.4	4.9	1.8
COT6.15	0.9	3.6	18.9	6.4
COT9.6	6.1	5.9	4.9	6.7
DU151.2	>50	>50	>50	>50
DU123.6	>50	>50	>50	>50
ConC	5	2.6	20.4	21.8
CAP228.2.00.5	9.5	9.6	2.2	1.8
CAP206.2.00.E8	3.8	22.1	2.1	1.8
CAP244.2.00.D3	2.3	15.2	5.2	5
IN8362.25	18.2	>50	20.1	1.1
IN0013095.211	0.2	0.1	3	1
No of viruses	32	32	32	32
% of viruses	84	84	81	84
Mean IC_{50} (nM)	6.6 ±8.1	9 ±14.3	7.9 ± 10.4	6.8 ± 10.3

A3. Comparison of the neutralising ability of FITC-conjugated CSIR1.1 aptamer and the parent CSIR1.1 aptamer.

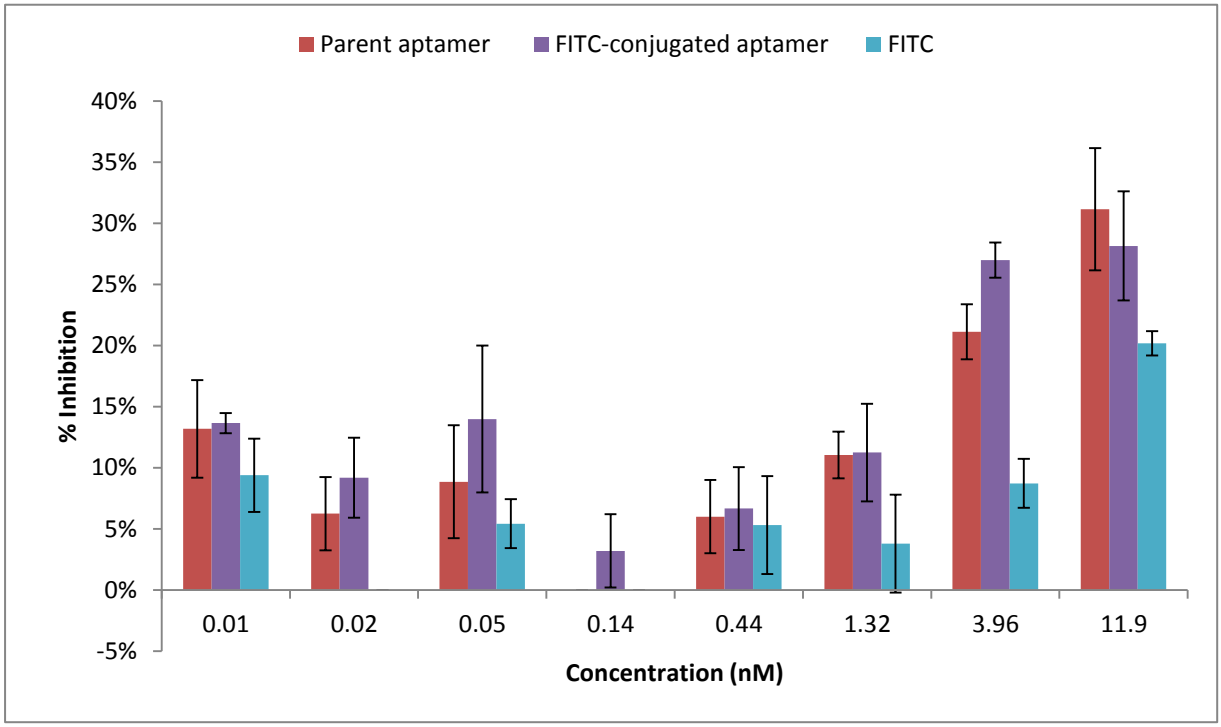


Figure A3.1: The percentage inhibition of conjugated and parent aptamer on TZM-bl cells infected with a CAP239.2.00.G3J pseudovirus at different concentrations. The negative control included the pseudovirus treated with the FITC only. The percentage inhibition was 31% for parent aptamer and 28% for the CAP239.2.00.G3J pseudovirus treated with FITC-conjugated aptamer at 11.9 nM. The inhibition of the CAP239.2.00.G3J pseudovirus by conjugated aptamer is similar to the inhibition exhibited by the parent aptamer.

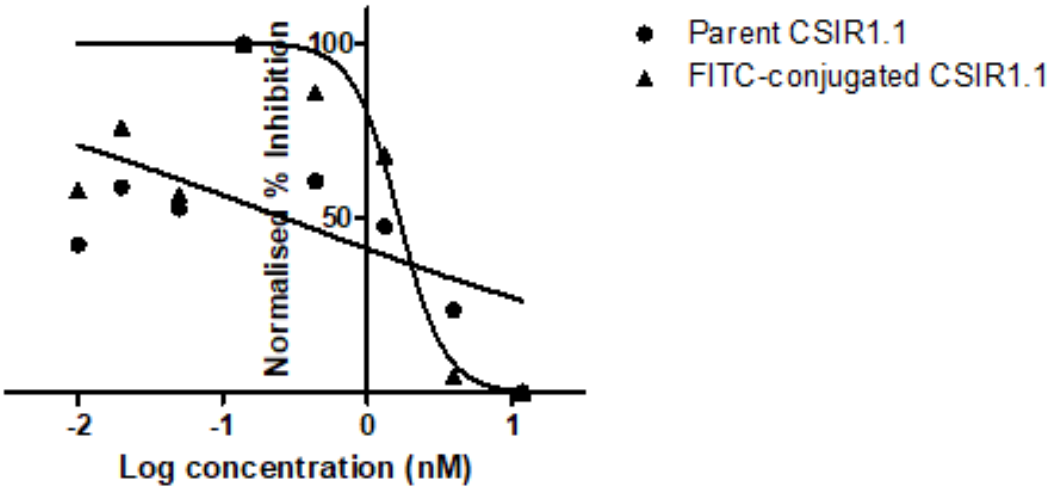


Figure A3.2: IC_{50} graph of the parent unconjugated CSIR1.1 aptamer and FITC-conjugated CSIR1.1 aptamer.

A4. Flow cytometry optimisation experiments

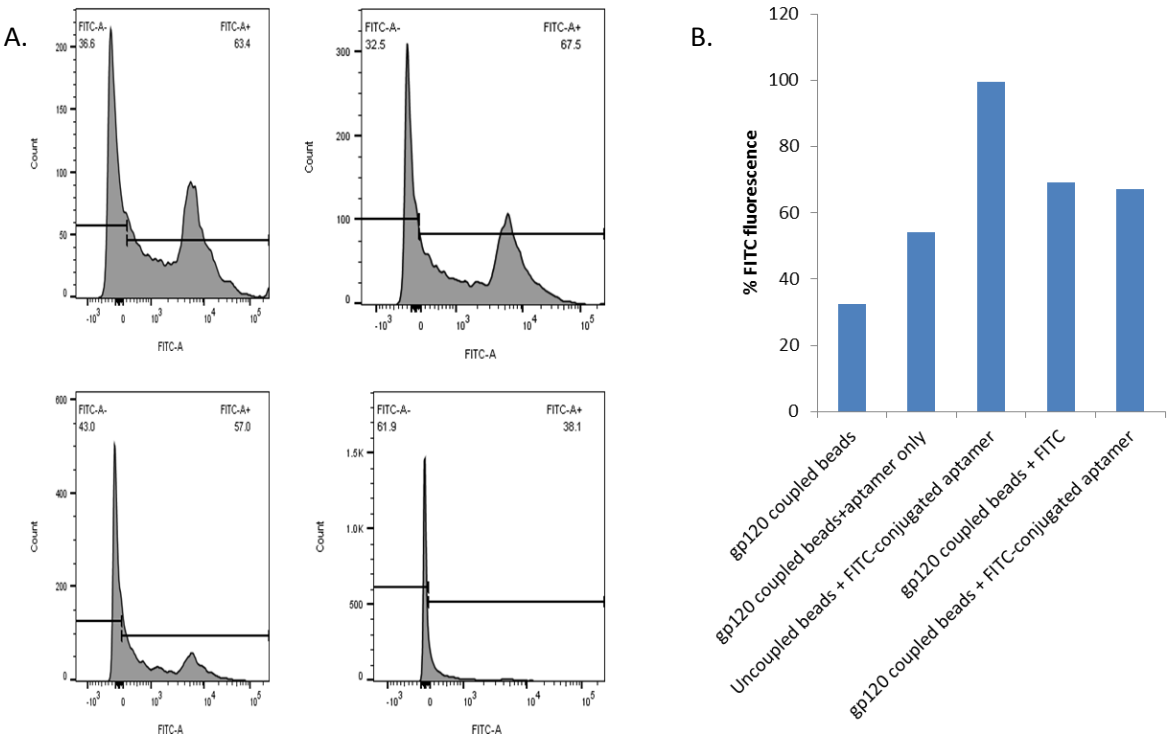


Figure A4.1: A bar graph showing the % FITC florescence of neutravidin beads stained with FITC-conjugated anti-gp120 CSIR1.1 aptamer, parent aptamer (CSIR1.1) and FITC only. In A4.1.A, representative flow cytometry histograms are shown while Figure A4.1B shows bar graphs representing this data. The gp120 coupled beads and uncoupled beads stained with FITC conjugated aptamer were included as controls. The neutravidin beads autofluoresce and this is shown by the 30% fluorescence. The aptamer bound non-specifically to the beads as uncoupled beads showed the highest fluorescence.

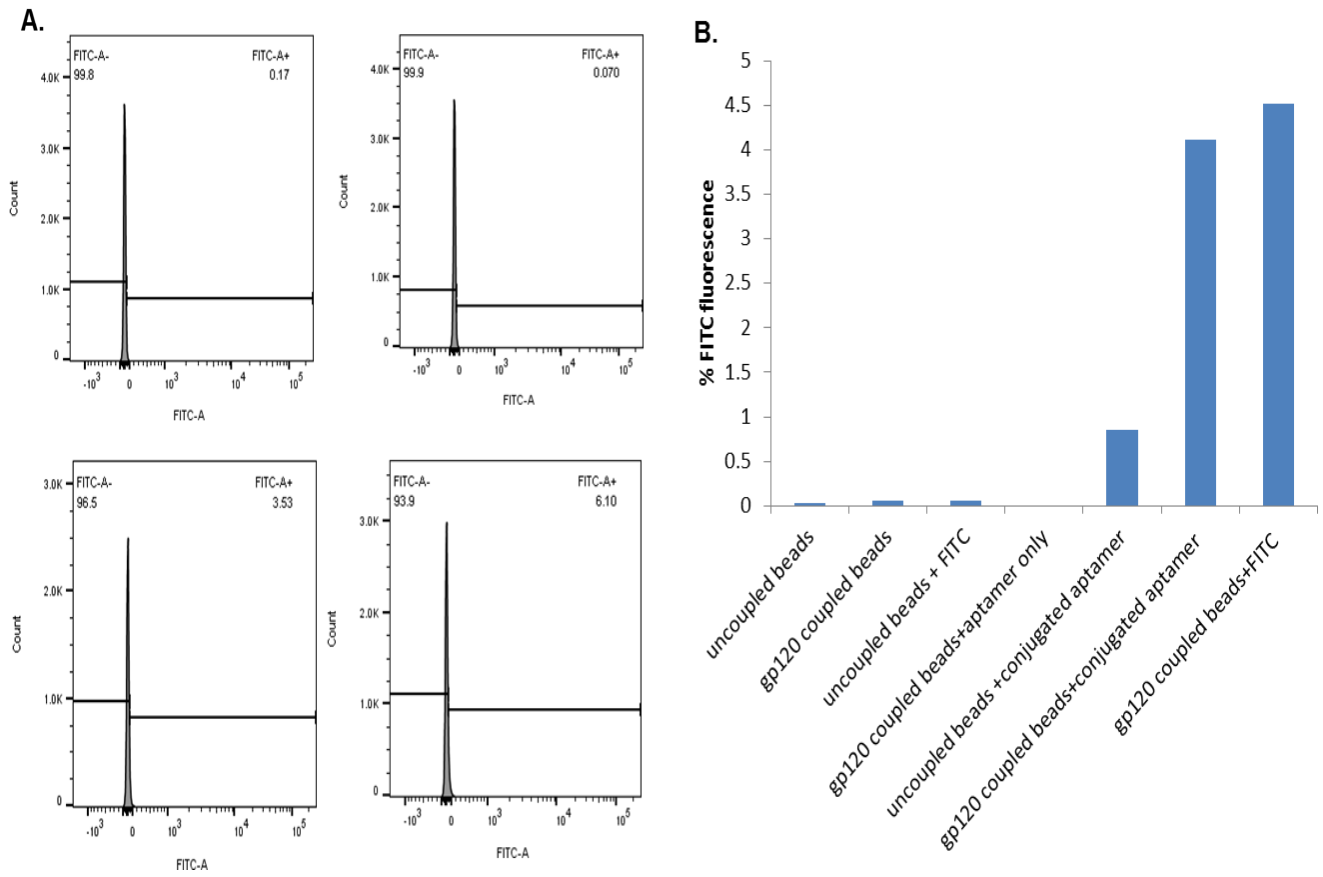


Figure A4.2: Fluorescence intensity of coupled and uncoupled latex beads stained with FITC-conjugated aptamer, parent aptamer and FITC only. In A4.2A, representative flow cytometry histograms are shown while Figure A4.2B shows bar graphs representing this data. The negative controls include beads only, coupled beads stained with FITC only, coupled beads treated with parent aptamer, uncoupled beads stained with FITC-conjugated aptamer and uncoupled beads stained with FITC only. The Latex beads do not autofluoresce. The binding of FITC to the coupled beads was non-specific.

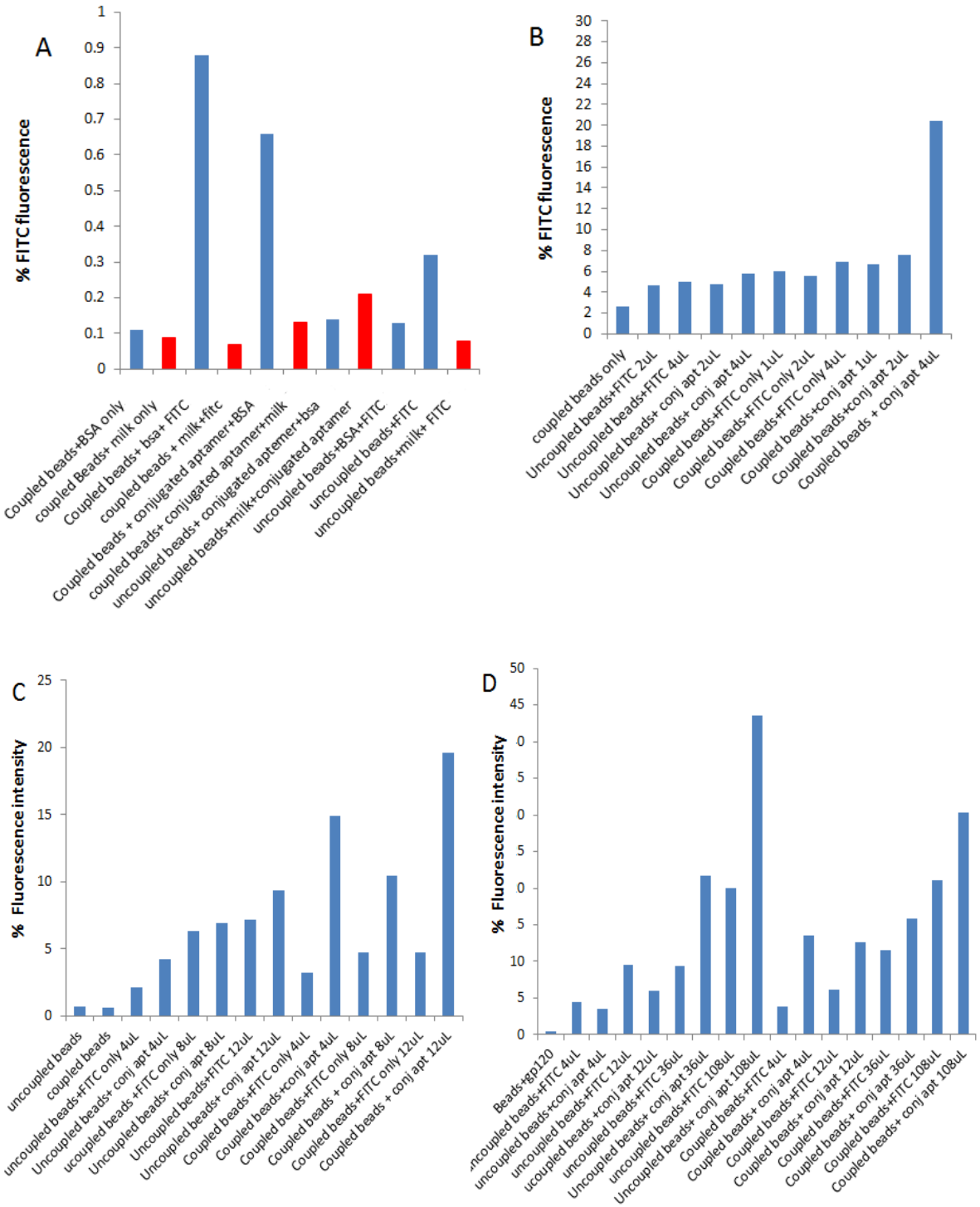


Figure A4.3: The optimisation of the blocking reagent, blocking time and volume of FITC and conjugated aptamer. A is a graph showing the optimisation of the coupled beads blocking reagent where milk was compared with BSA and the latter was seen to be the best in blocking. B, C and D show the optimisation results for the volume of FITC and FITC-conjugated aptamer. The 4 µl staining was seen to be the better volume to use as higher volumes showed non-specific binding.

A5. Optimisation of FITC-streptavidin concentration for gp120 polyclonal antibody

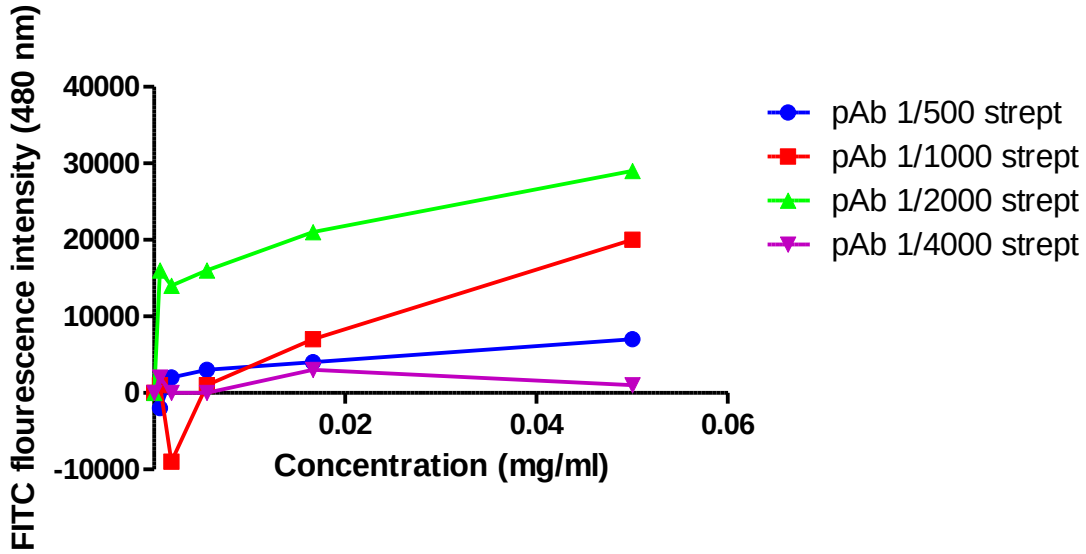


Figure A5.1: Capture assay for the determination of the optimal concentration of FITC-streptavidin to use with the gp120 polyclonal antibody. The 1 mg/ml FITC-streptavidin was tested at four different dilutions of biotin-conjugated antibody to streptavidin ratio: 1/500, 1/1000. 1/2000, 1/4000. The 1/2000 dilution was chosen as it gave better fluorescence with the antibody than the other dilutions.

APPENDIX B:

Table B1: The PCR master mix preparation showing the reagents, volumes and the final concentrations

PCR Reagents	100 µl	300 µl	500 µl	1000 µ	Final concentration
5 x Tag Polymerase buffer (Promega)	20	60	100	200	1×
25 mM $MgCl_2$	6	18	30	60	1.5 mM
10 mM dNTPs	2	6	10	20	0.2 mM
100 µM forward unmodified primer	1	3	5	10	1 µM
100 µM 5'-phosphate reverse primer	1	3	5	10	1 µM
5U/ µl Taq polymerase enzyme	0.5	1.5	2.5	5	2.5 U
DNA library ng/µl	1	3	5	10	12 ng
dH_2O	**68.5**	**205.5**	**342.5**	**685**	

B1.Optimising the number of cycles to use for amplifying DNA library

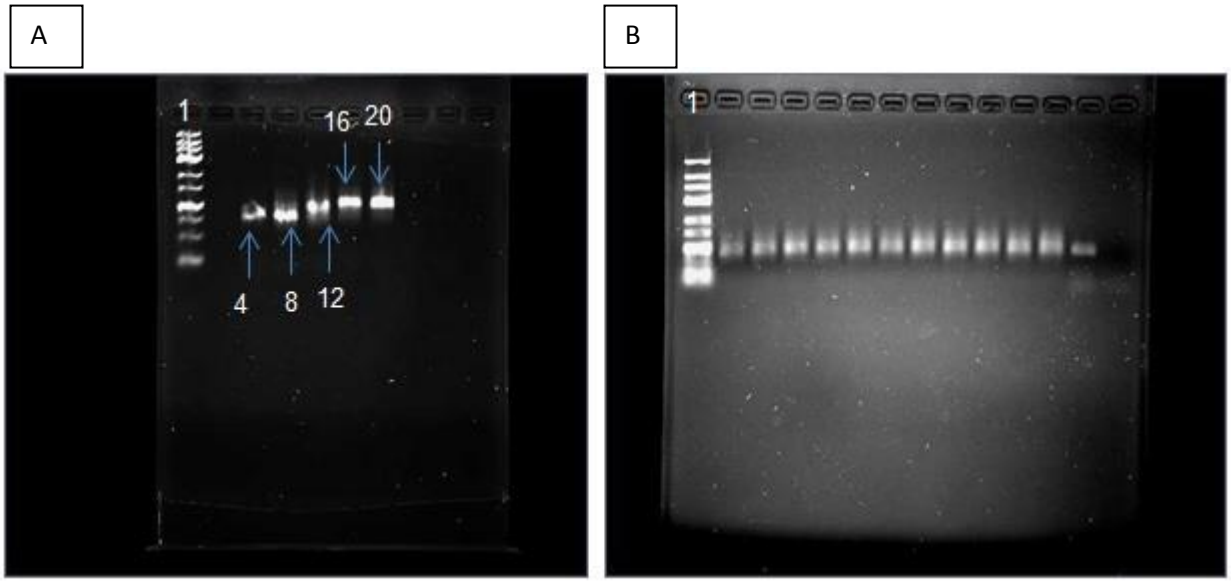


Figure B1.1: Optimisation of the number of PCR cycles for the ss-DNA library amplification. A. The gel shows the different cycles that were tested (indicated by the blue arrows). B. The gel shows the amplification performed with 8 PCR cycles using the same PCR product.

B2. Nanodrop concentration of library and ss-DNA from each round of selection

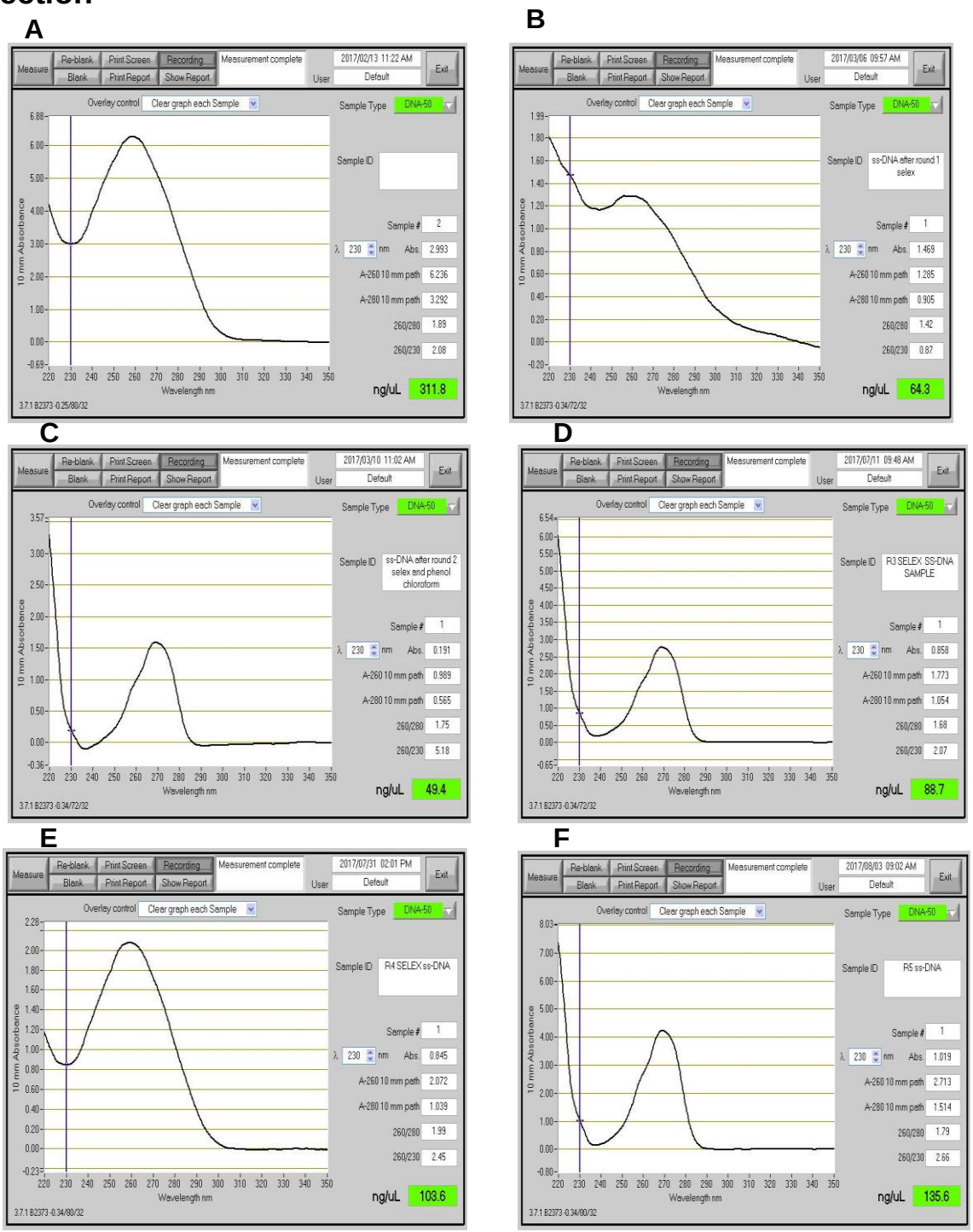
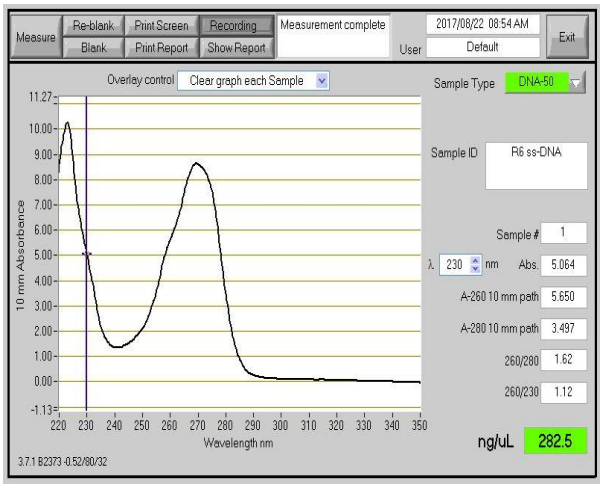
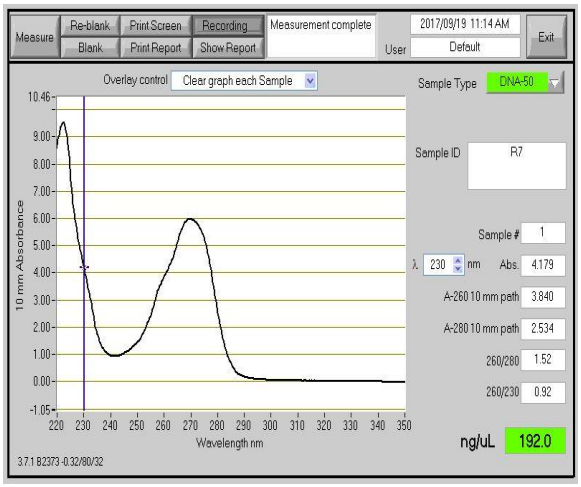


Figure B2.1: The concentration of the DNA library (A) and concentrations of the ss-DNA obtained for rounds 1 (B), round 2 (C), round 3 (D), round 4 (E) and round 5 (F) of selection after phenol: chloroform.

G



H



I

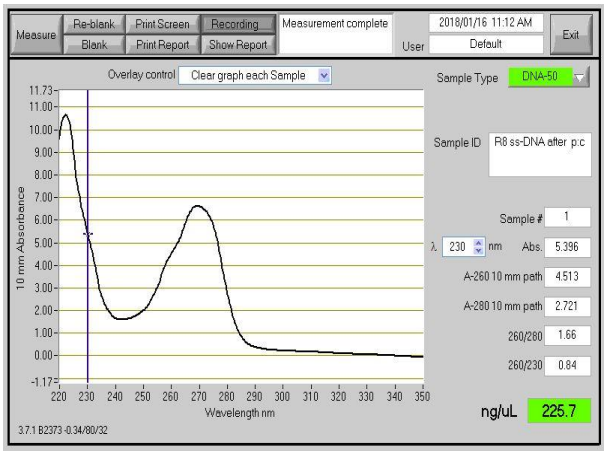


Figure B2.2: Concentration of ss-DNA for rounds 6 (G), round 7 (H) and round 8 (I) of selection after phenol: chloroform.

B3. Extra sequences obtained during deep sequencing of rounds 2, 3, 4, 5 and 8. Note that these sequences are not the complete number of sequences obtained for each round

Round 2 sequences

2	TTTTTTATGGGCTTGTGCTTCTTGCCTGTCCAGAAGCAGTACCCCAATG
2	CTACGGGGGTCACTGTGCGGTTCATAAGCACAGCAGTTAAAGGAAGGCC
1	TTTTTTTTTCAGTATCAAAGCTACTACCCGAAGTCTCATCTAGTTTGCA
1	TTTTTTTTTACGCATAGCCCCCTTAATGCGGTTAGTTATGTGTTGCCGT
1	TTTTTTTTCATGGTTTATTATCAGCCCTGTAGCAATGTAATGCGACACA
1	TTTTTTTTAATTCACACACTTGGCTTCGCTTACAAGTTCCCGGGGGGGT
1	TTTTTTTGTCGTGCGGACTGCACTCTTATCAACCTTTCATTGTTCCTCT
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1	TTTTTTTGCGACCAGGCAGAGGGGAAGGGGGATGAGGCGGAGTGGGTCG
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1 TTTTTTTAAGGTATTAGGGGTCGTCAAGGGGCAGCGGATACCATTTCTT

1 TTTTTTTAACCCACGAAAAAGTGTCGAATGTTCGCGTCTTAGATCGCCC

1 TTTTTTGTTGGTTTACTCCCACATAACGAAGACCTGTTTACGAAGTATC

1 TTTTTTGTTGGCTTCCGATCCATAAGCCGGCGCCTTCCCAACAAGTTAA

1 TTTTTTGTTAAATTTATCGACCTTCTGCGTTGAATGTATTAATGGCAAA

1 TTTTTTGTGTTTTACTTACATCTATTGGGCGCCTCTTGTAACGGCCCAG

1 TTTTTTGTGTCAGGACCATCGACCATGCGGACAGTCAAGTATTCACTAA

1 TTTTTTGTCGGTTCCTTTAACTTCAATGTGCACTTTTCAATCGTCATTC

1 TTTTTTGTAGTCCCTCAAATTGGGGTCAGATTTCCTATCGACCGACACT

1 TTTTTTGTACAATTTTGAAGTAGTTATGCATGGGGGTGAGGCGTAGATT

1 TTTTTTGTAAAACTGGACGGTCAGTATTTAAGGTCGTTTTTTGTTGCGC

1 TTTTTTGGTAGTAGAGCTGTGCTAGGTAGGATAGTCCAGTGTTACAGGG

1 TTTTTTGGACGGTTACGATTCGCAGGCCGCTTATTTGGTTATACTCGGG

1 TTTTTTGGACCCTTATAGATTCGTAAATCTTGTAGTCAAATCGGCCTCC

1 TTTTTTGCTTTATTTTCAATTTGACTTTGATCATCATGTTATGCTGCGA

1 TTTTTTGCTTCCGTGGAGCTTTACATAGCGGCATCCGGATCCTCACGGT

1 TTTTTTGCTTAGTTCCTCCCCTTGAACTCCTCTTTTGAACTCGCCTATA

1 TTTTTTGCTAGCTCTTTTTAACCTCCGCACTAAAACGTTTTCCTTGATA

1 TTTTTTGCGTTGTCTTTTATAGTCGGTTCGATAGCTGCGTGTACGTTTT

1 TTTTTTGCGTGGAACTTGCAACTTATCCCATCTTAGGAATCTTAATTAA

1 TTTTTTGATTAAGAGCCATTCTCGGTTAGTTAAAAGCATTATTAGGTAA

1 TTTTTTGAGCTCCTCGTATCTTCAGGGTCCACGCTACCTCTGTGGCGCA

1 TTTTTTGAATGCGTTCTCTCACCATTGTCTACTGCTCGCTATAAACAAT

1 TTTTTTGAAGTGAAATTGTGGTGTAGTAAGTCTCTTGGTGTTCTACTCG

1 TTTTTTGAAGATAGTAGTGTGCTCTATGGAAATCAGTAATAGACATATA

1 TTTTTTCTTTGATGCCTTTAGCCAATACCCGGATAAAGGCTCTATTTAC

Round 3 sequences

2 TGCAATATCCCGGCTTTTTGTAAATTAATTTCCTGTGTCGAACATCAAC

2 GTCCATACGCTTTGATTTCTTCTTATCATTATGAGCCCTTTTAAAATCT

2 CAATCTCTGTTTTGAGTACTATCAATCCTCATTTGCCGAACAATACACA

2 ATATTGGTATTTTTACGTATTATTATCTTCGGGATTGCTAGCCGCGATC

1 TTTTTTTTTGCCAGGCGTTCTGTTATGTTTGGTGGTTTCCCATACGTCG

1 TTTTTTTTTATACCTGTCCCGTATTCAGGTCTATTCACTTCTGCTACAC

1 TTTTTTTTTACAAATTCCGTCAACATTTTTTCCTTGTAGTTGGGACTAT

1 TTTTTTTTCGTATGAGTATTCTTATTCGCACTAAAATCCGATATTTATA

1 TTTTTTTGTTGCCTGTAATTCACGTTCTCGGTATTCTTACCTTAATCTC

1 TTTTTTTGTGCTTTGGAACCCTCCTTGTTTGAGATACATCCTATCGACC

1 TTTTTTTGTGACACCTCTATCTCATTTCTACATCTAGTTTACGCTTTTA

1 TTTTTTTGTAATTTCAAGGAAAGAATTTCTCCGTGTCATTTGTAATCAG

1 TTTTTTTGCTCTTTACATTCGGTCCCATCGAGATTTCTACGTATTTTGT

1 TTTTTTTGCCGTACACTCCAACAATCTTCTCGTAACCTCGTCATCCCTA

1 TTTTTTTGCACCTCGGAATTAATTGCCACCGCTCACCCTCTTTTACTAT

1 TTTTTTTGATTTTGCACAGAAATTTTATTAATATGTGCGTCGTAGTCTT

1 TTTTTTTCTTGTCTGCCACTAAATATTGAACTCCGACTCTTAGAGTGCT

1 TTTTTTTCTGCTCTCTTATCTTCACTGCTTTTTCGGCCAACACTTTTAT

1 TTTTTTTCCACACAATTTTTCGATTTTAAATTCCGCTTACTGGTGCGTC

1 TTTTTTTAGTCCCGTCCATTTATATTGCAGCCGTGCGATGTTTGCATCT

1 TTTTTTTAGACGACACTATACAAATATTGTTCTCCAGTTGCTATCGCTG

1 TTTTTTTACGTACATTTTATTATGTCATTGATATACCAAGCCTCGATGC

1 TTTTTTTACCTCTTAACTCATTGACTTACGATGAAGACCCAGATTTGTA

1 TTTTTTTACACGATATCCCGTTAGCTGTTATTATCACATCATCCTCTTC

1 TTTTTTTAATAATTATCCGGCACGTTCATACTATATTTAAAGACCTCGT

1 TTTTTTGTTTTCTAAGTCTTGGAGTGCTGAGTAAGTTCTGCCTCTCCTT

1 TTTTTTGTTTTATCAGTATCTCAGCGACTATACCGTACATCCAATTGTT

1 TTTTTTGTTCACAACCTCGCTCATAGCGCGTGTGTTCCTCTCAAACCTT

1 TTTTTTGGTTTACGGGTTGTATCTGTATCGATTCCTGTTTTTCGTTTCT

1 TTTTTTGGTAAAGTCGACCTTAGTCGCTACCGACTTTCGCTTTCTAGAC

1 TTTTTTGCGCATTTAAACGCTTGTTAATTCTCTTCCTTATCGAATGCAG

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1 TTTTTTGCCAGCCCTCGTTATCGGTGTTCCGAAATTACAGAATTTCTAG

1 TTTTTTGCATTCTCGCGTTTTATTCTGTTTTACCTGAGGAATTAATTTT

1 TTTTTTGCATGACTGCTGGTACCTTGAATAGTGAATCCTATTTTTCTCT

1 TTTTTTGCAACTTTTGTATCTGTGACTCGATGTTTATGTGCGTATTATT

1 TTTTTTGATCGTGGTTTCACGCAATGTTTATAATCTACTTCGGTTGTTA

1 TTTTTTGACTTAATCCTTCTATATCGTTACAACTTATCGACACGCAAAC

1 TTTTTTGACCACATATTTCGTTTTTTTTGCATAAGGACGTCATTAATCG

1 TTTTTTGAATTCCTTTATTTCTTGTAGAGTCGTATGAACCATAAGATAC

1 TTTTTTGAATACTGTTTCCTCGTGGCTGTTTCGGCAACATGTTGCTCGT

1 TTTTTTCTTCGATTACACAATGTTCCTCATCTTATCCACCTCAACGTCT

1 TTTTTTCTACCCGCCTGTAAACATTTGAATAACGTCCATCCATTTAAAT

1 TTTTTTCGTGGAATCCCGTAGCGCCAGTACCCCCAAAGCGCCTATCAAT

1 TTTTTTCGTCCTATTAGCTCACGTCTTACCATCTACCTTTATCCGATTT

1 TTTTTTCGTAGTTGCTATCGGGATTGTTGTAGTTACGATCCAGTCTTGC

1 TTTTTTCCTTGCCGCCAATCCGTCTCACTGTATATGGTCGGGTGAATTG

1 TTTTTTCCGTGTCATTAACCTTATTGCGCCATCACCCTTTATTTTCTCG

1 TTTTTTCCGTCTCTAGTGTGCGCGTGTCCTTCGAAAGCTCACAGTACGT

1 TTTTTTCCGTCCTAAACTTTTACTTTTCCATAGCTTTCCGACTTTATGG

1 TTTTTTCCCCGTACGTTTAAGTACTTGGTATCCCAACTTCGCCGCGGGT

Round 4 sequences

2 TTATCGTGATATGCTCCATCTATACGCATCATGTTAGAAGCCGATTCTC

2 TAGCCTTTACTACTTAAAAAGTCGAACTCTTGCGTGCGTCCTCCGTATT

2 GAGCTATTCTATGTTTCCCCCGTCCGCGGTTATCCTTTAGTCACGCCAT

2 CCCGTACCTTCCGTAATAATTTAGCCGTCCACGACCTGTTTTTCCCACA

2 CCCATCTTATACGTATATGGACTCATCTCGACCCCCGATAGGCTTGGTA

2 CACACACCTTAAATGCCCTGCTACTGCGCTGCTGGGTACACCCGTCCCT

1 TTTTTTTTTTTAAAAACGATCTTGTCTCGCCTTTAGTGACTTTGTCAGC

1 TTTTTTTTGGCAACCCCTGATTGTGTCCTCCTTCACGACGTAACCTCGT

1 TTTTTTTTGCCCGTAATTGTTATCAGTTGCTGCCATTCTTGGTATCTGG

1 TTTTTTTTGACAATTCGCTATGGTGATGTTAGTTCTGTACCTATTGGAT

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1 TTTTTTTTCATTGCAGACTCCTTTTGGCATTGACTGGGGGTGCCAATCG

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1 TTTTTTTAATTTTATGCTATCTTAATCCGGAGACTCGAATCTACTAAAG

1 TTTTTTTAATACTCTCGCATCCTTACTAATCGGTAGTGTCCCCTCTCCC

1 TTTTTTTAACTACCTCGCAAAGTTTCGTCACTGCTTCCGACTTGTTGAT

1 TTTTTTGTTTTTGTCTGGGGTATTTCTCTCTGCTTGAATTTTAACATGA

1 TTTTTTGTTCAGCACCCTACCGAAACATGTGCTCCTAATTTCTAGCGCG

1 TTTTTTGTGCCGATAATACTTCACTGATAATGTTTGTTTATCAGATCGA

1 TTTTTTGTATGTTAATAACGCTCCGCGCCTATTTATTCAGACAGTCCTA

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1 TTTTTTGGGCATTTATGCTTGCTACGTCTGGTCCGATACTGTTTGCGCG

1 TTTTTTGGCTTTATCCTAAATTGACGTCGAGGTACGTGTTTGGATACTG

1 TTTTTTGGACCTTTAGTAACTTGCATTTCATTTGCCCCCCGTTCTCTGT

1 TTTTTTGCCGGAAATTTTTAACCCTCTTCATCACTTAACCCAGTTATTC

1 TTTTTTGCCACTGGTTGTGATTCCTCATTCATGCATGACAAGTCAACAA

1 TTTTTTGCATATACTTCACATCGCGTTCGAGACAAGAGCTGGCCCGGGG

1 TTTTTTGATGTGTCATGGGGATTGACGAGCTGGTATACCTAGCTCTGTT

1 TTTTTTGACGCGCCAAGTACACTAGCTTACAGATATACTACATATTCTA

Round 5 sequences

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2 TGCCACTCATTTGAATCCCGCTAACCGACGGTGGCGTCTGTTAATGGTT

2 TGAGGTTAGAGTTCCTTACTATTTCACTGCTGCTTAGCAAGTCCATTAT

2 TAAATTGCTTTGTCTGCCAAAGACCCTTCATTAGCACTCTTCGCATTAT

2 GTAGCAAGTTTATTCTGATCAGTTGCTGTTGTTGTTTTTTGAGTCACCC

2 GCCTGCATCCTTTTTGAGCTTGAACTGGTCGCATACAATAACCGCACCC

2 CATTTCCTAAATTATCGATTTCGCATACAGTTATGTACCAGGACGCGTT

2 ATACGGTATATTATCCCTTGATTGTTAGCTGGCGGCCTTGTTAGTCATT

2 ACGCCGGACGTTTTAGTGATGTTGTTGTCACGGTTTATCGCGGTATCAT

2 AACCTCACCCTCGCTGTTCAATCACCTTAAAGTCCTTACTTTCTTCCTC

1 TTTTTTTTTGACCCCTAAAACCTATTGCTGTGTACCGCAGTATGATCCA

1 TTTTTTTTTCCTATTAATAAGCCGCTATCGACCAATCCAGCATGCATCC

1 TTTTTTTTGATAGGCCAATCCCGACGTTAGAAGGGGCCAGCTCACCGGC

1 TTTTTTTTCCCATATGCCCTTAACAAGTTTACTAGATTGCTCGCTGTAC

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1 TTTTTTTTCAACCCCACCAGAGGTACAATATTCCATCTATCGGACTCCA

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1 TTTTTTTAACAGGTACGTCTACACTGCACGCAAGAGCGCAATGTGCCTG

1 TTTTTTTAAACCGTCTACTCGTAGTAATATAGACCCCAATATCTCAACC

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1 TTTTTTGTTATGATTTTGGTCTTCACGGATGCGGAACCTACTCTTACTT

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1 TTTTTTCTTTTAGATTTGTGTGTTTTTTGAGATAGACATGTTTTATGAC

1 TTTTTTCTTCGTAATTCACCTTGATGACCTAGGGAACCTACTCACTGAC

1 TTTTTTCTTATCGCCACCGTACCTCGCTTAAGGTCACCTGATATCTGCA

1 TTTTTTCTGGGTCCTTGTCAAAGTATAACCTGGACACTTCATCATTCAC

APPENDIX C

C1. Ethical clearance

CSIR Research Ethics Committee
PO Box 395 Pretoria 0001 South Africa
Tel: +27 12 841 4060
Fax: +27 12 841 2476
Email:R&DEthics@csir.co.za

09 January 2017

Dear: **Dr Kabamba Alexandre**

Approval of Protocol: **Isolation of HIV-1 anti-gp120, anti-p24 aptamers for use as reagents in flow cytometry.**

This is to confirm that your Protocol reviewed by the CSIR REC has been approved. The reference number of this research project is **Ref: 184/2016**.

This approval is granted under the condition that:

1. The researcher remains within the procedures and protocols indicated in the proposal, as well as the additions made to the procedures and protocols as indicated in the responses submitted to the questions of the REC, particularly in terms of any undertakings made and guarantees given.
2. The researcher notes that **any deviations to the approved project/protocol must be submitted to the REC for approval before implementation**.
3. The researcher remains within the parameters of any applicable national legislation, institutional guidelines and scientific standards relevant to the specific field of research.
4. This approval is **valid for one calendar year from the date of this letter**.
5. The researcher submit bi-annual progress reports to the REC
6. The researcher immediately alert the REC of any adverse events that have occurred during the course of the study, as well as the actions that were taken to immediately respond to these events.
7. The researcher alert the REC of any new or unexpected ethical issues that emerged during the course of the study, and how these ethical issues were addressed. If unsure how to respond to these unexpected or new ethical issues as they emerge, the researcher should immediately consult with the REC for advice.
8. The researcher submit a short report to the REC on completion of the research in which it is indicated (i) that the research has been completed; (ii) if any new or unexpected ethical issues emerged during the course of the study; and if so, (iii) how these ethical issues were addressed.

We wish you all of the best with your research project.

Kind regards

Dr Shenuka Singh

(CSIR REC Chair)

Dr Sandile Ncanana

(CSIR REC Secretariat)

R14/49 Miss Kanyane Bridgett Malatji

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1611138

NAME: (Principal Investigator)	Miss Kanyane Bridgett Malatji
DEPARTMENT:	Surgery CSIR
PROJECT TITLE:	Isolation of HIV-1 Anti-gp120 and Anti-p24 Aptamers for Use as Reagents in Flow Cytometry
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Pascaline Fonteh
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	16/11/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in November and will therefore be due in the month of November each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 27/03/17

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

C2. Turn-it-in Report

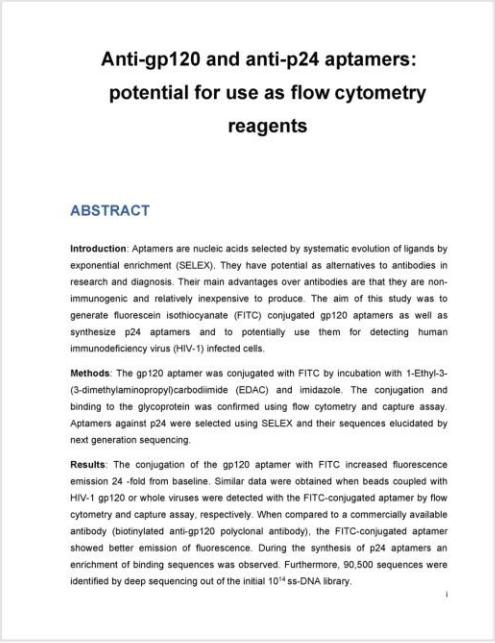
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Anti-gp120 and anti-p24 aptamers: potential for use as flow cytometry reagents

ABSTRACT

Introduction: Aptamers are nucleic acids selected by systematic evolution of ligands by exponential enrichment (SELEX). They have potential as alternatives to antibodies in research and diagnosis. Their main advantages over antibodies are that they are non-immunogenic and relatively inexpensive to produce. The aim of this study was to generate fluorescein isothiocyanate (FITC) conjugated gp120 aptamers as well as synthesize p24 aptamers and to potentially use them for detecting human immunodeficiency virus (HIV-1) infected cells.

Methods: The gp120 aptamer was conjugated with FITC by incubation with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and imidazole. The conjugation and binding to the glycoprotein was confirmed using flow cytometry and capture assay. Aptamers against p24 were selected using SELEX and their sequences elucidated by next generation sequencing.

Results: The conjugation of the gp120 aptamer with FITC increased fluorescence emission 24 -fold from baseline. Similar data were obtained when beads coupled with HIV-1 gp120 or whole viruses were detected with the FITC-conjugated aptamer by flow cytometry and capture assay, respectively. When compared to a commercially available antibody (biotinylated anti-gp120 polyclonal antibody), the FITC-conjugated aptamer showed better emission of fluorescence. During the synthesis of p24 aptamers an enrichment of binding sequences was observed. Furthermore, 90,500 sequences were identified by deep sequencing out of the initial 10^{14} ss-DNA library.

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