

DEVELOPMENT OF A MULTIPLEX HIV/TB POINT-OF-CARE DIAGNOSTIC ASSAY BASED ON THE MICROARRAY TECHNOLOGY

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DECLARATION

I declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Kanyane Bridgett Malatji

A handwritten signature in black ink, consisting of several overlapping loops and strokes, positioned above a horizontal line.

Day of 17 December 2023

ABSTRACT

HIV/AIDS mortality is caused by opportunistic illnesses/infections that take advantage of the weakened immune system in infected individuals. In Africa, the most common of these opportunistic illnesses include infection by *Mycobacterium tuberculosis* (*M.tb*) responsible for tuberculosis (TB). HIV co-infection with *M.tb* has negative implications for disease management given that each pathogen accelerates the morbidity caused by the other. The effective management of patients infected with both pathogens is restricted by the fact that their diagnosis is done separately. The situation is more difficult in remote areas where patients must wait for much longer to obtain their TB diagnostic results. In addition, the current diagnostic tests for the detection of TB such as chest X-ray and bacterial culture have a long turnaround time, are expensive to perform, and require sophisticated equipment and trained personnel. It is in this context that this project sought to develop an HIV and TB multiplex microarray-based assay for the detection of the two diseases using one test. The project used a 2.5 x 7.6 cm epoxy-coated glass slide as well as high-binding 96 well plates to which HIV-1 p24 and *M.tb* CFP10, ESAT6 and pstS1 antigens, known to be markers of active TB, were immobilized. The immobilized antigens were then incubated with anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies diluted in human serum to mimic physiological conditions where the antibodies would exist in the presence of other proteins. Detection of binding between the antigens and primary antibodies was achieved by means of secondary antibodies conjugated to either a fluorescence dye or horseradish peroxidase (HRP). In chapter two of the study, the immobilization of the HIV and TB antigens on the epoxy-coated glass slides as capture molecules of the HIV and TB antibodies diluted in human serum was performed. The antigen-antibody reactions detection were achieved by means of fluorescence dye conjugated secondary antibodies. This chapter also covered the sensitivity and specificity of the technology where the epoxy-coated glass slides were compared to the gold standard 96 well high-binding plates. Data showed that the HIV and TB antigen-antibody reactions were specific, and the slides were more sensitive relative to the 96 well high-

binding plates with limits of detection many folds lower. To be specific, the limit of detection from the slides averaged 0.954 ng/ml compared to 4474.6 ng/ml for the plates. The detection limit concentrations of the slides were lower than the reported physiological concentrations of HIV and TB antibodies in infected individuals. Chapter two also focused on the evaluation of the antigens' stability on the epoxy-coated glass slides by determining the optimal experimental pH buffer, temperature, storage condition (dry or wet), as well as the shelf-life. Data showed that the optimal pH and temperature for the HIV and TB antigens immobilized on the slides were pH 7.4 and 25 °C. Moreover, the antigens could be stored dry for at least 90 days without losing their function. Overall, this chapter showed that the epoxy-coated microarray slides performed better than the gold standard 96 well high-binding plates in terms of sensitivity; and that the immobilized antigens could remain stable for a long period, and do not require specialized storage conditions; thus, making the microarray technology a potential diagnostic tool for the multiplex detection of HIV and TB in the case of co-infection. Chapter three of the study focused on the proof-of-concept of the technology using human serum samples infected with HIV. The chapter showed that the technology could detect p24 antibodies in six out of seven samples infected with HIV, i.e., it detected p24 antibodies in 85.7% of samples known to be HIV positive. Furthermore, HIV negative samples also proved to be negative with this technology, thus no false positives were observed. Moreover, the technology was specific for HIV detection as no binding was observed on TB antigens. Therefore, these data support what was observed in the previous chapter when the HIV antibodies were spiked in normal human serum. Chapter four explored the application of the diagnostic technology for the point-of-care (POC) detection of HIV and TB antigen-antibody reaction, using HRP conjugated secondary antibodies, as well as the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid) (ABTS) and 3,3',5,5'-tetramethyl Benidine (TMB) substrates for colour change based endpoint. This chapter also covered the sensitivity and specificity of the immunoassay in the high-binding 96 well plates and on epoxy-coated glass slides. Similar to what was observed in the previous chapter, the HIV and TB antigen-antibody interactions were specific, and the epoxy-coated microarray slides were more sensitive than the 96 well high-binding plates with limits of detection averaging 815-folds lower than the plates. Nevertheless, both platforms were found to

be sensitive enough to be used for the POC detection of HIV and TB co-infection using visual inspection. Furthermore, the stability of the antigens in the 96 well high-binding plates using colour change detection was also evaluated. The antigens were found to be stable in the high-binding plates at different pH and temperature conditions; however, pH 7.4 and 25 °C were optimal. In addition, the antigens were stable when stored dry in the plates for a period of three months. In addition, between the two HRP substrates used, TMB was faster and more sensitive to the HIV and TB antigen-antibody reactions than the ABTS substrate, and the difference was statistically significant ($p < 0.05$). The importance of this chapter is that it eliminated the need for sophisticated equipment to detect the presence of HIV and TB antibodies, as the detection could be achieved by visual inspection. Overall, data in this chapter supported further development of the microarray technology for the POC HIV and TB co-infection diagnosis. Chapter five attempted to produce the CFP10, ESAT6, and pstS1 TB antigens in plants to reduce the cost associated with the current commercially available bacteria-produced antigens. These antigens were destined for use in the multiplex microarray-based HIV and TB diagnostic technology. *Nicotiana benthamiana* (*N. benthamiana*) was employed to express the proteins and the process involved techniques such as cloning, polymerase chain reaction (PCR), restriction digest, ligation, agroinfiltration, protein extraction, SDS-PAGE as well as Western blotting. The constructs were successfully cloned, PCR amplified, digested from the pUC57 vector, and ligated into the pEAQ expression vector, and agroinfiltrated into the leaves of the *N. benthamiana* plant. However, all the antigens were not successfully expressed in the plants. This may have been due to several reasons associated with the agroinfiltration process such as the agrobacteria and plant used, as well as factors such as pH and temperature employed during the expression. In chapter six, the implications of these findings, as well as future work that can complement the work reported in this thesis, are discussed. Overall, the data showed that the developed HIV and TB multiplex microarray-based technology can potentially be used for the detection of the two pathogens simultaneously and at POC without the need for sophisticated equipment. This is very important given that HIV and TB are diseases affecting mostly poor and developing countries in the world.

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DEDICATION

To my mother

Matobole Grace Malatji

Thank you for the prayers and support you have provided me throughout my studies, particularly this journey.

To my siblings

Mmotlana, Isaac, Foreman, Kholofelo, Zizi and Thabang Malatji

Thank you for supporting and believing in my dreams, particularly my sister Zizi who chased deadlines with me, ensuring I submitted all drafts on time during this journey.

A special dedication to late father

Mochai Juda Malatji

My number one fan. Thank you so much for instilling confidence in me, believing in me and most importantly inspiring and pushing me to where I'm today. RIP mmina Noko.

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ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid)
AGL-1	<i>Agrobacterium tumefaciens</i> strain AGL1
AIDS	Acquired immunodeficiency syndrome
AIR	Arrayed Imaging Reflectometry
Alere q	Alere q HIV-1/-2 Detect
ALEX	Allergy Explorer
ANOVA	One-way analysis of variance
Apa	Proline-rich antigen
bp	Base pairs
BSA	Bovine serum albumin
CarbArrayART	Carbohydrate microarray Analysis and Reporting Tool
CBA	Cytometric Bead Array
CCD	Charge Coupled Device
CFP10	Culture filtrate protein-10
DNA	Deoxyribonucleic acid
<i>E.coli</i>	<i>Escherichia coli</i>
ELISA	Enzyme-linked immunosorbent assay
ESAT6	Early secretory antigenic target protein 6
GOPS	3-glycidyloxypropyl trimethoxysilane
Gt	Goat
HIV	Human immunodeficiency virus

HRP	Horseradish peroxidase
IgG	Immunoglobulin G
kbp	Kilobase pairs
kDa	Kilodalton
LBA	<i>Agrobacterium tumefaciens</i> strain LBA4404
ml	Millilitre
Ms	Mouse
MS media	Murashige– Skoog (MS) media
<i>M.tb</i>	<i>Mycobacterium Tuberculosis</i>
<i>N. benthamiana</i>	<i>Nicotiana benthamiana</i>
NHS	N-hydroxysuccinimide
ng/ml	nanogram/millilitre
p24	Protein 24
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDITC	1,4-phenylene diisothiocyanate
PEG	Polyethylene glycol
POC	Point-of-care
PPDI	1,4-phenylene diisothiocyanate
pstS1	Phosphate-specific transport substrate binding protein-1
RCA	Rolling circle amplification
Rb	Rabbit
rpm	Rotations per minute

RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SPRi	Surface plasmon resonance imaging
TB	Tuberculosis
thio-NAD	Thionicotinamide-adenine dinucleotide
TMB	3,3',5,5'-tetramethyl Benzidine
μl	Microliter
V	Volts
Xpert	Cepheid Xpert HIV-1

CHAPTER ONE

LITERATURE REVIEW

Microarray-based multiplex diagnostic technologies and their applications

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Abstract

Microarrays are currently important tools in the high throughput profiling and characterization of different types of molecules particularly DNA, proteins, and carbohydrates. In many cases, this technology has allowed scientists to extract large amounts of data from small sample volumes. Furthermore, the microarray technology has advantages such as high sensitivity and specificity, they are inexpensive and have a short turnaround time. Multiplex microarray continues to develop with several different modifications being applied to enhance its performance in different applications. For example, it is currently being used in applications such as identifying underlying mechanism of drug responses, clinical diagnosis of bacteria such as meningitis, profiling mycotoxins, glycosidase activities as well as polysaccharide composition of plant species to name a few. In this review we give an overview of multiplex microarray technology and its applications in different fields. We also compare the different functionalized groups used in microarrays for screening, characterization, and profiling of different biomolecules.

1. Introduction

1.1. Microarrays origin and potential impact in diagnostics

Microarrays stem from a concept called ambient analyte immunoassay which was established by Roger Ekins (Ekins, 1989) and was later transformed into DNA microarray, a technology that determines the mRNA expression levels of thousands of genes in parallel (Chen et al., 2021a, Medina-Bastidas et al., 2020, Wu et al., 2021). The microarray technology usually involves the conventional immunoassay principles in which high affinity capture ligands are immobilized in parallel arrays in either a planar format or on a microsphere in suspension (Medina-Bastidas et al., 2020, Wang et al., 2022, Zhang et al., 2020a, Zhao et al., 2022a). The protein microarrays predominantly use antibodies, proteins, or peptides as binder molecules to capture circulating target proteins during incubation with biological specimens. The unbound proteins are washed off while the captured ones are detected by using several labeled reporter ligands such as dye conjugated secondary antibodies because of their sensitivity and selectivity (Gao et al., 2021, Khan et al., 2019, Ma et al., 2021, Thao et al., 2021). Though reporter proteins are the most used detection systems in microarrays, other label free methods have been applied to identify target molecules of interest. These include techniques such as quartz crystal microbalances, molecular imprinted polymer beads (Yarman et al., 2021, Bokeloh et al., 2022, Vaughan et al., 2018), spinning-disk microinterferometry (Varma et al., 2004), as well as plasmon resonance (Malinick et al., 2020, Pipatpanukul et al., 2018). However, these methods are expensive to perform, require sophisticated equipment and trained personnel.

Multiplex microarray refers to the miniaturization of different assays on one small plate or slide (Brittain et al., 2019, Liu et al., 2019a, Sauer, 2017, Sutandy et al., 2013). Microarray technologies are routinely applied in fields such as clinical diagnostics, environmental monitoring and food safety assessments; and offer advantages such as low probe consumption, low cost, short analysis time, increase in sample throughput, and the generation of high amounts of qualitative and/or quantitative information with a single test (Betancor et al., 2019, Brittain et al., 2019, Dincer et al., 2017, Klupfel et al., 2021, Savvateeva et al., 2021). Current immunoassay techniques based on single protein measurement can be financially and timewise costly. Therefore, it is of no surprise that multiplexing gained traction in the field of molecular biology and diagnostics. Since multiplex array is a fast-developing diagnostic technique that allows for the detection of multiple analytes in biological samples within a single experiment, they are an attractive solution for the analysis of biological responses in normal and diseased states (Charlarmroj et al., 2021, Khan et al., 2019, Zhang et al., 2021b). The success of a multiplex microarray depends on the efficient immobilization of different types of small molecules on a

solid support (Jorgensen et al., 2021, Zhu et al., 2016a), and the immobilization can either be through covalent or non-covalent interactions on functionalized surfaces (Li et al., 2021a, Yousefi et al., 2018).

Multiplex microarray can potentially have an immense impact on diagnostics for the development of inexpensive diagnostic tools. For example, Fan et al. reported the development of a surface plasmon resonance imaging (SPRi) biosensing assay for multiplex characterization of non-small cell lung cancer (NSCLC)-derived exosomes, using bioaffinity interaction of antibodies at different recognition sites (Fan et al., 2020). The SPRi array enabled the differentiation between normal lung and NSCLC cell-derived exosomes through precise identification of the exosomal protein pattern. In addition, the technique has been used for the simultaneous diagnosis of diseases such as HIV/AIDS, hepatitis C and A (Kranz et al., 2019). Moreover, it was also applied in the detection of multidrug resistance genes using recombinase polymerase amplification and microarrays (Warnt et al., 2021). Though multiplex assays require extensive validation when applied in diagnostic laboratories, they can be employed in early and differential diagnosis, disease staging as well as determination of disease prognosis (Tikhonov et al., 2020, Liu et al., 2019b, Liu et al., 2018a, Kumar et al., 2020).

2. Types of multiplex microarray assays

The most employed multiplex microarray system detects macromolecules, nucleic acids, proteins, or carbohydrates in biological samples (Zhou et al., 2019, Soares et al., 2021, Holz et al., 2018). Below, three forms of multiplex microarray assays and their application are discussed.

2.1. Nucleic acid Microarray

Nucleic acid microarray can be a collection of DNA molecules bound to a solid substrate used to measure the expression levels of a large number of genes as well as genotyping multiple regions of a genome (Oudeng et al., 2020, Wimmer et al., 2018, Zhang et al., 2020b). DNA microarrays enable the generation of a large quantity of genomic data with a single experiment (Alonso et al., 2018, Ma et al., 2020, Song et al., 2019, Stolpovsky et al., 2022). Nucleic acid microarray also has other use in research. For example, they are ideal tools for a wide range of diagnostic testing because of their stability, ease of synthesis, and high specificity for the target of interest (Yousefi et al., 2018). Their application in molecular and clinical biology include genetic mutational scanning and polymorphism detection, screening of disease and selection of antisense reagents and monitoring of gene expression to name only a few (Almugren and Alshamlan, 2019, Jia et al., 2022, Michalak et al., 2019, Petralia et al., 2021). Furthermore, they have been used in monitoring cancer therapy where the technology detects noncoding RNA for regulation of several disease related compounds (Zhao et al., 2022b).

Oligonucleotide microarray is the preferred form of nucleic acid microarray as they are inexpensive and have high specificity compared to long DNA molecules or cDNA microarrays (Figure 1) (Holz et al., 2018, Kappel et al., 2020, Lietard et al., 2018a). Another advantage of using oligonucleotides is that captured molecules can be amplified to facilitate quantification (Schaudy et al., 2023, Lietard et al., 2018b). Since oligonucleotides immobilization happens from an aqueous environment, a balance in the hydrophilic and hydrophobic characteristics of the solution is required in order to prevent a lower than needed number of probe sites per unit which can be caused by excessive droplet spreading (Qi et al., 2021, Sinyakov et al., 2021, Pirrung, 2002). The technique of immobilizing DNA on solid surfaces ought to be fast, covalent and reproducible in order to be compatible with automatic spotting instruments dispensing small drops of liquid on the surface (Alonso et al., 2018, Behzadi and Ranjbar, 2019, Chen et al., 2021a, Govarthanan et al., 2020). Nucleic acids can be immobilized on solid surfaces using physical adsorption or covalent linkage; however, with the former method, oligonucleotides tend to be unstable and liable to removal from the solid surface (Ventimiglia et al., 2022, Shi et al., 2015, Kunze et al., 2016). Thus, the latter is often preferred. The covalent linkage methods employ aldehyde coated slides as well as epoxy, amine, carboxyl, and N-hydroxysuccinimide-coated substrates (Cimaglia et al., 2018, Diaz-Betancor et al., 2019, Lietard and Somoza, 2019, Sinyakov et al., 2021, Yousefi et al., 2018). Moreover, the chemistry of covalent binding of DNA molecules on functionalized glass surfaces for the purpose of array formation must meet the following criteria: the coupling should involve one extremity of the DNA molecule and be long enough to eliminate unwanted steric interferences from the support (Bannister et al., 2018, Sinyakov et al., 2021, Zammattéo et al., 2000). To augment the DNA density and fluorescence signal and thus amplify the sensitivity of detection, dendrimeric structures that prevent steric hindrances and highly hydrophilic polyacrylamide gels which enable solution phase-type hybridization behaviour have been applied (Le Berre et al., 2003, Suriano et al., 2006). In recent studies, the most commonly used method of hybridization detection in DNA microarray is that which employs the use of fluorescence confocal microscopy because it offers high sensitivity and low background noise (Bannister et al., 2018, Lietard et al., 2018a, Qi et al., 2021, Stolpovsky et al., 2022).

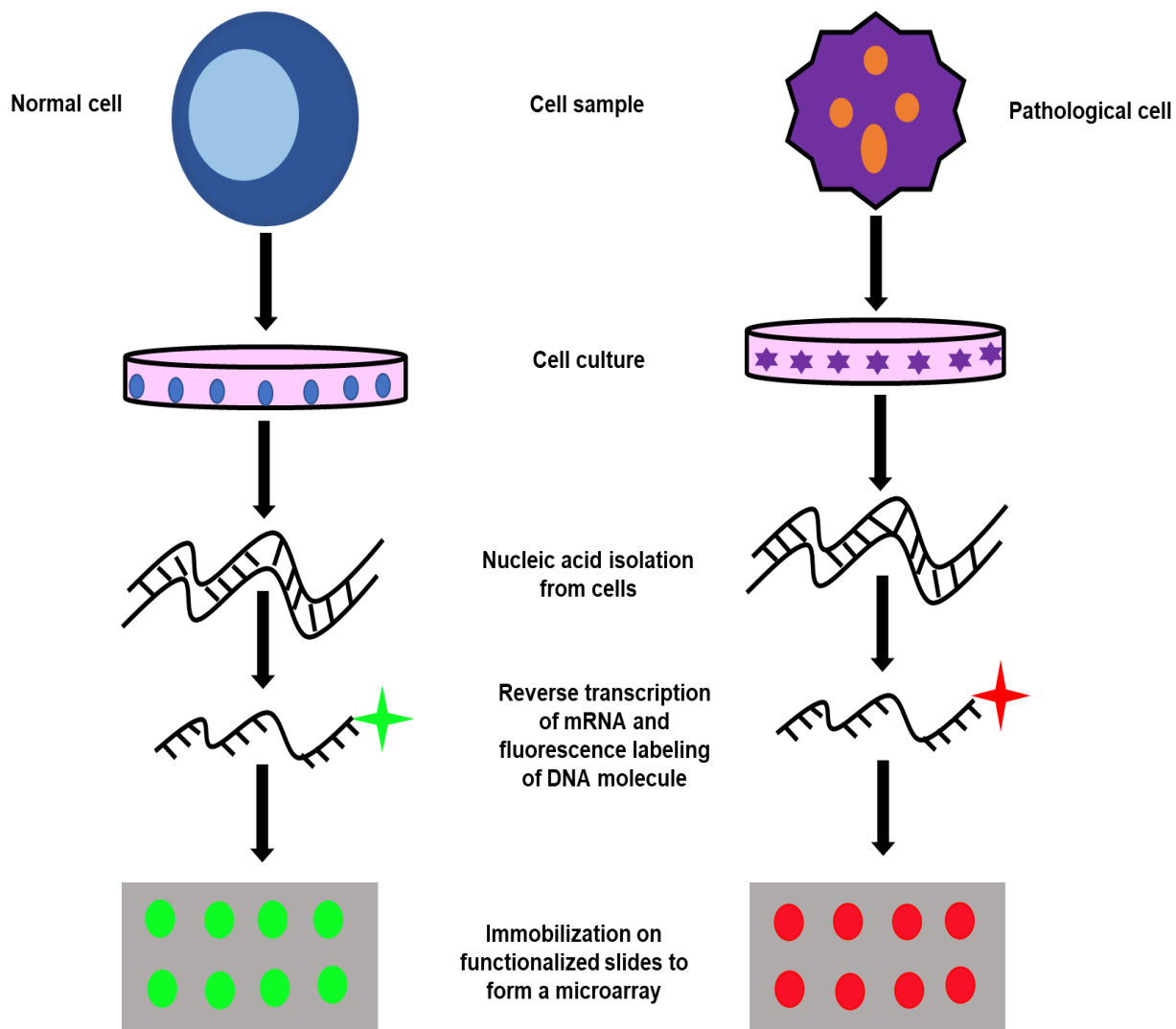


Figure 1: An illustration of nucleic acid microarray (DNA). The process starts with the culturing and isolation of nucleic acid molecules of interest from diseased or healthy cells. The isolated molecules are then subsequently labelled with a fluorescence dye (green or red symbols in the depiction) and immobilized on solid substrates for characterization and profiling. This immobilization commonly occurs through covalent binding.

2.2. Protein microarray

Protein microarrays are high-throughput methods that can be used to detect protein activities, interactions, and functions on a large scale (de Assis et al., 2021, Dorskaya et al., 2018, Kim et al., 2021). These microarrays can be useful for screening antibody responses against large panels of antigens and protein fragments to identify biomarkers (Berube et al., 2022, Du et al., 2021, Markwalter et al., 2021). In protein microarray, proteins of interest are immobilized on a solid surface while preserving their three-dimensional confirmation (Du et al., 2021, Ho et al., 2022, Rusmini et al., 2007, Sutandy et al., 2013). Since microarray individual spots comprise a large number of molecules within a very small surface area, the density of the active surface groups is the key factor allowing for the reduction of the amount of samples used and affecting the sensitivity of the array (Rusmini et al., 2007, Chiodi et al., 2021). Self-assembled monolayers of organosilicon are often used for coating glass slides, as they enhance the biocompatibility of the surface and shield proteins from denaturation during the immobilization step (Rusmini et al., 2007, Huang and Zheng, 2019, Lee et al., 2020). Glass slides coated with organosilanes, or nitrocellulose are also considered suitable for immobilization of proteins (Khan et al., 2019, Markwalter et al., 2021, Rusmini et al., 2007, Tulin et al., 2021). The most used form of protein microarrays involves planar surfaces. The power of protein microarrays instigates from the substantial specificity of antibodies for antigens and the current availability of polyclonal or monoclonal antibodies for different proteins (Gahoi et al., 2020, Ren et al., 2022).

Unlike the immunoassays or ELISA commonly performed in 96 well plates, which consume relatively large amounts of samples (Ellington et al., 2010), the protein microarray-based assays employ many folds smaller amounts of sample and usually have higher sensitivity (Brittain et al., 2019, Dincer et al., 2017, Klupfel et al., 2021). Several techniques have been employed to augment the sensitivity of multiplexed protein microarray including a rolling circle amplification (RCA) method. The technique uses the 5' end of an oligonucleotide primer attached to an antibody where the antibody-DNA conjugate binds to its target molecule and RCA occurs when a circular DNA interacts with the complementary primer. This is followed by the products detection with multiple fluorescent oligonucleotide probes. The result is a significant increase in sensitivity as this RCA enhanced signals remain localized at the microarray spot (Gao et al., 2019, Soares et al., 2021, Zhou et al., 2019).

2.3. Carbohydrate microarray

The commonly used carbohydrate microarray is one which employs glycans. These microarrays provide valuable information on immunological studies and have allowed for the discovery of novel binding epitopes of known endogenous immune receptors and enabled the discovery of natural ligands

that carry glycans (Gao et al., 2019, McQuillan et al., 2019). The printing efficiency of glycans on the microarray depends on the type of active slides, printing buffer and time used (Ruphecht et al., 2019). The carbohydrate microarray has been employed for example, to evaluate carbohydrate-protein interactions (Laigre et al., 2018, Ruphecht et al., 2019). They have also been used for the detection of bacterial pathogens exhibiting different mannose binding affinities such as *E.coli* and *Salmonella*; here particular mannose coated microarrays were used (Jing et al., 2020, Gao et al., 2019, Campanero-Rhodes et al., 2020). Furthermore, Kim et al., reported the development of a multivalent carbohydrate microarray using glycans immobilized on hydrazide-derivatized glass surfaces without the use of glycoconjugates and showed that the binding affinities of toxins and pathogens such as uropathogenic *E.coli* were dependent on the density of these glycans (Kim et al., 2018). Recently microarray consisting of glycans have been used for the label-free multiplex detection and discrimination between human and avian influenza viruses using Arrayed Imaging Reflectometry (AIR) (Zhang et al., 2021). Carbohydrate microarrays containing glycosylated fluorescence probes have been developed in an N-hydroxysuccinimide (NHS) ester-conjugated modified surfaces and have been reported to be highly useful for profiling glycosidase activities as well as for the determination of IC₅₀ values of glycosidase inhibition (Hyun et al., 2018). Lastly, carbohydrate antigen microarray comprising of greater than 400 glycoconjugates has been employed to determine IgM and IgG antibody specificities and concentrations as well as to profile anti-carbohydrate antibody repertoires (Luetscher et al., 2020, Nanno et al., 2021). For efficient analysis of carbohydrate microarray, a software has recently been developed called Carbohydrate microarray Analysis and Reporting Tool (CarbArrayART); this can be used for recording and storage of quantified array data and management of different types of slide-based glycan microarray experiments (Akune et al., 2022).

3. Three dimensional and planar microarray systems

Multiplex microarray immunoassays can be divided into two systems namely three-dimensional suspension microsphere and planar assays (Dincer et al., 2017, Medina-Bastidas et al., 2020, Wang et al., 2022). The three-dimensional suspension multiplex assay utilizes microspheres as the solid support to which different captured ligands are coupled covalently using reactive functional groups such as N-hydroxysuccinimide ester (Ellington et al., 2010, Yan et al., 2022). On the other hand, planar microarray involves the use of slides with molecules that are immobilized by functionalized groups such as nitrocellulose, epoxy, isothiocyanate, aldehyde and poly-L-lysine to profile and study the characteristics of target molecules. The two forms of microarrays are discussed in the subsections below.

3.1. Three dimensional microarrays

A three-dimensional label-free suspension multiplex assay was reported by Zhao et al., and Tsougeni et al., using molecularly imprinted polymer beads, which detects biomolecules without the use of immunological antibodies (Tsougeni et al., 2018, Zhao et al., 2009). Tsougeni and colleagues found that three-dimensional multiplex assay slides offered significant improvement in terms of spot intensity, homogeneity, and reproducibility relative to planar microarrays (Tsougeni et al., 2018). In addition, the three-dimensional slides showed higher signal to noise ratio over a range of protein concentration and a shelf life of one year without the need of specific storage conditions (Li et al., 2020b, Tsougeni et al., 2018).

Microsphere microarrays use classifications such as beads size, internal fluorophores, and flow cytometric principles to detect the captured molecules (Vafajoo et al., 2018, Kharati et al., 2020, Zattoni et al., 2022). This type of multiplex microarray was also used by Ellington et al., and Ekinins where they showed that miniaturization of immunoassays on this platform conferred lower limit of quantification because of improved signal-to-noise ratio and reduced reaction time due to shorter diffusion distances compared to conventional assays (Ekins, 1989, Ellington et al., 2010). Examples of three-dimensional microsphere microarrays also include Luminex techniques which are microbeads-based multiplex assays with the ability to measure multiple analytes at the same time in a single reaction (Keene et al., 2019, Liu et al., 2021a, Sackesen et al., 2019). These methods employ molecular reactions that take place on colour coded microbeads using different fluorescence intensities. Another multiplex method that uses beads is Cytometric Bead Array (CBA) which is a flow cytometry-based application that allows quantification of multiple proteins at the same time (Bai et al., 2019, Jin et al., 2020, Wei et al., 2018). The system utilizes the broad range of fluorescence detection offered by flow cytometry and antibody coated beads to efficiently capture and detect analytes (Mattsson et al., 2011, Tighe et al., 2013). In addition, a multiplex assay called Allergy Explorer (ALEX) was recently employed for identification of allergens (Heffler et al., 2018, Bojcukova et al., 2019, Buzzulini et al., 2019). ALEX array is an immunoassay for specific IgE to whole allergens and relevant molecular components and uses Charge Coupled Device (CCD) for detection (Bojcukova et al., 2019, Heffler et al., 2018).

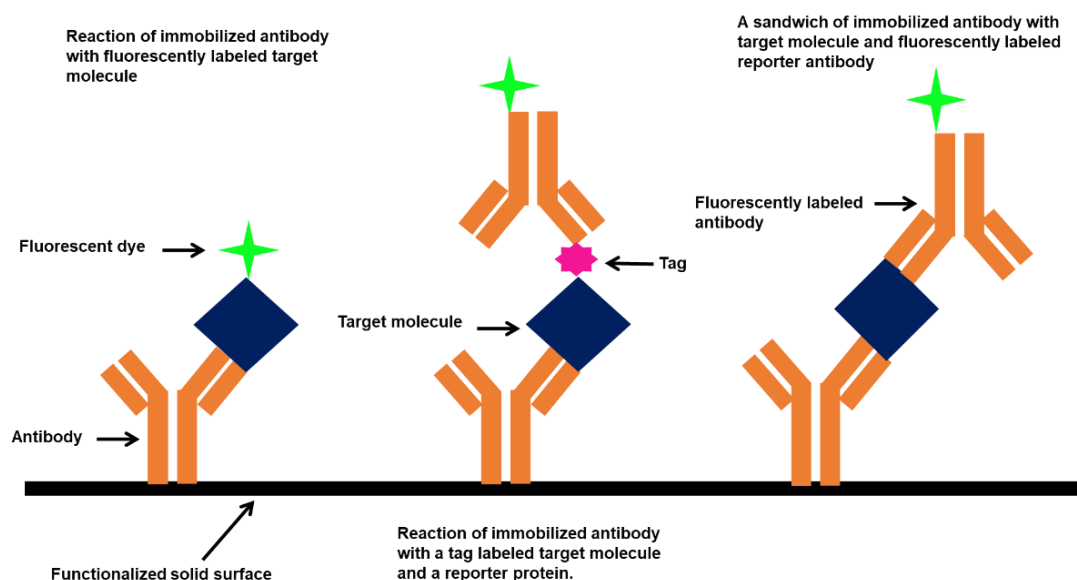
3.2. Planar microarray systems

Planar microarrays can be divided into analytical and functional microarrays (Sauer, 2017, Nakajima et al., 2018, Ma et al., 2021). An example of an analytical microarray is the antibody array which involves protein capture and detection using direct labeling (Haab, 2005, Sutandy et al., 2013). Analytical microarrays can be used to profile complex mixture of proteins to measure parameters such as binding affinities, protein expression levels and specificities (Bemetz et al., 2019, Sauer, 2017). However, these

types of microarrays have a number of limitations among them, the fact that they lack specificity in protein target labelling and have poor sensitivity for low-abundance proteins (Chandra et al., 2011, Sutandy et al., 2013, Zhang et al., 2020b). To circumvent these limitations, another format has been employed and uses the sandwich method in which two different antibodies are used to detect the target protein. The antibody which immobilizes the target protein on a solid phase is called the capture molecule, while the other dye labeled antibody, called the reporter molecule, produces the signal for detection (Ho et al., 2022, Khan et al., 2019, Ren et al., 2022) (Figure 2A). Using two antibodies substantially augments the specificity and sensitivity of the sandwich assay format to femtomolar levels (Chen et al., 2018b, Dincer et al., 2017, Hogan et al., 2019, Sutandy et al., 2013, Chen and Zhu, 2006).

Functional microarrays differ from analytical microarrays in that they are made of arrays containing full-length functional proteins (Nakajima et al., 2018, Ma et al., 2021). They enable the study of various biochemical properties of proteins such as protein-protein interaction, protein binding activities, protein-peptide interaction, and protein lipid interactions to name a few (Chen et al., 2021b, Govarthanan et al., 2020, Lin and Chen, 2018, Wang et al., 2019b) (Figure 2B). Functional microarrays are useful for the classification of protein functions, biochemical activities, enzyme-substrate relationships, as well as immune responses such as protein-protein binding (Feng et al., 2022, Hogan et al., 2019, Leday et al., 2018, Weng et al., 2019). Most functional microarrays use different types of functionalized solid surfaces which are capable of high-throughput, multiplexing, and have high sensitivity and specificity for protein measurements (Fabri-Faja et al., 2019, Normandeau et al., 2021, Yarman et al., 2021).

A.



B.

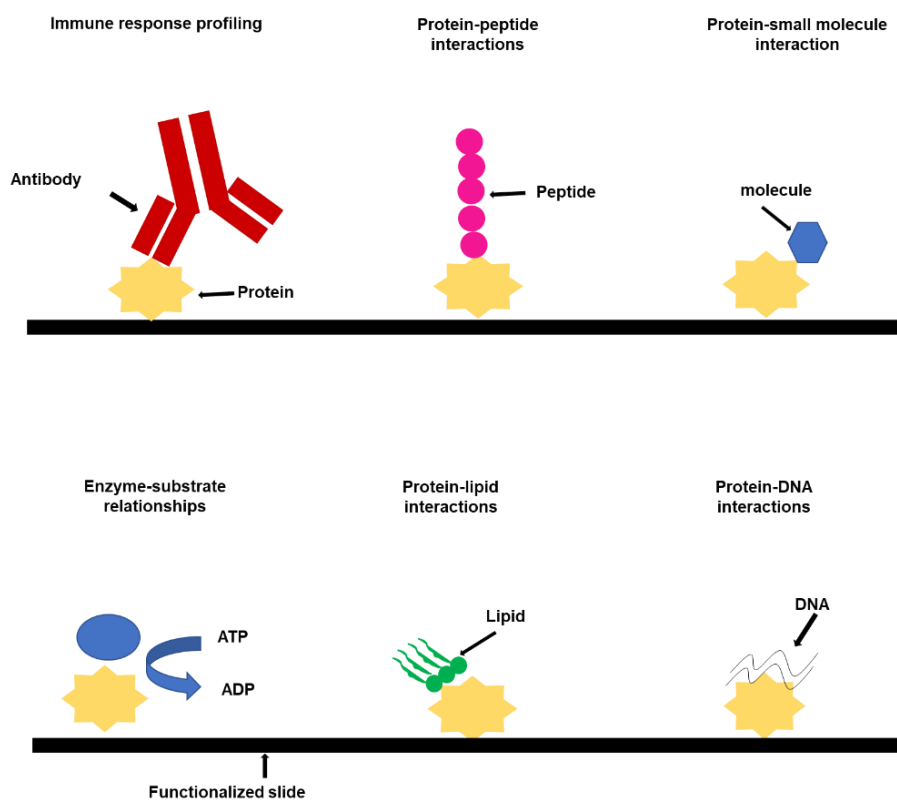


Figure 2: Illustration of analytical and functional planar microarrays. (A) Schematic representation of the analytical microarray system which involves the use of antibodies immobilized on solid substrates (black) to capture molecules of interest. The detection can be either through the use of labeled molecule (blue block and green star) or through another antibody (orange) labeled with a fluorescence dye (green) to form a sandwich immunoassay. The binding of the second antibody can either be via a tag (pink), or directly with the target molecule. **(B)** Shows functional microarray of biochemical properties

such as immune response profiling, protein-protein interactions, protein lipid interactions, protein-nucleic acid interactions, protein-enzyme interactions, protein-peptide interactions, and protein-small molecules interactions. Functionalized surface is shown in black; proteins are shown in yellow, peptides in pink, lipids in green, molecules in blue, and nucleic acid is represented by two black lines.

3.2.1. Different types of planar array functional groups

Microarray slides can be functionalized plastic or glass slides. This is because they are easy to handle and adaptable to automatic readers (Behzadi and Ranjbar, 2019, Connelly et al., 2017b, Robajac et al., 2020). Of the two surfaces, functionalized glass microscope slides are the preferred support for microarrays as they have low autofluorescence and are inexpensive (Das and Theato, 2016, LeBlanc et al., 2021). However, glass slides also have several disadvantages that include their low capacity for analyte binding and non-uniformity of spot size and intensity; thus, negatively interfering with the accuracy of quantitative analysis by microarray scanners software (Tsougeni et al., 2018).

Planar arrays can be developed with the use of robotics where printing of compounds can be readily achieved by contact printing utilizing solid split pins made of ceramic, steel or silicon (Zhang et al., 2021a, Chikae et al., 2021); or by non-contact printing using piezoelectric technology to eject uniform drops of antibody solutions to the surface of the slide (Summers et al., 2022, Martinez-Botas et al., 2021). Recent developments in planar multiplex microarray technology have shown that the technique has a wide variety of functional groups that can be used for immobilization such as nitrocellulose, aldehyde, ester derivatives, poly-L-lysine, isothiocyanate and epoxy. The functional groups and their application are discussed in detail in the next subsections and their comparison is outlined in Table 1.

3.2.1.1. Nitrocellulose and its applications in microarray

Nitrocellulose is a porous substrate which provides a 3-D network that augments the effective surface area to bind captured biomolecules resulting in higher protein fixation and increased signal intensity (Campanero-Rhodes et al., 2020, Guo et al., 2020, Yuan et al., 2019, Chen et al., 2022b). Glass slides coated with nitrocellulose have been used in microarray-based diagnostics for the evaluation and detection of *Streptococcus pneumoniae* capsular polysaccharides immunoglobulin G (IgG) in human sera, using 22 different *S. pneumoniae* capsular polysaccharides (Campanero-Rhodes et al., 2020). These slides were also used in the profiling of autoantibody biomarkers that serve as therapeutic targets in patients with psoriasis and psoriatic arthritis; the technique employed a variety of autoantigens such as rheumatic disease and skin antigens, inflammatory mediators, as well as putative autoantigens (Yuan et al., 2019). In addition, nitrocellulose slides were used for the identification of autoantibodies targeting

antigens in the trabecular meshwork as biomarkers for early detection of primary open-angle glaucoma (Beutgen et al., 2019).

Nitrocellulose coated slides also come with several limitations. For example, in multiplex protein microarray, these slides have a high amount of background autofluorescence in the visible spectrum utilizing laser excitation (Tighe et al., 2013, Chen et al., 2022b). They also result in poor immunoreactivity and inconsistent discrimination between samples, caused by their nonspecific interactions with bioprobes (Odinolfi et al., 2019, Chen et al., 2022b). Due to these limitations, Odinolfi and colleagues improved the use of the slides by introducing an easy method for peptide chemo selective bioconjugation onto nitrocellulose support; this was based on a clickable polymeric coating which allows efficient peptides bioconjugation and provides desirable anti-fouling properties to the nitrocellulose support (Odinolfi et al., 2019).

3.2.1.2. Epoxy-silane applications in microarrays

Epoxy-silane coating is a surface modification applied by many manufacturers of activated glass slides for protein and oligonucleotides microarrays (Chen et al., 2018a, Tsougeni et al., 2018). The epoxy-coated slides are reliable as they retain immobilized compounds better than most functional groups (Kalli et al., 2020) (Table 1). In addition, epoxy groups form strong bonds, with minimal chemical modification of the immobilized molecules (Behzadi and Ranjbar, 2019, Rusmini et al., 2007, Yousefi et al., 2018). The interaction between the coated slide and biomolecules such as proteins occurs through fast adsorption followed by intramolecular chemical attachment promoted by the epoxy groups on the glass surface (Feghali et al., 2020, Betancor et al., 2019). The reactive part of proteins that interacts with the epoxy on the solid surface is generally the amine group found on lysine and arginine since it is present on the exterior of proteins, forming stable amine bonds with the surface (Tulin et al., 2021, Pazitna et al., 2020) (Figure 3). During the coupling process, proteins are dissolved in a low ionic-strength buffer before being passed through the coated solid surface. The efficiency of immobilization depends on parameters such as the pH, ionic strength, concentration, and reaction time depending on the protein being studied (Yousefi et al., 2018). Furthermore, because of the epoxy glass slides stability and ability to retain molecules, immobilized biomolecules such as glycans can be stored for up to six months at room temperature (Nietzold et al., 2018).

The epoxy-coated slides have been used by researchers around the world in different fields. For instance, they have been applied in agriculture to study mycotoxins (Chen et al., 2018a), and were also used in lectin microarrays to select lectins that could be conjugated to ginger-derived glycoproteins (Zhao et al., 2018b). Epoxy-coated slides were as well used to implement an effective convergent synthetic strategy allowing access to an extended library of *Cryptococcus neoformans*

glucuronoxylomannan serotype A and D structures for the development of vaccines (Guazzelli et al., 2020). More recently, epoxy-coated slides were employed in SARS-CoV-2 studies to develop glycosaminoglycan (a glycan responsible for host attachment factor that promotes SARS-CoV-2 infection) microarray. This was done for high-sensitivity screening of the glycosaminoglycan-binding specificity of proteins and application in the analysis of the SARS-CoV-2 spike (S) protein (Ruano-Gallego et al., 2021, Watanabe et al., 2021).

Although epoxy slides are excellent for microarray applications, researchers have studied ways to improve their performance. One such study was performed by Batancor and colleagues where epoxy-coated slides were reacted with cross linked dextran hydrogels as the protective layer from degradation during the polymerization process (Batancor et al., 2019). In addition, the epoxy-coated slides have recently been improved for the immobilization of different immunoglobulin classes by including a cysteine-modified protein G on their surface, which enhanced their immobilization efficiency (Paul and Weller, 2020).

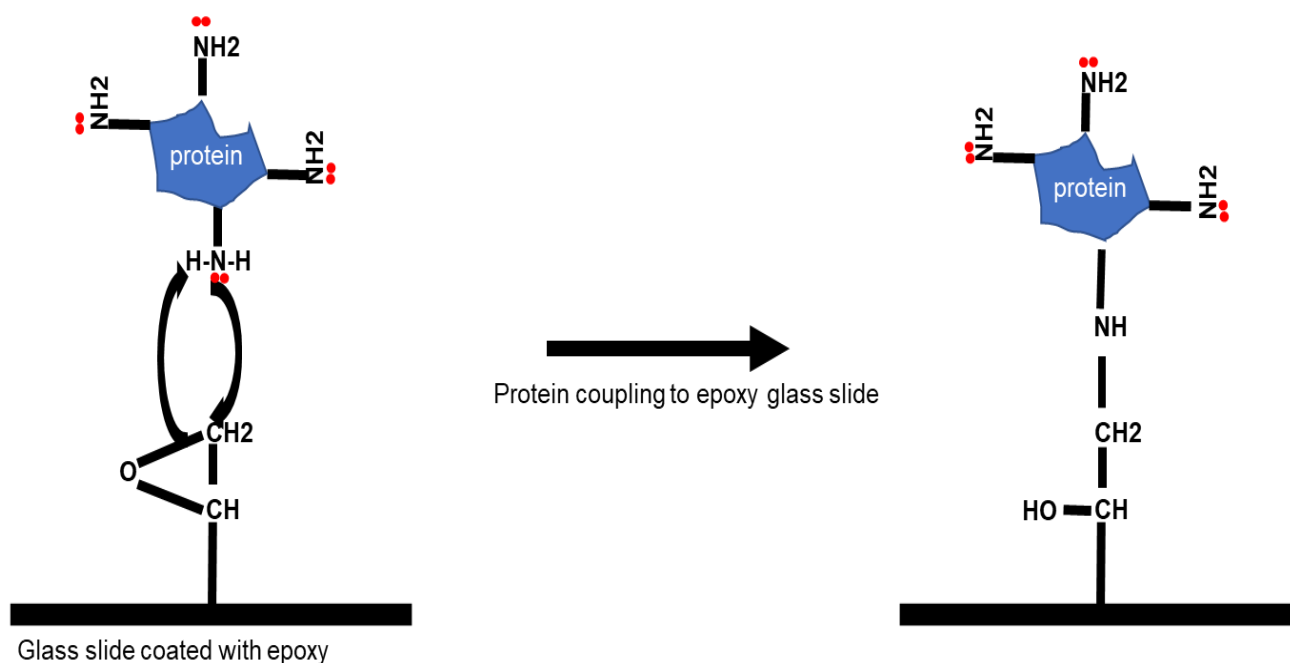


Figure 3: Illustration of the epoxy-coated glass slide interaction with proteins. The figure shows the epoxy group electrophilic carbon on the slide surface (black) being attacked by the lone electron pairs (red dots) from the protein (blue) amine group to form a covalent amine bond between the protein and the epoxy group.

3.2.1.3. Isocyanate functionalized slides

Unlike the above-mentioned functional groups, isocyanate offers non-selective functionalization to solid surfaces, making it a suitable functional group for libraries of natural products or compounds without a common residue for binding (Zhu et al., 2016b). This group can also be reacted with small molecules libraries containing either primary or secondary alcohols, arylamines, phenol secondary amine as well as carboxylic acid (Felsenstein et al., 2016, LeBlanc et al., 2021, Swain et al., 2021, Zhu et al., 2017) (Figure 4). In addition, isocyanate functionalized microarray slides can be used to identify small molecules that bind MicroRNA, such as in the study of microRNA -associated pathologies (Connelly et al., 2017b). Isocyanate coated slides created through the photo-activated cross-linking have advantages such as providing universal immobilization through carbon-based random insertions. However, they also have limitations that include their low immobilization efficiency for some molecules due to reduced reactivity (Zhu et al., 2016b). In addition, they have low reactivity to carboxylic acids and tertiary alcohols (Zhu et al., 2016b) (Table 1).

Previously, Felsenstein et al., used this technology to study small molecules that inhibit MYC transcription factor expression, a protein that plays a key role in cancer initiation, progression, and maintenance (Felsenstein et al., 2016). Furthermore, these slides have been used in cancer therapy monitoring studies to target Metastasis-associated lung adenocarcinoma transcript 1 identified as a prognostic biomarker of advanced metastatic non-small cell lung cancer (Zhao et al., 2022b). Moreover, isocyanate glass slides have been employed in monitoring the nuclear expression of triplex non-coding RNAs for therapeutic developments against Kaposi's sarcoma-associated herpesvirus (Swain et al., 2021).

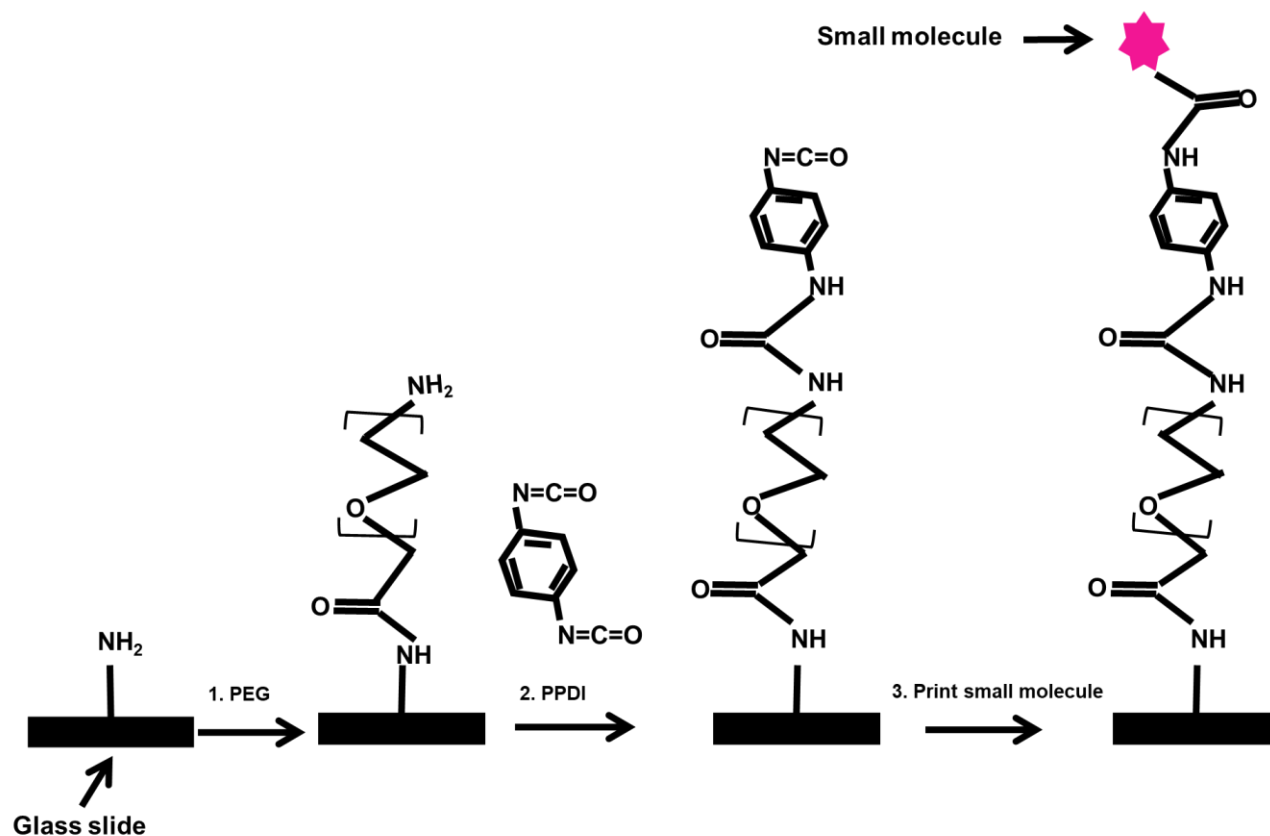


Figure 4: Illustration of the reaction between isothiocyanate coated glass slide and a small molecule. The isothiocyanate functionalized surface is prepared from an amine group containing glass slide. The amine slide is first incubated with polyethylene glycol (PEG) solution used as the spacer between the isothiocyanate chemical group and the surface. The process is then followed by the addition of 1,4-phenylene diisothiocyanate (PPDI) to add the phenyl-isothiocyanate residue to the slide. The latter is the part that reacts with molecules (shown in pink) to form covalent bonds. Adapted from Zhu et al., (Zhu et al., 2016b).

3.2.1.4. Ester derivatives microarray slides

Activated ester derivatives form part of the many different functional groups used to coat microarray slides. They are useful derivatives for covalent attachment of functional amine groups based on the stable amine bond formation which is the basis of many biological systems. The advantage of using activated esters is their diversity (Das and Theato, 2016, Lahiri et al., 1999) (Table 1). Activated esters such as N-hydroxysuccinimidyl esters are known to react with proteins or small molecules that have amino groups (Lahiri et al., 1999, Suriano et al., 2006) to form covalent bonds (Figure 5). This ester reactive group offers resistance to nonspecific adsorption of proteins, control over ligand density, and applicability to a wide range of ligands (Das and Theato, 2016). In addition, oligonucleotides also bind to the functional groups of active esters such as N,N-acryloxysuccinimide with their terminal amine groups, through a condensation mechanism to form an amide bond (Suriano et al., 2006).

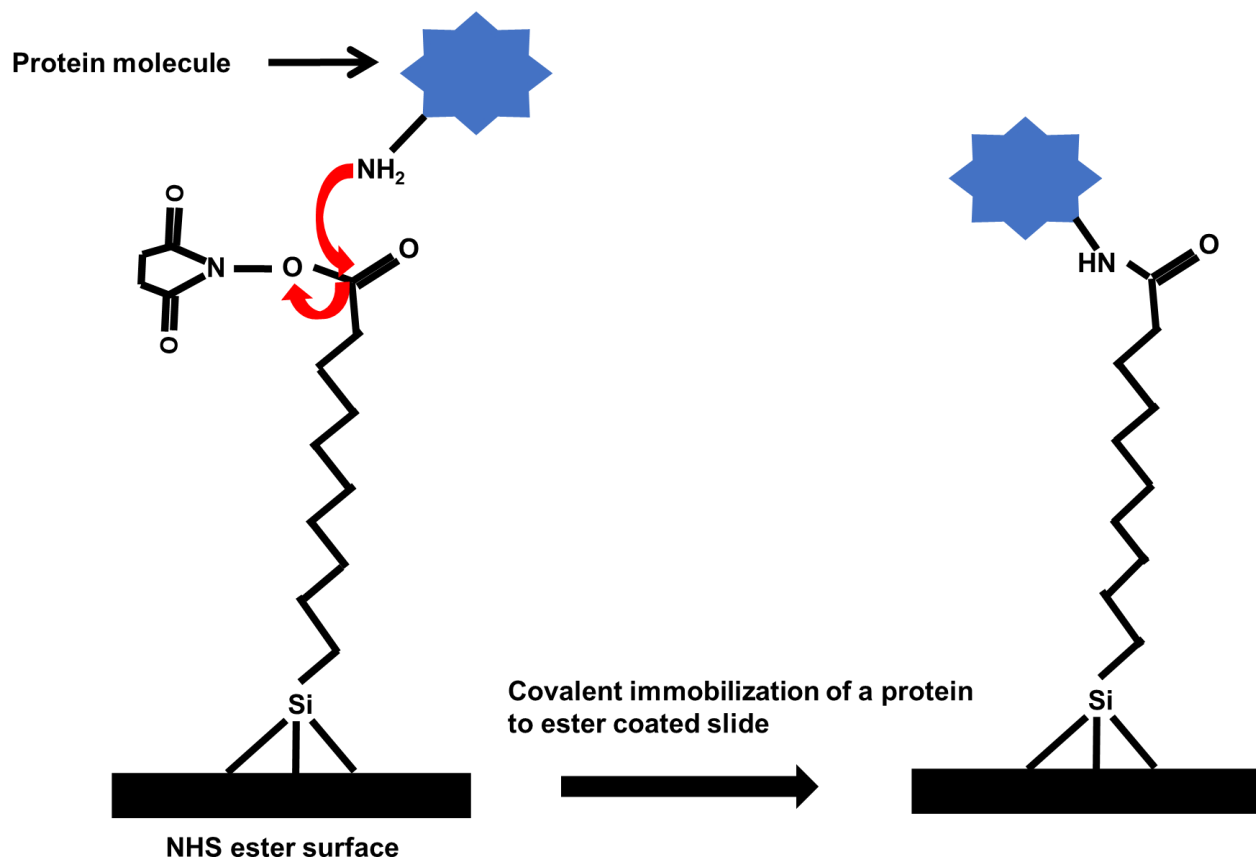


Figure 5: Illustration of the reaction between an ester coated slide and a protein. The lone pair of electrons from the amine group on the protein (blue) attacks the carbonyl group on the ester molecule coated slide (black) to form a covalent amide bond between the protein and substrate. The movement of bonds and lone electron pairs is indicated with red arrows.

3.2.1.5. Poly-L-lysine coated microarray slides

Poly-L-lysine coating through amine groups is also employed on microarray slides. This compound is an important water-soluble cationic polypeptide composed of naturally occurring L-lysine containing amine groups on the side chains (Xing et al., 2012). Like many other coating functional groups, poly-L-lysine reacts with the amine group found on proteins and oligonucleotides to form a covalent bond (Figure 6). To enhance the performance of conventional poly-L-lysine glass slides immobilization and hybridization, surface chemistry involving the use of 3-glycidyloxypropyl trimethoxysilane (GOPS)-a silane coupling reagent, was used together with pol-L-lysine by Wu et al., (Wu et al., 2005). These researchers showed that the immobilization and sensitivity was improved for the detection of SARS coronavirus by means of 60 mer oligonucleotide probes. In addition, Xing et al., used this chemistry to capture cancer cells where they found that the presence of GOPS and poly-L-lysine enhance the uniformity of the cancer cells adhered on the glass slides and can, therefore, be used in systems such

as ThinPrep for cervical carcinoma screening to augment the accuracy rate of diagnosis (Xing et al., 2012) (Table 1). This modification starts with coating slides with GOPS, followed by covalently coupling the GOPS modified slides with poly-L-lysine to form GOPS-poly-L-lysine. The slides are then activated with a 1,4-phenylene diisothiocyanate (PDITC) (Wu et al., 2005) (Figure 6).

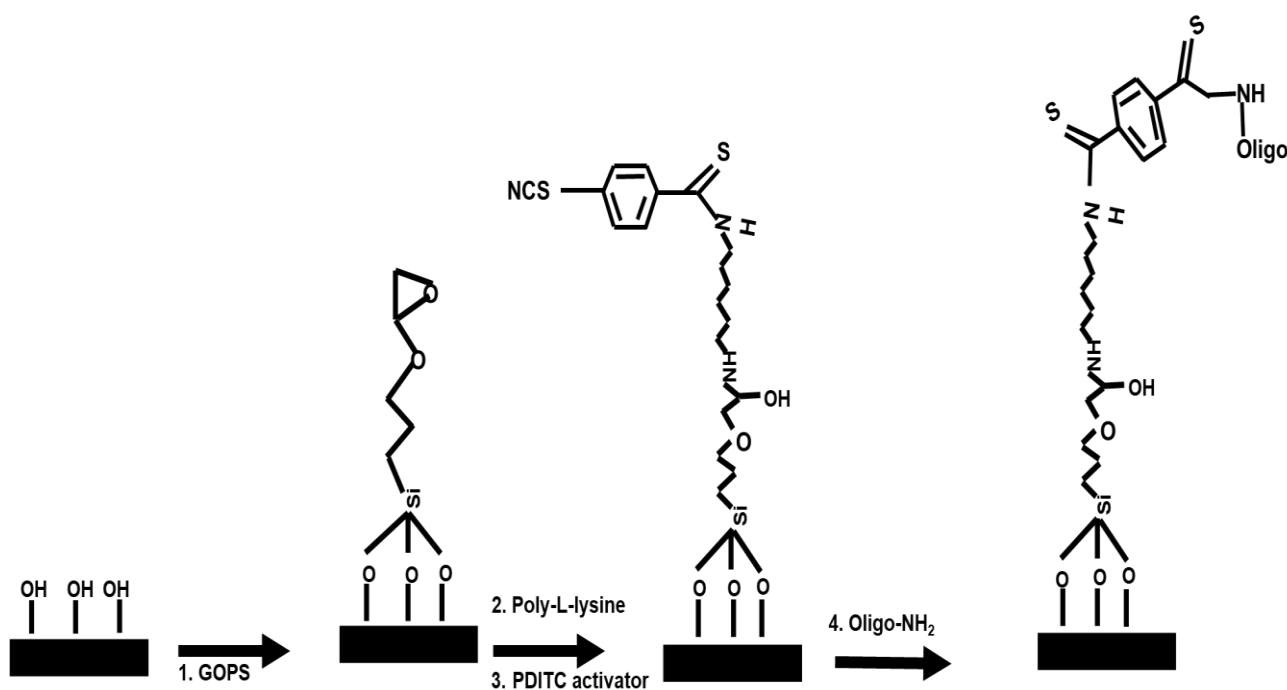


Figure 6: Illustration of the formation and activation of a poly-L-lysine slide and reaction with an oligonucleotide. The surface of the slide is first salinized using GOPS followed by covalent coating with poly-L-lysine. It is then activated with a 1,4-phenylene diisothiocyanate (PDITC) cross linker. The activated ploy-L-lysine reacts with the amine groups found on oligonucleotides to form covalent bonds during immobilization. Adapted from Wu et al., (Wu et al., 2005).

3.2.1.6. Aldehyde coated microarray slides

Like many other functional groups, aldehydes can also be used for coating microarray slides for immobilization of molecules containing the amine group such as proteins and nucleic acids. The chemistry of their reaction with amine involves the use of acidic conditions (Zammatteo et al., 2000), and employs nucleophilic attack of the carbonyl oxygen on the aldehyde by primary amine groups (Peelen and Smith, 2005) (Figure 7). Slides coated with aldehydes such as *n*-Hexacosal have been used to study the *in vitro* pre-penetration processes of *Blumeria graminis* using aerial plant organs (Hansjakob et al., 2010). In addition, these slides have been employed to investigate antibody-antigen interactions in protein microarray, where they were made with 3-aminopropyltriethoxysilane and

glutaraldehyde, a bis-aldehyde homobifunctional cross-linker (Liu et al., 2007). Aldehydes coated glass slides also have a number of limitations which include the fact that they are unsuitable for high-throughput screening studies, as it is the case with slides containing α -Oxo aldehydes (El-Mahdi and Melnyk, 2013) (Table 1).

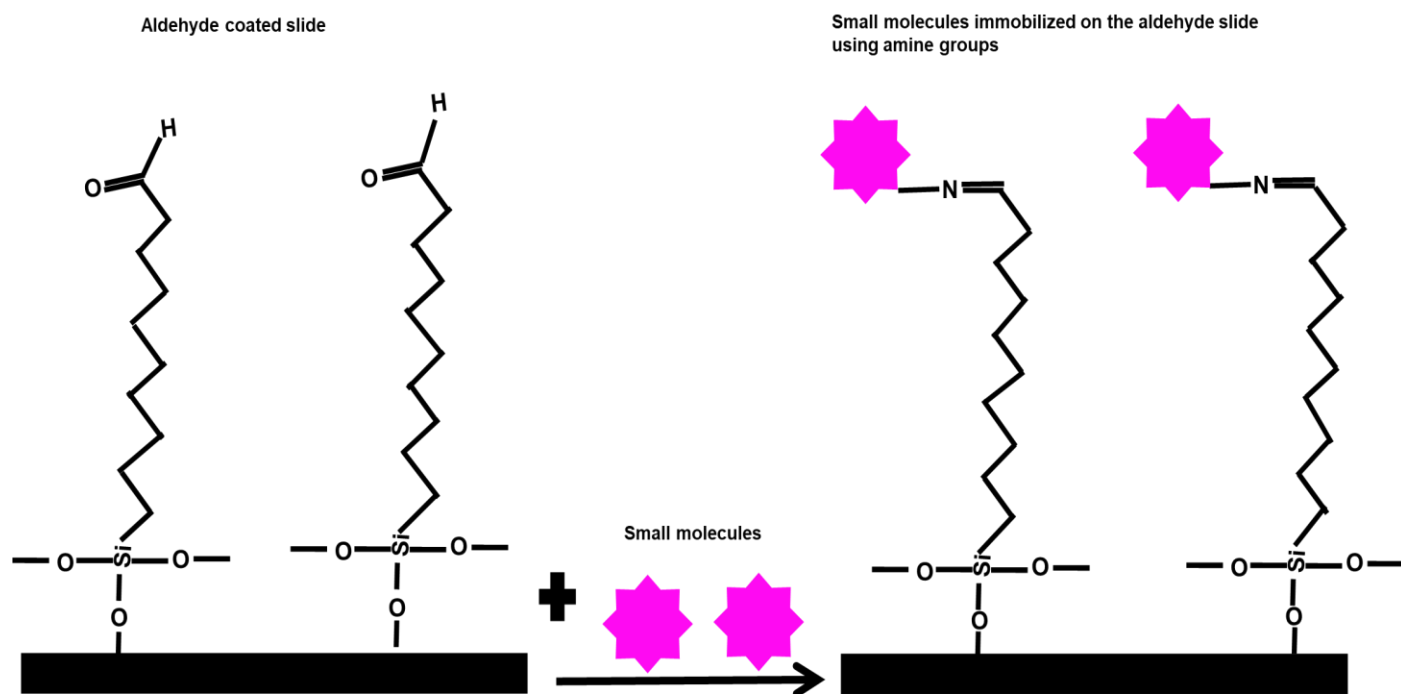


Figure 7: Illustration of the reaction between aldehyde coated glass slides with small molecules. The reaction between aldehydes on the slide surface (black) and primary amine groups on the small molecule (pink) involves the nucleophilic attack of the carbonyl oxygen by the amine group to form an amine bond between the molecules.

Table 1: The advantages and disadvantages of different functionalized glass slides commonly used in the microarray platform.

Function on slide	Bond type	Advantages	Disadvantages
Nitrocellulose	Non-covalent (Physisorption)	- High sensitivity and simple immobilization (Seurynck-Servoss et al., 2007) - Black surface nitrocellulose slides have low background autofluorescence (Walter et al., 2010) - Work well with colorimetric detection systems (Cretich et al., 2010) - Fluorescence detection from nitrocellulose achieves ease of data quantification (Tonkinson and Stillman, 2002) - High-binding capacity of biological samples without degradation (Tonkinson and Stillman, 2002) - High Sensitivity and reproducibility (Bondarenko et al., 1992) - Preserve the native protein conformation (Angenendt, 2005)	- White surface nitrocellulose has high background fluorescence (Cretich et al., 2010, Seurynck-Servoss et al., 2007, Walter et al., 2010) - Inability to be reprobated multiple times due to fragile nature of the polymer in membrane form (Tonkinson and Stillman, 2002) - Nonspecific binding of hydrophobic biomolecules which decreases sensitivity (Shakeri et al., 2019) - Few options for chemical immobilization (Shakeri et al., 2019) - Large variation in signal intensity (Angenendt, 2005) - Nitrocellulose permeability can complicate kinetics protein-antibody interactions due to slow diffusion of proteins through nitrocellulose (Southern et al., 1999)
Epoxy	Covalent	- Strong attachment of molecules (Angenendt, 2005) - Does not cause protein aggregation due to intramolecular loop formation and/or intermolecular interactions (Hong et al., 2007) - Stable bond formation with amine (Hong et al., 2007) - Covalent binding of proteins with different orientations and lack of auto-fluorescence (Aguilar-Mahecha et al., 2006) - High immobilization efficiency and long-term stability (Yousefi et al., 2018)	- Rapid evaporation of the liquid environment and close protein surface contact which may affect the three-dimensional structure (Angenendt, 2005) - High background signal (Tsougeni et al., 2018)
Isothiocyanate	Covalent	- Long term storage under ambient temperatures (Preininger et al., 2004) - Suitable for DNA and proteins immobilization (Preininger et al., 2004)	- low immobilization efficiency for some molecules (Zhu et al., 2016b) - low reactivity to carboxylic acids and tertiary alcohols (Zhu et al., 2016b)
Aldehyde	Covalent	- Does not cause protein aggregation due to intramolecular loop formation and/or intermolecular interactions (Hong et al., 2007) - Stable bond formation occurs if the bond is reduced by a reducing agent such as sodium cyanoborohydride (Hong et al., 2007)	unsuitable for high-throughput screening (El-Mahdi and Melnyk, 2013)
Poly-L-lysine	Covalent	- Strong cell adhesion and high accuracy (Xing et al., 2012) - Requires very low cell number (Mehta et al., 1983) - Sensitive and reduces noise in hybridization signal (Pourjahed et al., 2013)	- Cells on poly-L-lysine can easily agglomerate (Xing et al., 2012) - Can affect the adhesion behavior of different cell lines (Xing et al., 2012) - Low binding capacity and flat surfaces which result in sterical limitations in hybridization of target probes (Steel et al., 2000)

4. Recent development on functionalized microarray slides design

Recently researchers have developed microarrays with improved performance compared to existing platforms such as nitrocellulose. One such study involved the development of a thiol-ene micropillar scaffold sheets solid substrate with large pore size for fluorescence microarrays, and because of its unique surface chemistry it offers low autofluorescence and a stronger fluorescence intensity compared to nitrocellulose (Guo et al., 2020). Furthermore, a dextran hydrogel high-performance microarray has been reported to perform better than nitrocellulose and was used to covalently immobilize proteins, this to have high probe-loading and good biorecognition yields (Betancor et al., 2019). In addition to the functional groups above, dynamic magnetic nanomixers (Xiong et al., 2019), nanorod (Lu et al., 2021) and oxygen plasma micro-nanostructured poly(methyl methacrylate) have been reported to have a much lower detection limit and greatly reduce the spot-to-spot variation across a broad concentration range, providing more accurate assay results compared to nitrocellulose (Xiong et al., 2019, Lu et al., 2021, Koukouvinos et al., 2021).

5. Application of the microarray technology at point-of-care

Multiplex point-of-care (POC) testing is an on-site simultaneous detection of different analytes from a single specimen developed mostly for clinical diagnostics (Charlarmroj et al., 2021, Dincer et al., 2017, Zhang et al., 2022). POC multiplexing becomes more attractive when the detection of different analytes can be done in resource limited settings to significantly reduce the cost and time of diagnostics, (Dincer et al., 2017, Kubina and Dziedzic, 2020, Tsao et al., 2020, Zhang et al., 2022). POC microarray detection systems have been used for several decades and is still currently being employed and further developed by researchers to profile diseases and illnesses. For instance, Wu et al., developed a rapid, high throughput and highly sensitive POC smartphone-based microarray for the multiplex detection of mycotoxins (Wu et al., 2022). The smartphone-based microarray technology has been said to reduce the turnaround time of results from several hours with the conventional ELISA to 30 minutes and has sensitivity in the pg/ml range (Wu et al., 2022). In addition, a POC assay was employed to study and profile mycotoxins using a microarray lateral flow strip test (Charlarmroj et al., 2021). Furthermore, Dey et al. developed a POC label-free bacterial quantification system by means of a bioprinted microarray-based interferometric device which uses a small sample volume. This technology proved to be sensitive and has a sample-to-data turnaround time of 40 minutes (Dey et al., 2019). Multiplex colorimetric POC paper-based microarray tests have also been developed and used for the detection of influenza viruses such as IVA H1N1 and H3N2 in cell lysate and clinical samples (Lei et al., 2015, Oh et al., 2028). However, the use of microarray as POC platforms still has several challenges that must be addressed, such as the fact that they lack sample preparation functions (target biomarker enrichment), they have

complicated methods of substrate pre-treatment and require sophisticated instrumentation particularly for optical detection (Rosa et al., 2022).

6. Conclusion

Proteins, nucleic acids as well as carbohydrates microarrays have provided researchers with tools to simultaneously screen and profile thousands of molecules in qualitative and quantitative studies. These technologies have been used for the development of methods applied in research and clinical diagnostics, such as the investigation of gene expression and protein activities, as well as in the assessment of disease prognosis to name a few. The use of microarray platforms has gained traction in diagnostics and clinical research as they provide solutions to some of the current diagnostic methods challenges such as the fact that they require high sample volumes, have a long turnaround time, and can be expensive. The application of these platforms for the detection of multiple diseases at the same time solves some of the forementioned challenges as they use small sample volumes, have a short-turnaround time, and have the potential to significantly reduce the cost of diagnostics. These microarray technologies use different functionalized slides or microspheres by employing covalent and non-covalent interactions during the immobilization of molecules by means of chemical groups, among them epoxy-silane, aldehydes, esters, poly-L-lysine and nitrocellulose for the characterization and profiling of target compounds. The fact that various functionalized groups can be employed on microarray slides makes this platform broad as it can accommodate many diverse types of analytes. One of the most important applications of microarray technologies that researchers are working on is their use at POC for treatment monitoring and clinical diagnostics. This can considerably reduce the time between obtaining diagnostic results and initiation of treatment since the POC multiplex microarray assays are relatively fast. In addition, it also has the potential to significantly reduce the cost associated with diagnostics. This is particularly important for resource limited settings and developing countries as they often have many financial challenges with diagnostics and disease management.

7. Aim

Although there are diagnostic assays for the detection of HIV and TB currently employed such as the rapid HIV antibody tests, and state of the art TB GeneXpert test, there is still a need to develop technologies that can detect the two pathogens at the same time within a short period of time. The HIV rapid antibody test has high sensitivity and specificity, is reliable and fast, as results are obtained within 30 minutes and is also currently used worldwide for the detection of HIV. The TB GeneXpert is a nucleic acid amplification test for the fast diagnosis of TB by identifying *M.tb* DNA as well as resistance to rifampicin within a period of two hours (Sasikumar et al., 2019, Stevens et al., 2017a, Sagili et al., 2018). Although the mentioned HIV and TB tests have attractive characteristics, the fact that they diagnose the two pathogens separately propels the need to investigate the development of technologies that can detect both diseases at the same time within a reasonable timeframe. It is in this context that this project is aimed at the development of a user friendly, POC, fast and cheap multiplex microarray technology for the detection of HIV and TB given the relationship between the two diseases, and the attractive advantages of using multiplex technologies for disease diagnosis mentioned in the above sections of the literature review. The technology is also destined for use in remote rural areas, underserved communities as well as resource limited settings.

8. Study objectives

- To produce a planar array of immobilized HIV-1 p24 antigen; as well as *M.tb* CFP10, ESAT6, apa and pstS1 antigens on a glass slide for POC assay development.

- To determine *in-vitro* the sensitivity and specificity of the microarray based detection system for antibodies against HIV and *M.tb* antigens using a benchtop fluorescence microscope.
- To study *in-vitro* the sensitivity and specificity of the microarray based multiplex detection system for antibodies against p24, CFP10, ESAT6, apa and pstS1 antigens using colour change reaction as the detection system.
- To produce the *M.tb* antigens; CFP10, ESAT6, apa and pstS1 in plants and investigate their suitability for the microarray based multiplex detection system.

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CHAPTER TWO

DEVELOPMENT OF A MULTIPLEX HIV/TB DIAGNOSTIC ASSAY BASED ON THE MICROARRAY TECHNOLOGY

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Development of a Multiplex HIV/TB Diagnostic Assay Based on the Microarray Technology

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Abstract: Currently there are diagnostic tests available for human immunodeficiency virus (HIV) and tuberculosis (TB); however, they are still diagnosed separately, which can delay treatment in cases of co-infection. Here we report on a multiplex microarray technology for the detection of HIV and TB antibodies using p24 as well as TB CFP10, ESAT6 and pstS1 antigens on epoxy-silane slides. To test this technology for antigen-antibody interactions, immobilized antigens were exposed to human sera spiked with physiological concentrations of primary antibodies, followed by secondary antibodies conjugated to a fluorescent reporter. HIV and TB antibodies were captured with no cross-reactivity observed. The sensitivity of the slides was compared to that of high-binding plates. We found that the slides were more sensitive with the detection limit being 0.954 ng/ml compared to 4474.6 ng/ml for the plates. Furthermore, stability studies revealed that the immobilized antigens could be stored dry for at least 90 days and remained stable across all pH and temperatures assessed, with pH 7.4 and 25 °C being optimal. The data collectively suggested that the HIV/TB multiplex detection technology we developed has the potential for use to diagnose HIV and TB co-infection, thus, can be developed further for the purpose.

Keywords: HIV-1 p24; *M.tb* CFP10; *M.tb* ESAT6; *M.tb* pstS1; Multiplex microarray; diagnosis; Antibody; antigen

1. Introduction

An estimated 38.0 million people were living with HIV/AIDS globally with the annual number of deaths standing at 970 000 in 2021 and 25% were co-infected with *Mycobacterium Tuberculosis* (*M.tb*), the causative agent of TB (UNIAIDS, 2021). Albeit there are diagnostic tests currently available for HIV and TB, the diagnosis of these two diseases is still done separately and this can delay treatment in co-infected individuals, particularly because active TB diagnosis can take long (Tiewsoh et al., 2020, Rios-Hincapie et al., 2020). With HIV there is a rapid test for the detection of p24, in addition to the PCR test. However, the currently existing tests that rely on protein detection in TB infected blood such as the QuantiFERON and T-SPOT have challenges of low sensitivity and specificity, especially in HIV-infected individuals (Maeda et al., 2010, Bua et al., 2018, Zhu et al., 2014). Furthermore, there is also a GeneXpert test endorsed by the World Health Organization (WHO) for the detection of *M.tb* DNA and genetic mutations associated with resistance to the Rifampicin TB drug (Piatek et al., 2013, Stevens et al., 2017b). Although the test is reliable and fast as results are obtained within two hours, the technology is very expensive, cannot detect latent TB (which is important to identify and manage to avoid active disease from occurring) and is not sensitive in detecting TB in people infected with HIV as they have fewer bacteria in their sputum (Bajrami et al., 2018, Mechali et al., 2019, de Faria et al., 2021). Therefore, there is a need for alternative diagnostic technologies that will allow for the simultaneous detection of HIV and TB quicker, as well as the ability to distinguish between active and latent TB.

HIV-1 p24 is a significant marker in early HIV-1 detection as it is expressed in blood during infection (Zhang et al., 2011, Gray et al., 2018). Many p24 viral proteins are produced after infection during the acute infection phase, therefore, the detection of these antigen and its antibodies provides means to aid early diagnosis of HIV-1 infection (Parpia et al., 2010, Gray et al., 2018, Fitzgerald et al., 2017). The p24 antigen and its monoclonal antibodies are the key components of HIV-1 detection in most diagnostic kits (Ly et al., 2007, Gray et al., 2018).

M.tb culture filtrate protein (CFP-10) is one of the highly expressed early secreted *M.tb* proteins and is recognized as the most abundant antigen (Arlehamn et al., 2012, Li et al., 2013). The protein is known to be a major immunogenic protein of *M.tb* that stimulate host immune mechanisms by activating a protective T-cell response during infection (Kumar et al., 2010, Qiao et al., 2011, Borgström et al., 2020).

The *M.tb* Early Secreted Antigenic Target 6 kDa (ESAT6) is a major T-cell antigen and is also the main determinants of *M.tb* virulence during infection (Clemmensen et al., 2020, Liu et al., 2021d, Pathakumari et al., 2018). Since ESAT-6 is frequently recognized in disease, past *in vitro* studies have shown that the antigen is a potential diagnostic reagent highly specific for active TB (Sun et al., 2022, Hur et al., 2013, Zhang et al., 2015). Both ESAT6 and CFP10 proteins have been used to diagnose pulmonary and extrapulmonary TB in patients (Bekmurzayeva et al., 2013, Hemmati et al., 2011, Lagrange et al., 2014).

Phosphate-specific transport substrate binding protein-1 (pstS1) antigen is responsible for the uptake of phosphate by the *M.tb* from its environment and contributes to the intracellular survival of the pathogen (Ferraris et al., 2014, Tischler et al., 2013, Espinosa-Cueto et al., 2020). Moreover, the protein is highly immunogenic and induces Th1 immunity and antibody responses (Jiang et al., 2018, Dieli and Ivanyi, 2022). pstS1 protein has been used in the development of a number of commercial assays for the detection of TB (Alcaide et al., 2019, Li et al., 2015, Khurshid et al., 2017). The combined use of recombinant immunodominant CFP10, ESAT6 and PstS1 *M.tb* antigens increase the sensitivity and specificity of immunoassays as compared to using one antigen (de Araujo et al., 2014, Chen et al., 2022a).

Microarray technology refers to the miniaturization of multiple assays on one small plate or slide (Liu et al., 2019a, Sauer, 2017, Sutandy et al., 2013, Templin et al., 2002). There are different types of microarrays, which include analytical and functional microarrays (Sutandy et al., 2013). An example of an analytical microarray is the antibody array, which involves protein detection and concentration determination after antibody capture using direct protein labelling (Haab, 2005, Sutandy et al., 2013, Seidel and Niessner, 2008). The functional microarray is built using purified proteins to enable the study of various biochemical properties such as protein-protein interactions, protein binding activities,

protein-peptide interactions, and protein-lipid interactions to name a few (Chen et al., 2021b, Govarthanan et al., 2020, Lin and Chen, 2018, Wang et al., 2019b). Protein microarray technology allows for the characterization of hundreds of thousands of proteins in a highly parallel and high-throughput manner (Zhang et al., 2021b, Zhang et al., 2020b, Wu et al., 2021). Immunoassays take advantage of highly specific antigen-antibody recognition to build a protein detection system, whereas the microarray enables a rapid parallel and multiplex detection often requiring a small amount of sample (Ruano-Gallego et al., 2021, Khan et al., 2020). The immunoassay on microarrays can be very sensitive and specific, thus making it very reliable when studying or determining antigen-antibody interactions such as occurs in diagnostics (Pohlmann and Elssner, 2020, Fan et al., 2020, Cohen and Walt, 2019).

The specificity of antibodies to antigens is due to a defined region of antibody molecules containing the complementary determining region that interacts with antigens with high affinity (Zhao et al., 2018a, Mehraj, 2020). In addition, the binding between antigens and antibodies employs non-covalent interactions where the van der Waals, hydrophobic and electrostatic forces which are relatively weak and reversible are involved (Bhardwaj and Purohit, 2020, Gunasekara et al., 2022, Mehraj, 2020). The combined strength of all the non-covalent interactions between a single antigen-binding site of an antibody and a single epitope of the antigen determines the affinity of this interaction. Antigen-antibody binding are reversible under conditions such as ambient temperature, pH and time (Bhardwaj and Purohit, 2020, Mehraj, 2020).

Epoxy-coated slide chemistry is one of the commonly used systems for protein microarray as they are stable under various conditions and are reactive with several nucleophilic groups to form strong bonds with minimal chemical modification of the protein (Rusmini et al., 2007). For example, epoxy-coated glass slides have been used in cancer studies as well as in the detection of antibodies against SARS-CoV-2 for disease diagnostic purposes (Jian et al., 2021, Ruano-Gallego et al., 2021). The interaction between the coated slide and proteins occurs through fast adsorption followed by intramolecular chemical attachment promoted by the epoxy groups on the glass surface (Rusmini et al.,

2007). The part of proteins that reacts with epoxy on the solid surfaces is mostly the amine group, such as found on lysine residues which are present on the exterior of proteins, forming stable amine bonds with the surface (Shlyapnikov et al., 2021, Yousefi et al., 2018, Zhu et al., 2016b). During the coupling process, proteins are dissolved in a low ionic-strength buffer before being passed through the coated solid surface. The immobilization efficiency depends on parameters such as pH, concentration, and reaction time (Rusmini et al., 2007, Yousefi et al., 2018). Jorgensen et al., showed that epoxy substrates exhibit high stability in pH conditions of 6.0, 7.5 and 9.0 (Jorgensen et al., 2021), while Yousefi et al., showed that epoxy slides had the best functionality and produced high fluorescence at different temperatures such as 4 °C, 25 °C and 40 °C (Yousefi et al., 2018).

In this study, we developed a multiplex microarray technology on 2.5 x 7.6 cm epoxy-coated glass slide for the detection of HIV and TB co-infection using HIV-1 p24 antigen as well as *M.tb* CFP10, ESAT6 and pstS1 antigens known to be markers of active TB (Davidow et al., 2005, de Araujo et al., 2018, Khurshid et al., 2013, Watson et al., 2021). We found that the immobilized antigens captured corresponding primary antibodies in human serum. In addition, we proved that this interaction was specific and with sensitivity higher than what would be obtained on high-binding ELISA plates. Furthermore, this diagnostic technology performed optimally at room temperature and pH 7.4; and this performance was maintained for at least 90 days during the shelf-life experiments. In all, these data indicate that our diagnostic technology is potentially a solution for a quicker method of simultaneously diagnosing HIV-1 and TB in cases of co-infection.

2. Materials and Methods

2.1. Reagents

The HIV-1 p24 antigen and anti-p24 (Ms), anti-CFP10 (Rb), and anti-ESAT6 antibodies (Rb) were bought from Abcam (Cambridge, United Kingdom). The culture filtrate protein-10 (CFP10) antigen and early secretory antigenic target protein 6 (ESAT6) were obtained from BBI solutions (Caerphilly, United Kingdom). The phosphate-specific transport substrate binding protein-1 (pstS1) antigen and the pstS1 primary antibody (Ms) were bought from My BioSource (California, USA). The Goat (Gt) anti-Mouse (Ms) /anti-Rabbit (Rb) IgG superclonal secondary antibody, Alexa fluor 488 conjugates were obtained from Abcam (Cambridge, United Kingdom). The NEXTERION epoxysilane-coated glass slides were bought from Schott laboratories (Mainz, Germany), while the Corning polystyrene high-binding costar assay plates were purchased from Corning Inc. (New York, USA).

2.2. Immobilization of HIV-1 p24 as well as *M.tb* CFP10 ESAT6 and pstS1 antigens on epoxy coated glass slides.

Antigens (HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1) were diluted to 500 000 ng/ml in 1 × phosphate buffered saline (PBS) pH 7.4 (Thermo fisher scientific, Massachusetts, United States), with concentrations ranging from 500 000 ng/ml to 76.21 ng/ml. The different concentrations of each antigen were spotted (1 µl) on three NEXTERION epoxy-silane-coated glass slides and incubated for 90 minutes at room temperature. The slides were thereafter washed three times for 5 minutes in 1 × PBS pH 7.4, followed by blocking for an hour in 1% PBS-bovine serum albumin (BSA) (Sigma Aldrich, Missouri, United States). This was followed by washing the slides three times in 1x PBS for 5 minutes to remove excess BSA. The slides were then allowed to dry at room temperature for 15 minutes and bright-field images of the slides were captured using a cytation3 multimode reader (Biotek Instruments, Vermont, United States). To further profile and evaluate the immobilized antigens, the slides were incubated for an hour in a cocktail solution containing anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies at a constant concentration of 3906 ng/ml to determine if they could capture the antibodies in solution. Post-incubation with primary antibodies, the slides were washed and incubated for an hour in a cocktail solution of Goat (Gt) anti-Mouse (Ms)

/anti-Rabbit (Rb) IgG superclonal secondary antibody, conjugated to Alexa fluor 488, at a constant concentration of 100 ng/ml. The slides were thereafter washed and imaged using a cytation3 multimode reader. The sensitivity of the cytation 3 reader and limit of detection according to the manufacturer is as low as 0.25 fmol/well of fluorescein.

2.3. Determination of the optimal buffer pH, storage condition, temperature, and the shelf-life of immobilized HIV and TB antigens

To determine the optimal printing, washing and storage pH conditions for the HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens on the epoxy-coated glass slides, five different pH conditions adjusted using NaOH and HCl (Sigma Aldrich, Missouri, United States) ranging from 5.0 to 9.0 were evaluated on five epoxy-coated glass slides. To achieve this, the antigens were spotted in triplicates at a constant concentration of 31300 ng/ml in PBS at different pH followed by immobilization on the glass slides as mentioned above. Washing steps were performed as above also using the PBS at corresponding pH.

The determination of the optimal pH was followed by the evaluation of whether the immobilized antigens should be stored dry or in buffer. This was done by storing the immobilized antigens dry or in PBS (one slide each for three independent experiments) pH 7.4 for 24 hours and then studying their interaction with primary antibodies.

The next step was to determine the optimal temperature at which the immobilized antigens on the epoxy-coated glass slides could be stored. The different temperatures tested for the study were -20 °C, 4 °C, 15 °C, 25 °C and 37 °C tested using five epoxy coated glass slides and the experiment was repeated three times. To achieve this, immobilized HIV and TB antigens were incubated dry at -20 °C, 4 °C, 15 °C, 25 °C and 37 °C for 24 hours followed by the addition of primary and secondary antibody, and subsequent detection of fluorescence.

The immobilized antigens' shelf-life studies were also conducted. Jorgensen et al., showed that printed proteins for multiplexing can remain stable for up to three months (Jorgensen et al., 2021), therefore we assessed the HIV and TB antigens on the slide for a period of three months. This was done by storing immobilized antigens slides dry for 1 day, 15, 30, 45, 60, 75 and 90 days at room temperature using seven slides and the experiment was repeated in two independent experiments. All the slides for the different

conditions were incubated in a cocktail of the HIV and TB recombinant primary antibodies at a constant concentration of 3906 ng/ml. Following the incubation, the slides were washed and incubated in Gt anti-Ms/anti-Rb IgG superclonal secondary antibody, conjugated to Alexa fluor 488, at a constant concentration of 100 ng/ml for an hour at room temperature. After the incubation, the slides were washed, and fluorescence images were obtained using the cytation3 multimode reader.

2.4. *In-vitro* specificity and sensitivity of the microarray-based detection system for HIV and *M.tb* antibodies

The immobilization of the HIV and TB antigens for both the slides and plates was done as mentioned above, except that for the high-binding black with clear bottom polystyrene 96 well plates (Sigma Aldrich, Missouri, United States) 5 µl of the antigens per well was used (Three slides and high-binding plates were used and the experiment was repeated three times). This was followed by the addition of 4-fold serially diluted HIV and TB primary antibodies (for the slides, the series range was 250 000-0.954 ng/ml, while for the plates it was 250 000-20.0 ng/ml) in PBS or normal human serum containing the usual components found in serum such as proteins, including the different classes of antibodies that exist in healthy humans (Thermo fisher scientific, Massachusetts, United states, Catalog number 31876), and incubation for 2 hours at room temperature. To determine that each antigen specifically reacted with its corresponding primary antibody in solution, an anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibody cocktail solution missing one of the antibodies at a time was prepared and incubated with the immobilized antigens. Subsequently, the plate was washed three times with PBS for 5 minutes. Afterwards, 100 µl Gt anti-Ms/anti-Rb IgG superclonal secondary antibody conjugated to Alexa fluor 488, at a concentration of 100.0 ng/ml was added and incubated at room temperature for an hour. Post-incubation, the plate was washed as above, and fluorescence was measured using DTX 880 Multimode detector (Beckman Coulter Inc., California, Unites states) at a wavelength of 485 nm. The sensitivity and limit of detection for DTX 880 detector for fluorescein is reported by the manufacturer to be 50 fmol/well. SoftMax Proversion 6.4 (Molecular Devices, LLC) was used for data acquisition and

analysis of the plates. For the microarray slides, the detection was done using a cytation3 multimode reader, while ImageJ software was used for analysis.

Specificity between the HIV and *M.tb* antigens and the anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 recombinant primary antibodies was also investigated by immobilizing the antigens as aforementioned. The whole slide was then incubated with a solution containing one of the primary antibodies at a time at a constant concentration of 3906 ng/ml. For example, one slide of the immobilized antigens was incubated for an hour in a solution containing anti-p24 antibody only, while other slides were incubated with either anti-CFP10, anti-ESAT6 or anti-pstS1 primary antibody. After the incubation, the slides were washed and incubated with the secondary antibodies at a constant concentration of 100.0 ng/ml for an hour at room temperature. Then excess secondary antibodies were washed and the slide was imaged using Cytation3 multimode reader.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.4.0 (GraphPad Software, Inc.). Data are presented as mean $\pm$ standard deviation of the mean and each experiment was performed at least three times unless stated otherwise. Statistical significance was assessed using paired student T-test when comparing two means of the same groups under separate scenarios. One-way analysis of variance (ANOVA) test was also used to assess the statistical significance between multiple groups under different scenarios using Tukey's multiple comparison test. For all comparisons, *P values* less than 0.05 were considered statistically significant.

3. Results

3.1. Immobilization of antigens on epoxy-coated glass slides

The immobilization of antigens as capture molecules on functionalized glass slides is a well-developed system for microarrays in diagnostic and drug discovery (Tsougeni et al., 2018, Mujawar et al., 2013). In this study, microarray was developed by covalently immobilizing HIV-1 p24, *M.tb* CFP10, ESAT6 and pstS1 antigens on epoxy-coated glass slides. This was done to capture anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies in solution. The immobilization was achieved by reacting the epoxy group on the glass surface to amine groups on the antigens to form a covalent amine bond. Then we determined whether the immobilization of the TB and HIV antigens was successful as well as the optimal conditions for this immobilization. We demonstrated that the spot where the antigens were printed was stable on the slide by means of bright-field imaging even after washing with PBS. This was shown on the bright-field image obtained using scanning microscopy by the presence of clear spots where antigens were printed (Figure 1A). Furthermore, these spots were observed for all concentrations (500 000-0.228 ng/ml) and antigens printed, i.e., HIV-1 p24, as well as *M.tb* antigens CFP10, ESAT6 and pstS1. Thus, suggesting their interaction with the epoxy-coated glass slide. For negative control, PBS was spotted in place of antigens and as expected, no spots were observed for PBS after visualization with the microscope (Figure 1A). To investigate whether the HIV p24 and *M.tb* CFP10, ESAT6 and pstS1 on the glass slide could react with their specific antibodies, the immobilized antigens were incubated with a cocktail containing anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies. This was followed by the addition of a secondary antibody conjugated to Alexa fluor 488. The results showed that the coupled HIV and TB antigens could detect the primary antibodies even at the lowest concentrations of the antigens tested (3910 ng/ml). This was indicated by the presence of green fluorescence from all the spots on the slide after imaging (Figure 1B). The mean fluorescence intensity of the spots on the image was obtained using the ImageJ software. From the graph in Figure 1C, we observed that the fluorescence intensity decreased with decreasing concentration of the antigens when the concentrations of the primary and secondary antibodies were kept constant at 3906 ng/ml and 100.0 ng/ml, respectively. The antigen-antibody reaction that produced the highest

fluorescence intensity, at the highest concentrations tested, was ESAT6 followed by pstS1. HIV-1 p24 antigen-antibody reaction showed the lowest fluorescence intensity while *M.tb* CFP10 was the second lowest fluorescence intensity relative to the other antigens-antibody reactions (Figure 1C).

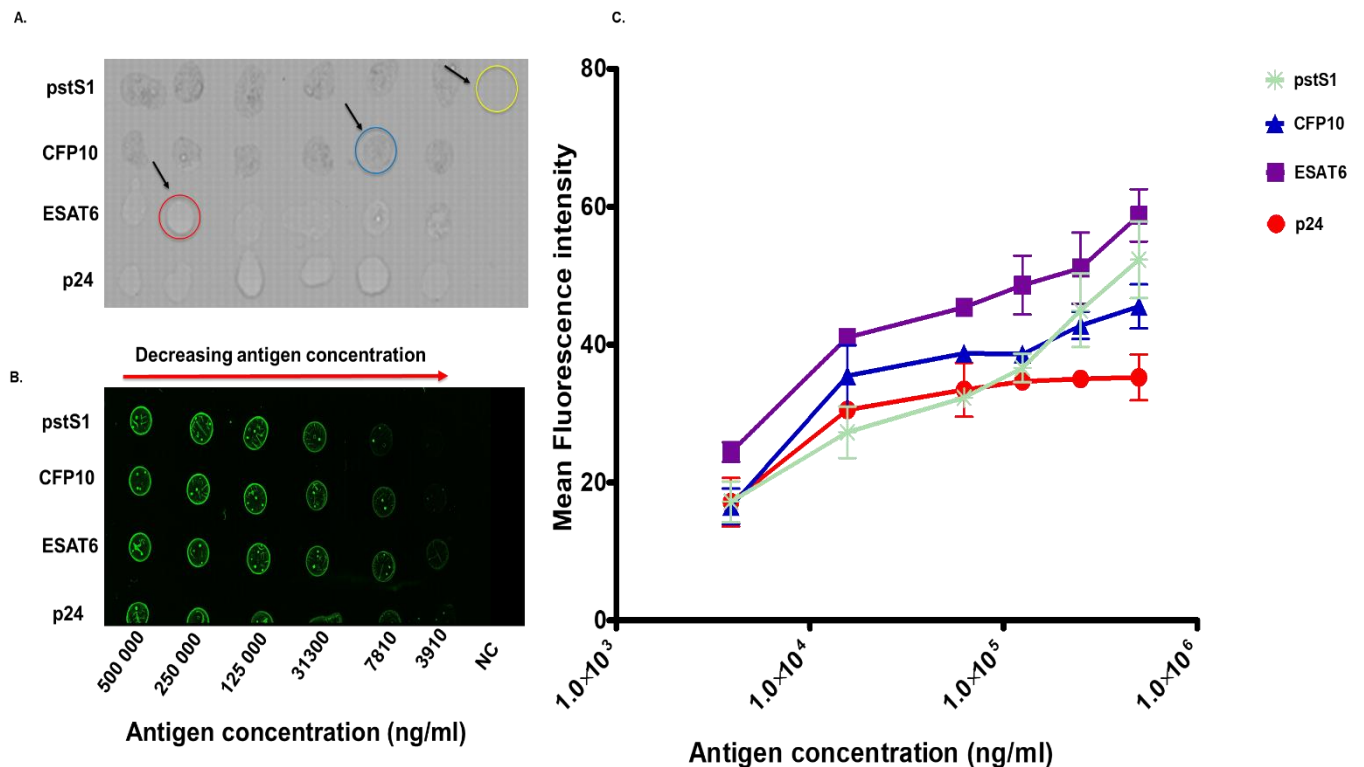


Figure 1: The immobilization of HIV-1 p24, *M.tb* CFP10, ESAT6 and pstS1 antigens on an epoxy-coated glass slide by covalent interactions. (A) The antigens were immobilized at different concentrations, PBS only spots were used as negative control (NC), and imaging was done using bright field. The antigen spots after washing and imaging are indicated with the red and blue circles, while yellow circles show the negative control. (B) The immobilized antigens were reacted with anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies and subsequently, secondary antibodies conjugated to Alexa fluor 488 were added and the slide imaged with a cytation3 multimode reader with Gen5 2.08 software. (C) Mean fluorescence intensity of the spots was measured with ImageJ and plotted. The concentration at which the antigens were assessed is also shown. Error bars are means $\pm$ standard deviation (n=3).

3.2. Investigation of the stability of immobilized antigens on the epoxy-coated glass slides

After showing that the antigens were immobilized on the microarray slide and could interact with the antibodies, we determined the optimal storage pH, temperature, and shelf-life of the immobilized antigens epoxy-coated slides. The pH studies were performed using PBS solutions at different pH values (pH 5.0, 6.0, 7.4, 8.0 and 9.0). The antigens were spotted five spots in a row (including the negative control PBS) in triplicates for each antigen at a concentration of 31300 ng/ml as this was one of the concentrations where we observed a strong reaction with the primary antibodies judging from the fluorescence obtained. After printing at the above-mentioned pH, the slide was blocked, followed by incubation with primary antibody at the corresponding pH. After addition of the secondary antibody, the slide was imaged. From the results obtained, the immobilized antigens (p24, ESAT6, CFP10 and pstS1) had similar interaction with primary antibody at all tested pH (pH 5.0, 6.0, 7.4, 8.0 and 9.0) (Figure 2). The highest fluorescence intensity observed at pH 6.0, 7.4, 8.0 and 9.0 were comparable while at pH 5.0 there was 2-fold decrease in intensity, and this was statistically significant ($p < 0.05$) (Figure 2). Therefore, we concluded that the optimal pH range for the antigen-antibody reaction on our epoxy-coated slides was 6.0-9.0.

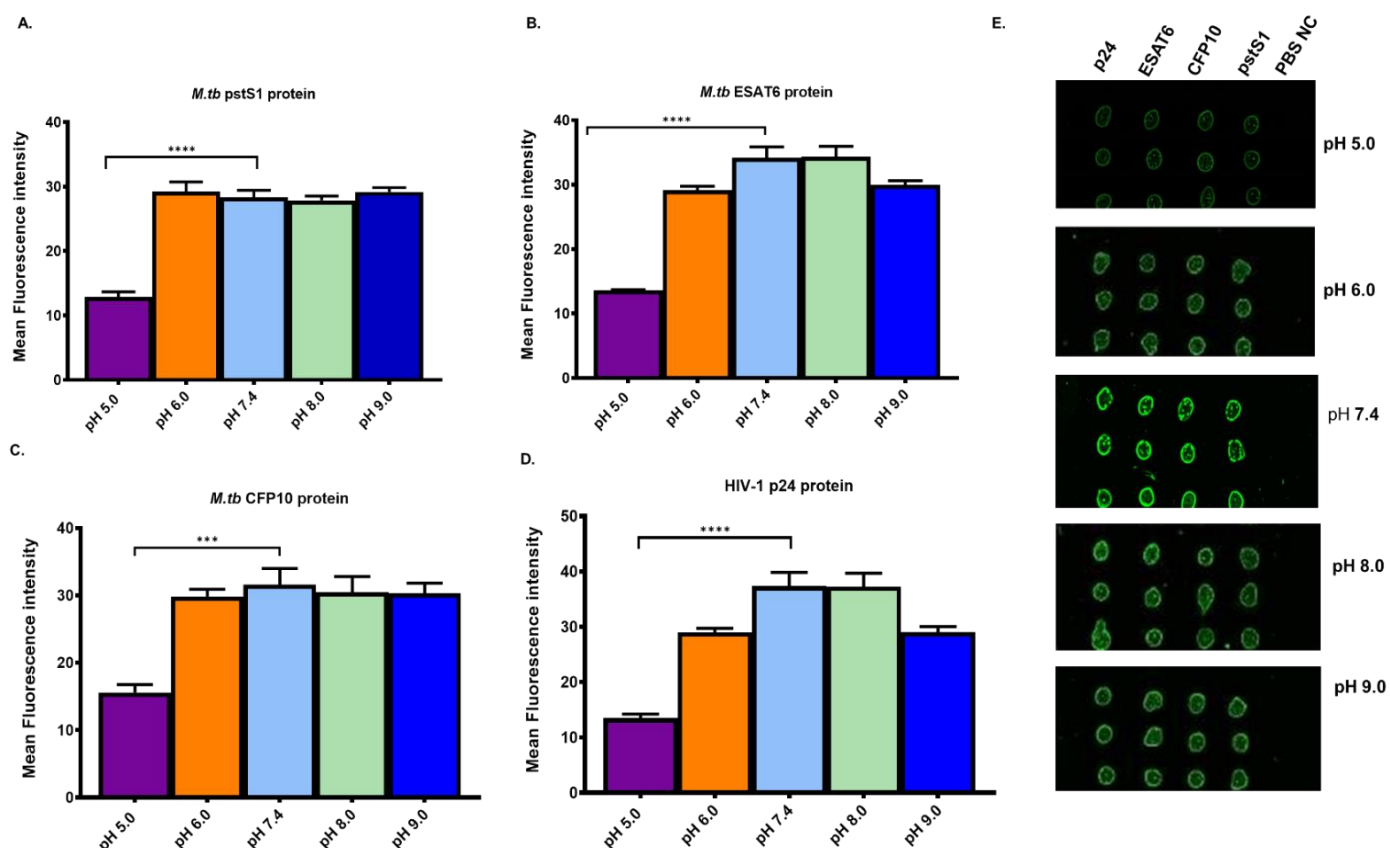


Figure 2: Effect of different pH on the immobilization of antigens on epoxy-coated glass slides. The antigens were printed and washed with PBS at different pH values. The bar graph shows fluorescence intensity obtained with *M.tb* pstS1 (A), ESAT6 (B), CFP10 (C) and HIV-1 p24 (D). Data represents mean $\pm$ standard deviation and error bars are standard deviation ($n=3$). Asterisks signifies statistical significance ($p<0.05$) between the fluorescence intensity of the highest reading and the lowest. (E) shows representative images obtained at different pH (pH 5.0, 6.0, 7.4, 8.0 and 9.0).

The optimal storage and reaction temperature for the immobilized antigens was also evaluated as temperature can play an important role in the stability of proteins on functionalized microarray slides (Yousefi et al., 2018, Jorgensen et al., 2021). This study was performed by immobilizing the antigens (Five spots in a row including the negative control in triplicates for each antigen) on the epoxy-coated glass slides in triplicates and incubating them at different temperature conditions (-20°C , 4°C , 15°C , 25°C , 37°C) for 24 hours. This was followed by washing with PBS at pH 7.4, which was one of the optimal

pH established previously. The antigens were reacted with a cocktail of their associated primary antibodies at the concentration of 3906 ng/ml and fluorescence was measured after the addition of secondary antibodies labelled with a fluorescence dye. The 3906 ng/ml primary antibody concentration used was determined experimentally to be optimal (Supplementary Figure A1). Based on the fluorescence intensities in Figure 3, the optimal temperature at which the p24, CFP10, ESAT6 and pstS1 antigens reacted with their antibodies was 25 °C, followed by 37 °C, 15 °C (except for p24 where 15 °C was the second-best temperature, followed by 37 °C), and 4 °C, while -20 °C had the lowest intensity. In fact, in general, the fluorescence intensity from slides stored at 25 °C was about 2-fold higher than the second-best storage temperature, and these differences were statistically significant ($p < 0.05$) for all antigens except pstS1 (Figure 3).

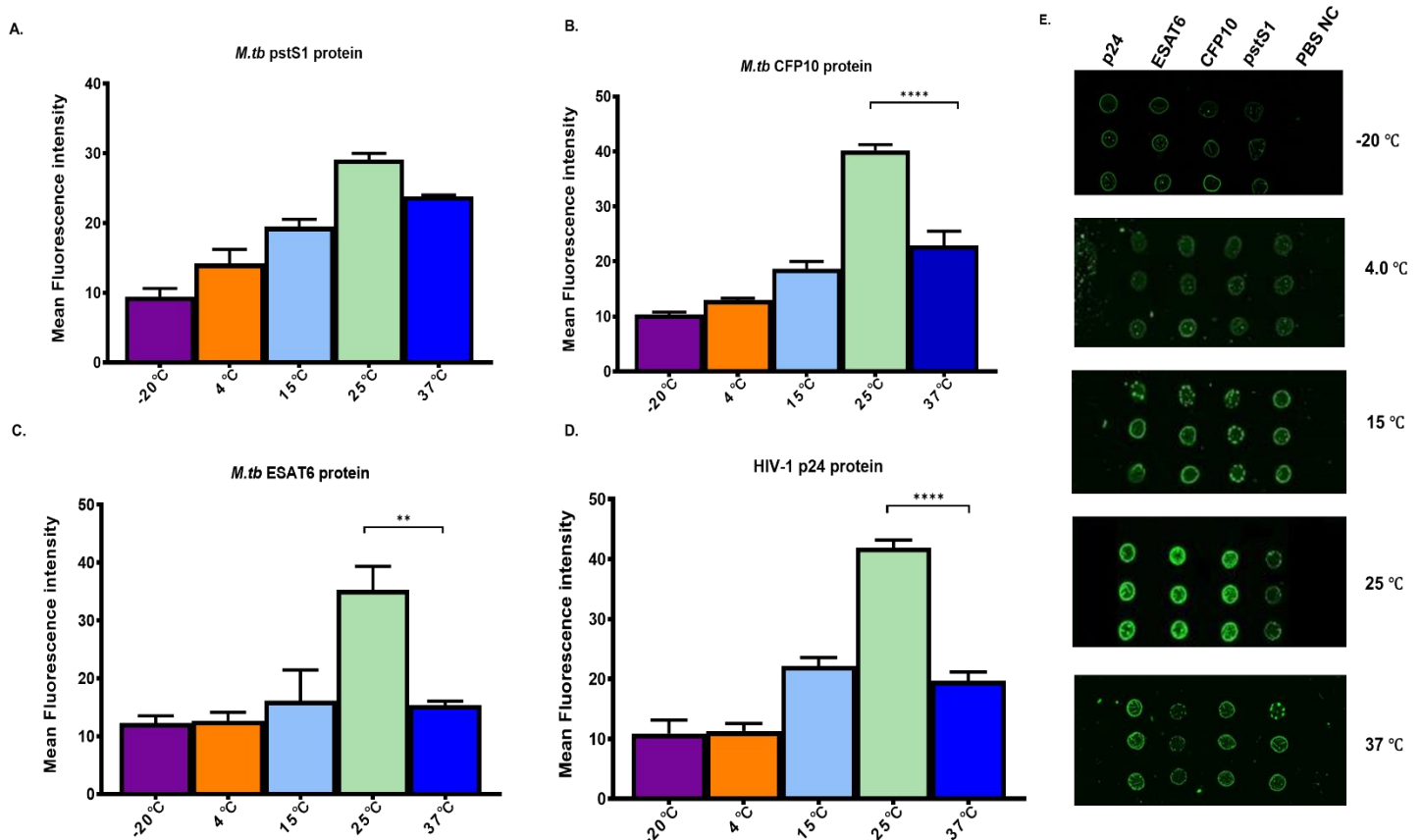


Figure 3: Evaluation of the optimal storage temperature for the immobilized antigens. The immobilized antigens were stored at different temperatures (-20 °C, 4 °C, 15 °C, 25 °C, and 37 °C) for 24 hours. The bar graphs of the fluorescence intensity

obtained from M.tb pstS1 (A) CFP10 (B), ESAT6 (C) and HIV-1 p24 (D) are shown. Data represents mean $\pm$ standard deviation (n=3) and asterisks indicate significant differences between the groups being compared. The slide images from which the bar graphs were plotted are shown in (E).

After studying the stability of the immobilized antigens at different pH and temperature conditions, the next step was to determine whether the immobilized antigens on the epoxy glass slides should be stored dry or wet. We investigated this at pH 7.4 and room temperature given that they were determined to be optimal. This was done by printing the HIV-p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens on glass slides followed by 24 hours incubation at room temperature in PBS pH 7.4, which represented the wet condition, or dry. Experiments were performed in triplicates in three independent experiments (three slides for each condition). From the results obtained, we observed fluorescence with both dry and wet slides (Figure 4). However, this intensity was lower for the antigens stored in PBS relative to the antigens stored dry (Figure 4B and C). In fact, the fluorescence intensity decreased by 2-fold for HIV-1 p24 and ESAT6, while the decrease was about 1.5-fold for CFP10 and pstS1. All these decreases were statistically significant ($p < 0.05$). Therefore, storage in the dry proved to be the optimal condition.

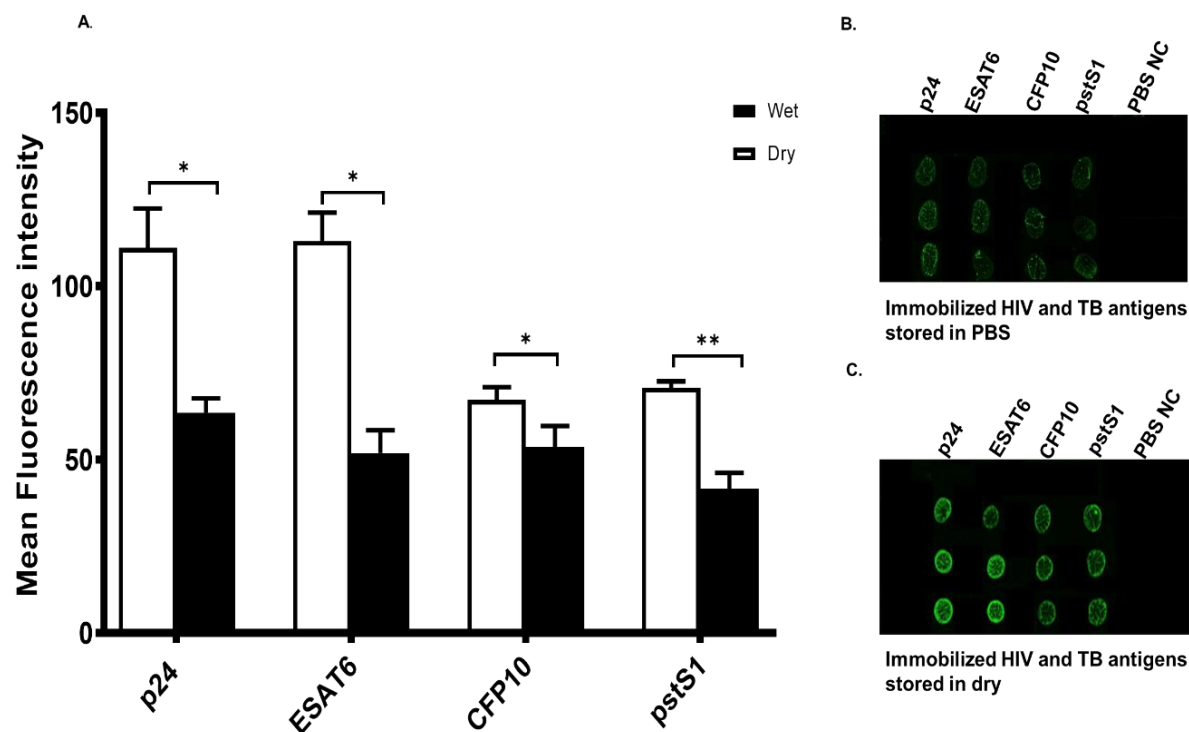


Figure 4: Evaluation of the stability in dry and wet storage conditions of the HIV and TB antigens immobilized on the epoxy-coated glass slide. The antigens were stored in PBS and dry at room temperature for 24 hours. (A) The mean fluorescence of each spot was measured and plotted. Data represent mean $\pm$ standard deviation ($n=3$) and asterisks shows significant difference between groups. (B) and (C) representative PBS and dry storage image, respectively, used to plot the graph in (A).

Lastly, we determined the shelf-life of the immobilized antigens on the epoxy-coated slides. This to get an indication of the durability of the immobilized antigens on the slide. To achieve this, seven slides were coated with the HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens at room temperature for an hour, washed and incubated dry (the optimal storage condition determined above) for different number of days. The incubation time points included day 1 to day 90 with fluorescence intensity readings after 15 days interval. Fluorescence was detected on all antigens from day 1 through day 90 after the incubation of the immobilized antigens with primary and secondary antibodies (Figure 5). However, a decrease in the mean fluorescence intensity, as measured using

the ImageJ software, was observed with longer periods of incubation, i.e., in most cases day 1 and day 15 yielded the highest fluorescence intensity while day 75 and day 90 had the lowest. In most cases these differences were statistically significant ($p < 0.05$). In fact, there was a progressive and consistent decrease in fluorescence intensity emitted from the slides as the shelf-life moved toward day 90. However, it is important to note that even at the day 90 there were still clear detection of all the antigens on the microarray slide, implying that our HIV/TB co-infection detection technology has the potential to last on the shelf for at least three months.

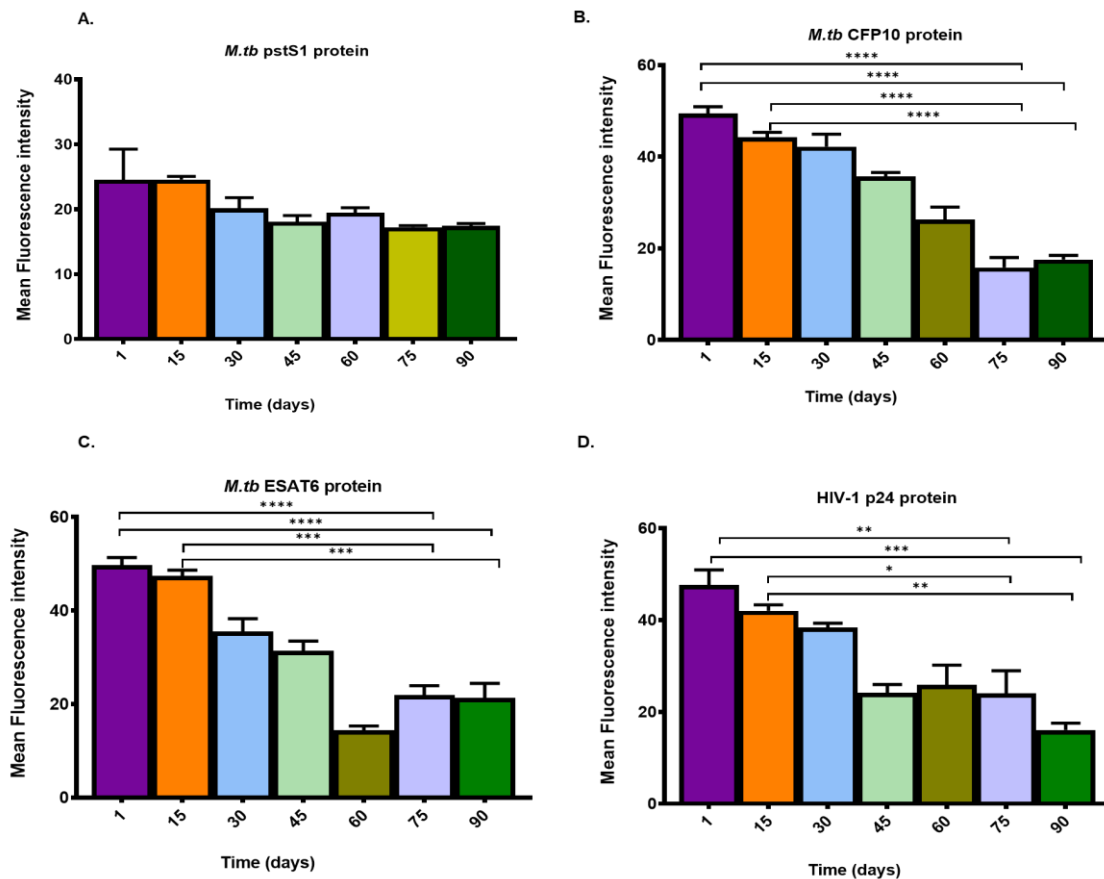


Figure 5: Evaluation of the shelf-life of the immobilized HIV and TB antigens on the epoxy-coated glass slides. The slides with immobilized antigens were stored dry at room temperature from day 1 to day 90, followed by addition of primary and secondary antibodies, with fluorescence reading after every 15 days. The bar graphs show mean fluorescence intensity obtained when slides with *M.tb* pstS1 (A), CFP10 (B), ESAT6 (C)

or HIV-1 p24 (D) were stored dry for 90 days. Data represents mean $\pm$ standard deviation ($n=2$) and asterisks signifies significant difference between the groups compared.

3.3. Comparison of the antigen-antibody reactions between the microarray epoxy-coated slide platform and the protein polystyrene high-binding plates

Next, we studied the sensitivity of the microarray-based multiplex technology. The assay was conducted by diluting antibodies in PBS or human serum and incubating them with the epoxy-coated slides containing immobilized antigens. The use of human serum was to determine how specific and sensitive the antigen-antibody reactions would be when the antibodies were in the presence of proteins that are commonly found in human blood. The concentrations evaluated for the HIV and TB antibodies on the epoxy slides ranged from 0.954 to 250 000 ng/ml where a 64-fold serial dilution was performed. The concentration range for the primary antibodies selected for this study is similar to what is commonly found in human blood during HIV and TB natural infections (Raux et al., 1999, Kimuda et al., 2017). From the results obtained, the lowest detection concentration of the primary antibodies against all antigens was determined to be 0.954 ng/ml for both PBS and human serum diluted antibodies (Table 1). The highest fluorescence intensity and the brightest spots were observed at the highest concentration of 250 000 ng/ml for all antigen-antibody interactions, and this intensity decreased with decreasing antibody concentration (Figure 6). No fluorescence was observed on the negative control spots where PBS was spotted instead of antigens. Comparable results were obtained when the primary antibodies were diluted in PBS, implying that proteins found in human serum are not likely to interfere with our diagnostic technology antigen-antibody interactions (Supplementary Figure A1).

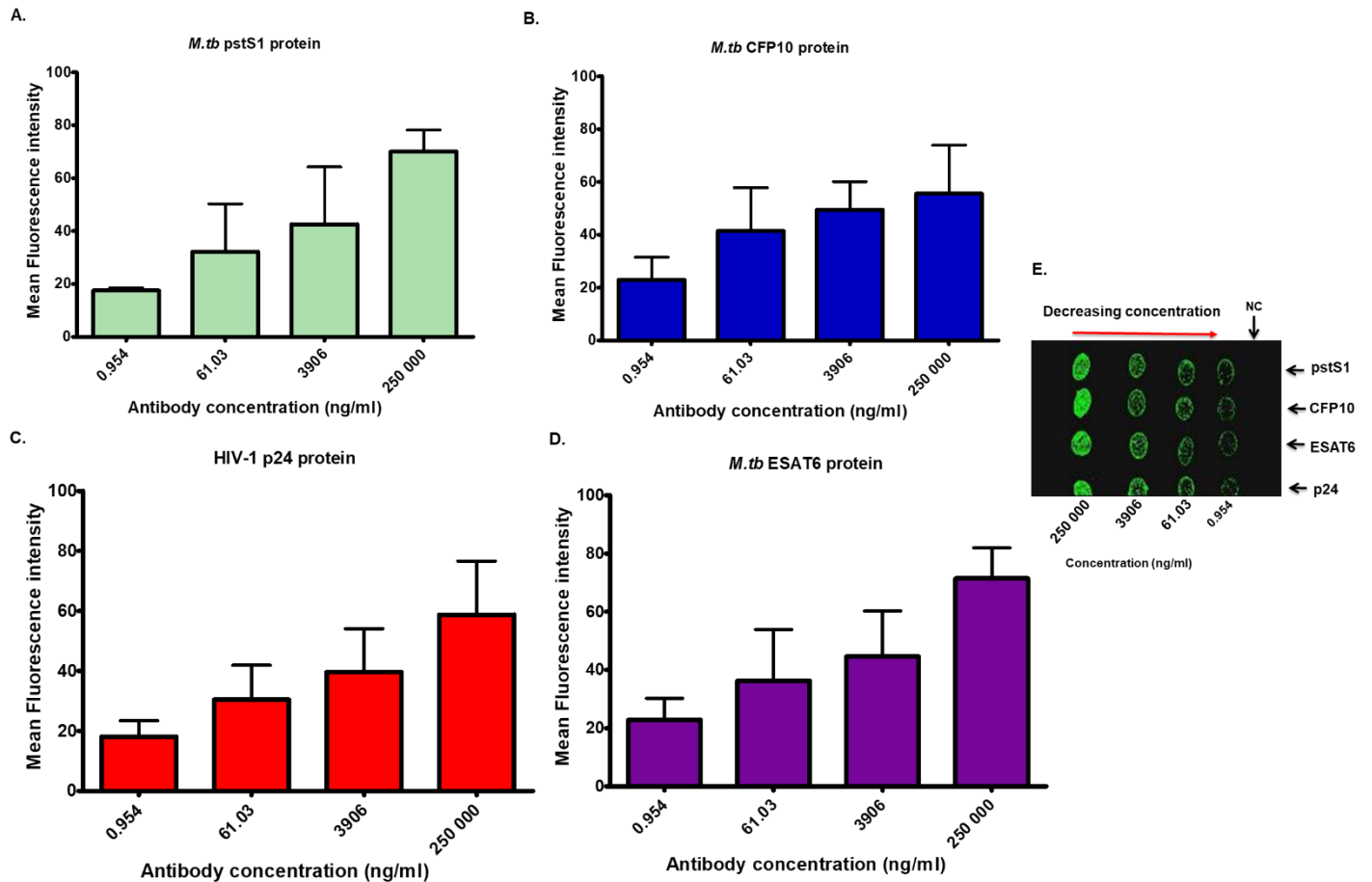


Figure 6: Evaluation of the sensitivity of the assay using epoxy-coated glass slides. Anti-pstS1, anti-CFP10, anti-p24 and anti-ESAT6 primary antibodies were diluted in human serum to mimic in vivo conditions. The antigen and secondary antibody concentrations were kept constant while primary antibody concentrations varied. Mean fluorescence intensity values obtained for pstS1 (A), CFP10 (B), p24 (C) and ESAT6 (D) are presented as bar graphs. Data represents mean $\pm$ standard deviation ($n=4$). (E) A representative image of the slide used to plot the bar graphs.

Since the use of high-binding plates for immunoassays is the gold standard (Jorgensen et al., 2021, Viitala et al., 2013), we replicated our microarray technology-based immunoassay (with primary antibodies diluted in PBS or human serum) in these plates to compare their performance. We observed that the limit of detection obtained from the high-binding plates was higher compared to the epoxy slides when the antibodies were

diluted in either PBS or human serum (Figure 6, supplementary Figure A1 and A2). To be precise, this limit in PBS was between 255 to 4094-fold higher using the plates compared to the microarray slide and the difference was statistically significant ($p<0.05$) (Table 1). When the assay was performed in human serum, the limit of detection for the plates was increased from 341 to 16373-fold compared to the microarray platform and the difference was statistically significant ($p<0.05$). Overall, the human serum spiked with HIV and TB showed higher limits of detection compared to the antibodies diluted in PBS due to the possibility of obstruction of binding in the former. In conclusion, our diagnostic technology on microarray slides showed significantly higher sensitivity than when the same assay was performed on the high-binding plates which are commonly used.

Table 1: A head-to-head comparison of sensitivity between the epoxy-coated glass slide and high-binding plates.

Primary antibody	Limit of detection concentration (ng/ml)			
	Primary antibodies diluted in PBS		Primary antibodies diluted in human serum	
	Epoxy-coated glass slides	Polystyrene high-binding microtiter plates	Epoxy-coated glass slides	Polystyrene high-binding microtiter plates
Anti-p24	0.954	244.1	0.954	976.5
Anti-ESAT6	0.954	244.1	0.954	325.5
Anti-CFP10	0.954	244.1	0.954	976.5
Anti-pstS1	0.954	3906	0.954	15620

Lastly, to determine the specificity of our diagnostic technology, i.e., the absence of cross reactivity, microarray slides containing all the antigens were incubated with a human serum cocktail of the primary antibodies missing one antibody at a time. This was done to see if the antigen corresponding to the absent antibody will bind to the remaining

primary antibodies in the cocktail. In another variation of this experiment, we incubated the four antigen coated microarray slides with only one antibody at a time to see if this antibody will react with the other antigens besides its own. In either experimental set-up, we observed no cross reactivity between the antigens and antibodies (Figure 7 and supplementary Figure A3). In all, these data suggest that our microarray-based technology is likely to perform with high specificity.

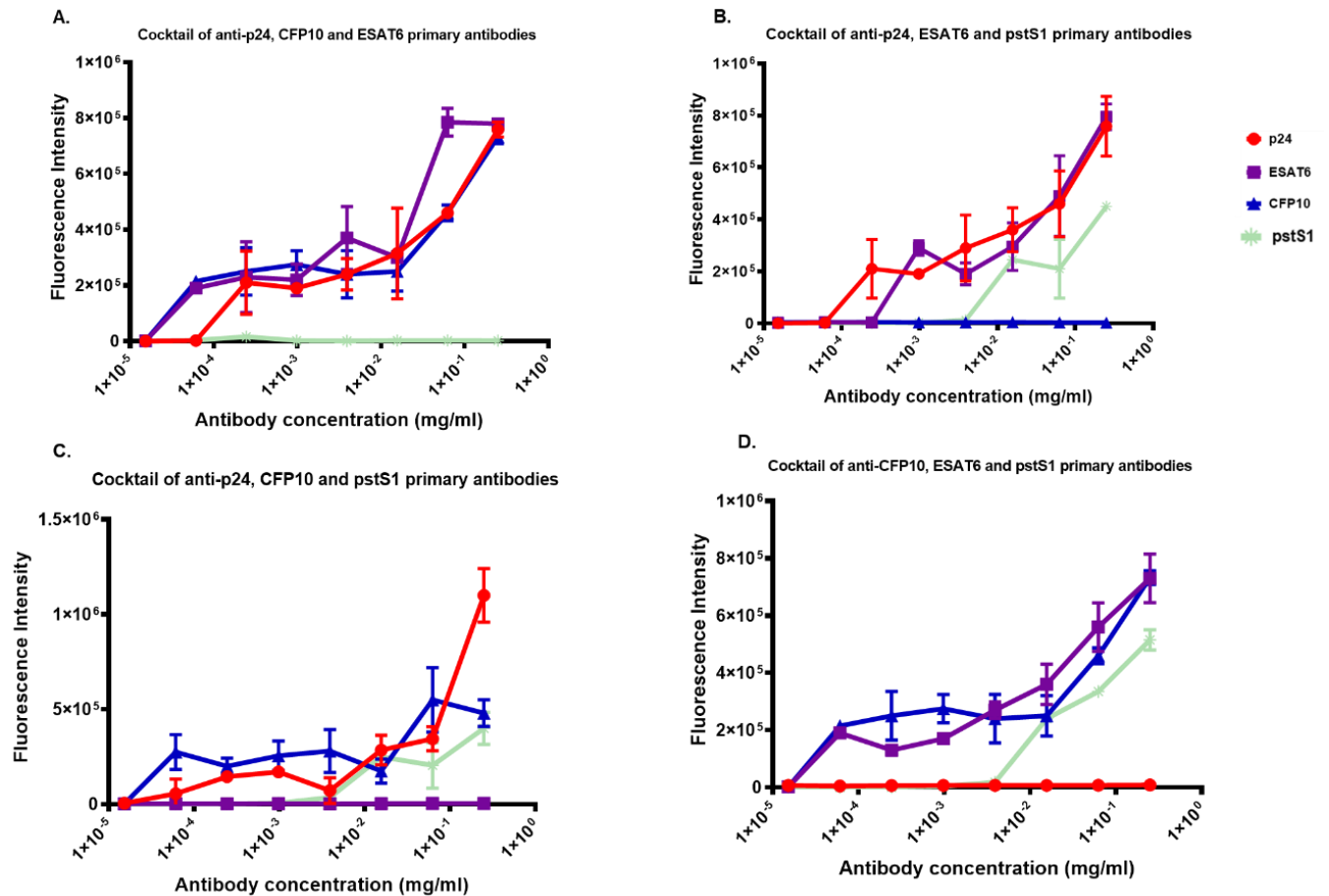


Figure 7: Microarray plates antigen-antibody reaction specificity in human serum.

Immobilized antigens were incubated with cocktails of primary antibodies missing one antibody at a time. Antigens and secondary antibody concentrations were kept constant while primary antibodies were serially diluted. (A) A line graph representing the fluorescence intensity obtained when immobilized antigens were incubated in a cocktail of primary antibodies missing the anti-pstS1 antibody; B, C and D are graphs for reactions missing anti-CFP10 or anti-ESAT6 or anti-p24, respectively. Data represents mean $\pm$ standard deviation (n=4).

4. Discussion

In this study, we sought to develop a multiplex technology for the detection of HIV and TB co-infection. Here epoxy functionalized slides were used to immobilize HIV-1 p24 as well as *M.tb* antigens CFP10, ESAT6 and pstS1 using covalent chemistry, and these were used to capture their respective antibodies in solution. The sensitivity and specificity of this multiplex microarray-based antibody-antigen reaction was then studied and compared to the gold standard platform, i.e., high-binding 96 well plate. We found that the antigen-antibody reactions on the microarray slide were significantly more specific and sensitive compared to results obtained in high-binding plates. In fact, the limit of detection of the primary antibodies using our microarray-based diagnostic technology was on average about 4690-fold lower compared to the plates. We have also shown that the microarray technology in this study is stable at different pH and temperature conditions as well as could be stored dry for at least three months. These findings suggest that our microarray-based technology has potential for use to diagnose HIV and TB co-infection and that the immunoassay conducted using this platform is likely to perform better than when commonly used high-binding plates are employed. These findings support further development of this technology for application in HIV and TB co-infection diagnostic.

The fact that the human serum we used was spiked with HIV and TB antibodies at a concentration that is commonly found in infected individuals (Raux et al., 1999, Kimuda et al., 2017) and were able to detect these antibodies using our microarray technology suggests that this technology is likely to work when actual human samples are used. Previous investigators have also spiked the analyte in human serum and found that the results could be replicated when actual human samples were used (Yeung et al., 2016, Wang et al., 2003, Toedter et al., 2008). Furthermore, the use of the human serum mimicked *in vivo* conditions where the HIV and TB antibodies could be present in the presence of other human proteins. The data obtained were compared to that of gold standard high-binding plates, and we found that the slides were more sensitive with the average limit of detection determined to be 0.954 ng/ml for antibodies against the four antigens, while for the plates this limit was on average 4474.6 ng/ml. Although, the limit of detection reported by other researchers for the detection of p24 antibodies in spiked blood (using p24 antigens immobilized on a chip and fluorescence as the mode of

detection) such as that reported by Liu et al., was as low as 17 pg/ μ l (Liu et al., 2021c), the limit of detection with the epoxy coated glass slides is still low enough to detect antibodies in human blood samples, albeit a lower detection limit would have been more favorable.

To explain the striking difference of the results obtained from the epoxy coated glass slides and high-binding plates, two platforms (slides and plates), it is important to note that on the slides the antigens are immobilized using a covalent bond while on the high-binding plates they are immobilized by adsorption using van der Waals, hydrophobic and/or electrostatic interactions (Bhakta et al., 2015). Since the covalent bond is stronger than the van der Waals, hydrophobic and/or electrostatic interactions (Liu et al., 2018b, Mallinson et al., 2003), the chances for some of the immobilized antigens being lost, for example by washing out during the assay, is much more reduced on the slides compared to the plates. It is also possible that the high sensitivity of the epoxy coated glass slides microarray technology compared to the high-binding plate was due to the fact that different instruments were used for the detection of fluorescence because of technical restrictions i.e., the slides were read using the cytation3 multimode reader while the plate DTX 880 Multimode detector was used for the high binding plates. In fact, the cytation 3 used to read the slides was 200 folds more sensitive than the DTX 880 plate reader according to the manufacturer specifications, which could possibly explain the difference in the sensitivity obtained with the two platforms. Nonetheless, the fact that we observed similar sensitivity when comparing PBS and human serum results for either the microarray slide or the microtiter plate indicates that the presence of other human proteins did not interfere with the antigen-antibody reaction on these two platforms. The similarity in this particular characteristic between the microarray slides and the high-binding plates was somewhat expected because the interaction between the antigen-antibody does not rely on the surface chemistry of the microarray slides or high-binding plates as long as the immobilized antigen is in the correct conformation. Lastly, we did not observe any cross reactivity between the antigen-antibody reaction on the microarray slides. This is consistent with numerous studies that have shown the high specificity of this reaction (Kapingidza et al., 2020, Cervia et al., 2021, Van Regenmortel, 2014). Also the high

specificity of protein-protein interactions on the microarray slide have been previously described, for example, Koukouvinos et al., studied the cross-reactivity of mycotoxins in a protein microarray and found that the antibody-mycotoxin reactions were highly specific (Koukouvinos et al., 2021).

Since proteins have three-dimensional structures and can easily lose their reactivity due to factors such as pH and temperature, they are prone to denaturation during printing and immobilization if the conditions are not ideal (Ellington et al., 2010). Our data suggests that the proteins captured on our microarray slides are likely to remain stable at room temperature and neutral pH. This is critical as routine laboratory diagnostics are commonly conducted at room temperature (Ndlovu et al., 2018, Shah et al., 2009, Lawn et al., 2011) and human blood has a pH of 7.4 (Kellum, 2000, Brunskill et al., 2012). Also, the fact that storage in dry conditions did not affect the performance of our microarray slide indicates that they can be stored without special requirements, which is very important, especially for resource poor settings. Here, it is worth noting that HIV and TB co-infection is a bigger problem in developing countries, which often have limited resources (Parsons et al., 2011, Karim et al., 2009). Lastly, we tested the shelf-life of our technology for three months, and during this period we observed that fluorescence could still be obtained on all the antigens spots printed on the slide even on day 90. This observation was similar to what was reported by Jorgensen and colleagues where they showed that printed antibody arrays could be stored for twelve weeks without losing their function in 96 well microtiter plates (Jorgensen et al., 2021). On the other hand, the widely used antibody HIV rapid test can be stored for a period of six months according to the manufacturer, which is double the storage time of our technology. However, it is possible that the actual shelf-life of the HIV/TB multiplex diagnostic technology is longer than three months as fluorescence was still observed at day 90. This could also offer more advantages in resource constrained settings, like what was reported by Asghar et al., where they showed that HIV antibodies can be stored at room temperature for a period of six month on an immune-functionalized device developed for possible use in resource limited settings for detection of HIV infection (Asghar et al., 2016). Thus, the shelf-life of our study must be proven experimentally beyond 90 days as it is desirable for developed

assay to have a long shelf-life to accommodate underserved communities (Asghar et al., 2016, Delaney et al., 2011, Naseri et al., 2022).

For coating the microarray slide we selected epoxy as the functional group, as it interacts with the amine group at the N-terminus of the protein for immobilization (Krizkova et al., 2015, Sauer, 2017). This ensured that the immobilized protein is free to interact with its target (Krizkova et al., 2015, Sauer, 2017). Yousefi et al. showed that the epoxy functional group performed better as a microarray slide coating agent when compared to other functional groups (Yousefi et al., 2018). This is a study to consider in future for this multiplex technology to see how the HIV and TB antigen-antibody reactions employed in this study behave when using other glass slide functionalized with groups such as aldehyde.

As for current limitation of the study, we think the data presented here can be complemented by those using actual human blood samples of HIV and TB co-infected patients. This being said, we are also aware that many investigators in the past used human serum spiked with antibodies, the same as we did here, for validation of technologies meant to detect biomarkers in human blood samples (Pultar et al., 2009, Mytych et al., 2009a, Mohammadi et al., 2018, Maple et al., 2004, Albrecht et al., 2008). In addition, the thorough comparison with other diagnostic methods for active TB such as *M.tb* culture or HIV PCR and Western blotting will be very useful in assessing our novel technology performance. Lastly as stated above, a longer storage study, particularly with the wet storage condition can be of benefit for further development of this technology.

In conclusion, we have shown that HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens can successfully be conjugated or printed on epoxy-coated glass slides at different pH and temperature conditions. We have also shown that the immobilized antigens on the slides can be stored for at least three months without compromising their function. Most importantly, these antigens were able to capture their complementary primary antibodies in human serum; and the multiplex microarray diagnostic technology showed high specificity and sensitivity when compared to immunoassay performed on high-binding plates. Thus, this is a promising diagnostic technology that requires further development for the detection of HIV and TB co-infection.

Author Contributions: Conceptualization, K.A. and C.T.; methodology K.A and K.M.; formal analysis and investigation, K.M.; data curation, K.M.; writing—original draft preparation, K.M.; writing—review and editing, K.A., C. T. and A.S; supervision, K.A., C. T. and A.S; funding acquisition, K.A. All authors have read and agreed to the submitted version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of the University of the Witwatersrand (protocol code M220866).

Data Availability Statement: The data sets used and/or analyzed during the present study are available from the corresponding author on request.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

5. Appendix A

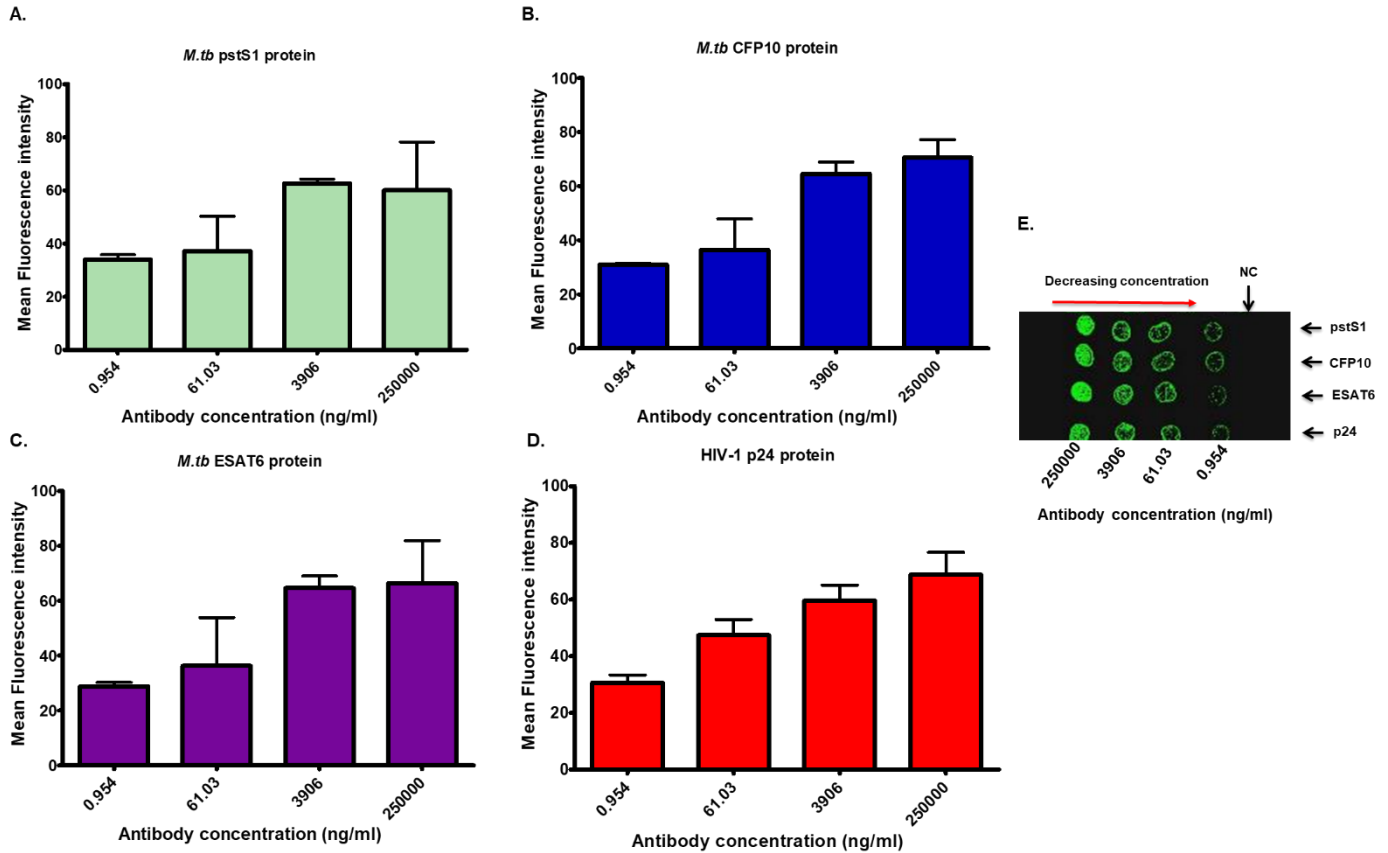


Figure A1: Evaluation of sensitivity of epoxy-coated glass slides assay in PBS. Primary antibodies were serially diluted in PBS while the antigen and secondary antibody concentrations were kept constant. Fluorescence intensity values for pstS1 (A), CFP10 (B), ESAT6 (C) and p24 (D) are presented as bar graphs. Data represent mean $\pm$ standard deviation ($n=4$). (E) A representative image of the fluorescence emitted on the microarray slide.

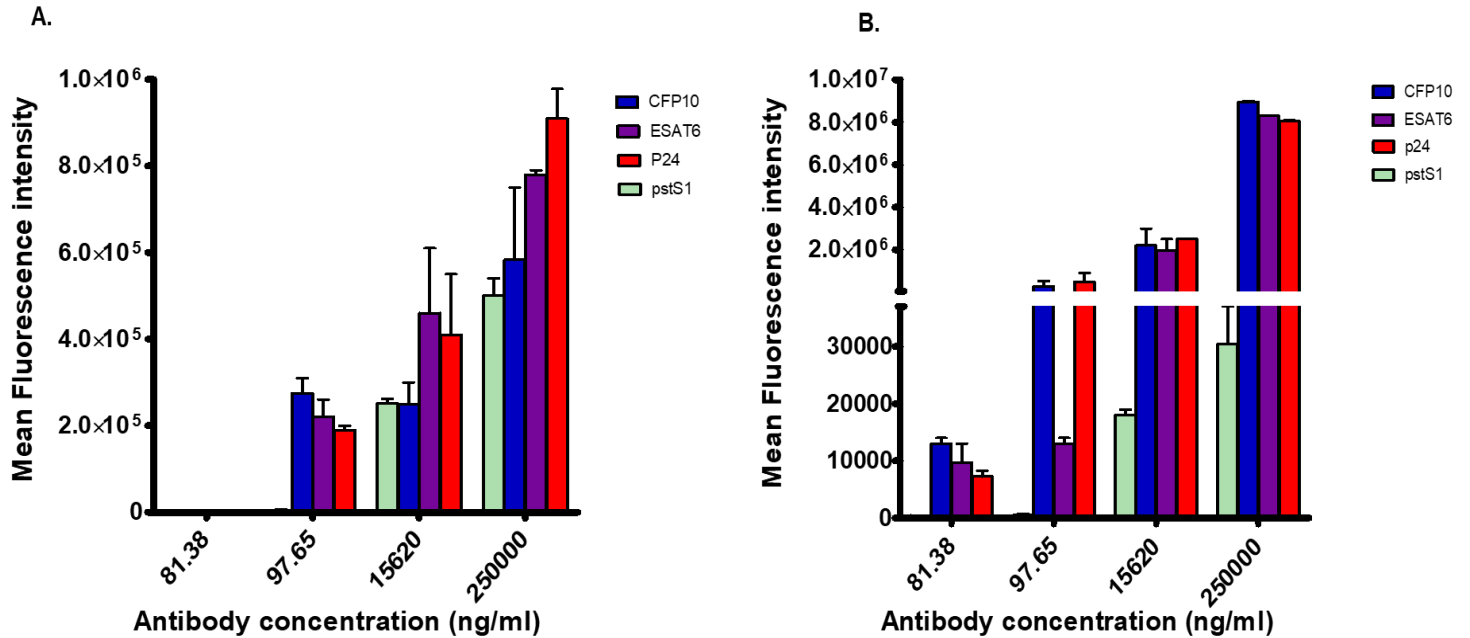
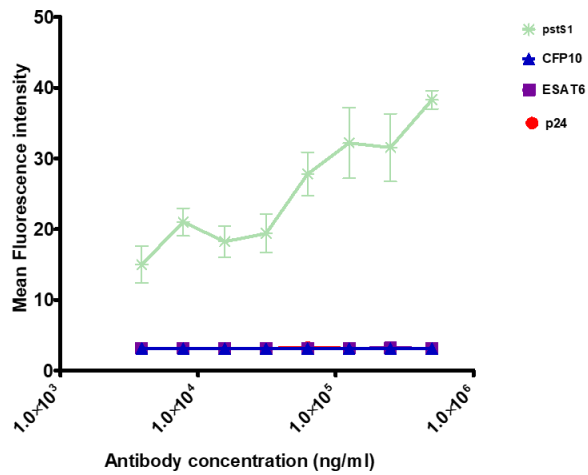
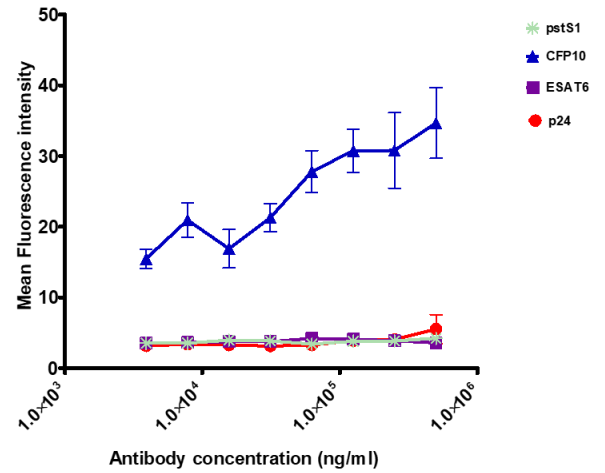


Figure A2: Determination of antigen-antibody reaction sensitivity for the microtiter plates. The concentration of immobilized antigen and secondary antibodies were kept constant while that of the primary antibodies varied. (A) shows the fluorescence intensity obtained when HIV and TB antibodies were diluted in PBS while (B) shows the intensity obtained when these antibodies were diluted in human serum. Error bars represent mean $\pm$ standard deviation ($n=4$).

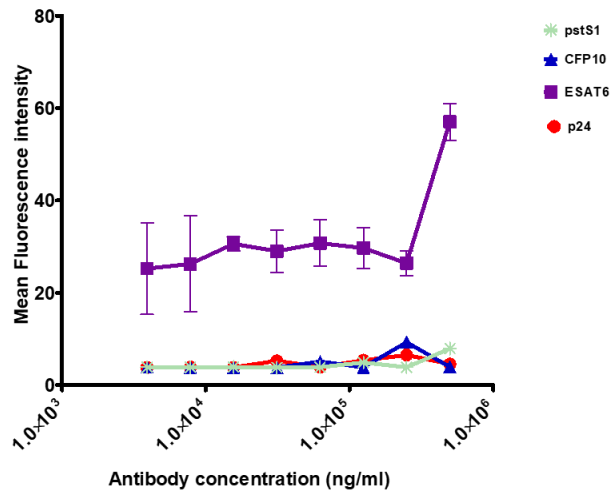
A.



B.



C.



D.

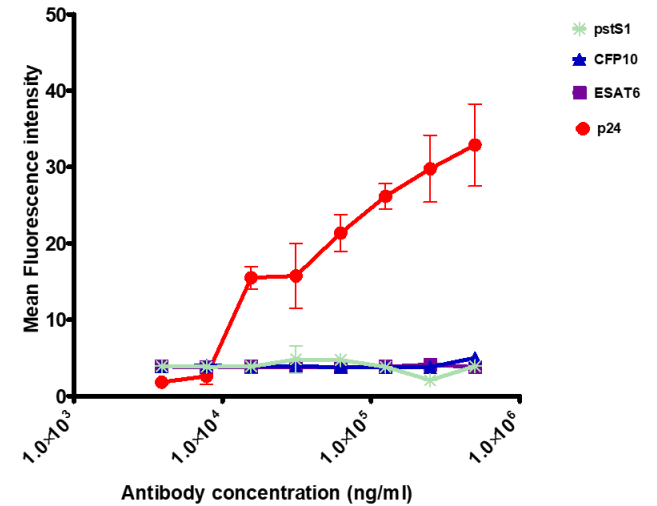


Figure A3: Alternative set-up of Microarray plates antigen-antibody reaction specificity in human serum. The immobilized antigens were incubated with the anti-p24 antibody (A), anti-ESAT6 antibody (B), anti-CFP10 antibody (C) or anti-pstS1 antibody (D) before the addition of the Alexa fluor 488 labelled secondary antibodies. Data represents mean $\pm$ standard deviation ($n=3$).

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CHAPTER THREE
MULTIPLEX MICROARRAY HIV/TB
TECHNOLOGY PROOF-OF-CONCEPT
USING HUMAN SERUM INFECTED WITH
HIV.

1. Introduction

Technological advances in molecular tests have become valuable for the detection of infectious agents that cannot be cultured or are difficult to grow in culture (Burd, 2010, Piddington et al., 2000). There have been efforts to develop easy to use, fast and accurate molecular diagnostic tools for different types of diseases. The use of microarray technology for the detection of many types of diseases at the same time is one of the laboratories developed techniques explored in diagnostics (Duarte and Blackburn, 2017, Sui et al., 2017, Feng et al., 2018). The performance specifications for laboratory developed tests to ensure precise and accurate results before the implementation of the test is of paramount importance (Burd, 2010, Braga et al., 2023, Genzen et al., 2017). The method characteristics that need to be accomplished include precision, accuracy, reference interval, reportable range, analytical sensitivity and specificity, to meet regulatory requirements (Burd, 2010, Lee et al., 2006, Braga et al., 2023, Rotgers et al., 2023). Furthermore, the performance validation of these test methods using human specimen with the target diseases is also important (Peters et al., 2007, Genzen et al., 2017).

In the previous chapter, the multiplex microarray technology for the detection of HIV and TB was tested for its specificity and sensitivity as well as stability under different conditions. The validation was performed with normal human serum spiked with the HIV and TB antibodies to mimic *in vivo* physiological conditions. It was important to also study the performance of the technology using actual human samples infected with HIV and TB. In this chapter the performance of the assay is evaluated using serum samples of seven patients infected with HIV and two uninfected controls. Although the aim of the study was to detect both HIV and TB antibodies in human samples simultaneously, obtaining human specimen (serum) of patients infected with both HIV and TB proved to be a challenge, therefore the samples used here were infected with HIV only for proof-of-concept purposes. Since the technology stability studies were reported in the previous

chapter, any factors such as pH and temperature that were deemed optimal were used to carry out the testing in this validation chapter.

The hypothesis here is that all the serum samples known to be positive for HIV will also be confirmed to be positive with the multiplex technology and no immobilized TB antigens will bind any antibodies and proteins in the serum samples. Any fluorescence observed on the p24 antigen spots immobilized on the slides would mean HIV antibodies are present in that serum sample. In addition, the lack of binding on any of the immobilized TB antigens would indicate the technology is likely to be specific. These results would indicate its ability to detect HIV and specificity potential of the technology.

2. Method and material

2.1. Material

The nine stored serum samples were obtained from the National Health Laboratory Services (NHLS) biobank (ethics clearance certificate number: M220866); seven of which were infected with HIV and two were uninfected controls. The following labels were used for the samples in this study: HIV serum sample A, B, C, D, E, F and G for the infected and H and I for the uninfected. The samples viral load, TB status and whether they were obtained from patients who were on treatment or not is unknown and information about the identity of the patients whose serum samples were used for the study was kept confidential. The Early Secretory Antigenic Target protein 6 (ESAT6) and Culture Filtrate Protein-10 (CFP10) antigens were obtained from BBI solutions (Caerphilly, United Kingdom). The pstS1 antigen was purchased from My BioSource (California, USA). The goat anti-human IgG conjugated to fluoresceine isothiocyanate (FITC) purchased from Abcam (Cambridge, United Kingdom). The epoxy coated glass slides were obtained from Schott laboratories (Mainz, Germany).

2.2. Method

To test the multiplex technology proof-of-concept using HIV infected serum samples, the slides were coated with the HIV-1 p24 antigen as well as the CFP10, ESAT6 and pstS1 *M.tb* antigens at a constant concentration of 31300 ng/ml. In total, the slides coated with the antigens were nine. These were incubated with different HIV infected serum samples and uninfected ones for an hour at room temperature. The washing and blocking conditions of the test were as previously mentioned in the chapters studying optimal conditions, i.e, the blocking was with 1% BSA, the wash buffer (PBS) was kept at pH 7.4 and all incubations were at room temperature. After the incubation and washing, the goat anti-human IgG-FITC secondary antibody was added and incubated with the slides for an hour at room temperature. After the hour incubation, the slides were washed three times for 5 minutes with PBS at pH 7.4. The slides were then read using cytation 3 multimode reader. Of note is that although the serum samples were only infected with HIV, the TB

antigens were included on the slide to check for specificity of HIV antibodies, i.e., to see if there would be any binding on the TB antigens spots.

3. Results

The seven HIV positive serum samples as well as the two uninfected samples were tested using the HIV and TB multiplex technology. The serum samples were incubated on the epoxy coated glass slides, followed by the addition of a secondary antibody conjugated to FITC for detection. The presence of fluorescence on the p24 antigen spots immobilized on the epoxy coated glass slides would indicate the binding of primary antibodies from the HIV infected serum samples. From the results obtained, out of the seven tested samples from HIV infected individuals, six samples showed fluorescence on the p24 antigen spots, and the mean fluorescence intensity of the spots varied between samples (Figure 1 A-F, Table 1). As expected, no binding was observed on the p24 antigen spots from uninfected serum samples (Figure 1 H and I). Furthermore, no binding was observed on the CFP10, ESAT6 and pstS1 TB antigens immobilized on the epoxy coated glass slide, thus no cross reactivity occurred during the assay performance (Figure 1).

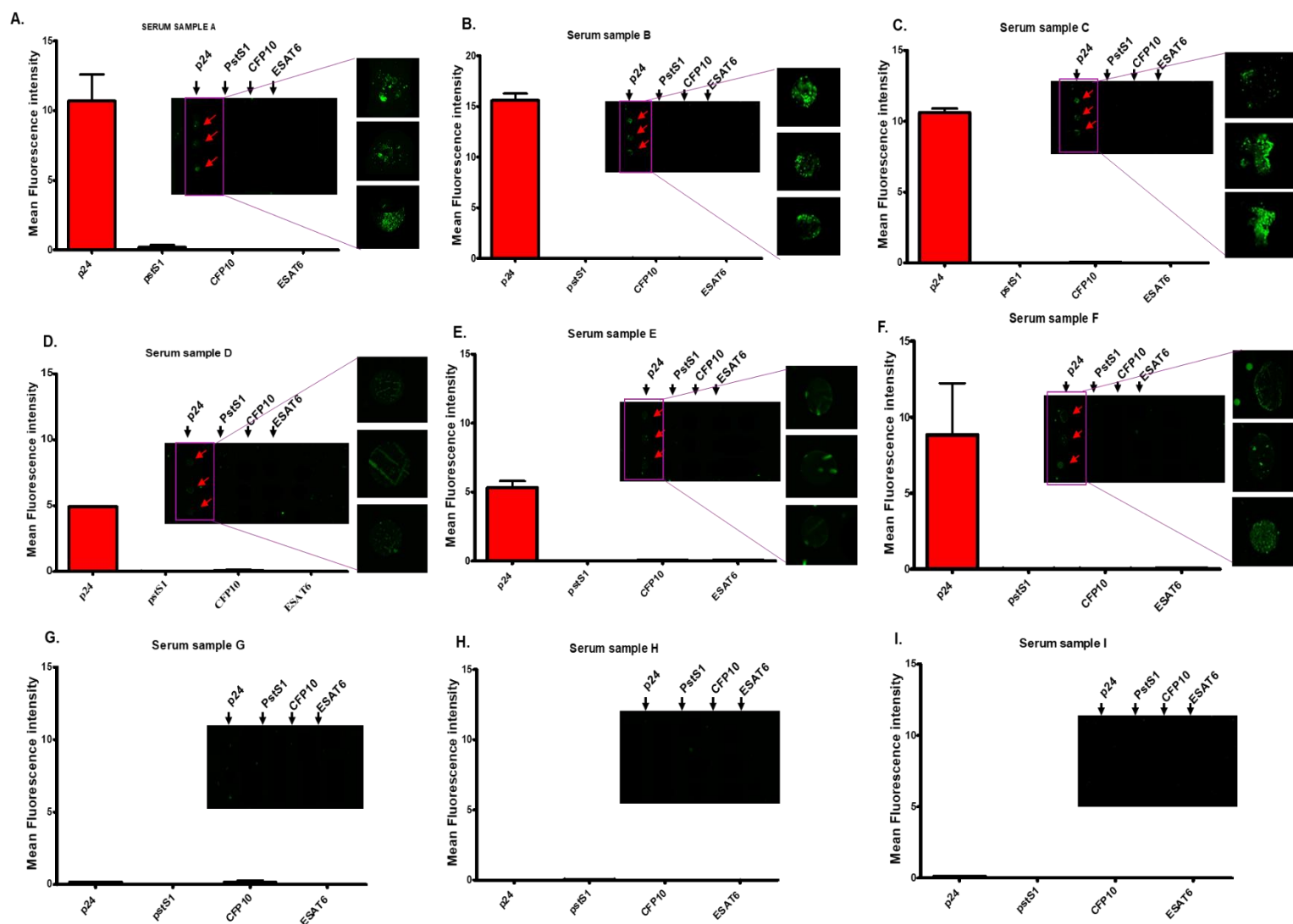


Figure 1: Investigation of the performance of the multiplex technology against HIV infected samples. The HIV infected serum samples (A-G) and uninfected controls (H-I) were incubated with the HIV p24 and TB antigens (CFP10, ESAT6 and CFP10) immobilized on epoxy coated glass slides. Goat anti-human IgG-FITC secondary antibody was used for infection signalling. Bar graphs were plotted from mean fluorescence of two independent experiments for each sample, after subtraction of the background. Error bars represent $\pm$ standard deviation of the mean.

Table 1: Serum samples test results obtained with the HIV-rapid test and the HIV/TB multiplex microarray technology.

Sample name	HIV rapid test	HIV/TB multiplex microarray technology
A	Positive	Positive
B	Positive	Positive
C	Positive	Positive
D	Positive	Positive
E	Positive	Positive
F	Positive	Positive
G	Positive	Negative
H	Negative	Negative
I	Negative	Negative

4. Discussion

The study aimed at developing a microarray technology that can detect HIV and TB simultaneously. In the previous chapter, the technology specificity and sensitivity were studied with limits of detection determined to be 0.000954 µg/ml when human serum was spiked with anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies to mimic *in vivo* conditions. In this chapter the technology's proof-of-concept, using serum samples infected with HIV, was explored.

The ability of the technology to detect HIV was tested using human serum samples infected with HIV as well as uninfected controls. The multiplex technology detected HIV infection from six out of seven (85.7%) samples, i.e., fluorescence was observed when the six samples were incubated with the p24 antigen immobilized on the glass slide. On the other hand, one sample known to be positive for HIV, tested negative. Moreover, the fluorescence intensity was different between the six serum samples that tested positive. One possible reason for this observation may be that the samples had different viral loads. Viral loads can differ from one person to the next as infections occur at different times between individuals (Hu et al., 2001, Sabin et al., 2000, Falasca et al., 2017). The fact that one sample (sample G) produced negative results with the technology may be because of low viral load and very low undetectable levels of antibodies in that samples. HIV antibodies are generated and maintained by the ongoing systemic expression of the HIV antigen and a decline in the antibody levels reflects lower levels of antigen production (Keating et al., 2017, Sajadi et al., 2011). Furthermore, because serum contains many other proteins and antibodies, the HIV antibodies may be obstructed by other proteins in the serum from binding to the p24 antigen (Alhajj et al., 2023, Shi et al., 2018, Leboffe et al., 2020), and we speculate this is likely to have much more pronounced effect in samples with lower antibody levels. Hence the observed low fluorescence from other serum samples such as sample G known to be positive for the virus or lack thereof, may be as a result of these potential obstructions. Moreover, the antiretroviral therapy status of the infected serum samples used for this proof-of-concept study was unknown. It is important to note that this may also have an effect on the detection of HIV antibodies in infected sera (Keating et al., 2019, Veldsman et al., 2021). As validation of this work, it was encouraging to note that the negative control uninfected serum samples showed no

binding and hence no fluorescence from the p24 spots. These data for HIV were consistent with what we observed with what was observed with spiked human serum, thus, it is very likely that similar results will be obtained with human sera infected with TB.

Like the previous chapter, the multiplex technology specificity for HIV was also tested in this chapter by including the TB antigens on the epoxy glass slides in addition to the p24 antigen and incubating with the HIV infected serum samples. The HIV antibodies and other proteins in the infected serum samples did not bind to the TB antigens as no fluorescence was observed on the spots where the CFP10, ESTA6 and pstS1 antigens were spotted. This finding supports the specificity studies performed in the previous chapter where we showed that the HIV and TB antibodies spiked in human serum reacted specifically to their respective antigens. Also, the fact that the uninfected negative control serum samples showed no binding to either the HIV or TB antigens spots on the slides further supports the specificity of this technology, i.e, even the other antibodies and proteins in healthy human serum did not bind non-specifically to the antigens on the slide. This aspect is of importance in multiplex technologies as several diseases are detected at the same time and cross reactivity can lead to false positive results (Wong et al., 2017, Tokarz et al., 2018, Kim et al., 2018). Therefore, the multiplex technology developed in this study is analytically specific for detecting HIV, i.e, the assay had the ability to detect only the intended target (HIV antibodies).

In conclusion, HIV infected serum samples were used as proof-of-concept for the HIV and TB multiplex microarray technology. This chapter has shown that out of seven positive serum samples, the technology was able to detect HIV antibodies in six samples. The negative control samples also proved to be negative using the multiplex technology, which meant no false positives were observed. Thus, the technology requires further development and future studies will involve using samples infected with both pathogens. Also, more detailed comparison with gold standard such as the rapid test used on the samples tested here is recommended. It would as well be interesting to test the technology at different stages of HIV and TB infections and explore the sensitivity studies at different stages of treatment.

5. References

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CHAPTER FOUR

DEVELOPMENT OF A POINT-OF-CARE COLOUR REACTION-BASED MULTIPLEX DIAGNOSTIC TECHNOLOGY FOR DETECTION OF HIV AND TB CO-INFECTION.

Development of a point-of-care colour reaction-based multiplex diagnostic technology for detection of HIV and TB co-infection.

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Abstract

Currently, there are point-of-care (POC) tests for the detection of HIV and *Mycobacterium tuberculosis* (*M.tb*), however none can detect these pathogens simultaneously. We developed a colorimetric multiplex HIV and active TB diagnostic technology, using HIV-1 p24 and *M.tb* CFP10, ESAT6 and pstS1 antigens immobilized in high-binding 96 well plates or on epoxy-coated microarray slides. These antigens were used to bind corresponding antibodies in human serum at concentrations commonly found in infected individuals. This was followed by the addition of HRP conjugated secondary antibodies using either 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid) (ABTS) or 3,3',5,5'-tetramethyl Benzidine (TMB) substrates. The sensitivity of the assay in the plates was compared to epoxy-coated slides and the latter had a better limit of detection, averaging 815-fold lower than the plates. Also, the TMB substrate produced better sensitivity than ABTS. Furthermore, we studied the specificity of the antigen-antibody reactions for this technology and determined that it was highly specific. Lastly, we investigated the stability of our technology at different pH, temperature, and storage conditions and found that it was optimal at pH 7.4, 25 °C, and dry storage for 1 to 90 days. Thus, the developed diagnostic technology has potential for the detection of HIV and TB co-infection at POC.

1. Introduction

Around 38.4 million people are living with HIV/AIDS globally with the annual number of deaths standing at 650 000 in 2021, of which about 25% were co-infected with *Mycobacterium tuberculosis* (*M.tb*), the causative agent of tuberculosis (TB) (UNAIDS, 2022) (Scott et al., 2017b, Bruchfeld et al., 2015). Although there are diagnostic tests currently available for HIV and TB, the fact that their diagnosis is done separately can delay treatment in co-infected individuals and, therefore, exacerbate the observed mortality rate (Tiewsoh et al., 2020, Rios-Hincapie et al., 2020).

Some of the laboratory diagnostics for HIV depend on the p24 antigen and antibody interactions (Gray et al., 2018). Sensitive p24 antigen detection methods include photoelectrochemical immunoassays (Zhao et al., 2015), ultrasensitive enzyme-linked immunosorbent assay (ELISA) coupled with thionicotinamide-adenine dinucleotide (thio-NAD) cycling (Nakatsuma et al., 2015), as well as ultrasensitive POC quantitative single-molecule transistor sensor assay (Macchia et al., 2020). There are also molecular tests used to diagnose HIV that rely on the polymerase chain reaction (PCR), and they include the Alere q HIV-1/2 Detect (Alere q) and Cepheid Xpert HIV-1 (Xpert) assays (Essajee et al., 2015, Hsiao et al., 2016, Jani et al., 2014). However, the commonly used test is the POC antibody based test as it is the most rapid and affordable, with result obtained within 30 minutes (Essajee et al., 2015). Nevertheless, this test also has disadvantages, among them the inability to accommodate infants and the occasional false positive results (Chavez et al., 2020, Jafa et al., 2007, Rifkin et al., 2012, Sherman et al., 2008).

M.tb infection can be diagnosed using chest X-ray, acid-fast bacilli smear and mycobacterial culture tests (Cheon et al., 2016, Gozes and Greenspan, 2019, Liu et al., 2020b). However, the chest X-ray technique is not specific for the diagnosis of pulmonary TB and requires highly trained personnel (Ryu, 2015). There are also molecular methods such as the Xpert, which are employed for the diagnosis of TB (Dewan et al., 2015, Tian et al., 2015). These techniques involve nucleic acid amplification for the detection of the *M.tb* genome (Bahr et al., 2018, Dewan et al., 2015). The GeneX-pert assay is WHO endorsed and allows for the peripheral detection of peripheral rifampicin resistance and the detection of drug-resistant TB as well as exponentially increasing the number of

treated TB cases. The technology also increased the sensitivity of TB detection in children, HIV-infected peoples and extrapulmonary TB with a turnaround time of between 0-6 days (Pooran et al., 2019, Nyaruhirira et al., 2022, Ng et al., 2016, Kwak et al., 2013). Although these methods are good at increasing the specificity as compared to other approaches of diagnostics, they have limitations such as long turnaround time, high cost, require sophisticated equipment, and are unable to distinguish between active and latent TB (Al-Zamel, 2009, Miranda-Schaeubinger et al., 2023). Nonetheless, previous investigators reported that the detection of antibodies against CFP10, ESAT6 and pstS1 TB antigens could be used to determine the presence of active TB in infected individuals (Davidow et al., 2005, de Araujo et al., 2018, Khurshid et al., 2013, Watson et al., 2021).

Multiplex analytical technologies are very useful for faster detection of multiple disease specific biomarkers as they can accommodate different types of molecules (Anfossi et al., 2018, Van Gool et al., 2020). The multiplex microarray is a fast-developing diagnostic technique that allows for the simultaneous detection of many analytes in biological samples within a single experiment and are an attractive solution for the analysis of biological responses in normal and diseased states (Kazakova et al., 2020, Zhao et al., 2022b, Zhang et al., 2022). Microarray-based sandwich immunoassays involves the use of microspots of capture molecules that are immobilized in a row or column onto a solid surface and exposed to samples containing corresponding binding molecules such as antibodies (Jian et al., 2021, Hogan et al., 2019, Fu et al., 2020). Current techniques such as enzyme-linked immunosorbent assay (ELISA) based on single protein measurement can be costly financially and timewise (Kranz et al., 2019). The most important benefits of multiplex array assays are the reduction of cost, sample consumption, rapid analysis of biomarkers, increase in sample throughput and the high amount of information generated by a single test (Zhu et al., 2017, Taghavian et al., 2018, Sauer, 2017). The technique has been used for the simultaneous diagnosis of diseases such as HIV/AIDS, hepatitis C and hepatitis A (Kranz et al., 2019). The power of immunoassays comes from the simplicity of the antibody-antigen reactions and the availability of polyclonal and monoclonal antibodies against many proteins (Nakajima et al., 2018, Ma et al., 2021, Li et al., 2021b).

Here we developed a colorimetric multiplex assay for the POC detection of HIV and active TB antibodies using HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens immobilized in 96 well high-binding polystyrene plates or on epoxy-coated glass slides. The stability of the technology in different pH, temperature and storage settings was evaluated to determine the optimal conditions. We found that both platforms were very sensitive and specific for the multiplex detection of HIV and TB, although, the epoxy-coated glass slides showed better sensitivity with their limit of detection being many folds lower than the high-binding 96 well plates. Furthermore, the comparison of TMB and ABTS for colorimetric detection showed that the former was the most suitable substrate for the POC application of our technology.

2. Method and material

2.1. Material and reagents

The phosphate-specific transport substrate binding protein-1 (pstS1) antigen and pstS1 primary antibody were purchased from My BioSource (California, USA). The Early Secretory Antigenic Target protein 6 (ESAT6) and Culture Filtrate Protein-10 (CFP10) antigens were obtained from BBI solutions (Caerphilly, United Kingdom). The HIV-1 anti-p24, *M.tb* anti-CFP10, *M.tb* anti-ESAT6 primary antibodies and HIV-1 p24 antigen were obtained from Abcam (Cambridge, United Kingdom). The Goat (Gt) anti-Mouse (Ms) /anti-Rabbit (Rb) IgG superclonal secondary antibody Horseradish peroxidase (HRP) conjugates were obtained from Abcam (Cambridge, United Kingdom). The NEXTERION epoxy silane coated glass slides were obtained from Schott laboratories (Mainz, Germany). The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid) (ABST) and 3,3',5,5'-tetramethyl Benzidine (TMB) colorimetric substrates were purchased from ThermoFisher Scientific (Massachusetts, USA). The Corning polystyrene high-binding costar assay plates came from Corning Inc. (New York, USA), while the slide hybridization cassette was purchased from Arrayit corporation (California, USA).

2.2. Determination of the optimal buffer pH, storage condition, temperature, and the shelf-life of immobilized HIV and TB antigens

All experiments performed in this chapter were conducted at CSIR next generation health cluster' synthetic biology and precision medicine center, array technology laboratory. The experiments were conducted three times in three independent experiments unless stated otherwise.

To determine the optimal printing, washing and storage pH buffer for the HIV-1 p24 antigen as well as *M.tb* CFP10, ESAT6 and pstS1 antigens, PBS at different pH (pH 5.0, 6.0, 7.4, 8.0 and 9.0) were assessed. The antigens at stock concentration of 1000000 ng/ml were diluted in these buffers to make the concentration of 250000 ng/ml. Five microliter of each antigen was added in triplicate to the wells of the high-binding 96 well plates and incubated at room temperature for 90 minutes to allow for adsorption. Negative controls included wells with PBS only without antigen. Post-incubation, wells were washed three times for 5 minutes each with 100 μ l of 1 $\times$ PBS at the various tested pH.

The plates were blocked thereafter with 3% bovine serum albumin (BSA) for an hour. Afterwards, they were washed three times with PBS at the corresponding pH conditions. This was followed by the addition of a cocktail of primary antibodies to HIV-1 p24, *M.tb* CFP10, ESAT6 and pstS1 at a constant concentration of 3906 ng/ml and incubation for an hour at room temperature. Thereafter, the plates were washed as described above and incubated for 90 minutes with 100 µl per well of secondary antibodies conjugated to HRP at the concentration of 100 ng/ml. Following the incubation, the wells were washed as above and 100 µl of ABTS substrate was added followed by incubation at room temperature for 45 minutes with agitation at 250 rpm. ABTS substrate is catalysed by HRP-H₂O₂ to produce a colorimetric signal (blue colour that can be visualized with the naked eye) (Zhang et al., 2018a). After the incubation with the ABTS, 50 µl of 1% Sodium Dodecyl Sulfate (SDS) stop solution was added to each well to end the reaction. Absorbance was then read at 450 nm using TECAN INFINITE F500 (Mannedorf, Switzerland).

After the evaluation of the optimal pH for the HIV and TB antigens immobilization, the next step was to determine whether the immobilized antigens could be stored dry or wet (in buffer). This was achieved by storing the immobilized antigens dry or in PBS pH 7.4 for 24 hours followed by incubation with primary and secondary antibodies and colour detection as aforementioned.

The determination of the temperature at which the immobilized antigens in the high-binding plates could be stored was accomplished by immobilizing them in the plates as mentioned above followed by incubation at -20 °C, 4 °C, 15 °C, 25 °C or 37 °C for 24 hours. Afterwards, the immobilized antigens were incubated with a cocktail of the HIV and TB primary antibodies, followed by secondary antibodies conjugated to HRP. Detection of binding was done as described earlier.

The immobilized antigens shelf-life was also evaluated to determine how long they can stay stable in the high-binding plate. Jorgensen et al., showed that printed proteins can remain stable for up to three months in microtiter plates (Jorgensen et al., 2021), thus the HIV and TB antigens in the plates were also evaluated for a period of three months. The immobilization of the HIV and TB antigens in the high-binding plates was achieved as

mentioned above. They were then incubated for 1, 15, 30, 45, 60, 75 and 90 days using nine plates at room temperature in two independent experiments. Colour change detection after the addition of primary and secondary antibodies was done as mentioned above.

2.3. Evaluation of the *in-vitro* sensitivity and specificity of the colorimetric multiplex technology for simultaneous detection of HIV and TB

The sensitivity of the colorimetric assay was performed in high-binding 96 well plates as well as on epoxy-coated glass slides using two HRP substrates, namely, ABTS and TMB. It is worth noting that the epoxy glass slides were clamped into a 96 well slide hybridization cassette during assay performance (Supplementary Fig. S1). To determine the sensitivity, the immobilization of the antigens on either the high-binding plates or the epoxy slides was done as mentioned above. This was followed by the addition of a cocktail of anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies diluted in normal human serum (Thermo fisher scientific, Massachusetts, United states, Catalog number 31876) or in PBS. The primary antibodies were serially diluted from 250000 to 15.3 ng/ml for the plates, and 250000 to 0.0149 ng/ml for the slides. These dilution series were inspired by what has been published on the HIV and TB antibodies concentrations in human blood (Raux et al., 1999, Kimuda et al., 2017). Human serum was used to dilute the primary antibodies to mimic *in vivo* conditions in an HIV and TB co-infected individual (Raux et al., 1999, Kimuda et al., 2017). To confirm the specificity of each antigen-antibody reaction, an anti-CFP10, anti-pstS1, anti-ESAT6 and anti-p24 primary antibody cocktail solution missing one of these antibodies at a time was prepared and incubated with all four immobilized antigens. Subsequently, the plates were washed three times with PBS for 5 minutes. Human serum or PBS containing no primary antibodies was added as the negative control to the immobilized antigens. Detection with secondary antibodies was done as above for the ABTS substrate. However, for TMB this was achieved by mixing 1:1 a solution of hydrogen peroxide (0.02% H₂O₂ in buffer) and peroxide substrate (0.4 g/l 3,3',5,5'-tetramethyl Benzidine in an aqueous solution). To each well, 100 µl of the TMB solution was added and incubated for 15 minutes, followed by colour change from clear to deep blue solution (that could be visualized with the naked eye) upon interaction with HRP in test wells. This was followed by the addition of a stop solution (2M

sulfuric acid) and reading was done immediately using TECAN INFINITE F500 at a wavelength of 450 nm.

2.4. Statistical analysis

The analysis was performed using GraphPad Prism version 9.4.0 (GraphPad Software, Inc.). Data are presented as mean $\pm$ standard deviation and each experiment was performed at least three times unless stated otherwise. One-way analysis of variance (ANOVA) test was used to assess the statistical significance between multiple groups under different scenarios using the Tukey's multiple comparison test. For all comparisons, *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Evaluation of the stability of immobilized antigens in the polystyrene high-binding plates

Since we already evaluated the stability of the HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens on the microarray slides (Malatji et al., submitted), here we began by only evaluating the stability of these antigens on the high-binding 96 well plates for comparison. To achieve this, HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens were immobilized as capture molecules in the wells of a 96 well high-binding plate, followed by the assessment of pH range at which the antigens were stable on the plate. The pH range tested was from 5.0-9.0. The detection of immobilized antigens was done using anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies; and secondary antibody conjugated to HRP, as well as the colour change reaction.

The data obtained showed that most antigen-antibody reactions were more effective from the neutral to basic buffer as compared to the acidic conditions (Fig. 1). This was shown by the high absorbance values obtained at pH 7.4, 8.0 and 9.0, while a 2-fold decrease in colour intensity was observed for pH 5.0 and 6.0 for most antigens and the difference was statistically significant ($p < 0.05$). Furthermore, the data also suggested that of all the pH, pH 7.4 consistently produced the highest colour intensity. Thus, the optimal pH at which the HIV and TB antigens were stable and gave the highest colour change reaction in the high-binding plates ranged from pH 7.4-9.0 with pH 7.4 being the most optimal. This was consistent with what we observed on the microarray slide (Malatji et al., submitted).

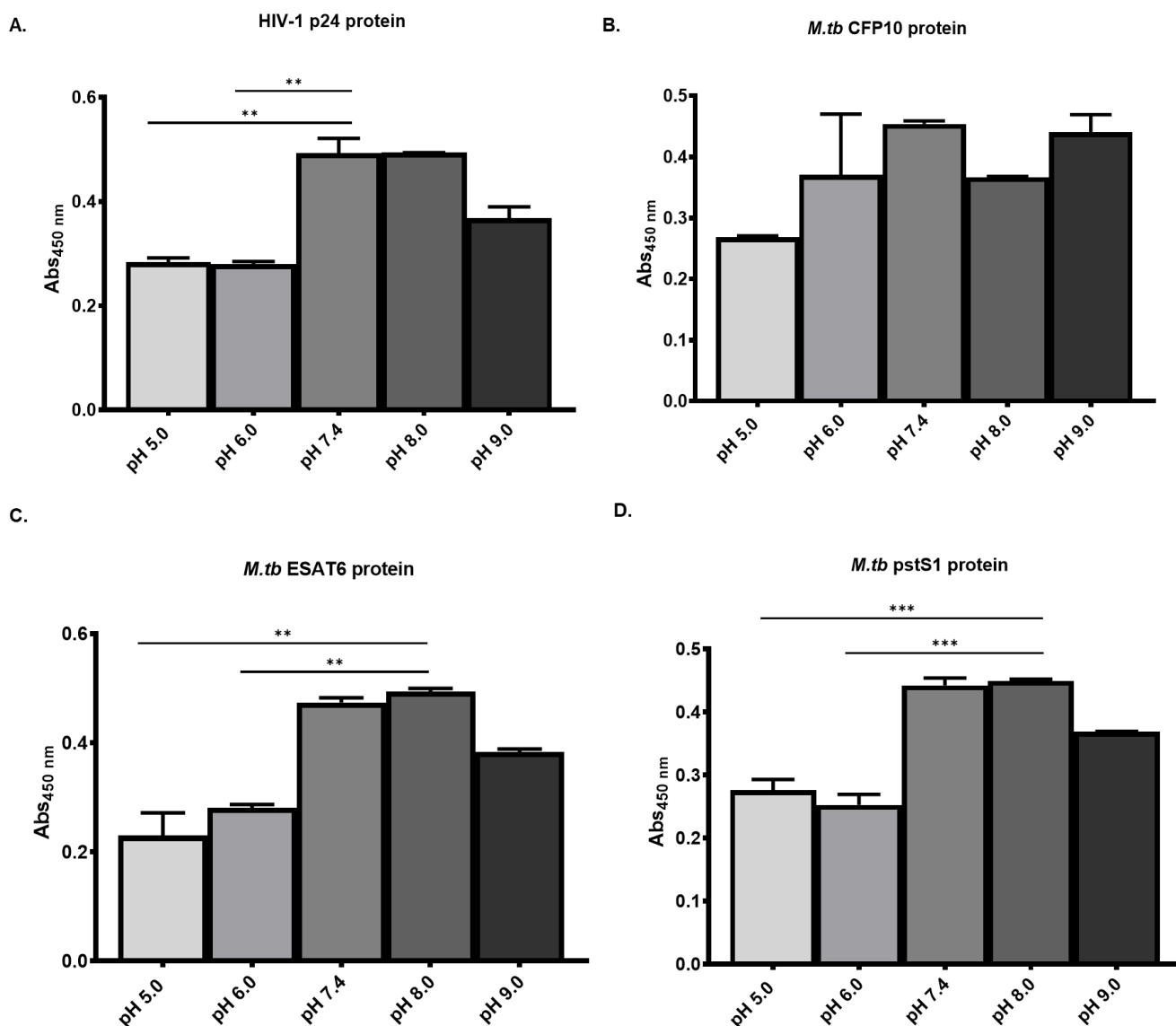


Figure 1: Evaluation of the optimal pH at which the HIV and TB antigens were stable. The antigens were adsorbed in high-binding 96 well plates using PBS at different pH (pH 5.0, 6.0, 7.4, 8.0 and 9.0). Colorimetric-based detection was achieved by means of secondary antibodies conjugated to HRP as well as the ABTS substrate. A, B, C and D show how the p24, CFP10, ESAT6 and pstS1 proteins, respectively, behaved under different pH conditions. Data represent the mean of three independent experiments and error bars are standard deviation of the mean. Asterisks signify statistical significance ($p < 0.05$) between pH being compared.

Given that temperature can also affect the stability of the immobilized proteins, we assessed the stability of the HIV and *M.tb* antigens, immobilized in the 96 well high-binding plate, at different temperatures. To accomplish this, different 96 well high-binding plates were used to immobilize HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens and stored at -20 °C, 4 °C, 15 °C, 25 °C or 37 °C for 24 hours. Colorimetric detection was achieved after the addition of primary antibodies as well as secondary antibodies conjugated to HRP. From the obtained results, the absorbance readings were around 0.4 at all temperatures tested except for -20 °C where we observed a 2-fold decrease in the reading and this difference was statistically significant when comparing it with the highest absorbance, which was at 25 °C ($p < 0.05$) (Fig. 2). It is worth noting that the optimal temperature was also 25 °C when evaluating the immobilization of the HIV and TB antigens on the epoxy-coated microarray slides (Malatji et al., 2023).

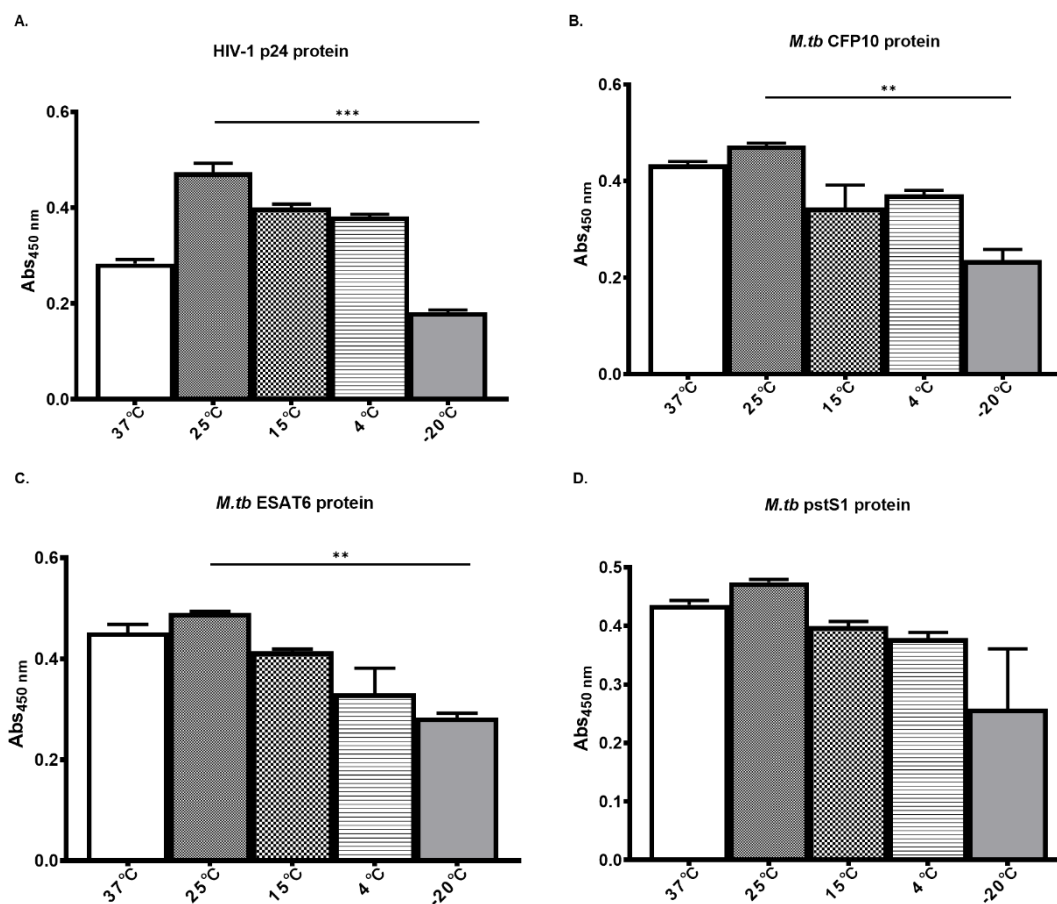


Figure 2: Determination of the optimal temperature at which the HIV and TB immobilized antigens were stable. The antigens were immobilized in the wells of a 96

well plate and incubated at different temperatures. Detection was achieved by means of secondary antibodies conjugated to HRP and the ABTS substrate. A, B, C and D show the absorbance obtained with p24, CFP10, ESAT6 and pstS1 antigens, respectively, at the different temperatures. Data represent the mean of three independent experiments and error bars are standard deviation of the mean. Asterisks represent statistical difference between compared temperatures.

The next step was to determine whether the antigens immobilized in the high-binding plates should be stored under dry or wet conditions. This was achieved by immobilizing the antigens in the plate and storing them in PBS pH 7.4 (wet) or dry, at room temperature (25 °C) for 24 hours. The detection was achieved by the addition of the HIV and TB primary antibodies as well as secondary antibodies conjugated to HRP. The data showed that the antigen-antibody reactions occurred under both conditions. However, the dry storage condition showed a higher colour intensity or absorbance relative to the wet condition, although generally the difference was not statistically significant (Fig. 3). This is consistent with what we observed for the microarray glass slides (Malatji et al., 2023).

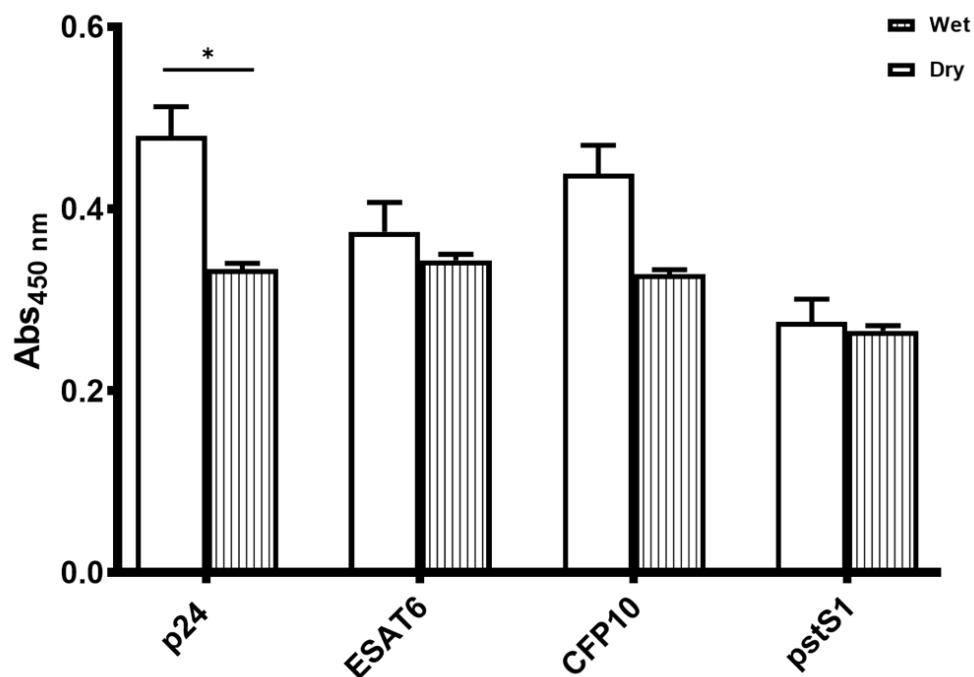


Figure 3: Evaluation of the HIV and TB antigens immobilization under dry and wet conditions. The antigens were stored in PBS pH 7.4 (wet) or dry, at room temperature

and then reacted with primary and HRP conjugated secondary antibodies after 24 hours incubation. The figure shows the absorbance obtained from the colour change reaction. Data represent mean $\pm$ standard deviation (n=3), while asterisks signify statistical significance between the compared storage conditions ($p<0.05$).

After the determination of the optimal storage conditions for the immobilized antigens, we assessed how long they could remain stable and active in the high-binding plates, i.e., their shelf-life. This was accomplished by immobilizing the antigens in the wells of the plate and incubating them dry at room temperature for 90 days with absorbance readings taken on day 1 and after every 15 days intervals. The absorbance readings were done by using primary antibodies specific to the immobilized antigens and HRP conjugated secondary antibodies. From the results obtained, absorbance was detected from day 1 through day 90 for all antigens (Fig.4). The trend observed was that the colour intensity decreased with an increase in the number of incubation days. Thus, there was a significantly higher colour intensity on day one compared to day 90 ($p<0.05$) (Fig. 4). However, the results indicate that the antigens could still bind to the antibodies for at least 90 days when stored at room temperature and under dry conditions. We reported a similar trend for the epoxy-coated microarray slides (Malatji et al., 2023).

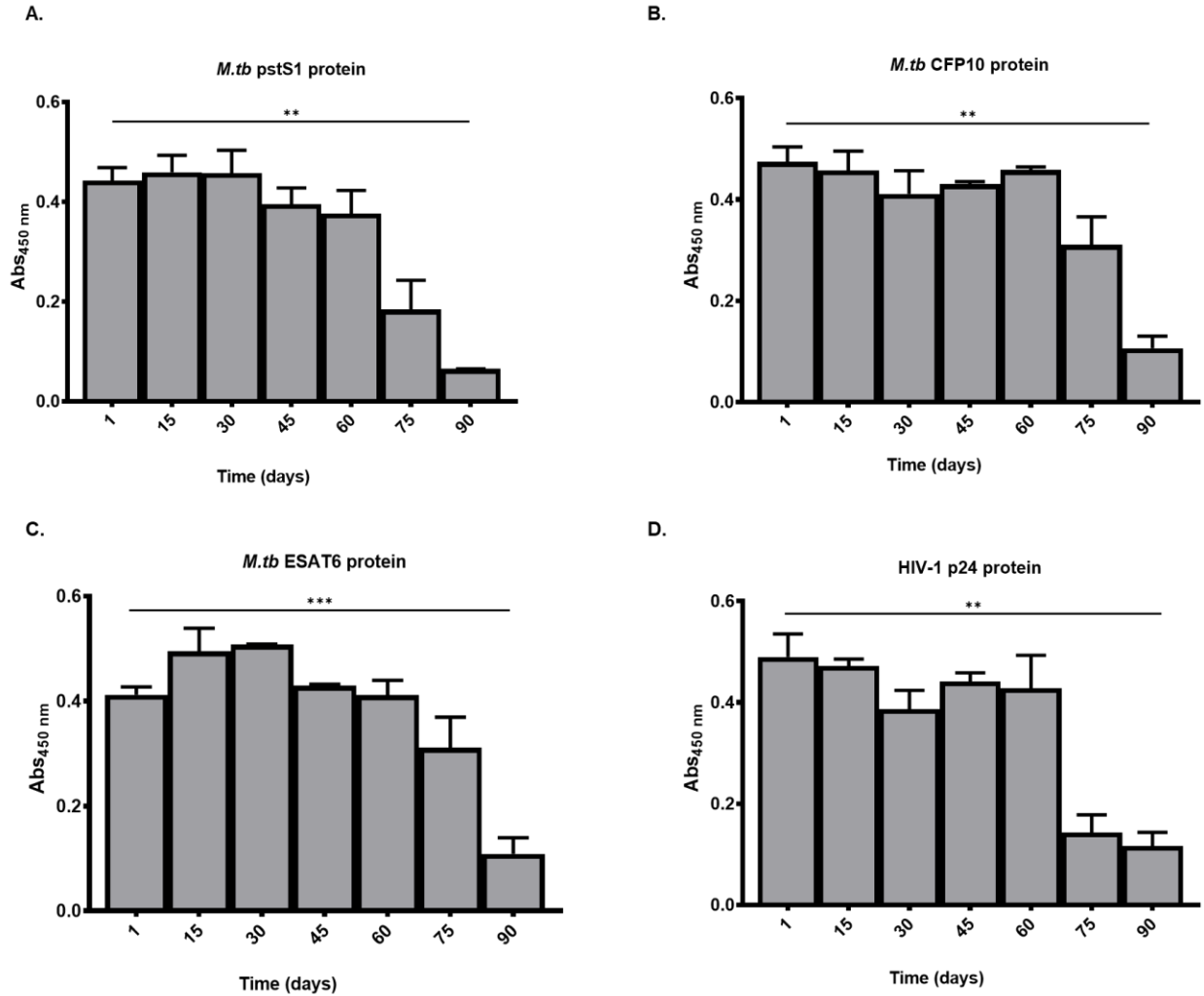


Figure 4: Evaluation of the shelf-life of the immobilized HIV and TB antigens in the 96 well high-binding plates. The antigens were immobilized in the wells of a 96 well high-binding plates and incubated dry at room temperature for up to 90 days, with colorimetric detection using primary and HRP conjugated secondary antibodies every 15 days interval. Then the absorbance obtained with each antigen was measured where A, B, C and D represent values obtained with pstS1, CFP10, ESAT6 and p24, respectively. Data represent mean of two independent experiments and error bars are standard deviations. Asterisks signify statistical significance between days being compared ($p < 0.05$).

3.2. Comparison of the sensitivity and specificity of the HIV and TB multiplex colorimetric immunoassay in 96 well high-binding plates and epoxy-coated glass slides.

The sensitivity of the colorimetric assay was determined by testing different concentrations of the HIV-1 and *M.tb* primary antibodies in a 4-fold serial dilution, ranging from 250000 to 15.3 ng/ml, in human serum using the high-binding 96 well plates. The use of the human serum was to mimic the expression of these primary antibodies in HIV and TB co-infected individuals, and the concentrations used to dilute the antibodies covered the range found in infected individuals (Raux et al., 1999, Kimuda et al., 2017). Colorimetric detection was achieved by means of secondary antibodies conjugated to HRP. Furthermore, given that the ABTS or TMB substrate can also affect the sensitivity of the colour change reaction as previously reported (Harpaz et al., 2020, Prasad et al., 2021), we compared detection using either of these substrates. The results showed that the TMB substrate generally produced higher colour intensity relative to the ABTS substrate (Fig. 5) and this difference was mostly statistically significant for the two highest concentrations when we did a head-to-head comparison ($p < 0.05$) (Table 1). In addition, the color development with TMB occurred after 15 minutes while for ABTS it occurred after 45 minutes, implying that using the TMB substrate was 3-fold faster than the ABTS substrate. Comparable results for the immunoassay, i.e., TMB showing better intensity than ABTS was also obtained when the primary antibodies were diluted in PBS at pH 7.4 (Supplementary Fig. S2). These findings were similar to what was reported by Harpaz et al., who showed that TMB gave more intense colour change reaction when compared to ABTS (Harpaz et al., 2020). As shown in Table 2, the lowest limits of detection for TMB were on average 203.4 ng/ml with CFP10 and p24 showing the lowest limit of 81,38 ng/ml. However, for ABTS the limit of detection was 427.2 ng/ml on average (Supplementary Table 1).

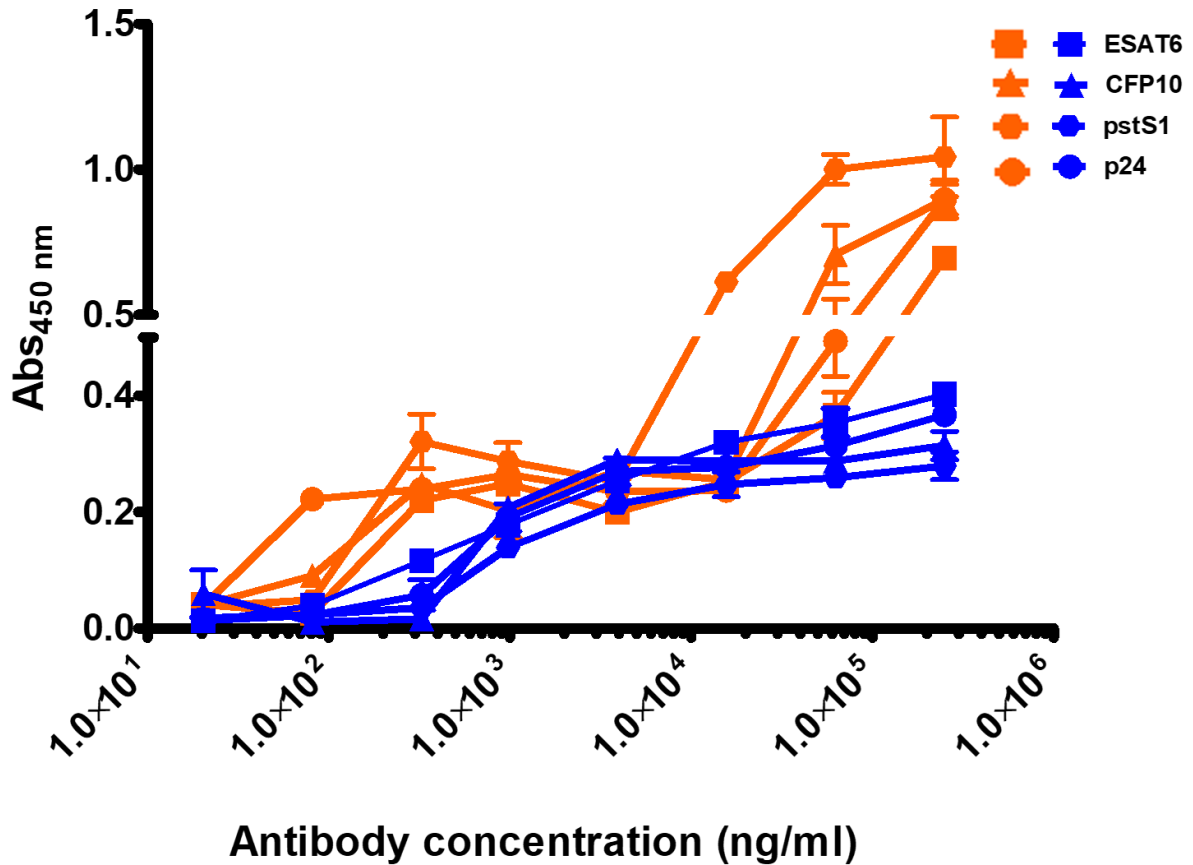


Figure 5: Evaluation of the antigen-antibody reactions sensitivity in the 96 well high-binding plates using primary antibodies serially diluted in human serum. The HIV and TB primary antibodies were diluted and mixed into a cocktail in human serum and incubated with immobilized antigens in a 96 well high-binding plates. Colorimetric detection was achieved using secondary antibodies conjugated to HRP with ABTS (blue) or TMB (orange) substrates. Data represent the mean of three independent experiments and error bars are standards deviation.

The experiment above was also replicated using epoxy-coated glass slides, however, the dilution series ranged from 250000 to 0.01491 ng/ml. Contrary to what we obtained with the plate, TMB and ABTS intensities were similar for the microarray slides (Fig. 6). Using TMB as the substrate we found that the limit of detection for the multiplex HIV and TB co-infection detection assay on the epoxy-coated microarray slides were consistently lower than the 96 well high-binding plates. To be specific, for ESAT6 and pstS1 this limit was 21845- fold lower when compared to the plate; for p24 it was 5461-fold lower, while for

CFP10 it was 85-fold lower. The fact that the slides had better sensitivity than the 96 well plates could also be observed when we detected colour change by visual inspection. For example, a clear blue colour could be seen on the slides at a much lower concentration, 61.0 ng/ml of the primary antibodies against the four antigens when compared to the 96 well high-binding plates where this detection was only down to 244.0 ng/ml (Supplementary Fig.S3). We observed a similar trend when ABTS was used as the substrate, i.e., the slides had consistently lower limits of detection than the plates (Supplementary Table S1).

Table 1: Determination of whether the comparison of TMB and ABTS absorbance readings were statistically significant.

Primary antibody Concentration (ng/ml)	HIV-1 p24		p-value	<i>M.tb</i> CFP10		p-value	<i>M.tb</i> ESAT6		p-value	<i>M.tb</i> pstS1		p-value
	ABTS	TMB		ABTS	TMB		ABTS	TMB		ABTS	TMB	
250000	0.3667	0.8972	<0.0001	0.314 4	0.896 1	<0.0001	0.402 4	0.694 8	<0.0001	0.2794 5	1.043 1	<0.0001
62500	0.3139	0.4335	0.0127	0.287 8	0.707 9	<0.0001	0.353 5	0.366 2	>0.999	0.2589 5	1.000 1	<0.0001
15620	0.2772	0.2369	0.9998	0.288 25	0.255 7	>0.999	0.320 1	0.251 9	0.6008	0.2476	0.613 9	0.0038
3906	0.2694	0.2136	>0.999	0.289 75	0.271 3	>0.999	0.254	0.199 8	0.9271	0.2135 5	0.253 1	>0.999
976.5	0.1906	0.2779	0.8696	0.207 6	0.202 4	>0.999	0.178	0.248 6	0.5509	0.1388	0.287 4	0.8021
325.5	0.0575	0.2317	0.0111	0.016 025	0.248 1	0.0010	0.116 6	0.220 1	0.0866	0.0358	0.321 2	0.0458
81.38	0.0226	0.2363	0.0042	0.010 865	0.090 5	0.8410	0.039 2	0.026 10	>0.999	0.0235	0.049 0	>0.999
20.34	0.0138	0.0368	>0.9999	0.059 52	0.040 2	>0.999	0.013 2	0.041 0	0.9997	0.0181 5	0.038 0	>0.999

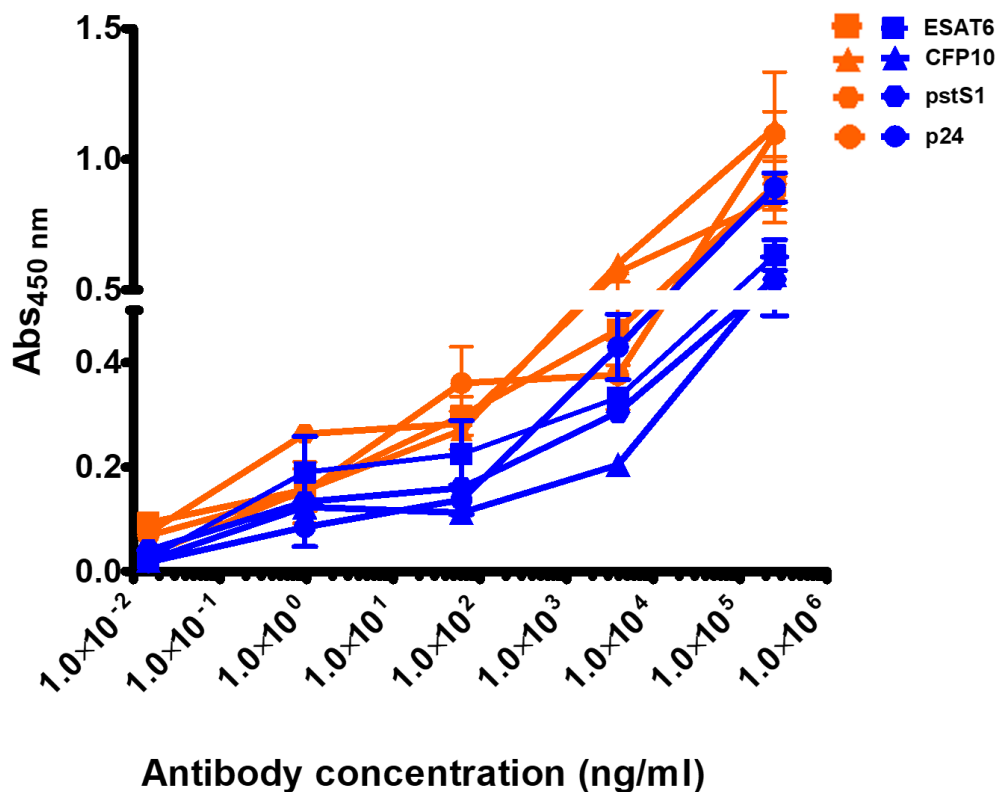


Figure 6: Evaluation of the antigen-antibody reactions sensitivity on the epoxy-coated glass slides using primary antibodies serially diluted in human serum. The anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies were serially diluted in human serum and mixed to form a cocktail. The absorbance obtained with the ABTS (blue) and the TMB (orange) substrates are shown. Data represent the mean of three independent experiments and the error bars are the standard deviation.

Overall, both the high-binding plates and slides proved to be suitable platforms for the HIV and TB POC multiplex immunoassay as they detected the primary antibodies at low concentrations. However, the epoxy-coated microarray slides showed much lower limits of detection than the plates, implying that they would be more suitable for POC application.

Table 2: The limit of detection concentrations for the HIV-1 p24 as well as *M.tb* CFP10 ESAT6 and pstS1 proteins obtained using 96 well high-binding polystyrene plates

Protein name	Limit of detection of HIV and TB primary antibodies in human serum (ng/ml)	
	TMB substrate	
	Epoxy slides	High-binding plates
HIV-1 p24	0.0149	81.38
<i>M.tb</i> CFP10	0.954	81.38
<i>M.tb</i> ESAT6	0.0149	325.5
<i>M.tb</i> pstS1	0.0149	325.5

To determine whether the antigen-antibody interactions were specific, i.e., there was no cross reactivity between antibodies and immobilized antigens, we mixed the primary antibodies diluted in human serum into a cocktail missing one antibody at a time. This was followed by incubating this cocktail with all four immobilized HIV and TB antigens. Given that TMB substrate showed better colour intensity in the 96 well high-binding plates compared to ABTS, it was used as the substrate in this experiment; the detection was then done by measuring absorbance. Data obtained in high-binding 96 well plates showed that no cross reactivity was observed, the immobilized antigens reacted specifically with their respective primary antibodies in solution i.e., there was absence of colour change in the well of the antigen that was missing its complementary antibody in the cocktail (Fig. 7). The same results were obtained when we repeated the experiments using the epoxy-coated glass slides (Fig. 8). Furthermore, no cross reactivity was apparent when we analyzed the high-binding 96 well plates or the epoxy-coated glass slides by visual inspection (Supplementary Fig. S4). Thus, the primary antibody-antigen

reaction in our multiplex immunoassay to detect HIV and TB co-infection is very specific with no detectable cross reaction.

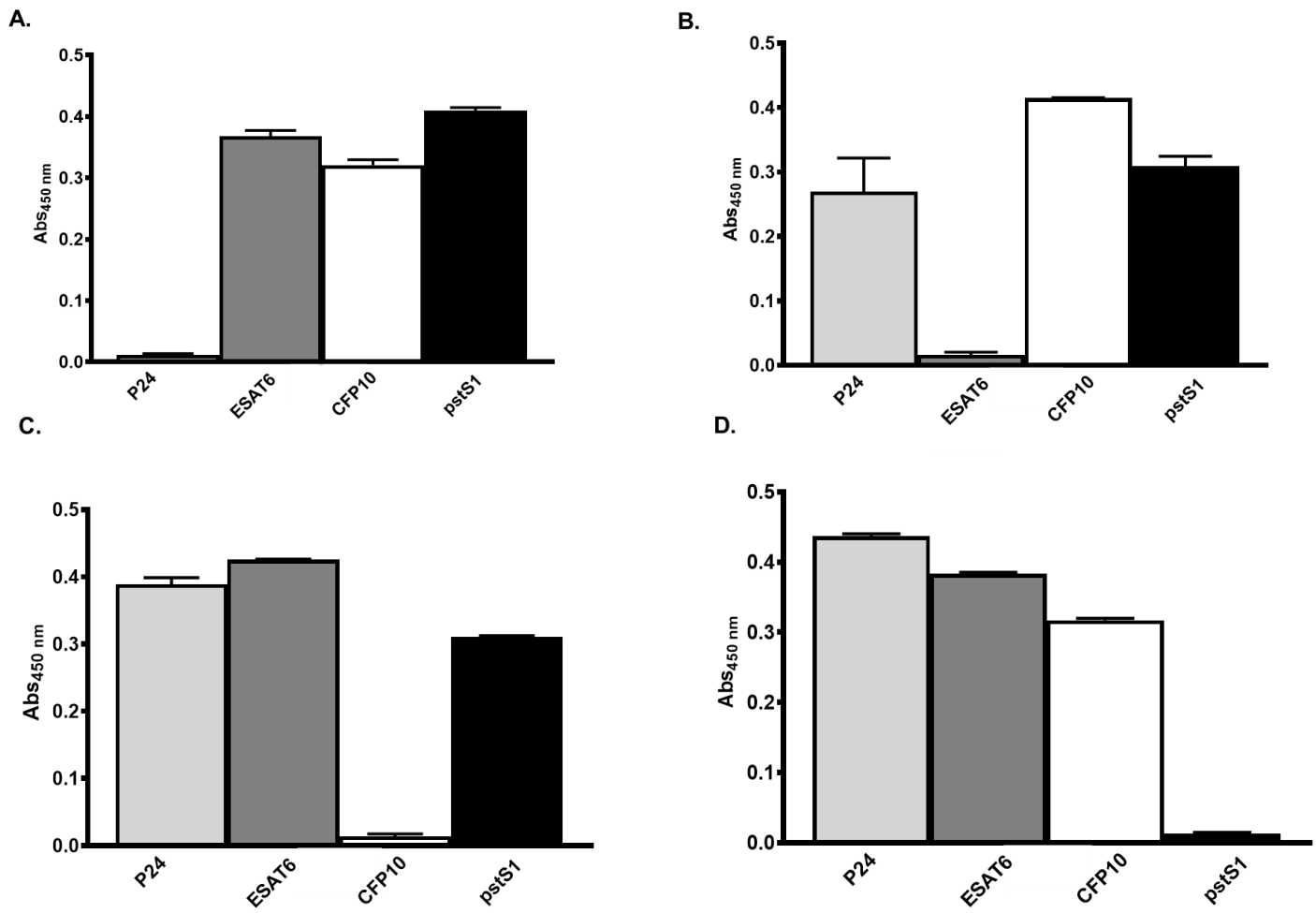


Figure 7: Evaluation of the specificity of the HIV and TB colorimetric assay in high-binding 96 well plates using a cocktail of primary antibodies in human serum. The anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 antibodies were combined into a cocktail missing one primary antibody at a time. (A), (B), (C) and (D) show the absorbance obtained when the immobilized antigens were incubated with a cocktail solution without anti-p24, anti-ESAT6, anti-CFP10 or anti-pstS1 respectively. Data represent mean of three independent experiments and errors bars are standard deviations.

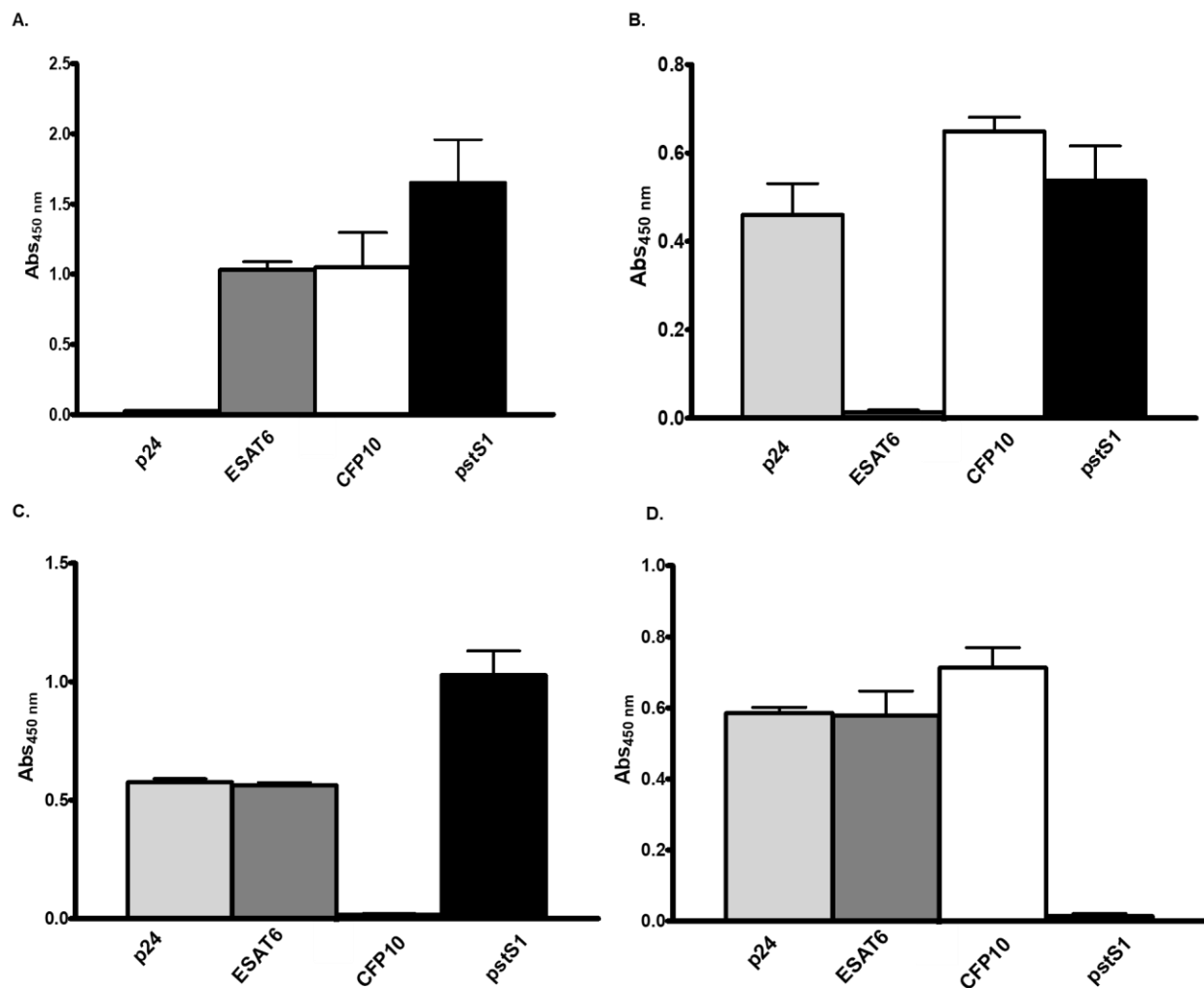


Figure 8: Evaluation of the specificity of the HIV and TB colorimetric assay in epoxy-coated slides using a cocktail of primary antibodies in human serum. *The anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 antibodies were mixed into a cocktail missing one primary antibody at a time. (A), (B), (C) and (D) show the absorbance obtained when the immobilized antigens were incubated with a cocktail solution exclusive of anti-p24, anti-ESAT6, anti-CFP10 or anti-pstS1 respectively. Data represent mean of three independent experiments and errors bars are standard deviations.*

4. Discussion

In this study we aimed to develop a multiplex assay for the colorimetric detection of HIV and TB at POC. We compared two different platforms for this purpose, i.e., the 96 well high-binding plates and the epoxy-coated glass slides. The detection end point was achieved using the HRP conjugated secondary antibodies and two HRP chromogenic substrates, namely the ABTS and TMB. The sensitivity and specificity of the immunoassay in the high-binding plates and epoxy-coated slides was evaluated. The data obtained showed that both systems were sensitive in the ng/ml range; this was irrespective of whether the detection was done using an instrument or the naked eye. In addition, they also showed high specificity for the HIV and TB antigen-antibody reactions. However, the slides were noticeably more sensitive compared to the high-binding plates with their limit of detection being on average 815-fold lower than the plates. We also demonstrated that the POC colorimetric multiplex assay developed here is stable at different pH and temperature conditions. Furthermore, our data indicated that the antigens could be stored immobilized dry in the plates for a period of at least three months without losing their reactivity with corresponding primary antibodies. We also found that the TMB substrate produced better sensitivity for the assay compared to the ABTS substrate. These findings suggested that the developed colorimetric assay, employing the 96 well high-binding plate or epoxy-coated glass slide, has the potential for use at POC for the detection of HIV and TB co-infection without the need for sophisticated instruments and trained personnel.

Using either the 96 well high-binding plates or epoxy-coated glass slides, the immobilized antigens were able to detect HIV and TB antibodies diluted in human serum at concentrations usually seen in infected patients (Raux et al., 1999, Kimuda et al., 2017). Furthermore, the colour change reaction could be observed with the naked eye at lower ng/ml concentrations, thus, implying that this colorimetric assay is likely to work when tested with human samples co-infected with HIV and TB for POC applications like what was reported by Gurmessa et al., where they showed that TB color change detection using TMB substrate and nanoparticles showed a 92% accuracy (specificity and sensitivity) in clinical samples (Gurmessa et al., 2021). Similarly, Ariffin et al., also

reported that a lateral flow colour change immunoassay for the detection of *M.tb* using infected patient specimen showed sensitivity and specificity to the pathogen (Ariffin et al., 2020). Specimen recommended by regulatory authorities for the validation of an assay or technology are those that contains the analyte of interest (in this case, HIV and TB antibodies) spiked with potential interfering substances, however, if the specimen containing the target analyte are not available, test specimen can be constructed by adding low concentrations of analyte with or without the potential interfering substances to negative specimen (Burd, 2010). Hence the use of normal human serum spiked with the HIV and TB antibodies in this study. Although spiked samples may come with limitations such as the fact that the analyte added to the negative specimen may not have the same properties as that found in physiological conditions (Westgard, 2002), they still give an indication of the expected performance outcomes of the assay.

The comparison of the sensitivity of the immunoassay on the 96 well high-binding plates and the epoxy-coated glass slides indicated that the slides performed many folds better than the plates. In fact, the average LOD for the slides was 0.249 ng/ml while for the plates it was 203.4 ng/ml. Analytical sensitivity at a low concentration limit is of paramount interest in molecular infectious disease diagnostics as it defines the ability of the test to diagnose disease (Burd, 2010). The LOD for the epoxy coated glass slides and the gold standard high binding plates were determined empirically, by testing serial dilutions of HIV and TB antibodies with known concentrations as advised by the Clinical and Laboratory Standards Institute (CLSI) regulatory authority for medical applications of molecular assays for LOD determination (Burd, 2010, Westgard, 2002). The LOD for multiplex assays must be determined for each analyte tested by the multiplex assay (Westgard, 2002), as was done for HIV and TB in this study. The difference in LOD between the plates and slides suggested that a platform using covalent binding for the immobilization of the antigens like the epoxy-coated glass slide is better for the POC application of our technology as compared to high-binding plates which use non-covalent bonds such as hydrophobic, van der Waals, and/or electrostatic interactions (Bhakta et al., 2015, Yousefi et al., 2018). Because covalent bonds are generally stronger than non-covalent ones (Liu et al., 2018b, Mallinson et al., 2003), it is possible that the higher sensitivity observed in the platform using these bonds, i.e., the epoxy-coated glass slides,

may be due to the fact that once the antigens are immobilized, fewer or none are lost during the experimental procedures when compared to a platform that relies on non-covalent bonds such as the plates. Molecular diagnostic methods have proven to be more sensitive than current gold standard methods of diagnosis, although the assessment of accuracy is challenging when the new techniques have lower LOD compared to the old methods (Burd, 2010, Chopra and Singh, 2020, Gupta and Kakkar, 2018).

According to the regulatory authorities, circumstances that contribute to infectious disease molecular assays non-specificity includes false positive results due to sample contamination, interfering substance or organisms that are genetically similar, therefore making specificity one of the parameters that needs to be evaluated for validation of molecular assays (Burd, 2010). The specificity of HIV/TB POC multiplex technology was determined by incubating the HIV and TB antigens with cocktails of HIV and TB antibodies spiked in normal human serum. Analytical specificity involves the ability of the assay to detect the intended target molecules (in this case HIV and TB) without being affected by cross-reactivity from other interfering molecules or specimen-related conditions (Burd, 2010). It was therefore important to spike the target HIV and TB target antibodies in human serum containing many other potential interfering molecules that could affect the performance of the assay as this is imperative according to regulatory bodies for laboratory developed assays (Burd, 2010, Westgard, 2002). The fact that the specificity of the HIV and TB antigen-antibody reactions on the epoxy-coated glass slides was similar to that in 96 well high-binding plates indicated that this aspect of the reaction was independent of the surface chemistry of both platforms. Furthermore, we evaluated the sensitivity of the immunoassay using either the TMB or ABTS substrates. Our data indicated that the limit of detection obtained with the ABTS substrate was higher than the TMB substrate implying that the latter was the more sensitive. This was consistent with what was previously reported (Harpaz et al., 2020, Li et al., 2012, Prasad et al., 2021). In addition, the reaction using TMB took 30 minutes less than the ABTS reaction. Since both are based on the oxidizing reaction of the HRP enzyme with the substrate in the presence of hydrogen peroxide, the more sensitive and faster TMB may be the result of its reaction being the more efficient (Harpaz et al., 2020, Vazquez-Alvarado et al., 2022). Moreover, we found that our HIV and TB immunoassay took 2 hours and 30 minutes to perform with

TMB. The turnaround time in our assay is more or less 5 times higher than the current reported HIV antibody tests such as Microfluidic-integrated multicolor immunosensor HIV-1 detection (Liu et al., 2020a) and TB reported naked-eye colorimetric assays such as that reported by Ng et al., its advantage is that it is likely to detect active TB after about 2 hours and 30 minutes, while the existing tests to confirm active TB can take weeks before results are obtained (Chan et al., 2000, Vittor et al., 2014, Ng et al., 2016). In conclusion, these data suggested that for POC application, our multiplex diagnostic technology would work better using the epoxy-coated glass slides as the platform and TMB as the substrate. Furthermore, because trueness or accuracy is the cornerstone of technology or method validation, the accuracy of the HIV/TB POC multiplex technology using the TMB substrate and epoxy coated glass slides would have to be evaluated to comparative methods that are in routine use for the detection of HIV and TB antibodies according to the Clinical and Laboratory Standards Institute. Although accuracy assessment is challenging for multiplex technologies, different types of comparative techniques may be used for each analyte if no multiplex technology that covers all analytes of interest is available (CLSI/NCCLS, 2008). However, since there are no available technologies in routine use for detection of TB antibodies in blood, the accuracy of the technology may be done using recovery studies where the assay can measure the TB antibodies in the analyte of interest (Blood/serum) when a known concentration is present in the specimen matrix and the amount of recovered analyte is compared to the initial known concentration of the analyte (Burd, 2010).

Since the stability of proteins in different conditions is important in the development of immunoassays (Deller et al., 2016, Capelle et al., 2007, Mezzasalma et al., 2007), it was essential to carefully choose the optimal experimental conditions for the immobilization of the HIV and TB antigens on the 96 well high binding plates as was done for the epoxy glass slides in chapter 2. The data obtained in our study indicated that the HIV and TB antigens could remain stable when immobilized in the 96 well high-binding plates at different pH and temperature conditions; however, they performed better at 25 °C and pH of 7.4. The same temperatures and pH were also seen to be optimal for the stability of the proteins and immunoassay on the epoxy-coated glass slides (Malatji et al., 2023). This was expected because most proteins are stable at neutral pH (Spears et al., 2018,

Kim et al., 2005), in addition to the fact that at warmer temperatures such as 25 °C the endothermic protein adsorption process is known to be stable (Li et al., 2020a, Wang et al., 2019a, Zhang and Ertbjerg, 2019, Bhakta et al., 2015). This is consistent with the storage conditions for most HIV rapid tests that have been reported be stored at room temperature up to 30 °C (Greenwald et al., 2006, Singh et al., 2013). Furthermore, the fact that the optimal pH was 7.4 makes our assay suitable for use to detect proteins in blood, which has a pH of around 7.0 (Kellum, 2000, Brunskill et al., 2012). Most HIV and TB assays such as the Naked-eye colorimetric *M.tb* detection using TMB (Ng et al., 2016) and multifluidic-integrated multicolor visual HIV-1 p24 detector (Liu et al., 2020a) are also performed at room temperature and neutral pH. Our data also demonstrated that the immobilized antigens could be stored either dry or in PBS. However, the fact that the colour intensity was higher when the antigens were stored dry compared to wet storage suggested that this latter condition may have some negative effects on the stability of antigens on the surface of the plate, and this is similar to what we reported for the epoxy-coated glass slide (Malatji et al., 2023). One explanation may be that some antigens on the solid surface may dissociate from it and rejoin the solution when stored for a long time in the buffer, which is not likely to happen when they are dried on the surface. This was consistent with the fact that we could store the immobilized antigens dry for up to three months and still be able to effectively detect the antibodies in human serum even at very low concentrations. This is similar to Jorgensen et al. who reported that immobilized proteins could be stored dry or in buffer at room temperature in the ELISA plates for up to 12 weeks without affecting the assay performance (Jorgensen et al., 2021). The shelf-life of our technology is half the shelf life of the rapid oral fluid HIV test which was reported to be six months (Facente et al., 2009). When developing a POC technology it is imperative to consider the stability of the technology to ensure prolonged storage of that particular device (Yetisen et al., 2013), thus the stability of the HIV and TB antigens on the epoxy coated glass slides/ high binding plates for POC applications was important as the use of the technology after the recommended shelf-life can potentially result in false positive/negative results (Facente et al., 2009). FDA approved or cleared assays are required to state the recommended storage condition of the developed technology as well as instructions of how the technology works (Regulations, 2010),

in this case, the above stability parameters would be included in the packaging insert of the technology when it has been validated and approved for use in HIV and TB diagnostics. In all, these data indicated that our multiplex HIV and TB co-infection detection technology would not require specialized storage conditions, making it suitable for use in both urban and rural or remote settings. This technology is also likely to be low cost due to its non-requirement for expensive equipment.

Future work for this technology will involve its validation using co-infected HIV and TB patient samples. This is probably one of the current study limitations and will form part of our follow-up investigation. However, it should be noted that previous investigators used human serum spiked with antibodies, similar to what we did here, to validate technologies intended for the detection of biomarkers (Albrecht et al., 2008, Mohammadi et al., 2018, Maple et al., 2004, Pultar et al., 2009, Mytych et al., 2009a). In the development of molecular assays, the CLSI regulatory authority also advises on the use of several spiked samples prepared from appropriate matrix using analytes of known concentrations within the range of the expected LOD (Burd, 2010). The matrix can be samples that tested negative for the target analytes, in this case HIV and TB antibodies, and the matrix should be from five or more specimen from different subjects (CLSI/NCCLS, 2004). Thus, to check the technology consistency, different samples from different individuals will be employed for the POC multiplex technology to meet the required regulations for assay development. Also, a study with extended storage, pH, and temperature range will be useful in providing a more detailed assessment of our technology.

In conclusion, we demonstrated that the colour change reaction-based HIV and TB multiplex immunoassay developed here is sensitive and specific in high-binding 96 well plates as well as on epoxy-coated glass slides, making them suitable for the detection of these two pathogens co-infection at POC. However, the technology performed better on the microarray slides than on the plates, opening an opportunity for miniaturization and multiplexing of a larger number of assays. Our stability studies indicated that this technology will be simple and likely inexpensive to store and perform, which is very important given that HIV and TB are diseases affecting mostly poor countries in the world.

Thus, our technology has a lot of potential for the simultaneous detection of HIV and TB co-infection, as well as for possible multiplexing with other assays and POC applications.

Author Contributions

Conceptualization, K.A. and C.T.; methodology K.A and K.M.; formal analysis and investigation, K.M.; data curation, K.M.; writing—original draft preparation, K.M.; writing-review and editing, K.A., C.T. and A.S; supervision, K.A., C.T. and A.S; funding acquisition, K.A.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data Availability Statement

The data sets used and/or analyzed during the present study are available from the corresponding author on request.

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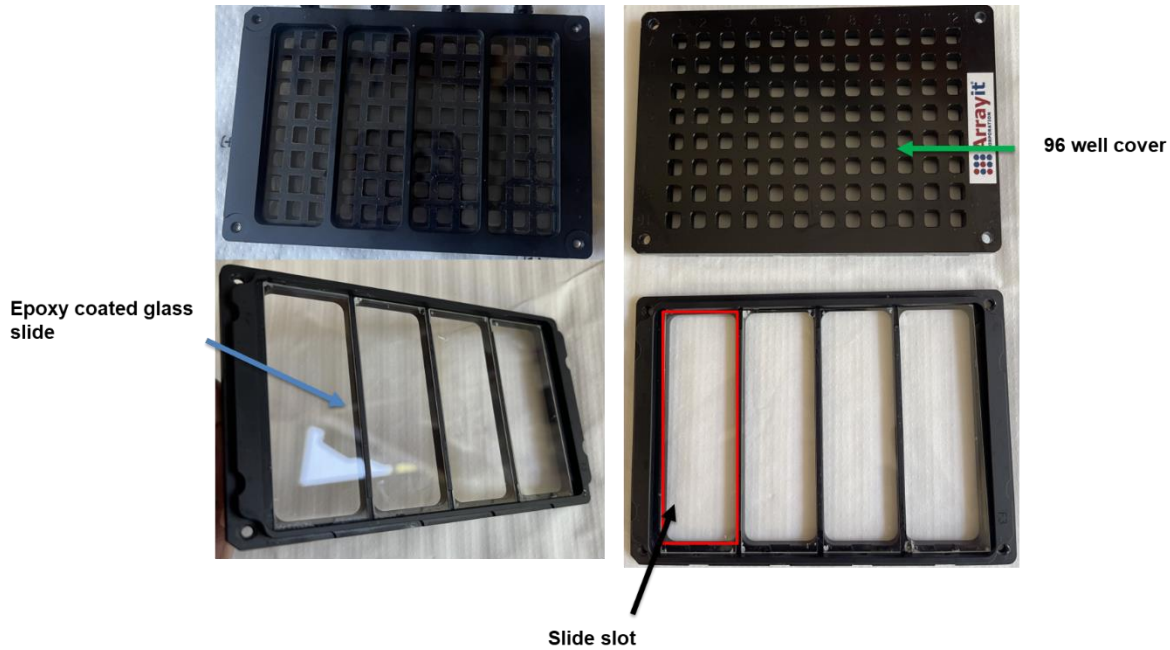
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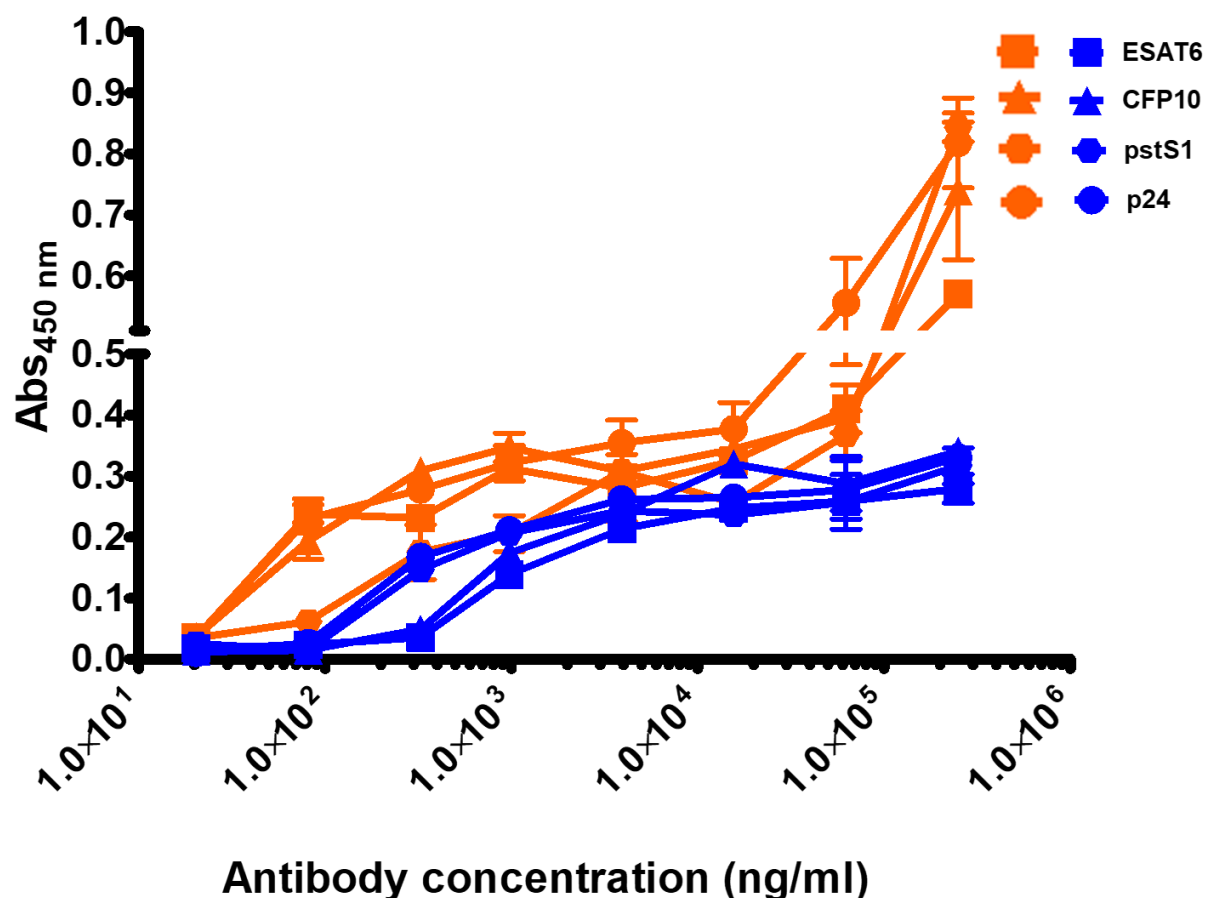
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6. Supplementary material

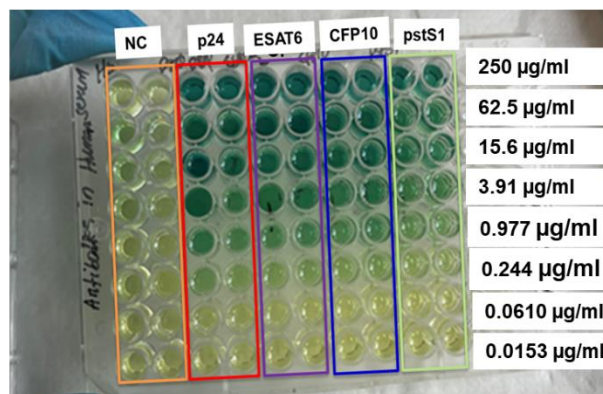


Supplementary Figure S1: An image of the 96 well hybridization cassette. The epoxy-coated glass slide indicated with the blue arrow was clamped into one of the cassette slots (indicated in red), this was to form a 96 well plate (see green arrow) with an epoxy-glass slide base in which the HIV and TB antigen-antibody reactions were performed.

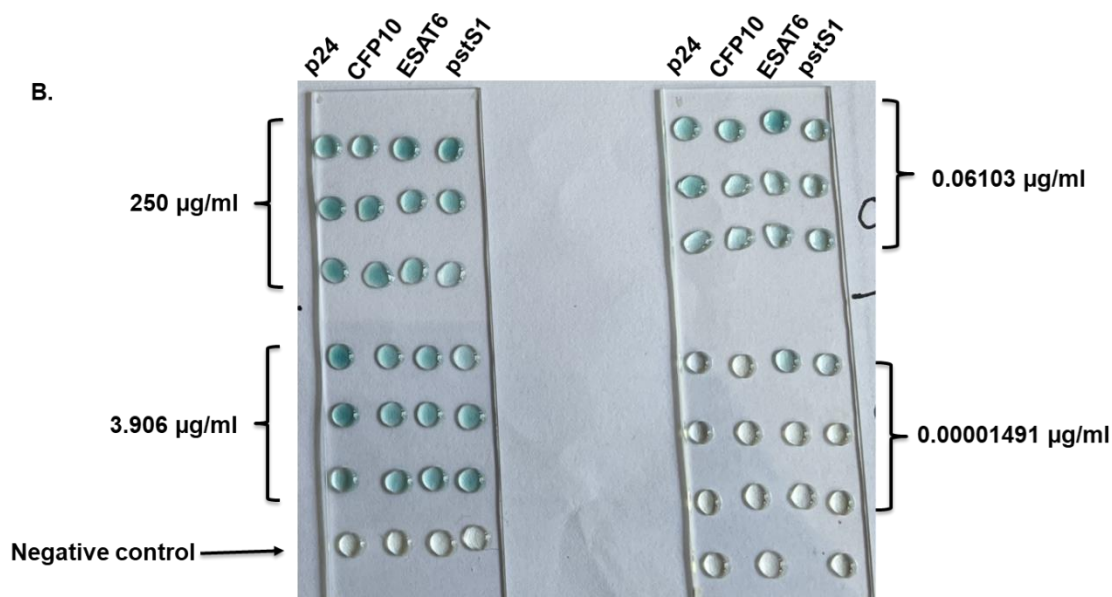


Supplementary Figure S2: Evaluation of the sensitivity of the antigen-antibody interaction in PBS. The anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies were serially diluted in PBS and mixed to form a cocktail and incubated with immobilized antigens in the 96 well high-binding plates. Colorimetric detection was achieved using secondary antibodies conjugated to HRP, with ABTS or TMB as substrate. Data represent the mean of three independent experiments and error bars are standards deviation.

A.



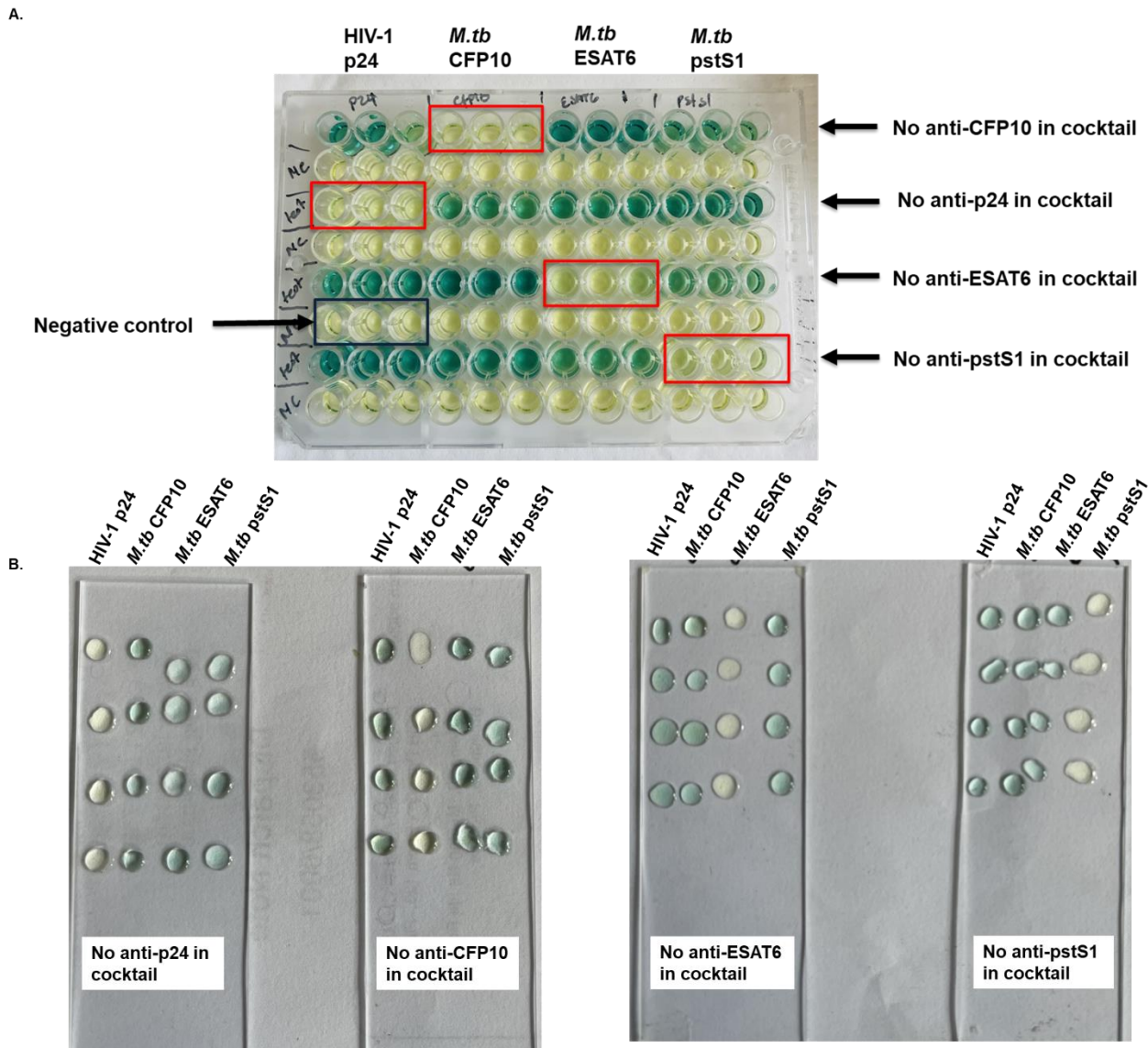
B.



Supplementary Figure S3: Evaluation of the sensitivity of the HIV and TB antigen-antibody immunoassay by visual inspection. HIV-1 p24, M.tb CFP10, ESAT6 and pstS1 primary antibodies diluted in human serum were incubated with their corresponding antigens on either the 96 well high-binding plate (A) or epoxy-coated glass slides (B), and the detection of the colour change reaction was done by visual inspection.

Supplementary Table S1: Head-to-head comparison of the limits of detection obtained on the epoxy-coated glass slides and high-binding plates using the ABTS substrate

Protein name	Limit of detection oh HIV and TB primary antibodies in human serum (ng/ml)	
	ABTS substrate	
	Epoxy slides	High-binding plates
HIV-1 p24	0.954	325.5
<i>M.tb</i> CFP10	0.954	976.5
<i>M.tb</i> ESAT6	0.954	81.38
<i>M.tb</i> pstS1	61.03	325.5



Supplementary Figure S4: Evaluation of the multiplex HIV and TB immunoassay specificity by visual inspection. The anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies were diluted in human serum and mixed into a cocktail missing one primary antibody at a time, (A) in 96 well high-binding plates, and (B) on epoxy-coated microarray slides. The images represent three experiments performed on separate days. They were done in triplicates for the high-binding plates and quadruplicates for the epoxy-coated slides.

CHAPTER FIVE

EXPRESSION OF THE *MYCOBACTERIUM TUBERCULOSIS* CFP10, ESAT6, APA AND PSTS1 ANTIGENS IN *NICOTIANA BENTHAMIANA* FOR USE IN THE HIV AND TB MULTIPLEX MICROARRAY-BASED TECHNOLOGY.

Chapter summary

Commercially available bacteria produced TB proteins for research purposes produced in bacteria can be expensive, with some antigens such as apa and pstS1 are difficult to obtain. It is in this context that we aimed at the expression of apa, pstS1, ESAT6 and CFP10 TB antigens, in *Nicotiana benthamiana* (*N. benthamiana*) for use in the HIV and TB multiplex microarray-based technology. This approach would reduce the technology cost since expression and purification of plant produced recombinant protein is relatively inexpensive. The expression of the proteins in *N. benthamiana* employed methods such as cloning and polymerase chain reaction (PCR) to amplify the construct DNA molecules; restriction digest and ligation for the digestion of the insert DNA molecules and ligation into an expression vector; agroinfiltration for the production of the proteins in *N. benthamiana*; protein extraction, SDS-PAGE as well as Western blotting for the isolation and identification of the expressed proteins. The antigen gene inserts were harboured in a pUC57 vector, and the presence on the *M.tb* DNA molecules on the vector was confirmed by PCR amplification using primers complementary to the genes of interest. In addition, the insert DNA molecules were successfully double digested from the vector and ligated into the pEAQ plant expression vector. Nonetheless, the agroinfiltrated recombinant pEAQ vector cloned in *Agrobacterium tumefaciens* strain AGL1 (AGL-1) and LBA4404 (LBA) did not successfully express the TB antigens as expected. Several factors may have affected the agroinfiltration and expression processes such as the ambient and leave temperatures, as well as natural plant cell functions against foreign substances such as agrobacteria to name a few. The plant production of the TB antigens will form part of the study future prospects. This will involve the use of different *N. benthamiana* growth conditions, assessment of other plant species, as well as employ different strains of agrobacteria to increase the possibility of a successful expression of the proteins. Furthermore, should they be expressed, the proteins will be immobilized on the epoxy-coated microarray slides together with the HIV-1 p24 antigen for the HIV and TB multiplex detection as detailed in the previous chapters.

1. Introduction

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* (*M.tb*) and has been identified as the first cause of death among human immunodeficiency virus (HIV) infected individuals (Bruchfeld et al., 2015, Scott et al., 2017b). High cost, long turn-around time, and use of sophisticated equipment and trained personnel are some of the limitations associated with TB diagnosis (Ryu, 2015, Cheon et al., 2016, Gina et al., 2017). This propels the need for the development of cheap and rapid TB diagnostic assays. The use of commercially available proteins produced in bacteria can be expensive relative to those produced in plants (Lim et al., 2022), therefore in this chapter, we aimed to produce the TB antigens in *Nicotiana benthamiana* (*N. benthamiana*) for use in the multiplex HIV and TB microarray-based technology.

Proteins can be expressed in various expression systems, these include bacterial, yeast, mammalian, insects, and plants systems. The microbial, mammalian and insect, however, have several disadvantages relative to the plant expression platforms. An example in bacterial system, is endotoxin contamination as well as inefficient folding of some secretory targeting domains is the main disadvantage (Della Cristina et al., 2015). For bacterial strains such as *Escherichia coli* (*E.coli*) very large proteins cannot be expressed, and these strains are not a good choice for proteins that require post-translational modifications (Demain and Vaishnav, 2009). Furthermore, the system is expensive to perform, thus proteins produced in bacterial systems available commercially are also costly (~ 500 USD per 100 µl) compared to 260 USD in plants. Although yeast expression of proteins has several advantages that include the fact that they are cost effective and have high yield, fungi such as *Pichia* cannot express proteins that require chaperonins for proper folding (Demain and Vaishnav, 2009). The mammalian expression system is a good choice to produce proteins requiring mammalian post-translational modification, however, the process has poor secretion and is expensive (Demain and Vaishnav, 2009, Wurm and Bernard, 1999, Nandi et al., 2016). Lastly, insect cells as hosts have advantages such as the fact that they are able to carry out complex post-translational modifications relative to other systems, however, they have the disadvantage of improper folding of proteins, incorrect glycosylation and low levels of expression (Demain and

Vaishnav, 2009). Due to all these disadvantages, the plant expression system was selected for the *M.tb* antigens.

N. benthamiana is a close relative of tobacco and is widely used for proteins production in plant (Pantazica et al., 2023, Zhang et al., 2023). This plant has transformation flexibility and the ability to support high levels of protein production in stable or transgenic expressions (Norkunas et al., 2018a, Lim et al., 2022). In addition, it has other advantages which make it desirable for research. For example, *N. benthamiana* has an immense susceptibility to plant viruses, can be used to produce virus vectors for endogenous genes silencing, and can use reporter genes to confirm plant expression (Bally et al., 2018, Yun et al., 2023, Smith et al., 2023, Bin et al., 2023). Furthermore, it is inexpensive, has a low risk of carrying human pathogens and has an advantage in its scalability and ability to glycosylate proteins (Bally et al., 2018). The *N. benthamiana* expression system usually comprises the gene of interest that is carried by recombinant agrobacteria and is delivered into the extracellular spaces by physical or vacuum infiltration in a process called agroinfiltration (Norkunas et al., 2018b, Singh et al., 2020). The most used part of the plant for agroinfiltration is the leaves as they have proven to have rewarding targets for agroinfiltration due to their high transformation efficiency (Bashandy et al., 2015, Joensuu et al., 2010). The *Agrobacterium* suspension can be infiltrated with a vacuum or a syringe to the parenchymal airspaces of the *N. benthamiana* plant (Bashandy et al., 2015). In previous studies, proteins that proved difficult to produce in microbial systems have been produced in *N. benthamiana* (Leuzinger et al., 2013, Liu et al., 2014). This plant has also been used to produce pharmaceutically active proteins or commercially interesting molecules (Qiu et al., 2014, Zerbe et al., 2014). For example, it has been used to produce antibodies against influenza, HIV, Ebola virus, hepatitis A and B to name a few (Bally et al., 2018, Singh et al., 2022). In this chapter we attempted to produce *M.tb* antigens ESAT6, CFP10, pstS1 and APA in *N. benthamiana* as it has been reported that the pathogen's antigens can successfully be produced in plants for vaccine development purposes (Frutos et al., 2008, Yadav et al., 2023).

2. Method and material

2.1. Constructs

The alanine and proline-rich antigen (apa) is a 47kDa protein (Modolo et al., 2018) encoded by 873 base pairs (bp). This protein was introduced in this expression chapter and not used in the previous chapters as it is not available commercially like the other TB antigens. Its expression was attempted here with the aim of increasing the sensitivity when used together with the other TB antigens for active TB detection. The Culture Filtrate Protein-10 (CFP10) has a weight of 10kDa (Wu et al., 2017) and is encoded by 315 bp, while the Early Secretory Antigenic Target protein 6 (ESAT6) is a 6.0 kDa molecule encoded by 309 bp (Liu et al., 2021b). Lastly, the Phosphate-specific transport Substrate binding protein-1 (pstS1) has 38 kDa and encoded by 1065 bp (Nisa et al., 2022). The constructs were cloned in a pUC57 vector of 2710 bp, into the lac operon gene (1) and were obtained from Genscript biotech (New Jersey, USA). The *M.tb* genes information is outlined in Table 1.

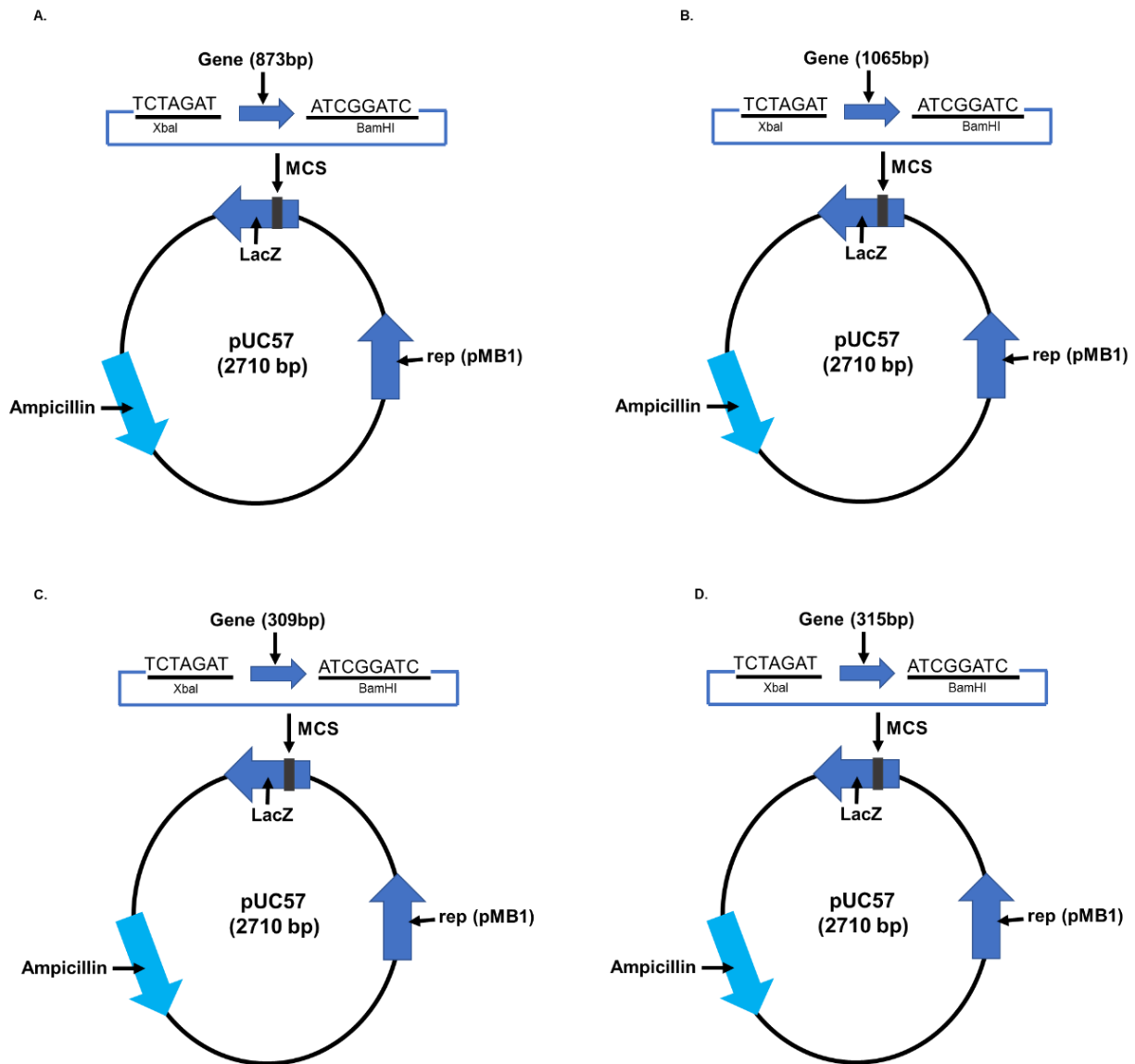


Figure 1: Map of the pUC57 vector with the *M.tb* *apa*, *pstS1*, *ESAT6* and *CFP10* DNA insert molecules used for genetic transformation. The *M.tb* *apa* (A), *pstS1* (B), *ESAT6* (C) and *CFP10* (D) genes were fused into the *lac* operon downstream of the *LacZ* gene on the 2710 bp pUC57 vector.

Table 1: *M.tb* CFP10, EAST6, pstS1 and Apa antigens gene information.

Gene Name		Restriction sites		Length (bases)		
	Primer Sequence	5' Sequence	3' Sequence		Host Expression organism	Cloning Vector
CFP10	Forward primer: TGGCCATACAAACGAC Reverse primer: CCCGGGAAATCCCC	ACCGGT	CCCGGG	303	<i>Nicotiana benthamiana</i>	pUC57
ESAT6	Forward primer: TGGCCATACCGACTAGG Reverse primer: CCCGGGAGCAAACATTCC	ACCGGT	CCCGGG	297	<i>Nicotiana benthamiana</i>	pUC57
Apa	Forward primer: TGGCCATACCTAGGTCTTGG Reverse primer: CCCGGGAGCAGGCAATG	ACCGGT	CCCGGG	861	<i>Nicotiana benthamiana</i>	pUC57
pstS1	Forward primer: TGGCCATACACACCAAGATTCTG Reverse primer: CCCGGGTGAAATTGTAGCAATC	ACCGGT	CCCGGG	1053	<i>Nicotiana benthamiana</i>	pUC57

2.2. Transformation of DH5- α bacteria with pUC57 plasmid encoding *M.tb* protein or with the pEAQ plasmid

All experiments in this chapter were conducted at CSIR chemicals cluster, future production plant science facility.

DH5- α competent cells (Thermo fisher scientific, Massachusetts, United States) were thawed on ice and 1 μ l of each construct was added to 50 μ l of the cells, and the mixture was incubated for 30 minutes on ice. After the 30 minutes incubation, the cells were immediately incubated in a 42°C water bath (heat shock) for 30 seconds. Afterwards, they were immediately transferred on ice for 2 minutes. Then 1 ml of SOC media (Thermo fisher scientific, Massachusetts, United States) was added followed by 1 hour at 37°C while shaking at 225 rpm in a shaking incubator. The transformed cells containing the *M.tb* genes in a pUC57 plasmid were then spread on agar plates containing 1 mg/ml of ampicillin or kanamycin (Sigma Aldrich, Missouri, USA) for the pEAQ vector for selection. The plates were then placed in a 37°C incubator overnight to allow cell growth. Post incubation, colonies were selected from the agar plates and each inoculated in 10 ml LB broth containing ampicillin for the pUC57 vector with *M.tb* gene, or kanamycin for the pEAQ expression vector. The culture was then incubated for 16 hours at 37°C in a shaking incubator.

2.3. Plasmid purification and restriction digest

Glycerol stocks of each of the constructs were prepared by mixing 1 ml of the cells containing constructs with 1 ml of 50% glycerol for future inoculations. The remaining cells were centrifuged for 5 minutes at maximum speed in a microfuge. The supernatant was discarded, and the plasmids were purified using the GeneJet plasmid miniprep kit (Thermo fisher scientific, Massachusetts, United States) according to the manufacturer's protocol. After purification, the *apa*, *pstS1*, *ESAT6*, *CFP10* and pEAQ expression vector DNA concentrations were determined using Nanodrop 1000 spectrophotometer (Thermo fisher scientific, Massachusetts, United States). Following plasmid concentration determination, the constructs were amplified by PCR. The PCR primers for each construct were as follows; CFP10- Forward primer: TGGCCATACAAACGAC, Reverse primer:

CCCGGGAAATCCCC; ESAT6- Forward primer: TGGCCATACCGACTAGG, Reverse primer: CCCGGGAGCAAACATTCC; Apa- Forward primer: TGGCCATACCTAGGTCTTGG, Reverse primer: CCCGGGAGCAGGCAATG and pstS1- Forward primer: TGGCCATACACACCAAGATTCTG, Reverse primer: CCCGGGTGAAATTGTAGCAATC. The PCR reaction was performed by mixing 3 µl of primer, 5 µl of PCR water, 2 µl of the template DNA (apa, pstS1, ESAT6 or CFP10) and 10 µl 2× Q5 master mix for a total volume of 20 µl for each construct. The PCR reaction conditions were as follows: initial denaturation at 98 °C for 2 minutes, denaturation at 98 °C for 45 seconds, annealing at 65 °C for 30 seconds, extension at 72 °C for 1 minute and final extension at 72 °C for 10 minutes. The amplification was performed on a C1000 Tm thermal cycler (Bio-Rad, California, United States). Post PCR, the samples were mixed with loading dye and run on a 1% agarose gel prepared by mixing 1 g of agarose into 100 ml of TAE buffer followed by the addition of 10 µl ethidium bromide. A 1 Kbp or 100 bp DNA ladders (New England Biolabs, Massachusetts, USA) were also loaded and the gel was run for 45 minutes at 120 V.

Following confirmation of the presence of the insert DNA in the pUC57 vector by PCR, the plasmids were double digested with SmaI and AgeI-HF restriction enzymes using the manufacturer's protocol (New England Biolabs, Massachusetts, USA). After the digestion of the construct DNA, the apa, pstS1, ESAT6 and CFP10 inserts were analysed on a 1% agarose gel to confirm digestion, and gel purification of the insert molecules was achieved using a gel purification kit according to the manufacturer's protocol (Thermo fisher scientific, Massachusetts, United States). The purified insert molecules were ligated into a pEAQ expression vector using T4 ligase enzyme according to the manufacturer's protocol (New England Biolabs, Massachusetts, USA). The pEAQ-insert complexes were sequenced to confirm the ligation of the plasmid and the *M.tb* genes.

After the ligation of the constructs into the expression vector, they were transformed into AGL1 and LBA agrobacteria strains (Figure 2). This was achieved by mixing the AGL1 and LBA *Agrobacterium* electrocompetent cells with 1.5 µl of the construct DNA and transferring into cuvettes on ice. This was followed by placing the cuvettes on an electroporation Biorad machine (Biorad, California, USA) at a voltage of 1.44k V/cm for 5

seconds. After the 5 seconds, 1 ml of LB broth was added to the cuvettes and mixed by pipetting. Afterwards, the reaction was transferred from the cuvettes to Eppendorf tubes and incubated at 30 °C for 3 hours while shaking at 200 rpm. Post incubation, the cells were centrifuged, and the supernatant was discarded while the pellet was resuspended with 100 µl of LB broth. The 100 µl of cells was then plated on agar plates and incubated at 28 °C for 2-5 days. From the colonies, inoculation was done, and glycerol stocks were prepared as mentioned above. Some of the cells were then used for agroinfiltration of *N. benthamiana*.

2.4. Plant growth

For the agroinfiltration, the *N. Benthamiana* seedlings were initially generated through plant tissue culture techniques. The seeds were seeded on Murashige–Skoog (MS) media on a plate, and then transferred to a vessel with the MS media after germination. Thereafter, the plant was allowed to grow for a few weeks before being transferred to soil. The plant growth was maintained at 25°C constant temperature and 16/8-hour light/dark cycle. During the light cycle, the photosynthesis photon flux density was $600 \pm 50 \mu\text{mol. m}^{-2}. \text{s}^{-1}$. Six-week-old *N. benthamiana* plants were selected for syringe agro-infiltration.

2.5. Agrobacterial syringe infiltration

For transient expression of the recombinant TB apa, pstS1, ESAT6 and CFP10 antigens, the AGL and LBA agrobacteria carrying the pEAQ-apa, pEAQ-pstS1, pEAQ-ESAT6 and pEAQ-CFP10 were infiltrated on the leaves of *N. benthamiana* using a 10 ml syringe. The agroinfiltration suspension was injected into the interstitial space of the leaves through a nick on the abaxial side (Figure 2). The agroinfiltrated leaves were left for 5 days prior to harvesting.

2.6. Protein purification

To purify and extract the protein from the *N. benthamiana* plant leaves, a protease inhibitor mixture was prepared by mixing 50 ml PBS and 5 µl of protease inhibitor cocktail (Sigma Aldrich, Missouri, United States) to prevent the plant protease enzyme from degrading the *M.tb* proteins in the plant. A 1:1 (weight to volume) of the buffer containing protease inhibitor and plant material in liquid nitrogen grinded using a pestle and mortar were agitated and mixed by vortexing. The mixture was centrifuged for 20 minutes at

maximum speed (12 000 rpm). The supernatant containing proteins was collected while the pellet was discarded. Samples were analysed by SDS-PAGE gels (Figure 2) (Thermo fisher scientific, Massachusetts, United States) and ran for an hour and 30 minutes at 195V. The SDS-PAGE gel was stained overnight with Coomassie blue dye after boiling the gel with the dye for one minute. The gel was destained with a destaining solution (200 ml Acetic acid, 100 ml isopropanol and distilled water) and incubated while shaking for 15 minutes.

2.7. Western blotting

The PVDF membrane (Biorad, California, USA) was briefly activated in methanol and equilibrated in Transfer buffer for 5 minutes. Sandwich papers were also soaked in transfer buffer prior to placing the membrane between them. Thereafter, the membrane and papers were transferred to a Biorad Trans-Blot Turbo instrument (Biorad, California, USA) for 20 minutes. The membrane was then removed from the sandwich papers and placed in 10% skim milk and incubated overnight while shaking. To the membrane, primary antibodies conjugated to Anti-polyHistidine–Peroxidase antibody, Mouse monoclonal (Sigma Aldrich, Missouri, United States) were added and incubated for 2 hours and 30 minutes while shaking to allow the antibodies to bind to the His tag present on the proteins of interest. Following the incubation, the membrane was washed three times for 10 minutes with PBS containing 0.1% tween-20 while shaking. The western blotting image was obtained using a Chemidoc (Biorad, California, USA) (Figure 2).

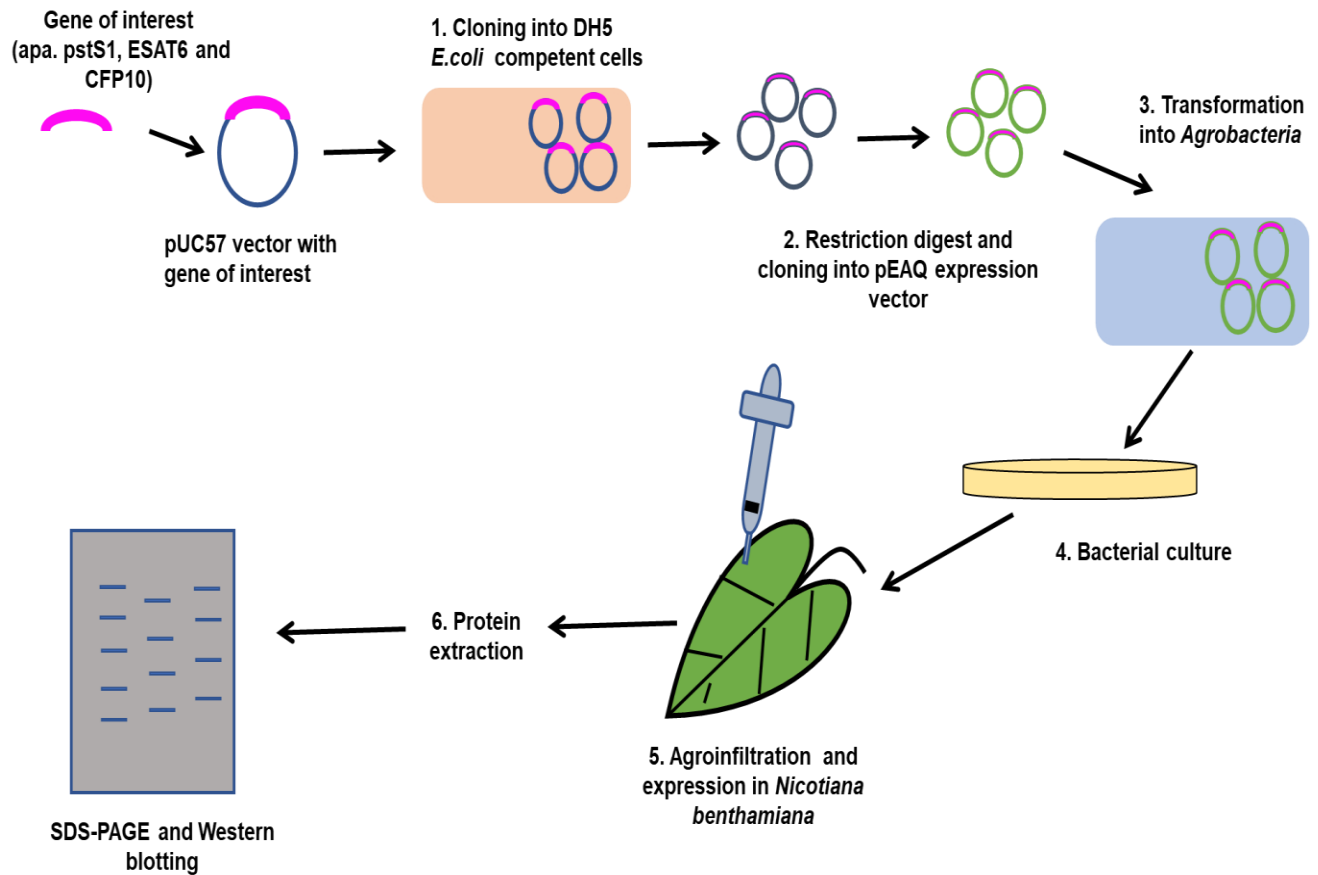


Figure 2: Illustration of the process involved in the expression of proteins in plants.

The *M.tb* antigens *apa*, *pstS1*, *ESAT6* and *CFP10* DNA insert molecules (pink) were cloned into a pUC57 vector (blue), transformed into *E.coli* (light orange), purified, digested, and ligated into a pEAQ expression vector (green) prior to cloning into AGL and LBA *agrobacteria* (light blue). This was followed by culture and agroinfiltration into *N. benthamiana* leaves. The process ends with protein extraction, SDS-PAGE, and western blotting.

3. Results

3.1. Plasmid purification of constructs

To confirm the presence of the *apa*, *pstS1*, *ESAT6* and *CFP10* sequences from the pUC57 vector, competent cells were transformed with the constructs, followed by plasmid purification. The purified DNA molecules were amplified by PCR (8 samples for each construct) using primers that were complimentary to sequences on the insert molecules. After PCR, the constructs were analysed on a 1% agarose gel to identify the molecules through electrophoresis. From the results obtained, the pUC57 vector contained the *apa*, *pstS1*, *ESAT6* and *CFP10* insert DNA molecules necessary for recombinant protein production (Figure 3). The DNA ladder was also loaded to confirm the size of the molecules. Confirming that the pUC57 vector did contain the *M.tb* genes of interest and could be used in further steps such as digestion.

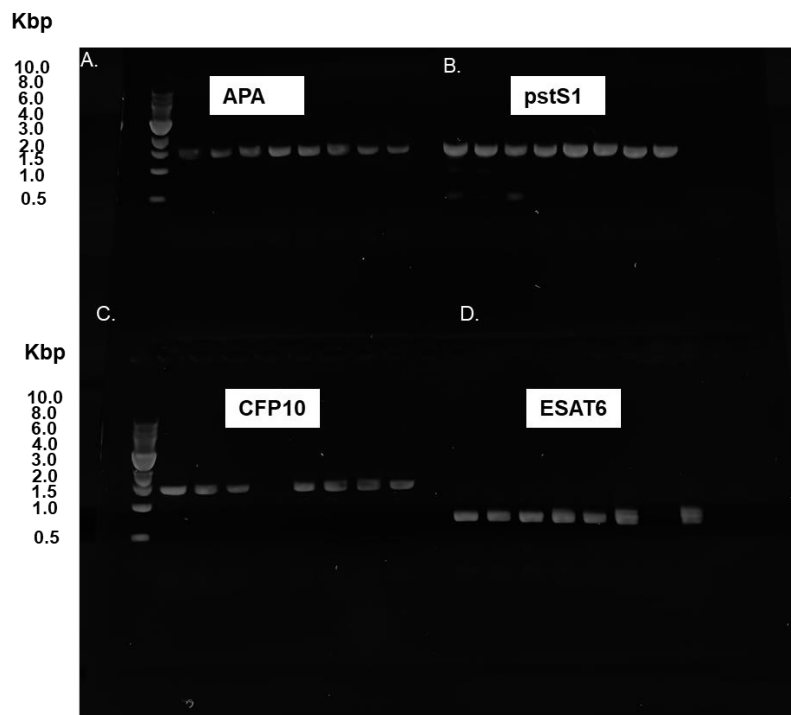


Figure 3: Agarose gel containing bands of the *apa*, *pstS1*, *ESAT6* and *CFP10* genes in the pUC57 vector. The *apa* (A), *pstS1* (B), *CFP10* (C) and *ESAT6* (D) genes were PCR amplified using 8 separate reactions prior to loading on a 1% agarose gel. A 1 kb

DNA ladder was loaded in the first well of the gels to confirm the sizes of the plasmid molecules.

3.2. Restriction digest

After the presence of the inserts in the pUC57 vector was confirmed, the genes were double digested using SmaI and AgeI restriction enzymes. The two enzymes recognized restriction sites flanking the insert molecules from which the plasmid could be cut. The pEAQ expression vector was also cut with the same enzymes to produce the same ends as the genes of interest. For a successful ligation to occur, the insert molecules and the vector ought to have the same sticky ends and this can be achieved by cutting these molecules with the same restriction digest enzymes (Yan et al., 2020, Zhang et al., 2018b, Hanlon et al., 2019). From the results obtained, the inserts were successfully cut from the pUC57 vector. This is shown by the presence of two bands in each lane for all the constructs (Figure 4). The cut pUC57 vector is the largest band and the inserts are situated below the vector. As expected in terms of size, the apa and pstS1 insert DNA molecules migrated slower than the CFP10 and ESAT6 as they had the highest number of bp. Shorter DNA molecules migrate through gels faster than longer ones (Green and Sambrook, 2019, Ivessa, 2013). The DNA ladders were also included to determine the size of the insert molecules. The pEAQ vector was also successfully cut from circular to a linear molecule (Figure 4). Therefore, the molecules could be used in the next step involving the production of recombinant pEAQ vectors.

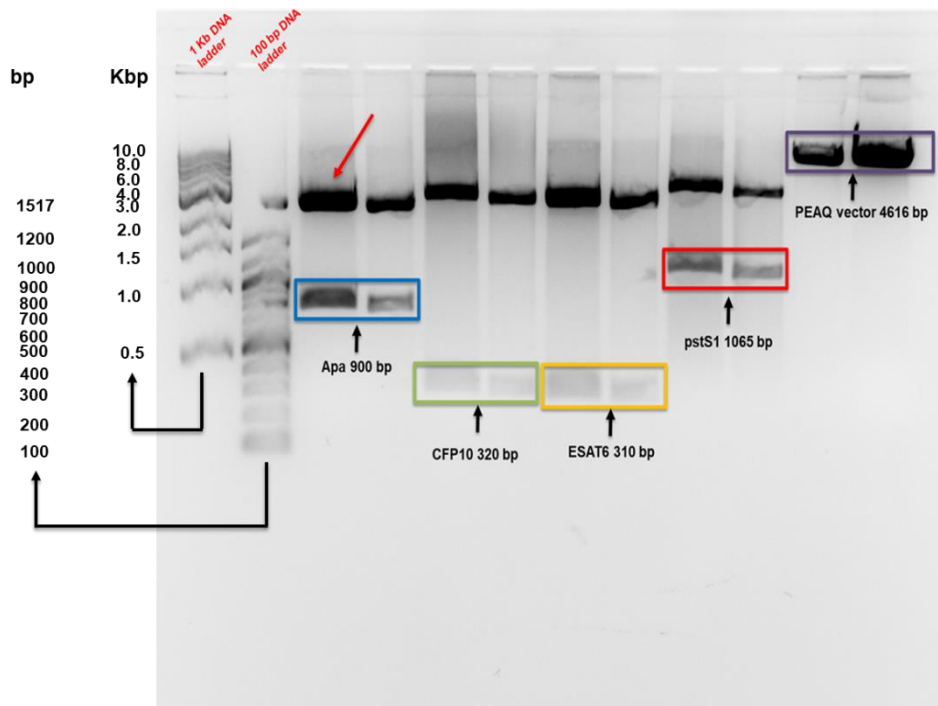


Figure 4: Agarose gel showing bands obtained after the double digestion of pUC57 and pEAQ vectors. The pUC57 vector containing the insert molecules (apa, pstS1, ESAT6 or CFP10) was double digested with *Sma*I and *Age*I restriction enzymes. This vector bands are indicated with the red arrow while the pEAQ vector is shown in the purple box with the circular plasmid on the left and the linearized one on the right. The 1 kbp and 100 bp DNA markers were also loaded.

3.3. Western blotting and Coomassie blue staining

After the digestion of the insert molecules from the pUC57 vector, they were ligated into the pEAQ expression vector and ligation was confirmed by sequencing (Appendix A). The pEAQ-insert complexes were cloned in LBA and AGL *Agrobacterium* and agroinfiltrated into the leaves of the *N. benthamiana* plant. The two *Agrobacterium* strains were used to determine which was best suited for the successful production of the proteins. The infiltrated leaves were harvested 7 days post infiltration, and the total soluble protein was extracted, and SDS-PAGE was performed to determine if the TB proteins had been expressed. Western blotting and Coomassie blue staining of the gels were performed to identify the extracted proteins. The expectation here was to observe bands corresponding

to the sizes of the TB apa, pstS1, CFP10 and ESAT6 antigens. However, from the results obtained, the bands seen on the Coomassie blue stained SDS-PAGE gel did not match the expected sizes of the antigens, i.e., no band corresponded to the 55, 38, 6 and 10 kDa apa, pstS1, ESAT6 and CFP10 antigens, respectively (Figure 5A). This suggests that the plant did not express the TB antigens. Furthermore, no protein bands were observed on the Western blot, further supporting the fact that the *M.tb* proteins were not expressed in the plant (Figure 5B).

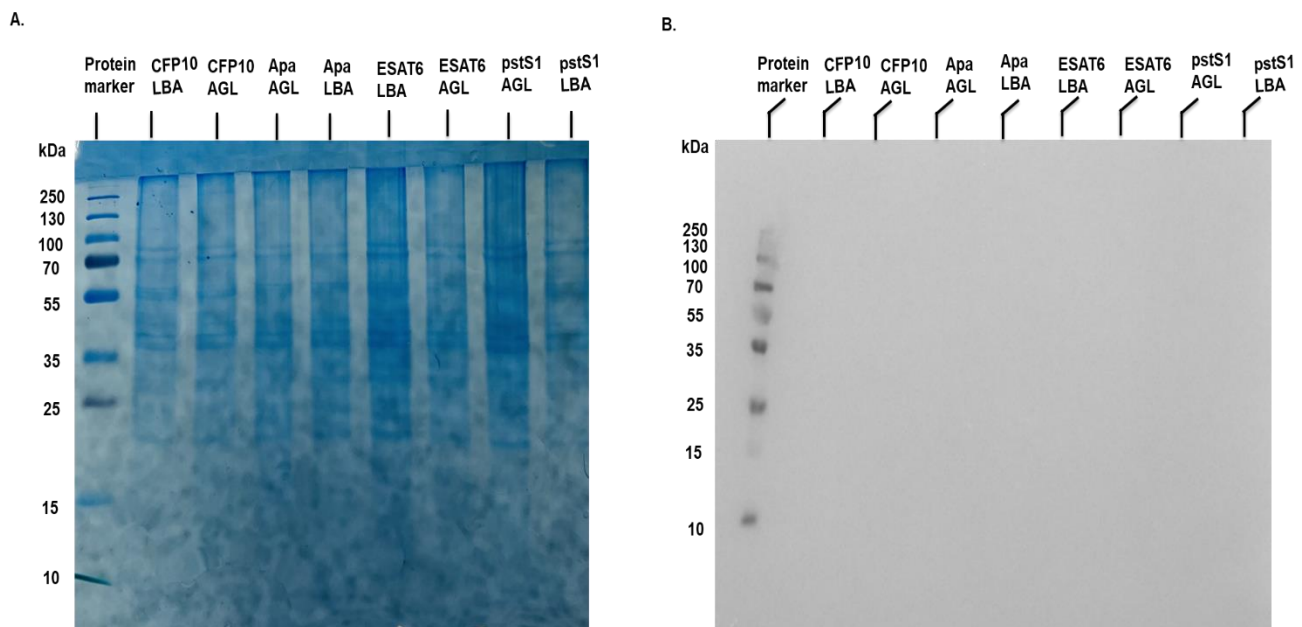


Figure 5: SDS-PAGE gel and Western blot image for the *M.tb* proteins after staining with Coomassie blue. The TB apa, pstS1, ESAT6 and CFP10 antigens were extracted from the *N. benthamiana* leaves and run on an SDS-PAGE gel (A) from which the western blotting (B) was performed. The protein ladder was also loaded.

4. Discussion

In this chapter, we aimed to produce the apa, pstS1, ESAT6 and CFP10 TB antigens in *N. benthamiana*. The expression of these proteins in the plants was performed by first cloning, purifying, and digesting the insert DNA molecules encoding the proteins of interest from the pUC57 vector and ligating them into the pEAQ expression vector. Agroinfiltration was performed using two different agrobacteria, namely; AGL and LBA employed to transfer the recombinant pEAQ vector into the leaves of the *N. benthamiana* plant (Norkunas et al., 2018b). The insert DNA molecules required for the expression of the TB antigens were successfully amplified by PCR and double digested using SmaI and AgeI restriction enzymes. In addition, the DNA molecules were ligated into the pEAQ expression vector and cloned in the AGL and LBA agrobacteria. Agroinfiltration was achieved by means of syringe injection into the plant leaves. Although the pEAQ vector containing the genes of interest were cloned in the agrobacteria, and infiltrated into the *N. benthamiana* plant, the TB antigens were not successfully expressed in the plant as expected. This was evident by the lack of bands corresponding to the molecular weight of the apa, pstS1, ESAT6 and CFP10 proteins on the Western blot and SDS-PAGE gel. Similar results of unsuccessful production of proteins in plants using the syringe infiltration method were reported by Abd-Aziz et al., where they showed that vacuum infiltration worked better for introducing agrobacteria in the plant species used (Abd-Aziz et al., 2020). The fact that there was little migration difference between the digested and undigested pEAQ expression vectors on the electrophoresis gel may have been due to insufficient pEAQ double digested plasmid (Figure 4), resulting in fewer linear pEAQ molecules for *M.tb* genes ligation. In addition, although the ligation of the insert DNA on the pEAQ vector was confirmed by sequencing, there may have been fewer pEAQ-insert complexes transformed into the AGL1 and LBA4404 *Agrobacteria* for infiltration into *N. benthamiana*, i.e., the plant cells were most likely unable to take in high copy numbers of the pEAQ-insert plasmid DNA molecules from the agrobacteria in the form of T-DNA through horizontal gene transfer (Figure 6) (Lacroix and Citovsky, 2019, Chen and Otten, 2017) since a single clone of each construct was used. This can be improved by first

optimizing the pEAQ expression vector double digestion as well as improving the ligation efficiency of the expression vector and the different *M.tb* genes.

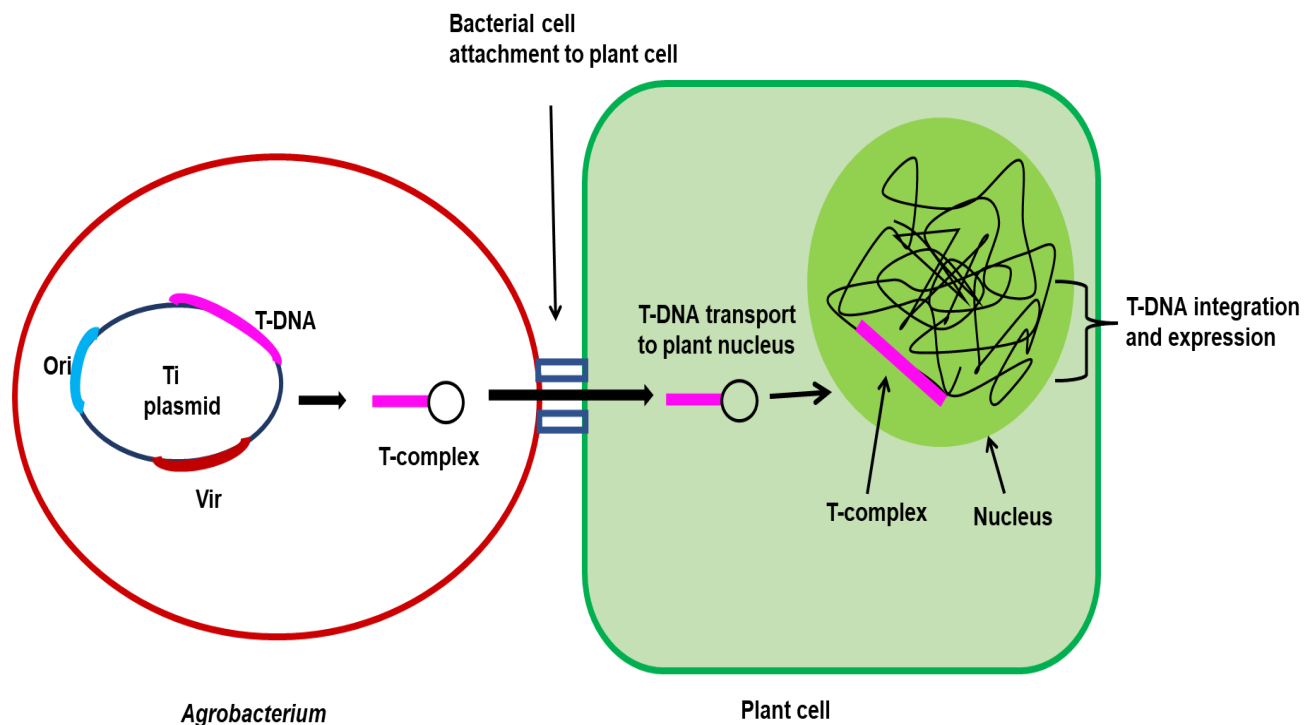


Figure 6: Illustration of the proposed interaction between the agrobacterium and the plant cell during DNA transfer. The DNA of interest harboured in the agrobacterium is transported to the plant cell nucleus for integration into the plant genome for expression using the plant cell mechanisms.

Other factors that may have affected the efficiency of the *agrobacterium* mediated transformation include the ambient and leaf temperatures, plant light source, pH and osmotic conditions, bacterial strains and density used as well as the co-cultivation time (Mondal et al., 2001, Dong and Mchughen, 1993, Matsuda and Dreher, 2004). Moreover, Ahlquist showed that after T-DNA transfer, the transgene can be affected by factors such as the natural plant response to pathogens, where transgene mRNA can be degraded in a targeted, systemic, and sequence-specific manner via post-transcriptional gene silencing (Ahlquist, 2002). Gene silencing suppressors such as p19 and P1/HC-Pro

greatly increase the expression of proteins by agroinfiltration; (Bally et al., 2018), therefore, the suppressors can be employed in future as one of the ways to support the production of the *M.tb* proteins. Furthermore, the expression of different genes may require different conditions (Papatheodorou et al., 2018), thus further optimization for more efficient production of the TB antigens in *N. benthamiana* ought to be explored.

In conclusion, using *N. benthamiana* for the expression of the TB apa, pstS1, ESAT6 and CFP10 antigens for use in the multiplex HIV and TB microarray-based detection was not successful. Several factors such as efficient double digestion of the pEAQ vector, growing conditions of *N. benthamiana*, as well as the possibility of silencing through post-transcriptional gene silencing may have played a role in the unsuccessful production of the proteins, thus, requiring future optimization to improve the expression. Other approaches that can be tried in future is the use of a different plant such as *Mucuna bracteate* which has been reported to have a better expression yield than *N. benthamiana* (Abd-Aziz et al., 2020). In addition, a different *agrobacterium* such as the *Agrobacterium tumefaciens* strain GV3101 which produces higher concentrations of proteins in conjunction with the *Mucuna bracteate* plant, two days after infiltration can as well be employed (Abd-Aziz et al., 2020). Lastly, vacuum infiltration instead of syringe infiltration could as well be explored as an alternative method.

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CHAPTER SIX

SUMMARY AND CONCLUSIONS

Given the mortality rate observed in HIV and TB co-infected individuals, it is important to efficiently manage both diseases sooner as each pathogen accelerates the morbidity caused by the other. The current technologies for the detection of TB are financially and time-wise costly, require trained personnel and sophisticated equipment and have a long turnaround time (Tiewsoh et al., 2020, Scott et al., 2017a, Rios-Hincapie et al., 2020). Unlike the TB diagnostic tests, the HIV antibody test has a short turnaround time of about 30 minutes and can be performed at point-of-care (POC) (Adams et al., 2017). However, the fact that the two pathogens are diagnosed separately can delay treatment, particularly for TB (Tiewsoh et al., 2020, Rios-Hincapie et al., 2020).

Microarray technology has offered the opportunity for high throughput multiplex detection of different infections using one test (Xiao et al., 2019, Weng et al., 2019, Nakajima et al., 2018). The platform is relatively fast and offers the advantage of obtaining diagnostic results for multiple diseases within a short period of time (Walter et al., 2010, Sutandy et al., 2013). This technology has been employed for the detection of a number of illnesses, however, it has not been explored for the simultaneous detection of TB and HIV. Therefore, this study sought to develop a multiplex microarray-based technology for the detection of the two diseases using HIV-1 p24, the commonly used protein in HIV detection (Sirivichayakul et al., 2021, Setty et al., 2019, Fox et al., 2011), as well as *M.tb* CFP10, ESAT6 and pstS1 antigens known to be markers of active TB (Watson et al., 2021, Khurshid et al., 2013, de Araujo et al., 2018, Davidow et al., 2005). The study took advantage of the reactivity of epoxy-coated glass slides with proteins, the reaction specificity between antigens and antibodies, as well as the use of reporter secondary antibodies conjugated to Alexa Fluor 488 or horseradish peroxidase. Firstly, the technology involved the detection of HIV-1 and TB antibodies spiked in 100 % human serum by means of fluorescently labeled secondary antibodies using a benchtop microscope. Secondly, we also investigated the possibility of detecting the presence of these pathogens at POC (Heggestad et al., 2023, Chuang et al., 2003, Fox et al., 2011, Wang et al., 2023), and this was achieved by colour change detection using horseradish peroxidase (HRP) conjugated secondary antibodies without the requirement of

sophisticated equipment for signal detection. Lastly, given that commercially available bacteria-produced antigens are expensive (approximately R10 000 (500 USD) for 100 µl), an attempt was made to express the TB antigens in plants for subsequent use in the HIV and TB microarray-based technology. This was particularly important because the technology is aimed at reducing the cost associated with current technologies for application in resource limited settings and developing countries.

Chapter two showed that the HIV-1 and TB antigens could successfully bind to the epoxy-coated glass slide (2.5 x 7.6 cm) using covalent interactions to form an amine bond. Since the specificity and sensitivity are paramount for diagnostic assay development to ensure no cross reactivity and determine the assay concentration limits of detection (Gibriel, 2014, Mbwana et al., 2007, Kucho et al., 2004), it was important to study the HIV and TB antigen-antibody reaction specificity and sensitivity. The findings show that the antigens reacted specifically with complementary antibodies in a cocktail solution prepared in human serum. In addition, the human serum spiked with HIV and TB antibodies were employed in the investigation of the sensitivity of the technology using different antibody concentrations. The sensitivity of the epoxy-coated slides for the HIV and TB antigen-antibody interactions was compared to that of the gold standard, i.e., high-binding 96 well plates. The immunoassay proved to be more sensitive on the slides with limits of detection many folds lower than that obtained with the plates. Also, this limit of detection was lower than the reported physiological concentrations of HIV and TB antibodies in infected individuals (Raux et al., 1999, Kimuda et al., 2017). This suggests that the technology developed here is likely to be sensitive enough to detect HIV and TB antibodies in blood of co-infected individuals. Though the study did not employ the use of actual human samples, uninfected human serum spiked with molecules of interest to simulate *in vivo* conditions has been used previously by many researchers to validate assay performances (Martinez-Perdiguero et al., 2014, He et al., 2017, Schneider et al., 2023, Mytych et al., 2009b, Mohammadi et al., 2018, Freitas et al., 2020) similar to what has been done in this study. Furthermore, because this is an assay development, it was paramount to study the stability of the antigens on the epoxy-coated glass slides at different conditions. The buffer pH, temperature and storage time can affect the performance of proteins in microarrays (Yousefi et al., 2018, Jorgensen et al., 2021).

Results from this study show that the antigens can remain immobilized and functional on the epoxy-coated glass slides at pH 7.4 and room temperature. Furthermore, the data showed that HIV and TB antigens can be stored immobilized dry on the slides for at least three months without losing their function and sensitivity for corresponding primary antibodies. For future prospects, though it has been shown that the epoxy-coated glass slides perform better in microarrays relative to other functional groups such as aldehyde (Yousefi et al., 2018), it would be interesting to test the HIV and TB immunoassay on glass slides coated with different functional groups to compare its performance relative to the epoxy-coated glass slides in terms of sensitivity and stability. It would also be of interest to include other diseases beyond HIV and TB on the microarray glass slides as they can accommodate thousands of different molecules for a high throughput multiplex detection (Ray et al., 2020, Connelly et al., 2017a, Wu et al., 2020). Lastly, assessing the validity of the technology using actual HIV and TB co-infected human samples would be recommended.

Chapter three demonstrated that the HIV and TB antigen-antibody reactions can be detected by visual inspection. The chapter aimed at investigating the multiplex POC detection of HIV and TB antibodies spiked in human serum using colour change as a means of signal detection using HRP conjugated secondary antibodies as well as ABTS or TMB substrates. This part of the chapter was particularly important because multiplex microarray diagnostic technologies ought to be POC for accessibility and rapid turnaround time of results without requiring sophisticated equipment (Fox et al., 2011, Appidi et al., 2023, Gao et al., 2023). Like the previous chapter, the specificity and sensitivity of the HIV and TB multiplex microarray technology was studied using uninfected human serum spiked with the HIV and TB antibodies. It was important to show that there was no cross reactivity between the HIV-1 and TB antigen-antibody reactions as non-specific binding can result in false positive results (Hakobyan et al., 2023, Ainoda et al., 2023, Quintana-Ortega et al., 2020, Slater et al., 2012). Results showed that the antigen-antibody reactions in this study were specific, i.e., the antigens reacted specifically with corresponding antibodies in a cocktail solution prepared in human serum. The HIV and TB immunoassay for the multiplex detection of their antibodies on the epoxy-coated glass slides was compared to the high-binding 96 well plates to choose a better suited platform

for the colour change detection assay at POC. Although both systems performed well, the epoxy-coated glass slides were better than the high-binding plates in terms of sensitivity. In addition, two substrates were also compared for colour change reaction, namely ABTS and TMB. The TMB substrate gave the lowest limit of detection for the antigen-antibody reactions and was three times faster than the ABTS. Moreover, the chapter investigated the stability of the HIV and TB antigens in the high-binding 96 well plates. Like what was observed on the epoxy-coated glass slides in the previous chapter, the antigens could be stored in the plates dry and at room temperature for a period of at least three months. Data suggested that although different pH, temperature, and storage conditions (wet or dry) could be used in the colour change detection immunoassay, the optimal reaction conditions were determined to be buffer at pH 7.4, incubation at room temperature and dry storage. In brief, the conclusion was that the epoxy-coated glass slides and the TMB substrate are better suited for the POC HIV and TB diagnostic technology developed here. Overall, the data obtained in this chapter showed that the development of the HIV and TB multiplex microarray technology as a POC application assay has a lot of potential as a cheap, user friendly and rapid test.

In chapter four, the aim was to express the *M.tb* antigens ESAT6, CFP10, pstS1 and apa in *N. benthamiana* for subsequent use in the HIV and TB multiplex microarray technology. Since commercially available bacteria expressed TB proteins can be expensive, in some cases like with apa not even commercially available, it was important to attempt producing the antigens in plants with the overall goal of making the assay cheaper. *N. benthamiana* is known to be inexpensive and highly efficient in protein expression (Miura et al., 2023, Smith et al., 2023, Kurotani et al., 2023). Although the insert *M.tb* genes were successfully amplified, restriction digested, ligated into a pEAQ expression vector, and cloned in AGL and LBA agrobacteria, the expression of the TB antigens was unsuccessful in the *N. benthamiana* plant. This may have been due to a number of reasons and factors involved in the expression of proteins in this plant. For example, inefficient cloning or infiltration used, the plant species and agrobacteria strain employed; as well as less optimal conditions such as the ambient and leave temperature, pH, and osmosis (Mondal et al., 2001, Dong and Mchughen, 1993, Matsuda and Dreher, 2004). Since it is important that the assay developed here is cheap, the TB antigens production in plants will be attempted

as part of the future prospects of the study using different plant strains such as *Mucuna bracteata* which was reported to perform better than *N. benthamiana* (Abd-Aziz et al., 2020). Different pH, osmosis, ambient and leave temperature, different strains of agrobacteria such as *Agrobacterium tumefaciens* strain GV3101, and more efficient cloning methods will as well be tried to attempt a more successful expression of the proteins.

The multiplex HIV/TB assay developed here is an important one which will certainly solve the issues faced by developing countries, underserved communities, and resource limited settings. As such it will be important to align the project with the regulations required for laboratory developed tests. This will ensure that the technology moves in the next phase of product development, validations, and clinical trials to test the validity and accuracy of the technology. Some of the regulatory standards that will be considered for this HIV/TB multiplex technology will include testing characteristics such as precision, accuracy, reportable range, analytical sensitivity, analytical specificity as well as reference intervals using infected specimen in order to meet the clinical laboratory improvement amendments federal regulatory standards (Burd, 2010). All these mentioned requirements for laboratory developed assay/technologies align and will be confirmed by the South African Health Products Regulatory Authority (SAPRA), prior to implementing and making the technology available in the South African market. Furthermore, the technology will be used together with a portable battery powered fluorescence reader currently being developed at the CSIR to complete the POC care part of the assay. Therefore, it will be important to ensure the portable fluorescence reader aligns with the epoxy coated glass slides and works as expected for the detection of HIV and/or TB antibodies in human specimens.

It does transpire that sometimes assays or technologies do not work as expected in terms of sensitivity. Should this occur with the HIV/TB multiplex technology, particularly for active TB as it is known that the sensitivity for the detection of the presence of this disease can be challenging in terms of sensitivity, the contingency plan would be to increase the concentration of the TB antigens on the slides. This will increase the number of TB antigen molecules available to react with the antibodies in positive specimens. In addition, to

promote the sensitivity of the assay, more TB antigens, particularly those known to be markers of active TB such as *apa*, would be added to the epoxy coated glass slides.

In conclusion, since there is currently no diagnostic test for the multiplex detection of HIV and active TB, it was important to develop a technology that can detect the two pathogens simultaneously, especially at POC. We have shown that the technology developed in this study could detect HIV and TB antibodies spiked in uninfected human serum and at very low concentrations, i.e., antibody concentrations as low as or even lower than those observed in HIV and TB infected individuals. Overall, the multiplex HIV and TB microarray-based technology developed in this study has the potential to offer the following advantages: it is sensitive and specific, is fast, user friendly, can detect the presence of both HIV and TB antibodies within 2 hours and 30 minutes and can be applied at POC without the need for sophisticated equipment and highly trained personnel. With these advantages, the technology is likely to benefit poor communities, under resourced laboratories as well as alleviate the challenges of HIV and TB diagnosis faced by developing countries. This technology warrants future development which will include testing using actual infected human samples and clinical trials based of required regulation for laboratory developed tests.

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APPENDICES

APPENDIX A

pEAQ-*M.tb* insert molecules complexes sequences

pEAQ-APA

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pEAQ-pstS1

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APPENDIX B

Chapter two published article



biosensors



Article

Development of a Multiplex HIV/TB Diagnostic Assay Based on the Microarray Technology

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Abstract: Currently there are diagnostic tests available for human immunodeficiency virus (HIV) and tuberculosis (TB); however, they are still diagnosed separately, which can delay treatment in cases of co-infection. Here we report on a multiplex microarray technology for the detection of HIV and TB antibodies using p24 as well as TB CFP10, ESAT6 and pstS1 antigens on epoxy-silane slides. To test this technology for antigen–antibody interactions, immobilized antigens were exposed to human sera spiked with physiological concentrations of primary antibodies, followed by secondary antibodies conjugated to a fluorescent reporter. HIV and TB antibodies were captured with no cross-reactivity observed. The sensitivity of the slides was compared to that of high-binding plates. We found that the slides were more sensitive, with the detection limit being 0.000954 µg/mL compared to 4.637 µg/mL for the plates. Furthermore, stability studies revealed that the immobilized antigens could be stored dry for at least 90 days and remained stable across all pH and temperatures assessed, with pH 7.4 and 25 °C being optimal. The data collectively suggested that the HIV/TB multiplex detection technology we developed has the potential for use to diagnose HIV and TB co-infection, and thus can be developed further for the purpose.

Keywords: HIV-1 p24; *M.tb* CFP10; *M.tb* ESAT6; *M.tb* pstS1; multiplex microarray; diagnosis; antibody; antigen



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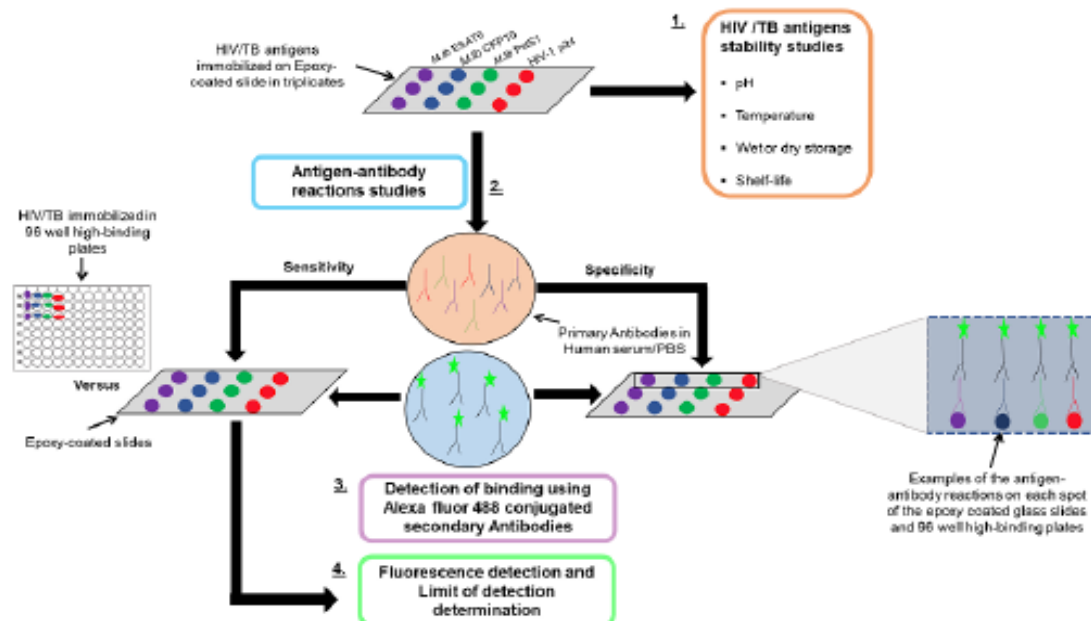
1. Introduction

An estimated 38.0 million people were living with HIV/AIDS globally with the annual number of deaths standing at 970,000 in 2021, and 25% were co-infected with *Mycobacterium tuberculosis* (*M.tb*), the causative agent of TB [1]. Albeit that there are diagnostic tests currently available for HIV and TB, the diagnosis of these two diseases is still performed separately and this can delay treatment in co-infected individuals, particularly because active TB diagnosis can take a long time [2,3]. With HIV, there is a rapid test for the detection of p24, in addition to the PCR test. However, the currently existing antibody test for TB suffers from low sensitivity and specificity, especially in HIV-infected individuals [4,5]. Furthermore, there is also a polymerase chain reaction (PCR) test available for TB detection, although this test cannot distinguish between active and latent TB, thus requiring the culturing of the bacteria to confirm the active infection, which can take time [6–8]. Therefore, there is a need for alternative diagnostic technology that will allow for the simultaneous detection of HIV and TB more quickly, as well as the ability to distinguish between active and latent TB.

Microarray technology refers to the miniaturization of multiple assays on one small plate or slide [9–12]. There are different types of microarrays, which include analytical and functional microarrays [11]. An example of an analytical microarray is the antibody array, which involves protein detection and concentration determination after antibody capture using direct protein labelling [11,13,14]. The functional microarray is built using purified proteins to enable the study of various biochemical properties such as protein–protein interactions, protein binding activities, protein–peptide interactions and protein–lipid interactions, to name a few [15–18]. Protein microarray technology allows for the characterization of hundreds of thousands of proteins in a highly parallel and high-throughput manner [19–21]. Immunoassays take advantage of highly specific antigen–antibody recognition to build a protein detection system, whereas the microarray enables a rapid parallel and multiplex detection, often requiring a small amount of sample [22,23]. The immunoassay on microarrays can be very sensitive and specific, thus making it very reliable when studying or determining antigen–antibody interactions such as occurs in diagnostics [24–26].

Epoxy-coated slide chemistry is one of the commonly used systems for protein microarray, as they are stable under various conditions and are reactive with several nucleophilic groups to form strong bonds with minimal chemical modification of the protein [27]. For example, epoxy-coated glass slides have been used in cancer studies as well as in the detection of antibodies against SARS-CoV-2 for disease diagnostic purposes [22,28]. The interaction between the coated slide and proteins occurs through fast adsorption followed by intramolecular chemical attachment promoted by the epoxy groups on the glass surface [27]. The part of proteins that reacts with epoxy on the solid surfaces is mostly the amine group, such as found in lysine residues which are present on the exterior of proteins, forming stable amine bonds with the surface [29–31]. During the coupling process, proteins are dissolved in a low ionic-strength buffer before being passed through the coated solid surface. The immobilization efficiency depends on parameters such as pH, concentration and reaction time [27,30].

In this study, we developed a multiplex microarray technology on a 2.5×7.6 cm epoxy-coated glass slide for the detection of HIV and TB co-infection using HIV-1 p24 antigen as well as *M.tb* CFP10, ESAT6 and pstS1 antigens, known to be markers of active TB [32–35], following the research strategy outlined in Scheme 1. We found that the immobilized antigens captured corresponding primary antibodies in human serum. In addition, we proved that this interaction was specific and with sensitivity higher than what would be obtained on high-binding ELISA plates. Furthermore, this diagnostic technology performed optimally at room temperature and pH 7.4, and this performance was maintained for at least 90 days during the shelf-life experiments. In all, these data indicate that our diagnostic technology is potentially a solution for a quicker method of simultaneously diagnosing HIV-1 and TB in cases of co-infection.



Scheme 1. A schematic diagram showing the representation of the research strategy used to develop the HIV and TB multiplex microarray technology. The strategy started with stability studies of the immobilized antigens followed by sensitivity and specificity studies.

2. Materials and Methods

2.1. Reagents

The HIV-1 p24 antigen and anti-p24 (Ms), anti-CFP10 (Rb) and anti-ESAT6 antibodies (Rb) were bought from Abcam (Cambridge, UK). The culture filtrate protein-10 (CFP10) antigen and early secretory antigenic target protein 6 (ESAT6) were obtained from BBI solutions (Caerphilly, UK). The phosphate-specific transport substrate binding protein-1 (pstS1) antigen and the pstS1 primary antibody (Ms) were bought from My BioSource (San Diego, CA, USA). The Goat (Gt) anti-Mouse (Ms)/anti-Rabbit (Rb) IgG superclonal secondary antibody, Alexa fluor 488 conjugates were obtained from Abcam (Cambridge, UK). The NEXTERION epoxy-silane-coated glass slides were bought from Schott laboratories (Mainz, Germany), while the Corning polystyrene high-binding costar assay plates were purchased from Corning Inc. (Corning, NY, USA).

2.2. Immobilization of HIV-1 p24 as Well as *M.tb* CFP10 ESAT6 and pstS1 Antigens on Epoxy-Coated Glass Slides

Antigens (HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1) were diluted to 500 µg/mL in 1 × phosphate buffered saline (PBS) pH 7.4 (Thermo Fisher Scientific, Waltham, MA, USA), with concentrations ranging from 500 µg/mL to 0.0762 µg/mL. The different concentrations of each antigen were spotted (1 µL) on the NEXTERION epoxy-silane-coated glass slides and incubated for 90 min at room temperature. The slides were thereafter washed three times for 5 min in 1 × PBS pH 7.4, followed by blocking for an hour in 1% PBS-bovine serum albumin (BSA) (Sigma Aldrich, Midland, MI, USA). This was followed by washing the slides three times in 1 × PBS for 5 min to remove excess BSA. The slides were then allowed to dry at room temperature for 15 min and bright-field images of the slides were captured using a cytation3 multimode reader (Biotek Instruments, Winooski, VT, USA). To further profile and evaluate the immobilized antigens, the slides were incubated for an hour in a cocktail solution containing anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies at a constant concentration of 3.906 µg/mL to determine if they could capture the antibodies in solution. Post-incubation with primary

antibodies, the slides were washed and incubated for an hour in a cocktail solution of Goat (Gt) anti-Mouse (Ms)/anti-Rabbit (Rb) IgG superclonal secondary antibody, conjugated to Alexa fluor 488, at a constant concentration of 0.10 µg/mL. The slides were thereafter washed and imaged using a cytation3 multimode reader.

2.3. Determination of the Optimal Buffer pH, Storage Condition, Temperature and Shelf-Life of Immobilized HIV and TB Antigens

To determine the optimal printing, washing and storage pH conditions for the HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens on the epoxy-coated glass slides, five different pH conditions, adjusted using NaOH and HCl (Sigma Aldrich, Midland, MI, USA), ranging from 5.0 to 9.0 were evaluated. Jorgensen et al. showed that epoxy substrates exhibited high stability in pH conditions of 6.0, 7.5 and 9.0 [36], hence the choice of the aforementioned conditions. To achieve this, the antigens were spotted in triplicate at a contact concentration of 31.3 µg/mL in PBS at different pH, followed by immobilization on the glass slides as mentioned above. Washing steps were performed as above, also using the PBS at corresponding pH.

The determination of the optimal pH was followed by the evaluation of whether the immobilized antigens should be stored dry or in buffer. This was done by storing the immobilized antigens dry or in PBS pH 7.4 for 24 h and then studying their interaction with primary antibodies.

The next step was to determine the optimal temperature at which the immobilized antigens on the epoxy-coated glass slides could be stored. Yousefi et al. showed that epoxy slides had the best functionality and produced high fluorescence at different temperatures such as 4 °C, 25 °C and 40 °C [30], hence our choice of −20 °C, 4 °C, 15 °C, 25 °C and 37 °C for this study. However, we used 37 °C as it is the optimal human body temperature instead of the 40 °C that Yousefi et al. used. To achieve this, immobilized HIV and TB antigens were incubated dry at −20 °C, 4 °C, 15 °C, 25 °C and 37 °C for 24 h, followed by the addition of primary and secondary antibody, and subsequent detection of fluorescence.

Studies were also conducted to determine the immobilized antigens' shelf-life. Jorgensen et al. showed that printed proteins for multiplexing can remain stable for up to three months [36]; therefore, we assessed the HIV and TB antigens on the slide for a period of three months. This was done by storing immobilized antigens slides dry for 1 day, 15, 30, 45, 60, 75 and 90 days at room temperature. All the slides for the different conditions were incubated in a cocktail of the HIV and TB recombinant primary antibodies at a constant concentration of 3.906 µg/mL. Following the incubation, the slides were washed and incubated in Gt anti-Ms/anti-Rb IgG superclonal secondary antibody, conjugated to Alexa fluor 488, at a constant concentration of 0.10 µg/mL for an hour at room temperature. After the incubation, the slides were washed, and fluorescence images were obtained using the cytation3 multimode reader.

2.4. In Vitro Specificity and Sensitivity of the Microarray-Based Detection System for HIV and *M.tb* Antibodies

The immobilization of the HIV and TB antigens for both the slides and plates was carried out as mentioned above, except that for the high-binding black with clear bottom polystyrene 96-well plates (Sigma Aldrich, Midland, MI, USA) we used 5 µL of the antigens per well. This was followed by the addition of 4-fold serially diluted HIV and TB primary antibodies (for the slides, the series range was 250–0.000954 µg/mL, while for the plates it was 250–0.020 µg/mL) in PBS or human serum, and incubation for 2 h at room temperature. To determine that each antigen specifically reacted with its corresponding primary antibody in solution, an anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibody cocktail solution, missing one of the antibodies at a time, was prepared and incubated with the immobilized antigens. Subsequently, the plate was washed three times with PBS for 5 min. Afterwards, 100 µL Gt anti-Ms/anti-Rb IgG superclonal secondary antibody conjugated to Alexa fluor 488 at a concentration of 0.10 µg/mL was added and incubated at room temperature for an hour. Post-incubation, the plate was washed as above, and fluorescence

was measured using DTX 880 Multimode detector (Beckman Coulter Inc., Brea, CA, USA) at a wavelength of 485 nm. SoftMax Pro version 6.4 (Molecular Devices, LLC) was used for data acquisition and analysis of the plates. For the microarray slides, the detection was carried out using a cytation3 multimode reader, while ImageJ v1.54f software was used for analysis.

Specificity between the HIV and *M.tb* antigens and the anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 recombinant primary antibodies was also investigated by immobilizing the antigens as aforementioned. The whole slide was then incubated with a solution containing one of the primary antibodies at a time at a constant concentration of 3.906 µg/mL. For example, one slide of the immobilized antigens was incubated for an hour in a solution containing anti-p24 antibody only, while other slides were incubated with either anti-CFP10, anti-ESAT6 or anti-pstS1 primary antibody. After the incubation, the slides were washed and incubated with the secondary antibodies at a constant concentration of 0.10 µg/mL for an hour at room temperature. Then excess secondary antibodies were washed and the slide was imaged using Cytation3 multimode reader.

2.5. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 9.4.0 (GraphPad Software, Inc., California, USA). Data are presented as mean ± standard deviation of the mean, and each experiment was performed at least three times unless stated otherwise. Statistical significance was assessed using paired Student *t*-test when comparing two means of the same groups under separate scenarios. One-way analysis of variance (ANOVA) test was also used to assess the statistical significance between multiple groups under different scenarios using Tukey's multiple comparison test. For all comparisons, *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. Immobilization of Antigens on Epoxy-Coated Glass Slides

The immobilization of antigens as capture molecules on functionalized glass slides is a well-developed system for microarrays in diagnostic and drug discovery [37,38]. In this study, the microarray was developed by covalently immobilizing HIV-1 p24, *M.tb* CFP10, ESAT6 and pstS1 antigens on epoxy-coated glass slides. This was done to capture anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies in solution. The immobilization was achieved by reacting the epoxy group on the glass surface with amine groups on the antigens to form a covalent amine bond. We then determined whether the immobilization of the TB and HIV antigens was successful, as well as the optimal conditions for this immobilization. We demonstrated that the spot where the antigens were printed was stable on the slide by means of bright-field imaging even after washing with PBS. This was shown on the bright-field image obtained using scanning microscopy by the presence of clear spots where antigens were printed (Figure 1A). Furthermore, these spots were observed for all concentrations (500–0.000228 µg/mL) and antigens printed, i.e., HIV-1 p24, as well as *M.tb* antigens CFP10, ESAT6 and pstS1, thus suggesting their interaction with the epoxy-coated glass slide. As a negative control, PBS was spotted in place of antigens, and, as expected, no spots were observed for PBS after visualization with the microscope (Figure 1A). To investigate whether the HIV p24 and *M.tb* CFP10, ESAT6 and pstS1 on the glass slide could react with their specific antibodies, the immobilized antigens were incubated with a cocktail containing anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies. This was followed by the addition of a secondary antibody conjugated to Alexa fluor 488. The results showed that the coupled HIV and TB antigens could detect the primary antibodies even at the lowest concentrations of the antigens tested (3.91 µg/mL). This was indicated by the presence of green fluorescence from all the spots on the slide after imaging (Figure 1B). The mean fluorescence intensity of the spots on the image was obtained using the ImageJ software. From the graph in Figure 1C, we observed that the fluorescence intensity decreased with decreasing concentration of

the antigens when the concentrations of the primary and secondary antibodies were kept constant at 3.906 $\mu\text{g/mL}$ and 0.10 $\mu\text{g/mL}$, respectively. The antigen–antibody reaction that produced the highest fluorescence intensity, at the highest concentrations tested, was ESAT6 followed by pstS1. HIV-1 p24 antigen–antibody reaction showed the lowest fluorescence intensity while *M.tb* CFP10 was the second lowest fluorescence intensity relative to the other antigen–antibody reactions (Figure 1C).

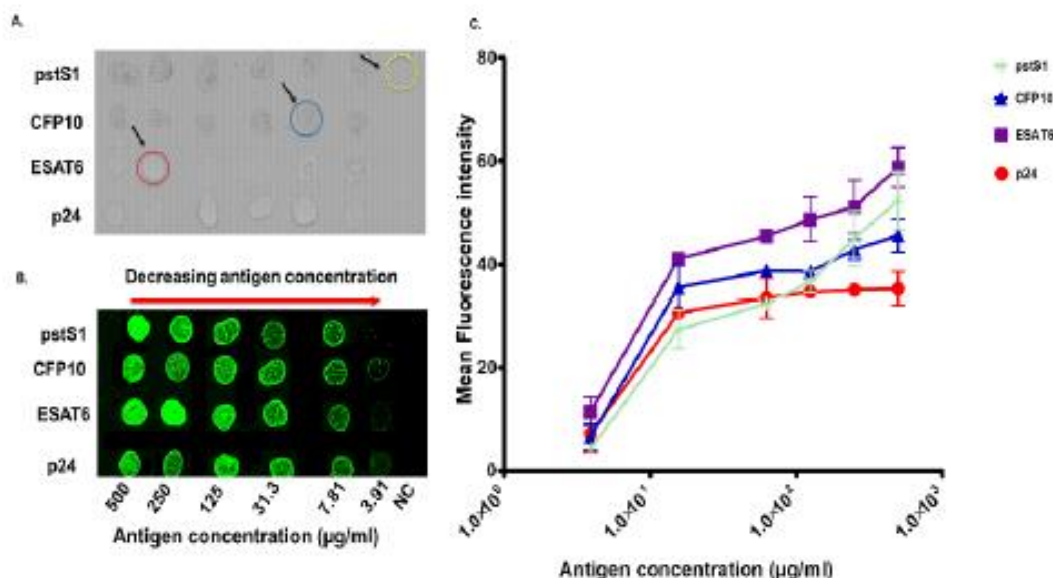


Figure 1. The immobilization of HIV-1 p24, *M.tb* CFP10, ESAT6 and pstS1 antigens on an epoxy-coated glass slide by covalent interactions. (A) The antigens were immobilized at different concentrations, PBS-only spots were used as negative control, and imaging was performed using bright-field microscopy. The antigen spots after washing and imaging are indicated by the red and blue circles, while yellow circles show the negative control. (B) The immobilized antigens were reacted with anti-p24, anti-CFP10, anti-ESAT6 and anti-pstS1 primary antibodies, and subsequently secondary antibodies conjugated to Alexa fluor 488 were added and the slide imaged with a cytation3 multi-mode reader with Gen5 2.08 software. (C) Mean fluorescence intensity of the spots was measured with ImageJ and plotted. The concentration at which the antigens were assessed is also shown. Error bars are means $\pm$ standard deviation ($n = 3$).

3.2. Investigation of the Stability of Immobilized Antigens on the Epoxy-Coated Glass Slides

After showing that the antigens were immobilized on the microarray slide and could interact with the antibodies, we determined the optimal storage pH, temperature and shelf-life of the immobilized antigen epoxy-coated slides. The pH studies were performed using PBS solutions at different pH values (pH 5.0, 6.0, 7.4, 8.0 and 9.0). The antigens were spotted at a concentration of 31.3 $\mu\text{g/mL}$ in triplicate, as this was one of the concentrations where we observed a strong reaction with the primary antibodies, judging from the fluorescence obtained. After printing at the above-mentioned pH, the slide was blocked, followed by incubation with primary antibody at the corresponding pH. After addition of the secondary antibody, the slide was imaged. From the results obtained, the immobilized antigens (p24, ESAT6, CFP10 and pstS1) had similar interaction with primary antibody at all tested pH (pH 5.0, 6.0, 7.4, 8.0 and 9.0) (Figure 2). The highest fluorescence intensities observed at pH 6.0, 7.4, 8.0 and 9.0 were comparable, while at pH 5.0 there was 2-fold decrease in intensity, and this was statistically significant ($p < 0.05$) (Figure 2). Therefore, we concluded that the optimal pH range for the antigen–antibody reaction on our epoxy-coated slides was 6.0–9.0.

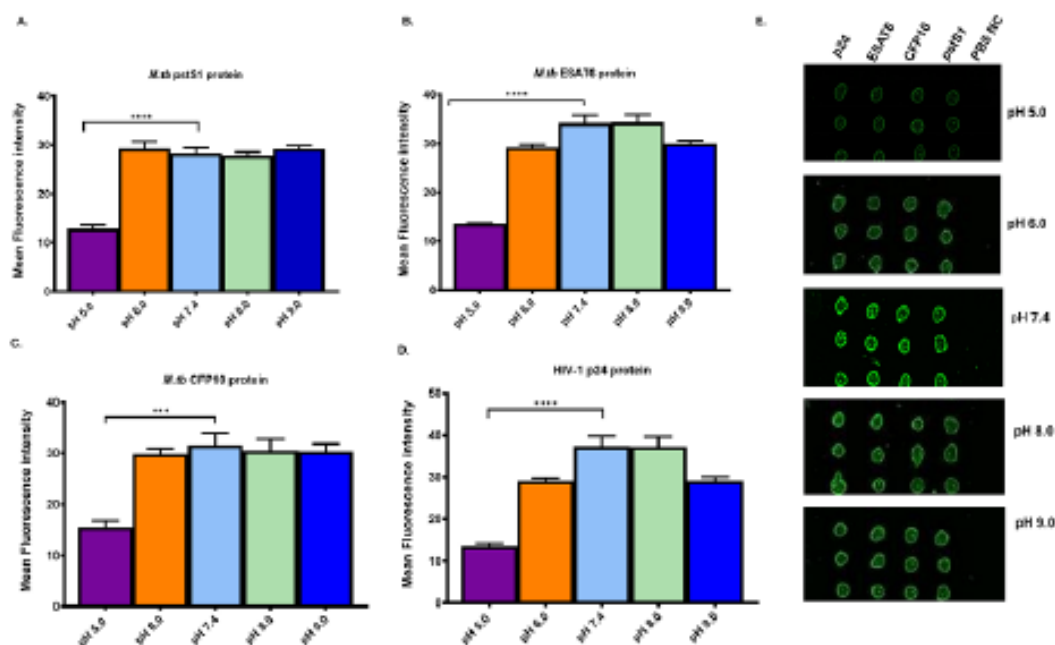


Figure 2. Effect of different pH on the immobilization of antigens on epoxy-coated glass slides. The antigens were printed and washed with PBS at different pH values. The bar graphs show fluorescence intensity obtained with *M.tb* pstS1 (A), CFP10 (B), ESAT6 (C) and HIV-1 p24 (D). Data represent mean $\pm$ standard deviation and error bars are standard deviation ($n = 3$). Asterisks signify statistical significance ($p < 0.05$) between the fluorescence intensity of the highest reading and the lowest. (E) shows representative images obtained at different pH (pH 5.0, 6.0, 7.4, 8.0 and 9.0).

The optimal storage and reaction temperature for the immobilized antigens was also evaluated, as temperature can play an important role in the stability of proteins on functionalized microarray slides [30,36]. This study was performed by immobilizing the antigens on the epoxy-coated glass slides in triplicate and incubating them at different temperature conditions ($-20\text{ }^{\circ}\text{C}$, $4\text{ }^{\circ}\text{C}$, $15\text{ }^{\circ}\text{C}$, $25\text{ }^{\circ}\text{C}$, $37\text{ }^{\circ}\text{C}$) for 24 h. This was followed by washing with PBS at pH 7.4, which was one of the optimal pH established previously. The antigens were reacted with a cocktail of their associated primary antibodies at the concentration of $3.906\text{ }\mu\text{g/mL}$, and fluorescence was measured after the addition of secondary antibodies labelled with a fluorescence dye. The $3.906\text{ }\mu\text{g/mL}$ primary antibody concentration used was determined experimentally to be optimal (Appendix A, Figure A1). Based on the fluorescence intensities in Figure 3, the optimal temperature at which the p24, CFP10, ESAT6 and pstS1 antigens reacted with their antibodies was $25\text{ }^{\circ}\text{C}$, followed by $37\text{ }^{\circ}\text{C}$, $15\text{ }^{\circ}\text{C}$ and $4\text{ }^{\circ}\text{C}$, while $-20\text{ }^{\circ}\text{C}$ had the lowest intensity. In fact, in general, the fluorescence intensity from slides stored at $25\text{ }^{\circ}\text{C}$ was about 2-fold higher than the second-best storage temperature, and these differences were statistically significant ($p < 0.05$) for all antigens except pstS1 (Figure 3).

After studying the stability of the immobilized antigens under different pH and temperature conditions, the next step was to determine whether the immobilized antigens on the epoxy glass slides should be stored dry or wet. We investigated this at pH 7.4 and room temperature, given that they were determined to be optimal. This was done by printing the HIV-p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens on glass slides followed by 24 h incubation at room temperature in PBS pH 7.4, which represented the wet condition, or dry. From the results obtained, we observed fluorescence with both dry and wet slides (Figure 4). However, this intensity was lower for the antigens stored in PBS relative to the antigens stored dry (Figure 4B,C). In fact, the fluorescence intensity was decreased by 2-fold for HIV-1 p24 and ESAT6, while the decrease was about 1.5-fold for

CFP10 and pstS1. All these decreases were statistically significant ($p < 0.05$). Therefore, storage in the dry proved to be the optimal condition.

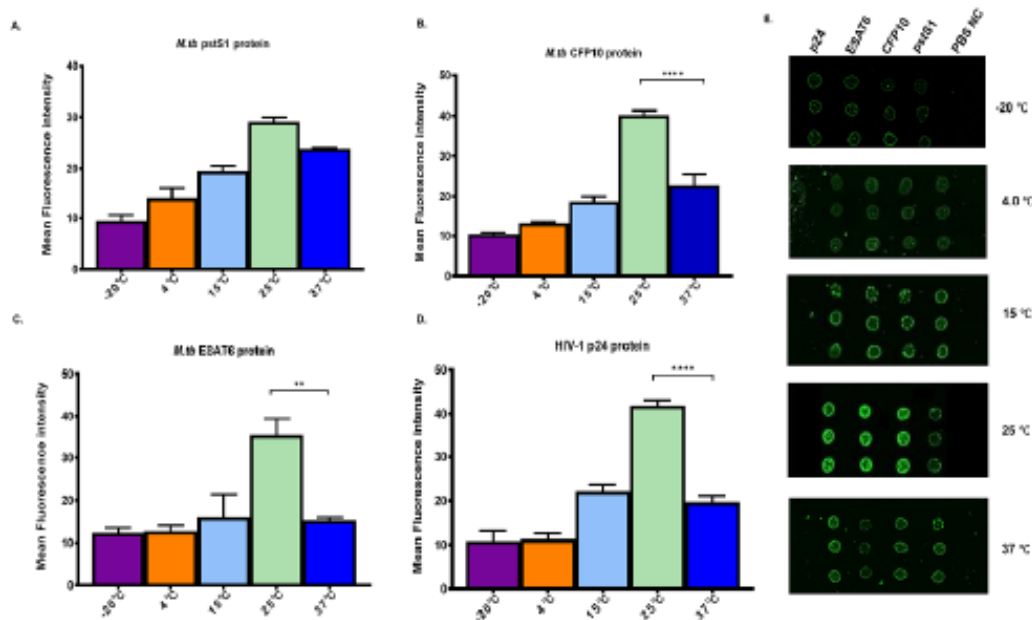


Figure 3. Evaluation of the optimal storage temperature for the immobilized antigens. The immobilized antigens were stored at different temperatures (−20 °C, 4 °C, 15 °C, 25 °C and 37 °C) for 24 h. The bar graphs of the fluorescence intensity obtained from *M.tb* pstS1 (A), CFP10 (B), ESAT6 (C) and HIV-1 p24 (D) are shown. Data represent mean $\pm$ standard deviation ($n = 3$) and asterisks indicate significant differences between the groups being compared. The slide images from which the bar graphs were plotted are shown in (E).

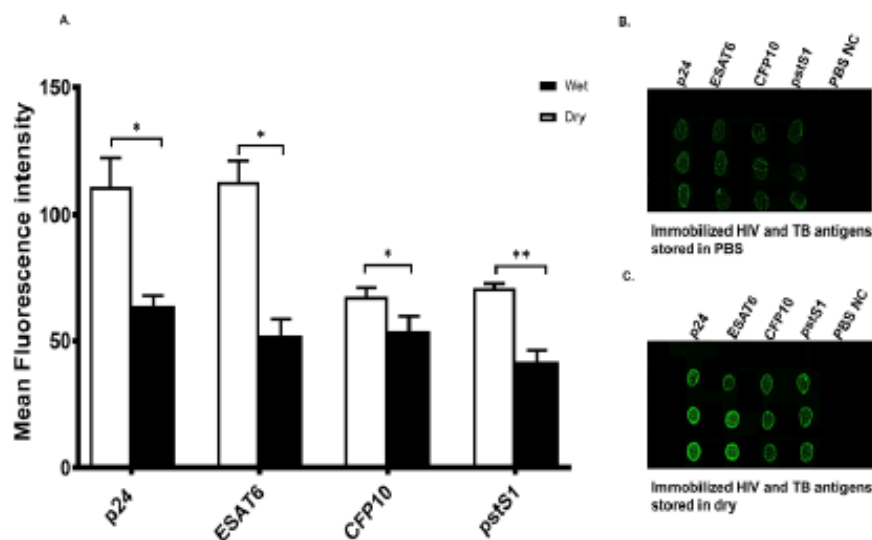


Figure 4. Evaluation of the stability in dry and wet storage conditions of the HIV and TB antigens immobilized on the epoxy-coated glass slide. The antigens were stored in PBS and dry at room temperature for 24 h. (A) The mean fluorescence of each spot was measured and plotted. Data represent mean $\pm$ standard deviation ($n = 3$) and asterisks show significant difference between groups. (B) and (C) representative PBS and dry storage images, respectively, used to plot the graph in (A).

Lastly, we determined the shelf-life of the immobilized antigens on the epoxy-coated slides, in order to obtain an indication of the durability of the immobilized antigens on the slide. To achieve this, seven slides were coated with the HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens at room temperature for an hour, washed and incubated dry (the optimal storage condition determined above) for different numbers of days. The incubation time points included day 1 to day 90 with fluorescence intensity readings at 15-day intervals. Fluorescence was detected on all antigens from day 1 through day 90 after the incubation of the immobilized antigens with primary and secondary antibodies (Figure 5). However, a decrease in the mean fluorescence intensity, as measured using the ImageJ software, was observed with longer periods of incubation, i.e., in most cases day 1 and day 15 yielded the highest fluorescence intensity while day 75 and day 90 had the lowest. In most cases, these differences were statistically significant ($p < 0.05$). In fact, there was a progressive and consistent decrease in fluorescence intensity emitted from the slides as the shelf-life moved toward day 90. However, it is important to note that even at day 90 there was still clear detection of all the antigens on the microarray slide, implying that our HIV/TB co-infection detection technology has the potential to last on the shelf for at least three months.

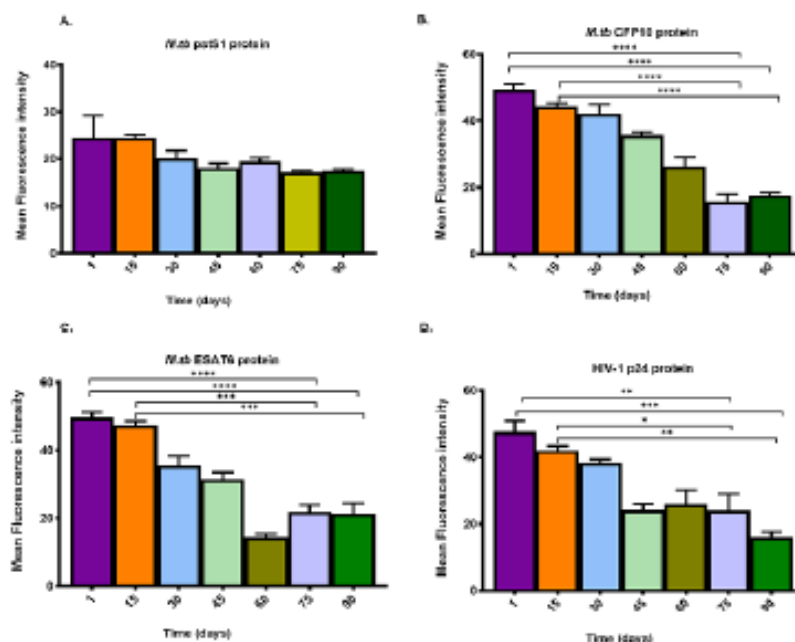


Figure 5. Evaluation of the shelf-life of the immobilized HIV and TB antigens on the epoxy-coated glass slides. The antigens were stored dry at room temperature from day 1 to day 90, followed by addition of primary and secondary antibodies, with fluorescence readings after every 15 days. The bar graphs show fluorescence intensity when *M.tb* pstS1 (A), CFP10 (B), ESAT6 (C) or HIV-1 p24 (D) were stored dry for 90 days. Data represent mean $\pm$ standard deviation ($n = 2$) and asterisks signify significant difference between the groups compared.

3.3. Comparison of the Antigen–Antibody Reactions between the Microarray Epoxy-Coated Slide Platform and the Protein Polystyrene High-Binding Plates

Next, we studied the sensitivity of the microarray-based multiplex technology. The assay was conducted by diluting antibodies in PBS or human serum and incubating them with the epoxy-coated slides containing immobilized antigens. The use of human serum was to determine how specific and sensitive the antigen–antibody reactions would be when the antibodies were in the presence of proteins that are commonly found in human blood. The concentrations evaluated for the HIV and TB antibodies on the epoxy slides ranged from 0.000954 to 250 $\mu\text{g/mL}$, where a 64-fold serial dilution was performed. The

concentration range for the primary antibodies selected for this study is similar to what is commonly found in human blood during HIV and TB natural infections [39,40]. From the results obtained, the lowest detection concentration of the primary antibodies against all antigens was determined to be 0.000954 $\mu\text{g/mL}$ for both PBS- and human serum-diluted antibodies (Table 1). The highest fluorescence intensity and the brightest spots were observed at the highest concentration of 250 $\mu\text{g/mL}$ for all antigen–antibody interactions, and this intensity decreased with decreasing antibody concentration (Figure 6). No fluorescence was observed on the negative control spots where PBS was spotted instead of antigens. Although the linearity of the antibody binding curve was observed to be unexpectedly better when antibodies were diluted in human serum than in PBS (Appendix A, Figure A1), in general comparable results (same limit of detection) were obtained in both cases. This implies that proteins found in human serum are not likely to interfere with our diagnostic technology antigen–antibody reactions.

Table 1. A head-to-head comparison of sensitivity between the epoxy-coated glass slide and high-binding plates.

Primary Antibody	Limit of Detection Concentration ($\mu\text{g/mL}$)			
	Primary Antibodies Diluted in PBS		Primary Antibodies Diluted in Human Serum	
	Epoxy-Coated Glass Slides	Polystyrene High-Binding Microtiter Plates	Epoxy-Coated Glass Slides	Polystyrene High-Binding Microtiter Plates
Anti-p24	0.000954	0.061	0.000954	0.9765
Anti-ESAT6	0.000954	0.06138	0.000954	0.9765
Anti-CFP10	0.000954	0.06138	0.000954	0.9765
Anti-pstS1	0.000954	15.62	0.000954	15.62

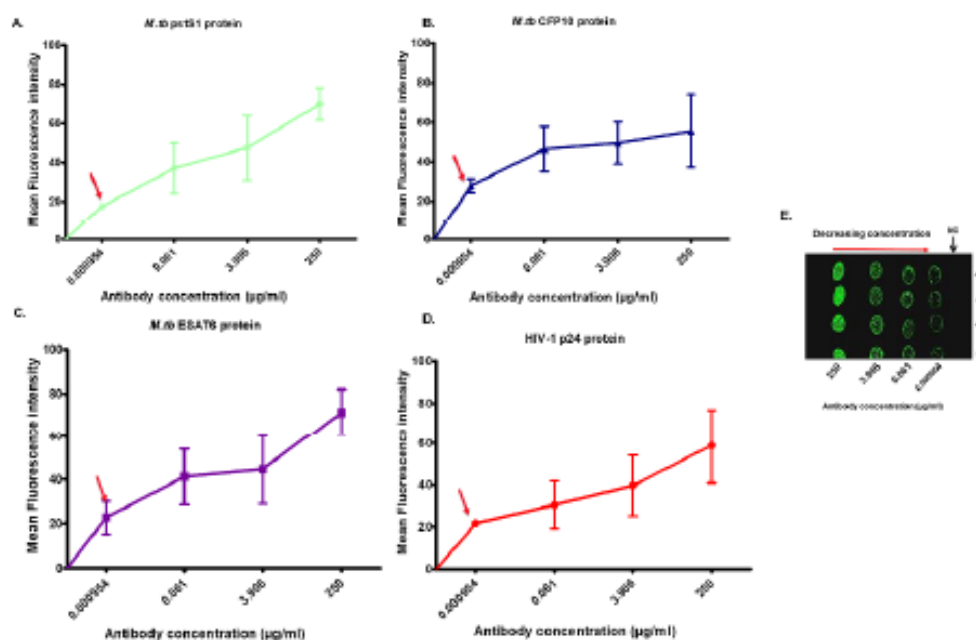


Figure 6. Evaluation of the sensitivity of the assay using epoxy-coated glass slides. Primary antibodies were diluted in human serum to mimic in vivo conditions where there are many other proteins present. The antigen and secondary antibody concentrations were kept constant. Fluorescence intensity values for pstS1 (A), CFP10 (B), ESAT6 (C) and p24 (D) are presented as line graphs for the different antibody concentrations, and the limit of detection is indicated by the red arrow. Data represent mean $\pm$ standard deviation ($n = 4$). (E) A representative image of the slide used to plot the graphs.

The limit of detection was determined to be the lowest concentration of the primary antibody to give a fluorescence signal after subtraction of the background fluorescence (fluorescence emitted by the negative control spot).

Since the use of high-binding plates for immunoassays is the gold standard [36,41], we replicated our microarray technology-based immunoassay (with primary antibodies diluted in PBS or human serum) in these plates to compare their performance. We observed that the limit of detection obtained from the high-binding plates was higher compared to the epoxy slides when the antibodies were diluted in either PBS or human serum (Figure 6, Appendix A, Figures A1 and A2). To be precise, this limit in PBS was between 64 to 16,373-fold higher using the plates compared to the microarray slide, and the difference was statistically significant ($p < 0.05$) (Table 1). When the assay was performed in human serum, the limit of detection for the plates was lowered by 1023 to 16,373-fold compared to the microarray platform and the difference was statistically significant ($p < 0.05$). In conclusion, our diagnostic technology on microarray slides showed significantly higher sensitivity than when the same assay was performed on the high-binding plates which are commonly used.

Lastly, to determine the specificity of our diagnostic technology, i.e., the absence of cross-reactivity, microarray slides containing all the antigens were incubated with a human serum cocktail of the primary antibodies, missing one antibody at a time. This was done to see if the antigen corresponding to the absent antibody would bind to the remaining primary antibodies in the cocktail. In another variation of this experiment, we incubated the four antigen-coated microarray slides with only one antibody at a time to see if this antibody would react with the other antigens besides its own. In either experimental set-up, we observed no cross-reactivity between the antigens and antibodies (Figure 7 and Appendix A, Figure A3). In all, these data suggest that our microarray-based technology is likely to perform with high specificity.

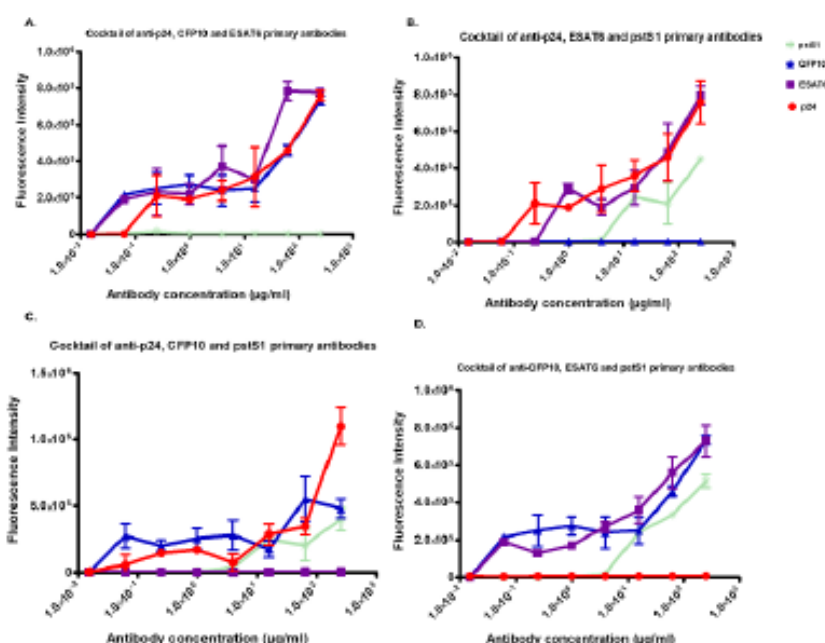


Figure 7. Microarray plates' antigen-antibody reaction specificity in human serum. Immobilized antigens were incubated with cocktails of primary antibodies, missing one antibody at a time. Antigens and secondary antibody concentrations were kept constant, while primary antibodies were serially diluted. (A) A line graph representing the fluorescence intensity obtained when immobilized antigens were incubated in a cocktail of primary antibodies missing the anti-pstS1 antibody; (B–D) graphs for anti-CFP10 or anti-ESAT6 or anti-p24, respectively. Data represent mean $\pm$ standard deviation ($n = 4$).

4. Discussion

In this study, we sought to develop a multiplex technology for the detection of HIV and TB co-infection. Here, epoxy functionalized slides were used to immobilize HIV-1 p24 as well as *M.tb* antigens CFP10, ESAT6 and pstS1 using covalent chemistry, and these were used to capture their respective antibodies in solution. The sensitivity and specificity of this multiplex microarray-based antibody–antigen reaction was then studied and compared to the gold standard platform, i.e., high-binding 96-well plates. We found that the antigen–antibody reactions on the microarray slide were significantly more specific and sensitive compared to results obtained in high-binding plates. In fact, the limit of detection of the primary antibodies using our microarray-based diagnostic technology was on average about 4690-fold lower compared to the plates. We have also shown that the microarray technology in this study is stable at different pH and temperature conditions, as well as able to be stored dry for at least three months. These findings suggest that our microarray-based technology has potential for use to diagnose HIV and TB co-infection. Currently it is likely to be the first to do so, and the immunoassay conducted using this platform will probably perform better than when compared to commercially available kits that use the high-binding 96-well plates. These findings support further development of this technology for application in HIV and TB co-infection diagnostic.

The fact that the human serum we used was spiked with HIV and TB antibodies at a concentration that is commonly found in infected individuals [39,40] and that we were able to detect these antibodies using our microarray technology suggests that this technology is likely to work when actual human samples are used. Previous investigators have also spiked the analyte in human serum and found that the results could be replicated when actual human samples were used [42–44]. Furthermore, the use of human serum mimicked in vivo conditions where the HIV and TB antibodies could be present in the presence of other human proteins. The data obtained were compared to that of gold standard high-binding plates, and we found that the slides were more sensitive, with the average limit of detection determined to be 0.000954 $\mu\text{g/mL}$ for antibodies against the four antigens, while for the plates this limit was on average 4.637 $\mu\text{g/mL}$. To explain the striking difference of the results obtained from these two platforms, it is important to note that on the slides the antigens are immobilized using a covalent bond while on the high-binding plates they are immobilized by adsorption using van der Waals, hydrophobic and/or electrostatic interactions [45]. Since the covalent bond is stronger than the van der Waals, hydrophobic and/or electrostatic interactions [46,47], the chance of some of the immobilized antigens being lost, for example by washing out during the assay, is greatly reduced on the slides compared to the plates. It is also possible that the high sensitivity of the microarray technology compared to the high-binding plate was due to the instrument used for the detection of fluorescence, as the slides were read using the cytation3 multimode reader while for the plates we used the plate reader DTX 880 Multimode detector. Nonetheless, the fact that we observed similar sensitivity when comparing PBS and human serum results for either the microarray slide or the microtiter plate indicates that the presence of other human proteins did not interfere with the antigen–antibody reaction on these two platforms. The similarity in this particular characteristic between the microarray slides and the high-binding plates was somewhat expected, because the interaction between the antigen–antibody does not rely on the surface chemistry of the microarray slides or high-binding plates as long as the immobilized antigen is in the correct conformation. Lastly, we did not observe any cross-reactivity between the antigen–antibody reaction on the microarray slides. This is consistent with numerous studies that have shown the high specificity of this reaction [48–50]. Also, the high specificity of protein–protein interactions on the microarray slide has been previously described; for example, Koukouvinos et al. studied the cross-reactivity of mycotoxins in a protein microarray and found that the antibody–mycotoxin reactions were highly specific [51].

The above being said, similar ELISA-based technologies, such as the electrochemical impedimetric ESAT6 immunosensor [52], gold-nanoparticle-based lateral flow TB

immunoassay [53] and HIV p24 detection using combined immunoprecipitation and digital ELISA [54], have generally reported limits of detection less than ours of 0.000954 µg/mL (Table A1); however, our assay still has the main advantage of being able to detect HIV and TB at the same time. In addition, the combination of the selected three TB antigens is likely to enable our assay to detect active TB [32–35].

Since proteins have three-dimensional structures and can easily lose their reactivity due to factors such as pH and temperature, they are prone to denaturation during printing and immobilization if the conditions are not ideal [55]. Our data suggest that the proteins captured on our microarray slides are likely to remain stable at room temperature and neutral pH. This is critical, as routine laboratory diagnostics are commonly conducted at room temperature [56–58] and human blood has a pH of 7.4 [59,60]. Also, the fact that storage in dry conditions did not affect the performance of our microarray slide indicates that they can be stored without special requirements, which is very important, especially for resource-poor settings. Here, it is worth noting that HIV and TB co-infection is a bigger problem in developing countries, which often have limited resources [61,62]. Lastly, we tested the shelf-life of our technology for three months, and during this period we observed that fluorescence could still be obtained on all the antigens spots printed on the slide even on day 90. Thus, it is possible the actual shelf-life of this diagnostic technology is longer than three months, which could offer more advantage in resource constrained settings. However, this must be proved experimentally by conducting experiments beyond the time frames measured in this study.

For coating the microarray slide, we selected epoxy as the functional group, as it interacts with the amine group at the N-terminus of the protein for immobilization [10,63]. This ensured that the immobilized protein is free to interact with its target [10,63]. Yousefi et al. showed that the epoxy functional group performed better as a microarray slide coating agent when compared to other functional groups [30]. Thus, it is unlikely that different functional groups would have performed better than the epoxy group used in this study. However, this study can still be replicated in future with these functionalized groups to compare the performance of the assay.

As for current limitations of the study, we think the data presented here can be complemented by those using actual human blood samples of HIV and TB co-infected patients. This being said, we are also aware that many investigators in the past used human serum spiked with antibodies, the same as we did here, for validation of technologies meant to detect biomarkers in human blood samples [64–68]. In addition, a thorough comparison with other diagnostic methods for active TB such as *M.tb* culture or HIV PCR and Western blotting will be very useful in assessing our novel technology performance. Lastly, as stated above, a longer storage study as well as a wider range of pH and temperature studies could be of benefit for further development of this technology.

In conclusion, we have shown that HIV-1 p24 as well as *M.tb* CFP10, ESAT6 and pstS1 antigens can successfully be conjugated or printed on epoxy-coated glass slides under different pH and temperature conditions. We have also shown that the immobilized antigens on the slides can be stored for at least three months without compromising their function. Most importantly, these antigens were able to capture their complementary primary antibodies in human serum, and the multiplex microarray diagnostic technology showed high specificity and sensitivity when compared to immunoassay performed on high-binding plates. Thus, this is a promising diagnostic technology that requires further development for the detection of HIV and TB co-infection.

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Appendix A

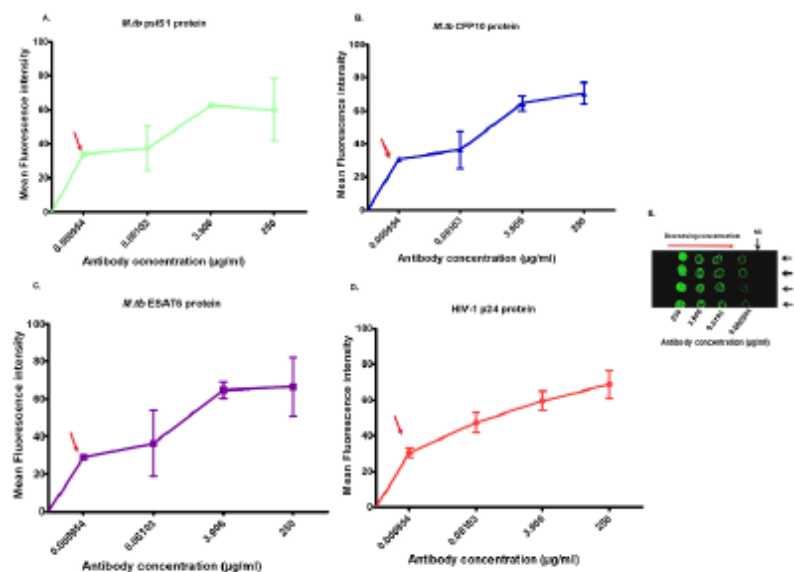


Figure A1. Evaluation of sensitivity of epoxy-coated glass slides assay in PBS. Primary antibodies were serially diluted in PBS while the antigen and secondary antibody concentrations were kept constant. Fluorescence intensity values for pStS1 (A), CFP10 (B), ESAT6 (C) and p24 (D) are presented as line graphs, and the limit of detection is indicated by the red arrow. Data represent mean $\pm$ standard deviation ($n = 4$). (E) A representative image of the fluorescence emitted on the microarray slide.

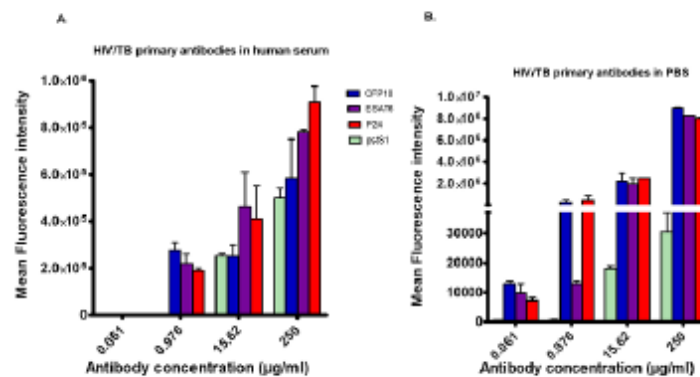


Figure A2. Determination of antigen-antibody reaction sensitivity for the microtiter plates. The concentrations of immobilized antigen and secondary antibodies were kept constant while that of the

primary antibodies varied. (A) shows the fluorescence intensity obtained when HIV and TB antibodies were diluted in human serum, while (B) shows the intensity obtained when these antibodies were diluted in PBS. Error bars represent mean $\pm$ standard deviation ($n = 4$).

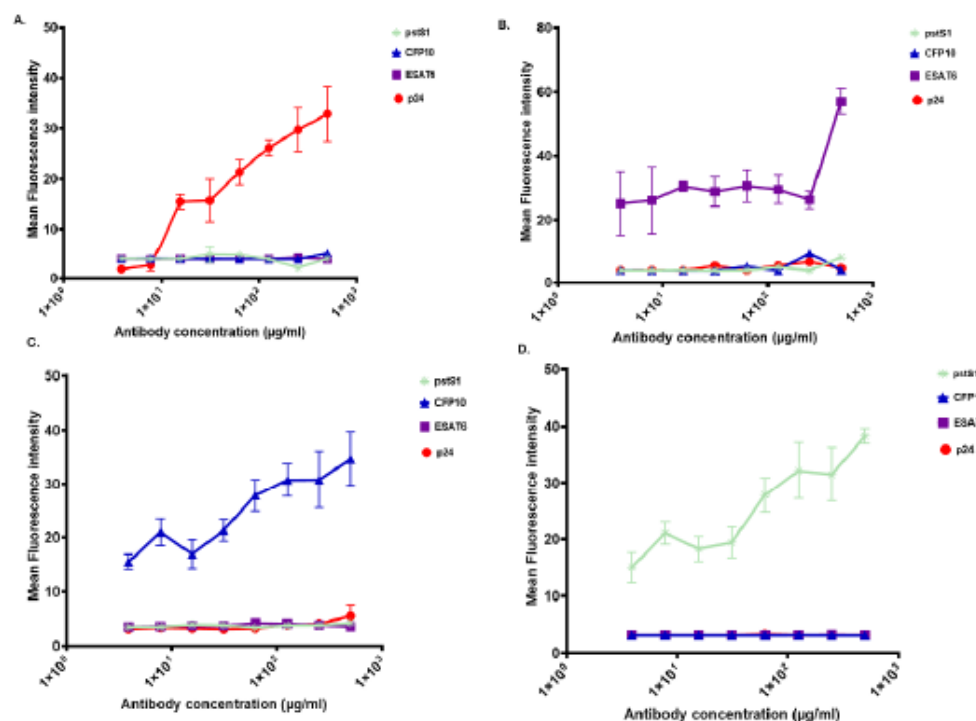


Figure A3. Alternative set-up of Microarray plates' antigen-antibody reaction specificity in human serum. The immobilized antigens were incubated with the anti-p24 antibody (A), anti-ESAT6 antibody (B), anti-CFP10 antibody (C) or anti-pstS1 antibody (D) only, before the addition of the Alexa fluor 488-labelled secondary antibody. Data represent mean $\pm$ standard deviation ($n = 3$).

Table A1. Head-to-head comparison of the multiplex HIV/TB microarray technology reported here and ELISA-based technologies in the literature for the detection of HIV or TB.

Method	Used Material	Target Matrix	Limit of Detection (LOD)
Multiplex HIV/TB microarray technology	-CFP10, ESAT6, pstS1 and p24 antigens and primary antibodies -Alexa fluor-conjugated secondary antibodies for fluorescence detection	-Human serum	-CFP10, ESAT6, pstS1 and p24 antibodies LOD of 0.000954 µg/mL
Electrochemical impedimetric ESAT-6 immunosensor [52]	-ESAT6 proteins and monoclonal antibodies -Label free (uses Ultrasensitive electrochemical immunosensors)	-Mice serum samples	-ESAT 6 protein LOD of 7.0×10^{-6} µg/ml
Gold Nanoparticle (AuNps)-based Lateral flow TB immunoassay [53]	-ESAT6 and CFP10 antibodies and antigens -AuNps-conjugated antibodies for colorimetric labelling	-Non-sputum samples	-ESAT6 antigen LOD of 0.0000625 µg/mL -CFP10 antigen LOD of 0.00769 µg/mL
HIV p24 detection using combined immunoprecipitation and digital ELISA [54]	-p24 antigen and p24 monoclonal antibodies -Biotin-labelled anti-p24 antibody	-Cell lysate	-p24 protein LOD of 5.0×10^{-9} µg/mL

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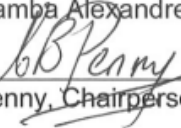
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APPENDIX C

Ethics clearance certificate

R14/49 Miss Kanyane Malatji

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M220866 MED22-05-046

NAME: Miss Kanyane Malatji
(Principal Investigator)
DEPARTMENT: School of Pathology
Council for Scientific and Industrial Research (CSIR)
PROJECT TITLE: Development of multiplex HIV/TB Point of Care
diagnostic assay based on the microarray Technology
DATE CONSIDERED: 26/08/2022
DECISION: Approved unconditionally
CONDITIONS:
SUPERVISOR: Dr Kabamba Alexandre and Dr Christina Thobakgale
APPROVED BY: 
Dr C Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL: 15/09/2022

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 August and will therefore be due in the month of August each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).



Principal Investigator Signature

31 October 2022

Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX D

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ABSTRACT

HIV/AIDS mortality is caused by opportunistic illnesses/infections that take advantage of the weakened immune system in infected individuals. In Africa, the most common of these opportunistic illnesses include infection by *Mycobacterium tuberculosis* (*M. tb*) responsible for tuberculosis (TB). HIV co-infection with *M. tb* has negative implications for disease management given that each pathogen accelerates the morbidity caused by the other. The effective management of patients infected with both pathogens is restricted by the fact that their diagnosis is done separately. The situation is more difficult in remote areas where patients must wait for much longer to obtain their TB diagnostic results. In addition, the current diagnostic tests for the detection of TB such as chest X-ray and bacterial culture have a long turnaround time, are expensive to perform, and require sophisticated equipment and trained personnel. It is in this context that my project sought to develop an HIV and TB multiplex microscopy-based assay for the detection of the two diseases, using one test. The project used a 2.5 x 7.5 cm epoxy-coated glass slide as well as high-binding 96 well plates to which HIV-1 p24 and *M. tb* CFP10, ESAT6 and *poB1* antigens, known to be markers of active TB, were immobilized. The immobilized antigens were then incubated with anti-p24, anti-CFP10, anti-ESAT6 and anti-*poB1* primary antibodies diluted in human serum to mimic physiological conditions where the antibodies would exist in the presence of other proteins. Detection of binding between the antigens and primary antibodies was achieved by means of secondary antibodies conjugated to either a fluorescence dye or horseradish peroxidase (HRP). In chapter two of the study, the immobilization of the HIV and TB antigens on the epoxy-coated glass slides as capture molecules of the HIV and TB antibodies diluted in human serum was performed. The antigen-antibody reactions detection was achieved by means of fluorescence dye conjugated secondary antibodies. This chapter also covered the sensitivity and specificity of the technology where the epoxy-coated glass slides were compared to the gold standard 96 well high-binding plates. Data showed that the HIV and TB antigen-antibody reactions were specific, and the slides were more sensitive relative to the 96 well high-binding plates with limits of detection many folds lower. To be specific, the limit of

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