LATERAL FLOW NUCLEIC ACID BIOSENSOR FOR THE DETECTION OF SEXUALLY TRANSMITTED DISEASES

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ABSTRACT

Nucleic acid detection is of central importance for the diagnosis and treatment of genetic diseases, infectious agents, bio-warfare agents, and drug discovery. Nucleic acid testing for diseases is exclusively performed in laboratories using high-end instrumentation and personnel. However, this has developed the need for point of care diagnostics which can provide near-patient testing in a clinic, doctor's office, or home. Such diagnostic tools can prove advantageous when rapid response is required or when suitable facilities are unavailable. Compared to equivalent methods used in laboratories, point of care testing is more affordable, as it eliminates the need for expensive instrumentation and skilled labor. One option involves the use of lateral flow assays. Pre-fabricated strips of dry reagents activated upon fluid application are already used in diagnostics, such as to ascertain pregnancy. Nucleic acid based detection assays on lateral flow offer several advantages over traditional microbiological detection methods.

In this work we introduce a lateral flow biosensor that can combine the optical properties of nanoparticles (such as gold nanoparticles) with conventional immunoassay techniques to deliver a simple platform for rapid analysis of DNA with high sensitivity and selectivity. The quick 30 minute assay provides a platform to detect multiple nucleic acids with high efficiency achieved via chromatographic separation sandwich-type DNA hybridization reactions. Captured gold nanoparticles on the device can provide qualitative analysis by observing the color change to red and a semi-quantitative analysis via a strip reader. The biosensor was applied to the detection of human genomic DNA directly with high sensitivity and selectivity. The work was further expanded to detect *Chlamydia trachomatis* and *Neisseria gonorrhoeae* samples using nucleic acid amplification to generate large numbers of target copies. Improvements were made in the preparation of the biosensor to enable detection of Human Papilloma Virus Type-16. The

clinical samples obtained were amplified using PCR for direct detection on the lateral flow biosensor without interference from other HPV types (e.g. HPV 18). The feasibility of the biosensor shows great potential for further development to assure its use in point of care diagnosis. The promising properties of the biosensor are reported in this dissertation.

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DEDICATION

This dissertation is dedicated to my parents **Ranveer Singh Baloda** and **Vinod Kumari** in appreciation to their infinite love and moral support throughout my life. Their encouragements and blessings motivated me every day to fulfill my ambition of earning a doctoral degree in Biochemistry.

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LIST OF ABBREVIATIONS

µ…….Micro (concentration)

AuNP…….Gold Nanoparticles

ATP……....Adenosine Triphosphate

BSA……Bovine Serum Albumin

CT……..*Chlamydia trachomatis*

DNA……...Deoxyribonucleic Acid

HPLC…….High Performance Liquid Chromatography

HPV……...Human Papilloma Virus

M……....Molar

n…….Nano (concentration)

Na_3PO_4…….……..Sodium Phosphate

NG……..*Neisseria gonorrhoeae*

NTC……Non Template Control

O.D. ……...Optical Density

RPM……...Rotations per Minute

RT……..Room Temperature

STIs……Sexually Transmitted Infections

PBS…….Phosphate Buffered Saline

CHAPTER 1. INTRODUCTION

This section provides the introduction about detection of multiple samples of nucleic acids on Lateral Flow Biosensors and its application on a real sample for *C. Trachomatis*, *N. Gonorrhea* and HPV 16 analyses, respectively. This chapter also discusses DNA-based biosensors, traditional and current methods to detect samples of nucleic acids, an introduction to sexually transmitted infections, and current methods to diagnose *Chlamydia trachomatis, Neisseria gonorrhoeae* and HPV-16.

1.1. DNA Detection

The fundamental basis of the detection of specific nucleic acid sequences is base pairing between complementary strands of DNA or RNA. However, at high temperatures (e.g. 90°C to 100°C) the complementary strands of DNA denature or separate from each other, resulting in two single strands of DNA. If the denatured strands of DNA are incubated under appropriate conditions they can result in re-hybridization to form a double stranded molecule. This nucleic acid hybridization provides a platform for the detection of DNA or RNA sequences that are complementary to nucleic acid sequences isolated as a desired target. One of the traditional methods to detect specific sequences of nucleic acid was developed by Edward M. Southern in 1970s. The technique, southern blotting, combines the transfer of DNA fragments from an agarose gel to a filter membrane and subsequent detection by labelled probe via hybridization.

According to Thurmon (2014), DNA detection includes a group of procedures in which DNA extracted from the cells of the patient is tested in lab for changes suspected of causing a disease. DNA detection is also used to collect other data essential for appropriate health care. Assuring the accuracy of disease diagnosis is the most essential reason for DNA detection, as several diseases can exist in a form that is identifiable using these methods. DNA detection is

also important in determining liability for financial assistance and for inheritance legalities. Kuhr (2000) mentioned that the reliability and rapid detection of small fragments of RNA/DNA has become highly essential in diagnosis of molecules.

Umek et al. (2001) stated that electrochemical technologies have revealed the critical importance of offering viable solutions to the challenge of detection. Individually addressable arrays of microelectrodes have been described for diagnosis of molecules which can be combined directly with microfluidics and microelectronics systems to realize benefits in multiplex detection and miniaturization. Additionally, Li et al. (2003) described that elements of nanoscale sensing can be integrated to obtain ultrahigh sensitivity. Nano-electrodes, specifically a carbon nanotube-based nano-electrode array, can be combined into an EC system for ultrasensitive detection of chemicals and DNA. Therefore, it can be inferred that DNA detection plays an essential role in different fields related to health care.

1.1.1. Methods to detect DNA

According to the BBC (2013), forensic, genetic and parental testing look for differences in genetic markers between two biological samples because all body cells contain similar DNA, so samples can be taken from anywhere on the body: including hair follicles, blood, skin and other fluids of the body. DNA is separated first from the cells and millions of copies are made using a method known as PCR (polymerase chain reaction); it utilizes a naturally existing enzyme to copy a particular expansion of DNA repeatedly. Having numerous copies of DNA makes the genetic code easier to examine.

There are numerous methods to detect DNA. One such method is DNA sequence identification. Nam, Stoeva and Mirkin (2004) identify another method of DNA detection: the method of polymerase chain reaction itself. PCR has had a powerful influence in the medical and

biological communities. It revolutionized medical diagnostic systems that depend on quantifying and detecting DNA. However, PCR is criticized for its costly, complex, labor-intensive and time-consuming process, and wider target range of DNA quantification after amplification. According to Gaylord, Heeger and Bazan (2002), DNA sequence identification methods are of huge economic and scientific interest. Their application involves examination of genetic mutations, medical diagnostics, specific genomic techniques and gene delivery supervision. Wang (2000) has mentioned that cationic organic dyes such as thiazole orange and ethidium bromide discharge when intercalated inserted into the double stranded DNA grooves, and serve as direct hybridization probes of DNA. Schork et al. (2000) described the difficulties in simultaneously labeling two sites of DNA, resulting in reduced yields, greater costs and individually labeled impurities which reduce detection sensitivity. Most of the motivation behind developing DNA detection methods is to enhance economic and procedural factors for estimating strand-specific hybridization that uses homogenous fluorescence assays easily, with reduced DNA alterations, and developing amplification of signals.

Bustin (2002) has mentioned that numerous assays of DNA detection have been expanded using molecular fluorophores, radioactive labels, electrochemical tags, chemiluminescence schemes, and labels based on nanostructure. Makrigiorgos et al. (2002) stated that it would be beneficial for a researcher to expand a methodology that permits for PCR-like amplification of signal and eliminates its disadvantages. A biological bar code-based approach can detect the targets of DNA in addition to targets of protein. According to Nicewarner-Pena et al. (2001), a novel approach to detect DNA has been developed that offers greater selectivity with a sensitivity that is comparable to numerous PCR based approaches without the requirement for enzymatic amplification. The major benefit of the DNA bio bar code

approach over conventional microarray sandwich assays is that the complete assay can be performed in three to four hours regardless of target concentration. The system has a dynamic range and is ideal for multiplexing.

Gillet, Mori, van Esbroeck, van den Ende, and Jacobs (2009) have identified the biosensor as the analytical instrument used in analyte detection–the mixture of the biological component with a physicochemical detector. DNA biosensors involve the use of DNA as sensory devices that could theoretically be used in medical diagnostics, agriculture, and forensic science and in environmental clean-up, food analysis, the study of bimolecular reactions and their interactions, drug development, crime detection, medical diagnostics, environmental field monitoring, quality control, industrial process control, detection systems for biological warfare agents, and the manufacturing of pharmaceuticals and replacement organs. Mathur et al. (2012) has mentioned that there are four types of DNA-based biosensors: optical, piezoelectric, DNA chips and electrochemical biosensors. Piunno et al. (1994) noted that optical biosensors of DNA are based on fiber optics, which transduces an emission signal to a fluorescent label and can conduct light from one place to another via internal inflections. The advantages of optical biosensors are the sensitivity of optical approaches and the use of portable instruments; however, they suffer the disadvantage of the high cost associated with the equipment. According to Kerman, Kobayashi and Tamiya (2004), electrochemical biosensors of DNA are useful for sequence-specific DNA biosensing. The inherent miniaturization of electrochemical components and advanced micro-fabrication techniques make biosensors a powerful tool for DNA identification. Lazerges et al. (2006) discussed the piezoelectric biosensor of DNA as a mass-sensitive tool which relies on quartz crystals that oscillate at defined frequencies when oscillation voltage is applied. Attention has been given to development of the piezoelectric method due to its low cost, real-time label-

free detection, sensitivity and simplicity. Arora and Malhotra (2008) described DNA biochips as solid, small devices which themselves are microscopic slides, but can be nylon membranes or silicon chips onto which sequences from thousands of different genes are attached or immobilized at fixed places.

1.2. Lateral Flow Nucleic Acid Biosensor

According to Henderson (2000), Lateral Flow Biosensors is a technique that is used to detect proteins, small molecules and viral antigens. This enables the rapid POC (point of care) diagnosis of infections and diseases like malaria, dengue fever and HIV. The lateral flow biosensor technique also detects cardiac markers, cancer etc. The lateral flow format uses a sandwich-like structure such as that used in immunoassay: it has two antibodies that are bound to perform sandwich-style analysis. One antibody, mAb, is bound, initially non-covalently, in a horizontal stripe on a narrow strip of nitrocellulose. The nitrocellulose is void of protein to avoid vague observance of analyte and other proteins. The analyte and a second labeled antibody (classically, it is labeled with colloidal gold) are permitted to stream up nitrocellulose.

Edwards (2006) has mentioned that the existing lateral flow (immuno-) assay (LFA) methods are the association of a fluid model, or its extort surrounded the analyte besides a slip of polymeric material thus fleeting separated zones wherever molecules have been naïve of further or fewer brief communication with analyte. Several layouts have been described for LFAs. These layouts are chosen on the basis of the desired analyte. Mudanyali, Dimitrov, Sikora, Padmanabhan, Navruz, and Ozcan (2012) have described several possible layouts for the lateral flow of nucleic acid, especially in third-world countries. These tests are acceptable for biomedical applications since there is no need to keep the strips refrigerated. They have a long

shelf life with little variation in the quality of batches. LFAs are intended for single-use applications, so there is no risk of prior-use contamination.

Carter and Cary (2007) have identified a method to detect DNA using LFAs. Lateral flow detection of RNA or DNA amplification reaction products offers a method of simplifying detection of nucleic acids. Dineva et al. (2005) has mentioned that lateral flow devices have been fabricated predominantly using more than one capture line, permitting more than one analyte. As a step toward improving information detection of lateral flow nucleic acids, the researchers have developed nitrocellulose patterning methods that allow microarray characteristic densities to be met on compatible substrates of lateral flow. Thus, the lateral flow microarray method develops sequence-specific detection, opening the door to increasingly multiplexed implementations for a vast number of assays, well-suited for point of care and other field applications.

1.3. Sexually Transmitted Infections

Sexually transmitted infections (STIs) also referred to as sexually transmitted diseases or venereal diseases. These infections are primarily passed from one person to another sexually. STIs can spread through vaginal, anal or oral sex, or through some other non-sexual means, such as the sharing or re-use of sub-dermal needles. About 25 different types of STIs are known that show a broad range of symptom severity, and in some cases are asymptomatic. Certain STIs can also be transmitted from a pregnant woman to her child in utero. The symptoms of STIs vary, but common symptoms include: site-specific pain, unusual lumps or sores, pain during urination, itching and/or unusual discharge. Most STIs can be cured easily, but if these infections are left untreated, they can result in severe symptoms and long term damage (e.g. infertility). Some common and most wide-spread STIs are: Chlamydia, gonorrhea and genital warts, which are discussed in the following sections in more detail.

1.3.1. *Chlamydia trachomatis*

Chlamydia is a common sexually-transmitted disease caused by the infection of *Chlamydia trachomatis* (CT). According to Geisler (2011), CT is a gram-negative bound intracellular micro-organism that usually infects squamocolumnar epithelial cells. CT generally involves the bacterial strains of the genus *Chlamydophila* and *Chlamydia*. CT can be differentiated into 18 serologically variant strains on the basis of monoclonal antibody-based typing assays. *Chlamydia trachomatis* is the cause of infections with numerous severe problems. Because most of the patients affected by CT are asymptomatic, such that an essential proportion of them remain unidentified, the disease can cause infectious outbreak problems. *Chlamydia trachomatis* is conveyed through the mucous membranes of the rectum, urethra, throat and conjunctive tissues. Similarly, Hammerschlag (2011) has noted that CT infections infect the urethra, cervix, uterus, epididymis, salpinges and nasopharynx. It is usually reported that bacterial sexually-transmitted disease in the U.S. is a major cause of sterility in women.

Chlamydial trachomatis infection influences other diseases as well, including: pneumonia, pneumonitis, conjunctivitis and afebrile pneumonia syndrome. According to Manavi (2006), CT has been reported to cause similar STI issues in the United Kingdom. In fact, untreated chlamydial infection cases can lead to PID (pelvic inflammatory disease), infertility, chronic pain and ectopic pregnancy in women. During labor, untreated chlamydia infection can be transmitted to the newborn and cause pnuemonitis and conjunctivitis in infants. Therefore, an infected mother can infect her baby during vaginal delivery. Untreated chlamydia infections in men can lead to epididymo-orchitis. Because of the long-term influence of chlamydial infection to young adults' health, it is essential to identify and treat affected patients and their partners.

La Montagne et al. (2004) noted that, although screening programs handled by genitourinary medicine physicians are important, it is essential that urologists, general practitioners and gynecologists have adequate training and knowledge to handle chlamydia infections and its associated health and reproductive problems. Watson et al. (2002) has mentioned that the asymptomatic nature of chlamydia infection makes screening important to manage the public health concerns of this infection. Jang et al. (1992) stated that, because of the seriousness of the problems of CT infection and their impacts on health and economics terms, other countries—including France, the United Kingdom, Finland and the Netherlands—have taken action to lower the prevalence of CT infection. To be efficient, a national screening program must make a precise diagnostic test accessible. It is essential that any test utilized in a national screening program can be used in a primary care setting without the requirement for costly training.

1.3.2. *Neisseria gonorrhoeae*

Knapp et al. (1994) identifies *Neisseria gonorrhoeae* (NG) as a bacterium that is transmitted sexually and is characterized by a symptom spectrum ranging from asymptomatic infection to pelvic inflammatory disease. Gonorrhea is another gram–negative, infectious bacterium and a fastidious diplococcic that can develop and multiply rapidly in mucous membranes, particularly those of the anus, mouth and throat, and the fallopian tubes, uterus and cervix of the female reproductive tract. Uncomplicated gonorrhea infects nearly 650,000 people every year. Fox et al. (1997) has described that the commonness of *Neisseria gonorrhoeae* in the United States and abroad, particularly in developing and under-developed countries, has been reduced in past two decades. Presently however, greater rates of infection have been discovered due to development of antimicrobial-resistant gonococci. The infection of NG can be prevented

using safe sexual practices and condoms. According to Hook and Handsfield (1999), the NG infection is sometimes asymptomatic among females; if untreated, *Neisseria gonorrhoeae* infection can lead to pelvic inflammatory disease, ectopic pregnancy, chronic pelvic pain and tubal infertility. NG generally causes symptomatic urethritis among males, and occasionally presents in epididymitis. Rarely, local infection spreads to affect an acute dermatitis tenosynovitis syndrome, which can lead to meningitis, endocarditis or arthritis. *Neisseria gonorrhoeae* can cause serious conjunctivitis which can result in blindness if left untreated, and can rarely cause sepsis with related meningitis, arthritis or endocarditis. According to the study of Unemo and Dillon (2011), NG can affect a newborn's eyes during delivery through the infected mother's birth canal, which can result in blindness.

Globally, *Neisseria gonorrhoeae* public health concerns are similar to those for human immunodeficiency virus (HIV) and other bacterial STIs. Recognition of cases and efficient treatment with antibiotics are primary approaches for control and prevention of NG. However Buve, Gourbin and Laga **(**2008) have mentioned that there are new global concerns considering new gonorrhea incidents in several countries, coupled with the prevalence of greater resistance to antimicrobial agents previously used for treatment, coupled with with lowered resistance and/or susceptibility to modern antimicrobial agents such as extended spectrum cephalosporins and azithromycin. Therefore, Tapsall et al. (2009) notes that NG may become untreatable in some circumstances, wherein the organism has developed as a "superbug." International efforts to gather data on developing trends in antimicrobial susceptibility, coupled with national, regional and international surveillance of epidemiological features and the spread of *Neisseria gonorrhoeae* have become a priority of public health efforts. However the availability of performing reliable, phenotypal, antimicrobial testing of susceptibility has been reduced because

NAATs (nucleic acid amplification tests) are rapidly replacing the culture for gonorrhea diagnosis.

1.3.3. Human papilloma virus

Human papilloma virus (HPV) is among the most common sexually transmitted infections. HPV is so common that almost all sexually active men and women get it at some point in their lives. According to the CDC (2012), human papilloma viruses are small, double-stranded viruses of DNA that affect the epithelium. More than 100 types of human papilloma viruses have been identified, distinguished by their outer capsid protein L1 genetic sequences. Among the strains of HPV known, certain varieties cause health problems such as warts and cancers. Those HPV that affect the cutaneous epithelium create warts in the skin. However, nearly forty strains affect mucosal epithelia. in addition to causing cervical cancer, infection of mucosa by HPV is associated with anogenital cancers, such as cancer of the vagina, vulva, penis, and anus.

According to Munoz et al. (2006), HPV is often not the sole contributing factor to the development of cervical cancer. The long-term use of hormonal contraceptives, tobacco use, and co-infection ofHIV have been recognized as cofactors to the development of cervical cancer with HPV; co-infection with HSV-2 (herpes simplex virus type 2) and CT, some dietary deficiencies and immuno-suppression are other likely cofactors. Bhatla and Joseph (2009) state that HPV epidemiology in cervical cancer formed the basis for determining the two most prevalent kinds of HPV globally: HPV 16 and HPV 18, which, they state, must be included in first prophylactic vaccines against HPV. Compared to most viral vaccines that are based on attenuated virus, a vaccine for HPV was not feasible before because there was no efficient system of culturing the virus. Roden et al. (1996) described that the human response to genital infections of HPV is

characterized mainly by local cell mediated immunity and is related to lesion regression, which would then prevent future infection with a similar genotype of HPV. The HPV L1 vaccines of virus-like particles that have been developed commercially protect versus HPV 16 and HPV 18, which together account for 70% of cervical cancers globally. Roden, therefore, determined that an HPV vaccine could be developed based not upon the viral DNA, but the protein coat that surrounds it.

Bharti et al. (2009) described both conventional and alternative therapeutic methods for the treatment of HPV. Even after the identification of the causal relationship between cervical

cancer and HPV, the absence/presence of the virus does not influence treatment strategies, which are primarily anti-cancerous. Conventional therapies involve traditional Western cancer therapies, such as. Molano et al. (2003) described alternate therapies of HPV, involving immunotherapy, photodynamic therapy and treatment with cytotoxic agents. Though excision is the most preferred process for genital warts, topical cytotoxic preparations like trichloro-acetic acid and podophyllin acid are used in Europe and the USA. Most of these therapies reveal inconsistent or no antiviral response against HPV. Similarly Nagai et al. (2000) noted that in immunotherapy, interferons are the only antiviral drugs approved for benign HPV-related lesion therapy. Photodynamic therapy is a new treatment for various premalignant dysplasias and malignant tumors, as well as for non-cancerous indications. Thus, it can be inferred that a considerable number of HPV infected immunosuppressed individuals cannot be handled with greater cost RNA interference technique or through immunotherapies.

1.4. Detection of Sexually Transmitted Infections

Hook et al. (1997) reported that *Neisseria gonorrhoeae* and *Chlamydia trachomatis* are two of the most widespread sexually transmitted pathogens in the world, with high infection

rates among female sex workers in developing countries, a considerable ratio of whom have asymptomatic infections. Traditional lab identification of these infections is performed by culture for NG and antigen or cell culture detection of CT. Presently nucleic acid amplification tests (NAATs) are widely used, and have revealed a high degree of sensitivity predictive ability for NG and CT infections. Numerous studies—Schepetiuk et al. (1997), Steingrimsson et al. (1998) and Loeffelholz et al. (1992)—have revealed that NAATs are more exact than previous standard tests for *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. However, it is known that some substances in clinical specimens may be related to inhibition of amplification, and NAATs may provide false positive outcomes. Presently accessible commercial NG and CT DNA tests of amplification involve LCR (ligase chain reaction), SDA (strand displacement amplification) and PCR (polymerase chain reaction). According to Kehl et al. (1998), a major barrier for comparative studies of varied commercialized nucleic acid amplification tests for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in genital swabs is the incapability of different specimen gathering and transportation of specimens. To permit a scientifically valid comparison and a proper head to head estimation of various diagnostic assays, entire tests must be performed on a similar type of specimen, and involve a considerable number of true-positive specimens.

According to Black (1997), chlamydia culturization is regarded as the "gold standard" method, as it has nearly 100% specificity. Culture methods can be used for antibiotic testing of susceptibility of transmission. The method relies on specimens' inoculation on a monolayer of cells susceptible to CT. The drawbacks of culturization include its reduced 100% sensitivity and that it relies on laboratory proficiency. Similarly, the non-culture processes involve enzyme immunoassay (EIA), NAATs, nucleic acid hybridization technologies and direct fluorescent

antibody (DFA). EIA uses enzymes to detect Lipopolysaccharide on chlamydial elementary bodies. Enzyme immunoassay can generate false positive outcomes, and are less sensitive than the method of chlamydial culture. DFA is based on the direct visualization of chlamydial elementary bodies through combination of monoclonal antibodies to MOMP molecules on the membrane. Chernesky et al. (1986) determined that DFA is highly specific, but is not applicable for large numbers of specimens because it is labor intensive and time consuming. The techniques of nucleic acid hybridization predict rRNA of chlamydia with a complementary probe of DNA.

The invention of NAAT has revolutionized the identification of chlamydial infection. These assays detect and amplify the sequences of nucleic acid distinct to CT in specimens and have greater specificity and sensitivity than the techniques described above. These assays do not require feasible micro-organisms, and some can predict the existence of an individual nucleic sequence in the specimen. Lee et al. (1995) described that the drawbacks of NAATs are their price, their lowered performance in the presence of inhibitors in urine samples and the requirement to freeze urine specimens to increase test performance.

According to Ng and Martin (2005), discharge from the urethral meatus is required for NG detection. If a post-pubertal patient has no such discharge, an intra-urethral swab can be used for gonococci detection. To maximize the detection of organisms, samples must be gathered from patients who have not voided for at least two hours. The swabs are used to prepare a smear, culturing on proper media or for transport to other laboratories. Tabrizi et al. (2011) noted that NAATs can be used for NG detection in samples from extra-genital sites such as the rectum and pharynx, which harbor greater number of species of NG and possibly *N. meningitidis*. The performance features of NAAT assays and platforms require precise evaluation. This is achieved by using well-characterized isolates of varied species of *Neisseria gonorrhoeae* and clinical

samples. Some of the former studies have estimated that recent generation nucleic acid amplification test systems of gonococci—such as Gen-Probe Aptima Combo 2, CT/NG assay on m2000, ProbeTec GC Qx assay and Roche COBAS-4800 NG/CT test--have shown high concordance compared to other potential targets of nucleic acid amplification tests. Smith et al. (1992) mentioned that ligase chain reaction (LCR) was approved by the FDA for *Neisseria gonorrhoeae* detection in urine. LCR was considered the best choice for detecting gonococcal culture, because it is highly sensitive for men and women. Its evaluation for discrepant analysis is 100 percent accurate for both women and men. Stary et al. (1997) described that the use of LCR or PCR for NG detection in rectal specimens has been researched in one study, but had not yet been approved by the FDA. In a subsample of men and women for whom a urogenital specimen was positive by culture or by ligase chain reaction, LCR detected NG in rectal samples in 10 of 22 women and 6 of 47 men, while culture did not detect any infections. Nolt et al. (1994) described that the new nucleic acid detection tests, particularly ligase chain reaction, provide some benefits over culture, such as flexibility in source of sampling, handling of samples and speed of results, all of which encourages their use. LCR may predict numerous infections where the conditions for a culture's optimal performance are absent. In more formal laboratory settings where such circumstances are well maintained, the ratio of benefits of LCR to culture may be lower.

According to the study of Jampasa et al. (2014), different techniques have been enhanced and used for the identification of HPV infections. Presently, the most widely-used technologies for diagnosis and screening of HPV infection are Pap smear test, PCR with genetic primers and digene Hybrid Capture assay. However, Villa and Denny (2006) described that these technologies have some drawbacks: a reduced specificity for HPV and a sensitivity which

requires time consuming expertise and costly and complicated instrumentation. Therefore these methods are not suitable for nations with limited personnel and resources. Fu et al. (2004) identified new methods of detection such as fluorescence and piezoelectric spectroscopy and acoustic wave have been used for HPV detection, but these suffer the need for costly instruments as well. However, electrochemical detection of sequences related to HPV has been developed. It requires little storage space, has greater sensitivity, portability, and reduced cost and simplicity, which makes electrochemical detection a strong contender for a POC (point of care) DNA diagnostic process. According to Vilaivan et al. (2011), a new conformationally-restricted PNA system of pyrrolidinyl with alpha-beta peptide backbone derived from two acpcPNA (aminocyclopentanecarboxylic acid) was developed. AcpcPNA displays a powerful affinity for binding and a greater specificity towards a complementary target DNA than PNA or DNA. Ananthanawat et al. (2009) noted that, because of these properties, acpcPNA has been used as a sensor probe to identify target DNA in combination with different techniques of detection, such as matrix-supported ionization/desorption time of flight mass spectrometry, surface plasmon resonance and fluorescence microscopy.

For the testing of HPV in real samples, various methods have been developed since the turn of the century. Buger et al. (2011) described that both RNA and DNA tests can be used for HPV detection, where the RNA tests detect gene expression (which is similar to detection of cancer), and DNA tests detect the absence or presence of an HPV virus genome. Most essentially, HPV E7 and E6 are products of a viral gene recognized as being overexpressed and deregulated following integration of the virus in a host genome. According to Zur & Hausen (2002), over-expression allows for malignancy in cancers related to HPV. The two viral oncogenes promote growth of irregular cells by deactivating tumor suppressor proteins pRB and

p53. It is mentioned that the detection of E6 and E7 may permit a better differentiation between HPVs that are temporary and those that will advance to cancer during primary screening. Malloy et al. (2000) has mentioned that, in cervical specimens, direct HPV detection may provide a complement or alternative to population-based cytological screening. Schiffman et al. (2000) and Wright et al. (2000) described that test results of HPV are more sensitive than Pap smears in predicting severe dysplasia in older women. In some cases, women with positive tests for HPV still have Pap tests or other diagnostic processes that turn up negative for the virus. Pavonen et al. (2010) noted that HPV-18 and HPV-16 AS04 adjuvant vaccine offered security against lesions of CIN2+ that were related with HPV-18 and HPV-16, as well as lesions that are associated with non-vaccine treatments. Although the significance of regular Pap or HPV tests in unvaccinated and vaccinated women must be emphasized, the vaccination of HPV has the potential to lower the event of pre-cancer and cervical cancer substantially, as well as the rate of cervical excision procedures and colposcopy referrals.

1.5. Problem Identified

The detection of nucleic acids is critical for the diagnosis of bio-warfare agents, genetic diseases, and other infectious agents. In general, traditional technologies, methods and strategies for detecting nucleic acids are labor intensive and time consuming (Zeng, Lie, and Fang, 2013). The advent of modern technologies and methods, including the RT-PCR (real time polymerase chain reaction), isothermal strand displacement reaction based on chemical or fluorescence methods, SPR (surface plasmon resonance) and DNA microarrays provide sensitive and fast tools for the detection of nucleic acids. However, these methods still require highly trained people and complicated instrumentation. So, the burden is on researchers to identify strategies and methods which are fast, low cost, sensitive and easy to use for the detection of nucleic acids,

and offer a POC diagnosis for sexually transmitted infections like *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and HPV-16. This study intends to describe the method of detection of multiple nucleic acids based on the Lateral Flow Biosensor and its applications.

1.6. Aims and Objectives of the Study

The primary aim of this study is to detect multiple nucleic acid samples simultaneously on a Lateral Flow Biosensor. The secondary objective is to apply the bio-sensing platform to the detection of PCR-amplified products and to explore its application for real sample detection of sexually transmitted infections like *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and HPV-16.

1.7. Significance of the Study

In biomedicine, detection systems for nucleic acids are critical for several applications in order to handle the process of monitoring and diagnosis of illnesses such as genetic material based disease detection and infectious diseases (Drummond, Hill, and Barton, 2003). Successful treatment of bacterial infection requires particular identification of the causative agent. Traditionally, identifying the bacterial pathogens by is the crucial element of the diagnostic process (Mothershed and Whitney, 2006). Improvement in traditional methods and approaches like automation of blood culture systems requires laboratories to adopt NAATs (nucleic acid amplification tests) in order to identify pathogens reliably and rapidly (Kaltsas, Want and Cohen, 2005).

The benefit of nucleic acid-based testing includes high throughput, stability, and reduced time to diagnosis and accurate results. Apart from these, NAATs provide additional benefits in specific organism detection, scenarios of low detection limits (e.g. a single cell) and rapid results. The recent advances in NAAT methods and approaches to detect bacteria provide increased specificity and sensitivity over traditional microbiological techniques.

The Lateral Flow Biosensor developed in this work allows the selective and sensitive detection of multiple DNA targets in a complex mixture of nucleic acids. The biosensor has the capacity to provide fast and sensitive results. Its nucleic acid based testing method has great potential to increase accuracy and speed of nucleic acid based STI detection. The lateral flow device provides great selectivity towards other infectious agents as well, allowing it to be a potential platform for point of care diagnosis and clinical use.

1.8. Limitations of the Study

This study is exclusively focused on detection of nucleic acid samples only. The work is also limited to real sample *C. trachomatis*, *N. gonorrhoeae* and HPV 16 detection, and no other agents are taken into consideration.

CHAPTER 2. EXPERIMENTAL TECHNIQUES

A variety of experimental techniques are described in this chapter. The topics covered under this section include the preparation of gold nanoparticles, a method to conjugate thiolated DNA oligonucleotides to gold nanoparticle colloid solution, and preparation of a Lateral Flow Biosensor including the modification and assembly of each component. A description of each technique that was used in this research project is presented hereafter.

2.1. Gold Nanoparticle Preparation

Gold nanoparticles are generally prepared in a wet reaction by the reduction of $HAuCl_4$ (chloroauric acid). After dissolving chloroauric acid, the solution is stirred with continuous heating. A reducing agent (citric acid) is added that causes Au^{3+} ions to be reduced to neutral gold atoms. Continuing to heat with stirring reduces more gold ions to gold atoms until the solution becomes saturated and precipitates in the form of sub-nanometer particles. To produce particles of a larger size, the amount of reducing agent is lowered so that the rate of gold ion reduction is decreased.

Gold nanoparticles (AuNP) with an average diameter of 15 nm ± 3.5 nm were prepared according to the method reported by Liu et al. (2009). In detail, a round-bottomed flask was cleaned thoroughly with aqua regia ($3HCl:HNO_3$) followed by rinsing with double-distilled water. For the synthesis procedure, 100 ml of 0.01% $HAuCl_4$ prepared with ultrapure water was boiled with vigorous stirring followed by the addition of 4.5 ml of 1% trisodium citrate. Boiling was continued for an additional ten minutes after the solution turned wine red. The heating source was removed and the red colloid solution was stirred while cooling for another 15 minutes. The resulting AuNP solution was stored in dark bottles at 4 °C for future use. The AuNP solution was characterized by an absorption maximum at 520 nm to validate the

uniformity of the particles and the expected red color of the particles was captured via a camera as shown in Figure 1. A concentrated solution of 15 nm gold nanoparticles was further used to conjugate with DNA oligonucleotides.

Figure 1: Image of 15 nm sized colloidal gold nanoparticle prepared using citrate reduction method.

2.2. Gold Nanoparticle DNA Conjugates

This section outlines the preparation of conjugates between short synthetic oligonucleotides (20 to 40 bases in length) and gold nanoparticles (15 ± 3 nm in diameter). These conjugates are formed between aqueous gold colloid solutions and synthesized short nucleic acid sequences modified with a free thiol or a disulfide group at either end. The AuNPs functionalized with multiple oligonucleotides are stabilized against flocculation and precipitation at high ionic strength or temperatures. The HPLC purified thiol functionalized oligonucleotide can be purchased from Integrated DNA Technologies, California. The gold colloid solution was concentrated fivefold before conjugation. Briefly, 5 ml AuNP solution was distributed in five

tubes equally. The solution was centrifuged at 12000 rpm for 15 minutes at room temperature. The pellet from all tubes was collected and re-suspended in 1 ml double distilled water before using for conjugation. AuNP conjugation reactions were carried out by adding approximately (33 µg/ml) 1 O.D. DNA probe to 1 ml of the 5-fold concentrated AuNP solution. After standing at 4 °C for 24 hours, the solution was subjected to "aging" by the slow addition of NaCl to a concentration of 150 mM, and 15 µl of 1% sodium dodecyl sulfate (SDS) to stabilize the AuNPs. The solution was allowed to stand for another 24 hours at 4 °C, and the excess of reagents were removed by centrifugation for 12 minutes at 12000 rpm. The conjugated solution was washed with PBS buffer three times. The supernatant was discarded, and the red pellet was re-suspended in 1000 µl of eluent buffer containing 20 mM Na_3PO_4, 5% BSA, 0.25% Tween-20, and 10% sucrose. The resulting red colored conjugate solution can be stored at 4°C until further use.

2.3. Preparation of Streptavidin-Biotin DNA Conjugates

This section includes the conjugation of DNA oligonucleotides to streptavidin via biotin. Streptavidin is a protein composed of four identical subunits each with a high affinity binding site for biotin. The bond formation between the two is very rapid and is unaffected by a wide range of pH or temperature changes. To facilitate successful conjugation of synthetic oligonucleotide to streptavidin, biotin-labeled DNA sequences (20 to 40 bases) were purchased from Integrated DNA technologies, CA. The oligonucleotide solution (~50 nmol) was added to 300 µl of 1.67 mg/ml streptavidin solution and diluted with PBS to make the final volume 500 µl. The mixture was incubated for 1 hour at room temperature (RT). The excess DNA probes from conjugates were removed in three washing steps with a centrifugal membrane filter (cutoff 30000, Millipore) at 6000 rpm for 20 minutes each. Finally, PBS was added to make the final volume 500 µl and stored at 4°C until further use.

2.4. Preparation of Lateral Flow Biosensor

The Lateral Flow Biosensor consists of four components: a sample pad, a conjugate pad, a nitrocellulose membrane and an absorbent pad (Figure 2).

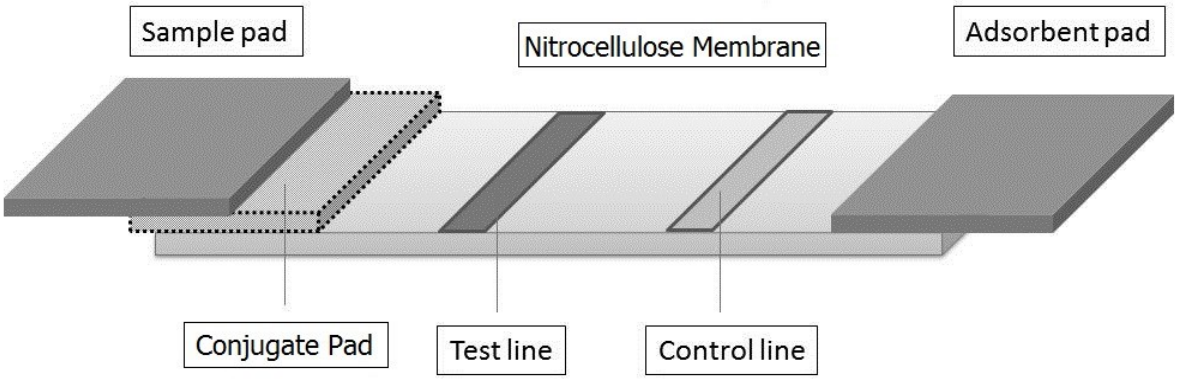

Figure 2: Schematic representation outlining the assembly of a typical Lateral Flow Biosensor.

All components are mounted on a common backing layer (typically an inert plastic) using the Clamshell Laminator (Biodot, CA). The sample application pad (17 mm × 30 cm) made from cellulose fiber (CFSP001700, Millipore, Billerica, MA) was saturated with buffer (pH 8.0) containing 0.25% Triton X-100, 0.05 M Tris-HCl, and 0.15 M NaCl for 1 hour at room temperature. The treated sample pad was dried at 37 °C for its assembly on the backing layer with other biosensor components. The conjugate pad (8 mm × 30 cm), a glass fiber (CFSP001700, Millipore, MA) was prepared by dispensing a desired volume of AuNP-DNA conjugates solution onto the glass fiber pad with the dispenser Airjet AJQ 3000, dried at RT. Nitrocellulose membrane (25 mm × 30 cm) was dispensed with DNA probes at different locations to form test and control zones. Biojet BJQ 3000 was used to dispense each conjugate on the nitrocellulose membrane with a distance of about 2 mm between each zone. Figure 3

shows the image of the Airjet AJQ 3000/Biojet BJQ 3000 used for dispensing conjugates on different membranes.

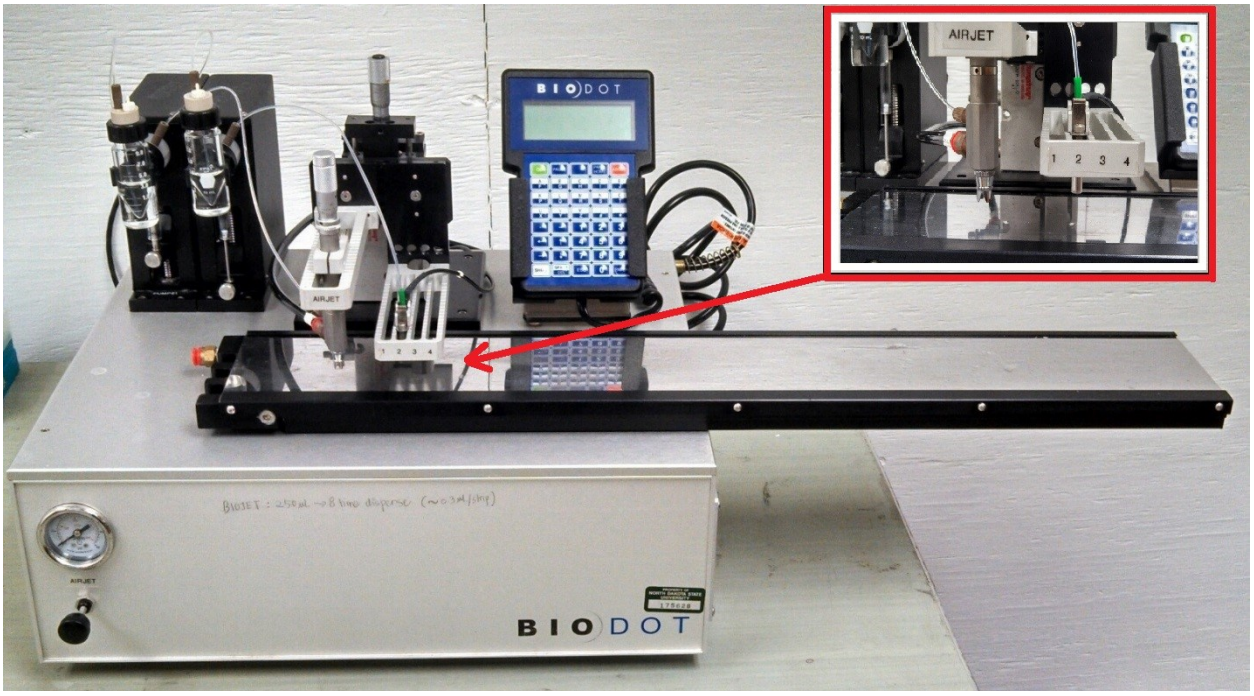

Figure 3: Image of Airjet / Biojet dispenser. The inset shows the zoomed section of the outlet used for dispensing different solutions connected to stock containers via air pump.

The individual, dried membranes are finally assembled on the backing layer to finish the biosensor preparation. The sample pad, conjugate pad, nitrocellulose membrane, and absorption pad (cellulose fiber sample pads (CFSP001700)) were assembled on plastic adhesive backing (60 mm × 30 cm) laminated cards (HF000MC100) obtained from Millipore, Billerica, MA. Each part overlaps 2 mm to ensure the solution migrates through the strip during the assay. The biosensor (3mm width) was assembled and cut using the Clamshell Laminator and the Guillotine cutting module CM 4000 shown in Figure 4. The Guillotine cutting module CM 4000 was used to cut the biosensor of 3 mm width and stored at 4 °C until further testing. The Lateral Flow Biosensor can be stored for 6-8 months at 4 °C without any significant loss in sensitivity, giving it a good shelf life.

Figure 4: Image of a fully automatic workstation, Guillotine cutting module CM 4000 that uses data input to cut biosensor to a desired width and speed, providing high-quality precision cuts.

CHAPTER 3. DETECTION OF MULTIPLE NUCLEIC ACIDS

This section outlines a historical introduction to different techniques and methods developed for the detection of nucleic acid targets in a sample mixture. Then this chapter focuses on the detection of multiple DNA targets on a Lateral Flow Biosensor. The high sensitivity achieved via this simple and rapid test provides high selectivity and point of care analysis of DNA targets from a sample mixture.

3.1. Introduction

Since the 1990's, the detection and analysis of DNA has become more important in fields such as genetics, pathology, and food safety. The realization of DNA diagnostics in general demands innovative analytical tools capable of providing high speed, low cost and simple methods (Fritzsche and Tanton, 2003). One such technology, DNA microarrays, can conduct multiple DNA sequence tests simultaneously on a single sample. Nevertheless, this remarkable technology of microarray has its limitations. The readout and fabrication of the test must be miniaturized in order to obtain easy readout without false responses from non-target sequences. In addition to high sensitivity provided by microarray techniques, combination with florescence detection allows for multicolor labeling of single-stranded DNA targets, providing highly sensitive readouts. But these tests also have the disadvantages of expensive labeling and instrumentation. Such demands have encouraged the development of new diagnostics: DNA sensors and high density DNA arrays. These analytical techniques provide a great tool for rapid and low cost detection, but lack high sensitivity and selectivity (Ramsay 1998). Although high sensitivity can be achieved with amplification (e.g. PCR amplification of samples), providing small-sample detection down to single cell or a few copies of genomic DNA, effective discrimination against mismatch/non-complementary nucleic acids and other biomolecules are

also required for effective analysis and reliable point of care testing (Wang, 2000 and Christopoulos, 1999).

3.2. Brief Literature Survey

In last decade, advances in technology have driven a number of research groups to develop DNA detection methods using lateral flow assays: prefabricated strips containing dry reagents that are activated upon application of the fluid sample. Such assays are important for diagnostic purposes of not just small DNA targets, but to determine pregnancy, infection, contamination and abuse of illicit drugs (Schuurs et al., 1980; Gelder et al., 1993). These assays usually provide results in 20-30 minutes where a binary output (positive/negative) is sufficient. Recently, the sensitivity and selectivity of such assays has been improved by combining thin-layer chromatography, antigen-antibody or DNA/RNA specific reactions, and/or labeling of the target analyte with a recognition element (e.g. fluorophoric tag) (Ullman et al., 1980; Hockmeyer et al., 1983; Lee et al., 2006). Lateral flow test strips have been well established in an attempt to develop highly accurate results cheaply. A typical lateral flow assay format contains a surface layer to carry sample solution along the strip via the conjugate pad. This helps the mixture encounter the test/control zones of the membrane moving further up to the absorbent pad. The detection label is usually made of a colored or fluorescent material nanometers in size. Commonly used labels that allow unobstructed flow through the membrane are often colloidal gold (Kapil et al., 2002; Torii et al., 2007; Yang et al 2006; Haasnoot et al., 1998), latex (Smith et al., 1997), selenium (Gordon et al., 1993), quantum dots (Mattoussi et al., 2004), carbon (Carlson and Lonnberg, 2001) or liposomes (Baeumner et al., 2004, Price et al., 2004, Wauchope and Ho 2002; Chen et al., 2008; Huang and Ho 2005). The first application of a lateral flow test was the assay for pregnancy determination (Schuurs et al., 1980). The speed and visual output of

the result to the naked eye provided a one-step, low cost analysis. The sensitivity of this study's Lateral Flow Biosensors can be further enhanced by the use of nanomaterial labels and novel signal amplification strategies (Wang, 2005).

Since the emergence of nanotechnology is opening new doors for analytical chemistry, nanoparticles are garnering considerable worldwide interest because of their unique chemical and physical properties Niemeyer, 2001, Mirkin et al., 1998). The power of such nanoparticles is greatly enhanced and utilized in recognition and electrochemical assays. The rising interest in tailoring and functionalizing the surfaces of nanoparticles has a profound impact on bio analytical applications in the field of DNA detection. Due to their great potential, nanoparticle-based testing relies on optical detection using gold nanoparticles. The hybridization-induced cross-linking of gold colloidal particles due to surface oligonucleotides triggers a red to purple color change in solution. Such assays have the ability to simultaneously analyze multiple target molecules in a sample mixture (Mirkin and Storhoff, 1999; Willner, 2002). High sensitivity can be obtained by using fluorophore tags or raman-active dyes (Mirkin et al., 2000, 2001, 2002). Nanoparticles offer an elegant way to recognize DNA with high sensitivity and specificity using electrochemical-based detection or amplification strategies involving tags and dyes (Nie et al., 2001, Neimeyer, 2001). High sensitivity provided by nanoparticles can be combined with selectivity and the easy application of lateral flow devices to develop an elegant, simple detection platform for multiple DNA targets.

In this chapter, further focus is now provided on the use of gold nanoparticles on a disposable Lateral Flow Biosensor for the sensitive and point of care (POC) detection of multiple nucleic acid targets. This work combines the unique optical properties of gold nanoparticles and highly efficient separation on a chromatographic platform. The sandwich-type DNA

hybridization reactions performed on the lateral flow device enable a fast detection method without multiple incubation, separation and/or washing steps compared to conventional techniques.

3.3. Lateral Flow Biosensor

The Lateral Flow Biosensor was designed in the manner described in section 2.4 with slight modifications. The sample application pad was saturated with a buffer and dried before assembling on the backing layer of the lateral flow device. The AuNP-DNA conjugates were also prepared according to the procedure described in the previous chapter. Since the work focuses on multiple target detection, two sets of AuNP-DNA conjugates were prepared using Thio Probe 1 and Thio Probe 2, described below. A desired volume of AuNP-DNA conjugate mixture was dispensed using the Airjet 3000 on the glass fiber membrane used as the conjugate pad during biosensor assembly. The nitrocellulose membrane was dispensed with three conjugates of streptavidin-biotin DNA conjugates at different sections of the membrane, identified as test zone 1, test zone 2 and the control zone. The streptavidin-Biotin DNA conjugates were prepared using the Test Probes 1, 2 and 3, described below. The different zones of the nitrocellulose membrane were dispensed using Biojet 3000 with desired volumes which can be achieved by dispensing multiple cycles. The Lateral Flow Biosensor was assembled by combining the individually-developed strip components.

The following sequences designed for the Lateral Flow Biosensor were purchased from Integrated DNA Technologies, CA.

Target 1

5’-ATGAAGAAGGCCTTCGGGTTTGTGTGGGAAGGGAGTAAAGTTAATACC-3’

Test Probe 1 (Test line for Target 1)

5’-Biotin/GTCAATGAGCAAAGGTATTAACTTTACTCCCTTCC-3’

Thio Probe 1 (Target 1)

5’-ThioMC6-D/CTGAAAGTACTTTACAACCCGAAGGCCTTCTTCAT-3’

Target 2

5’-AGACCATCCTGGCTAGTCTGTTGTCTCTACTAAAAATA-3’

Test Probe 2 (Test line for Target 2)

5’-Biotin/CGCCCGGCTAATTTTTTGTATTTTTAGTAGAGAC-3’

Thio Probe 2 (Target 2)

5’-ThioMC6-D/GGGGTTTCACCGTGTTAGCCAGGATGGTCT-3’

Probe 3 (Control line)

5’-ATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAG-3’

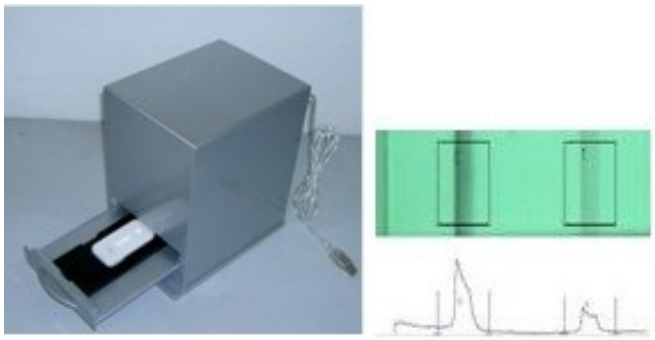

Figure 5: Analyzer DT 1030 to read color intensity of the red band on the Lateral Flow Biosensor.

3.3.1. Test procedure

Sample solution for testing was prepared by diluting a desired concentration of target DNA in ¼ SSC, 4%BSA. 100 µl solution was slowly applied to the sample pad of the Lateral Flow Biosensor slowly using a micropipette. The test/control zones on the nitrocellulose membrane were observed for the appearance of one or more red bands at the test/control zones

for 20 minutes. For quantitative results, the strip was inserted into the strip reader DT1030 (Figure 5), the optical intensities of the test lines and the control line were recorded simultaneously by using the "GoldBio strip reader" software.

3.3.2. Working principle of the Lateral Flow Biosensor

The principle of detection is based on an on-strip sandwich DNA hybridization reaction (Figure 6). The detection solution containing desired concentrations of the target DNA is applied to the sample application pad. Via capillary action, the solution moves toward the conjugate pad on the strip resulting in hybridization of target DNA to their complementary DNA probes conjugated on AuNPs. The conjugate probe sequence is designed to hybridize with half of the sequence for each target DNA introduced. The solution containing target DNA-conjugate DNA hybrid migrates further on the lateral flow strip and interacts with the probes immobilized on the test/control zone of the nitrocellulose membrane.

The DNA probes are immobilized on the test or the control zone via interaction between nitrocellulose membrane and the streptavidin conjugated to biotin DNA (Test Probes 1 or 2, respectively). Each test zone contains DNA oligonucleotides complementary to the second half of their respective DNA targets. The migration of the target-conjugate hybrid towards the test zones results in the formation of the sandwich complex with their complementary probes on the membrane. The sequence-specific binding of the three oligonucleotides on the test zone results in the appearance of a red band due to the accumulation of AuNP labeled with DNA oligonucleotides. The intensity (color) of the red band increases with the amount of target DNA present in the sample solution. If one or both target nucleic acids are not present in the test solution, only one line is observed on the lateral flow biosensor, denoted as the control zone.

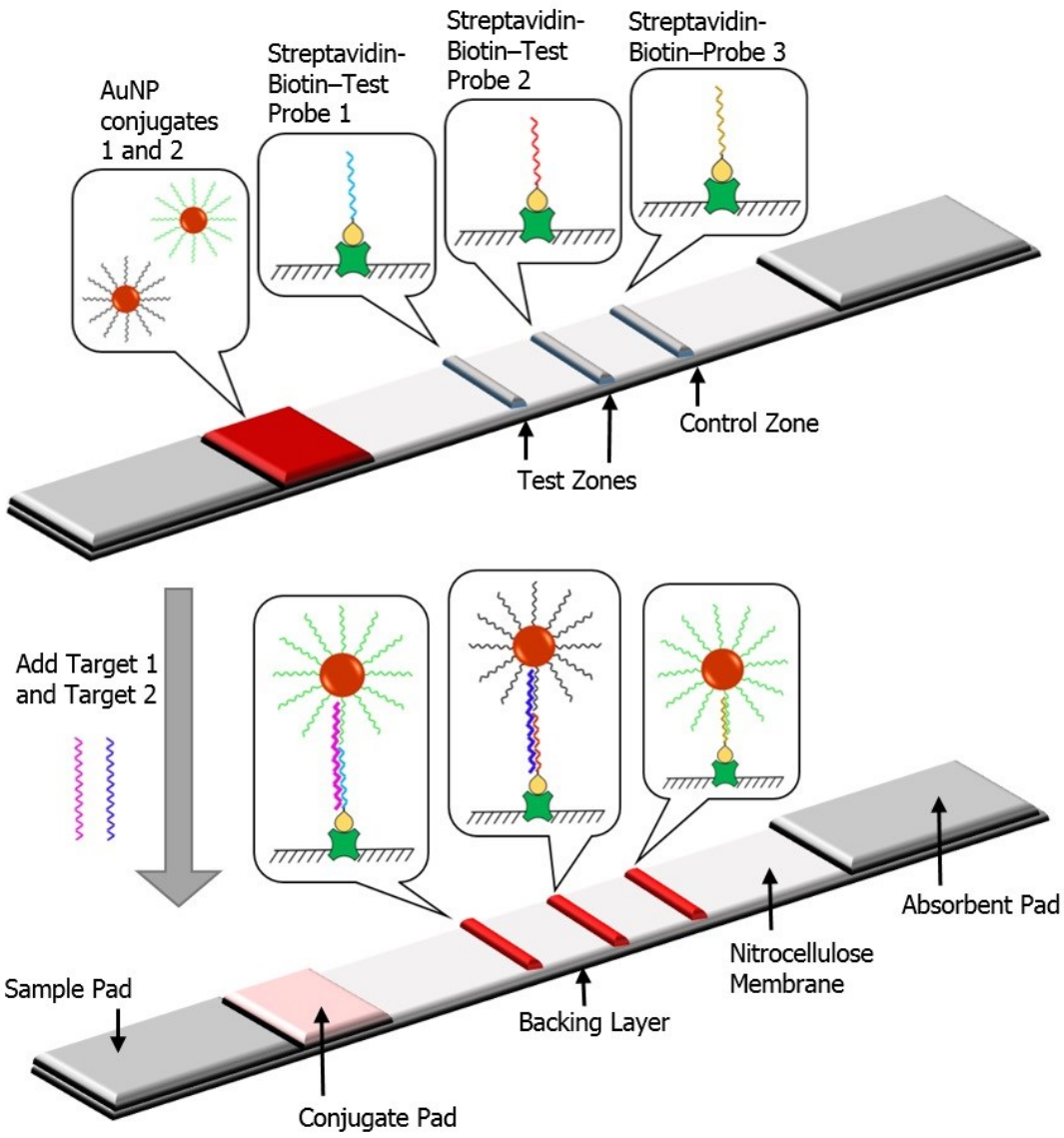

Figure 6: Schematic illustration of the working principle on a Lateral Flow Biosensor for the simultaneous detection of two nucleic acid targets.

The control zone contains immobilized oligonucleotides that are fully complementary to the oligonucleotides labeled on the gold nanoparticles. The free, un-hybridized AuNPs pass over the test zone and form double stranded complexes on the control zone of the strip. This results in appearance of a red band at the control zone even in the presence or absence of either/both targets. This confirms the proper function of the lateral flow biosensor.

3.4. Optimizations of Different Parameters

The formation of red band on the test zones and the control zone were used for the qualitative and quantitative analysis of the target DNA. Various conditions were optimized to improve the performance of the biosensor.

Different running buffers were employed for the detection of nucleic acid targets as shown in Figure 7. The optimization was performed in the presence of 50 nM concentration of Target 1. Best results were obtained in the presence of buffers BSA and SSC.

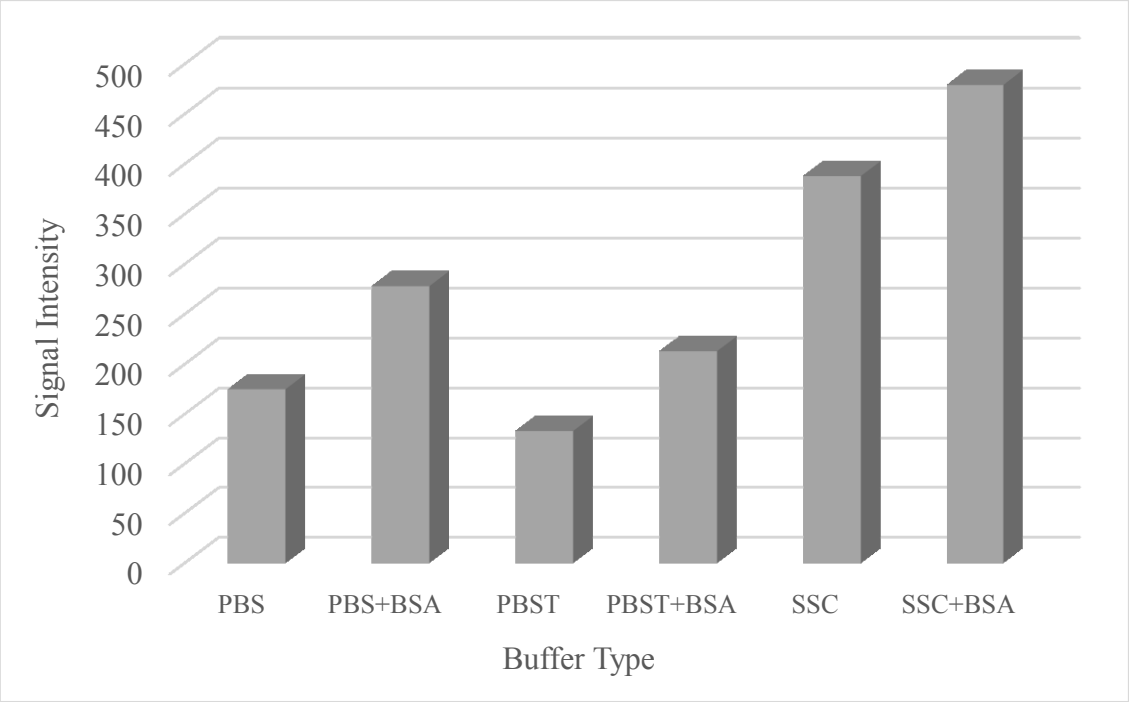

Figure 7: Bar graph comparing the effect on signal intensity on using different running buffers in the presence of 50 nM target DNA 1.The SSC buffer, well known for its ability to successfully enhance DNA hybridization, was used in combination with BSA. The running buffer capability was further improved by individually studying the amount of SSC and BSA in the running buffer. Figure 8 shows different concentrations of SSC diluted from the stock solution of 20x concentrated buffer.

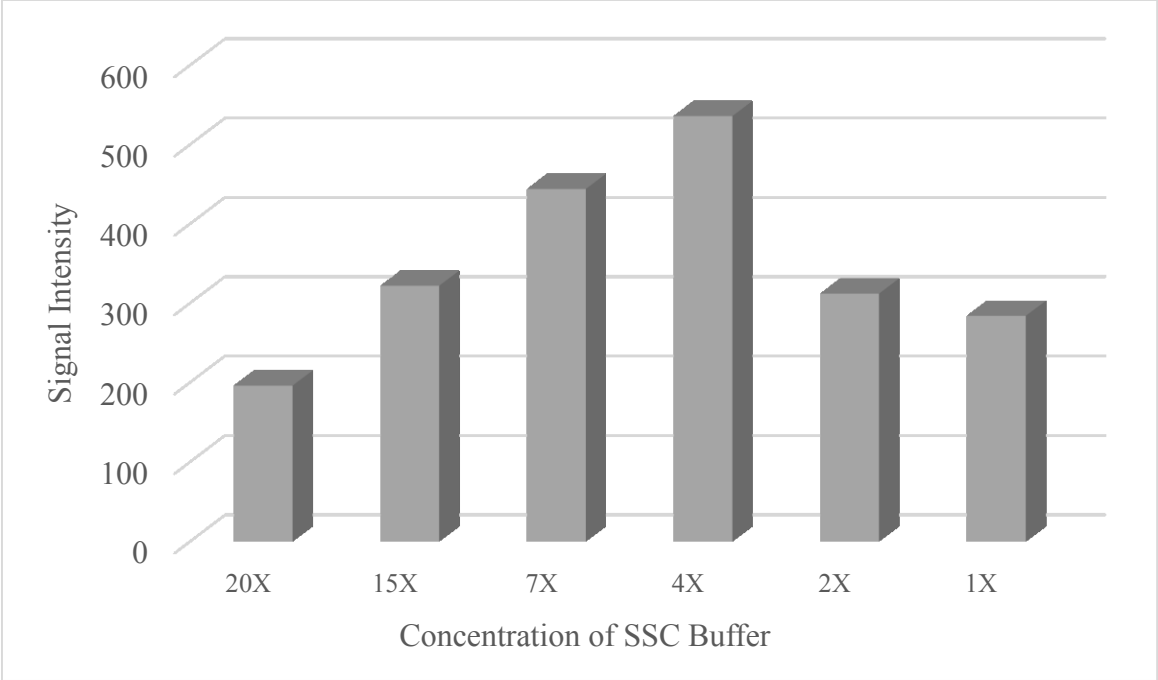

Figure 8: Graph showing different dilutions of 20X SSC buffer employed as running buffer in the presence of 1% BSA.

Different tests with same target concentration gave different signal response with varying concentration of SSC. 4X was chosen as the optimum as concentrated SSC had obstructed movement and resulted in high background in sample reading. The second component of the buffer, BSA, was also optimized (Figure 9). Different percentages were added to obtain the best signal for 50 nM target concentration. The test was performed in combination with 4X SSC and 4% BSA was determined to provide the best results. Addition of BSA in the buffer removes unwanted adsorption of AuNP conjugates on the nitrocellulose membrane. 4% BSA was chosen as an optimum amount used for the running buffer that gave the best signal versus background noise. Higher amounts of BSA gave false responses for control conditions, in the absence of target DNA due to accumulation/adsorption of AuNP conjugates on the test zones.

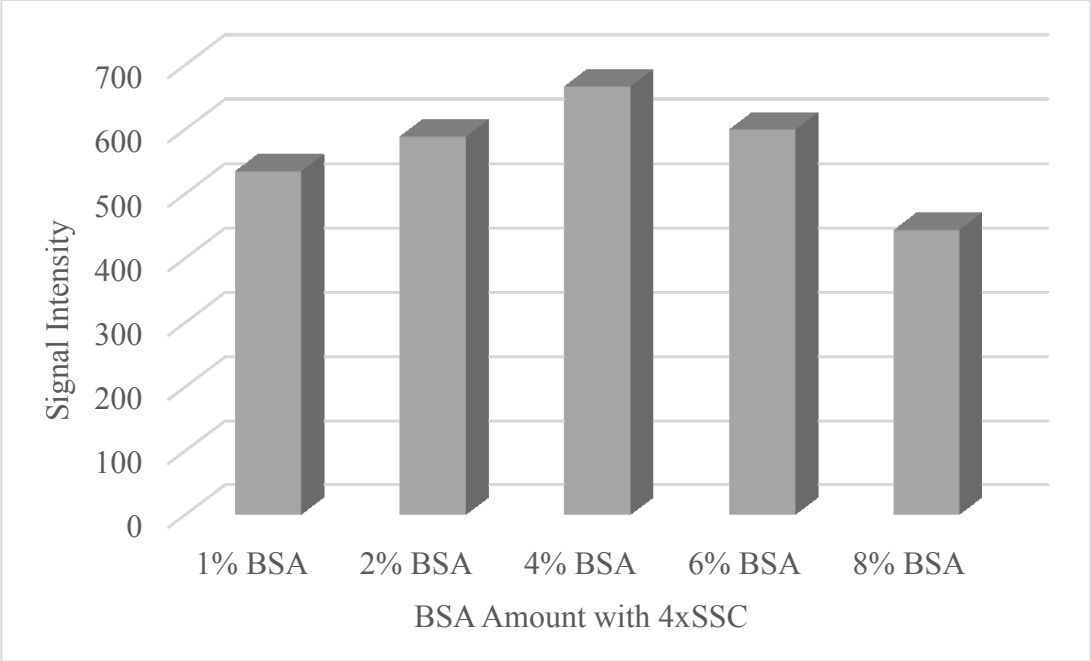

Figure 9: Bar graph illustrating the different percentage of BSA used in the running buffer with 4 times diluted SSC buffer for a target concentration of 50 nM.

Further optimizations were performed on the lateral flow strip by changing the amount of Thiolated DNA probe labeled on the AuNP. As shown in Figure 10, different concentrations (0.1 OD, 0.5 OD, 1.0 OD, 2.0 OD and 3.0 OD) of DNA probe were added to the AuNP for the preparation of nanoparticle conjugates. Increased concentrations increased the signal intensity on the Lateral Flow Biosensor up to 1.0 OD. Higher concentrations of DNA probe for AuNP conjugates did not significantly affect the signal response. Hence, 1.0 OD DNA for 1 ml AuNP was chosen as the optimal amount of oligonucleotide for the preparation of each conjugate.

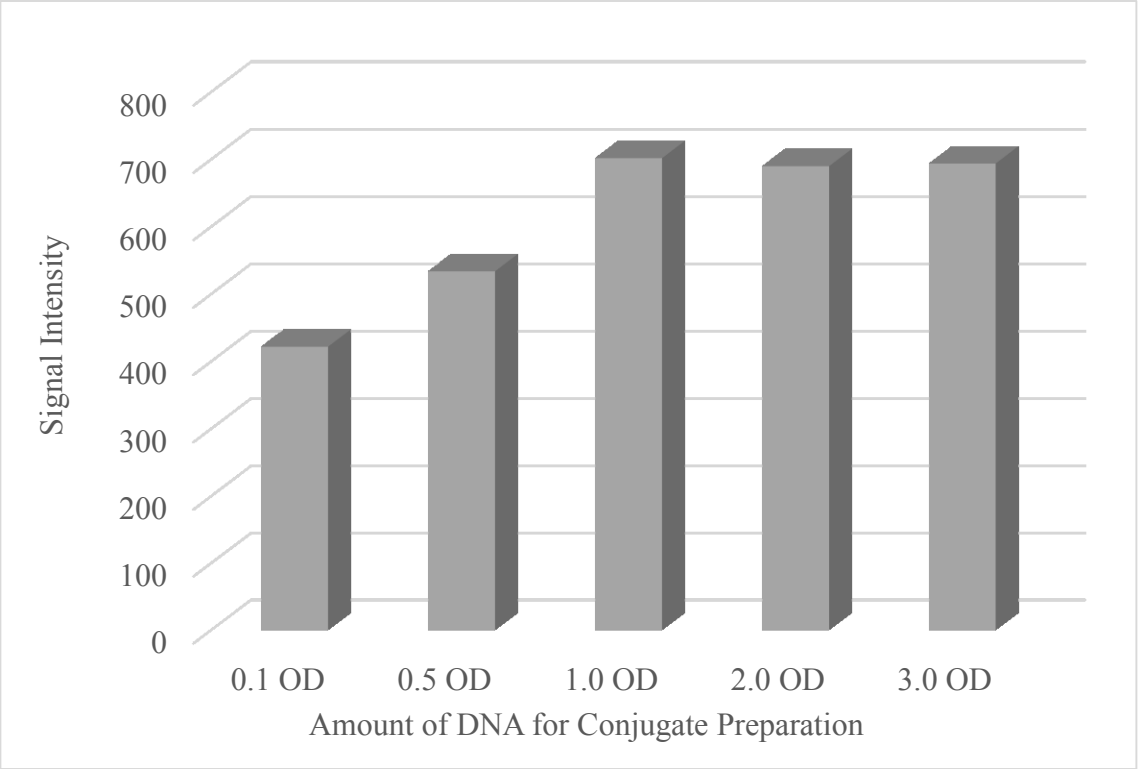

Figure 10: Graph showing different amount of oligonucleotide labelled on 1 ml AuNP solution during conjugate preparation used on the lateral flow device.

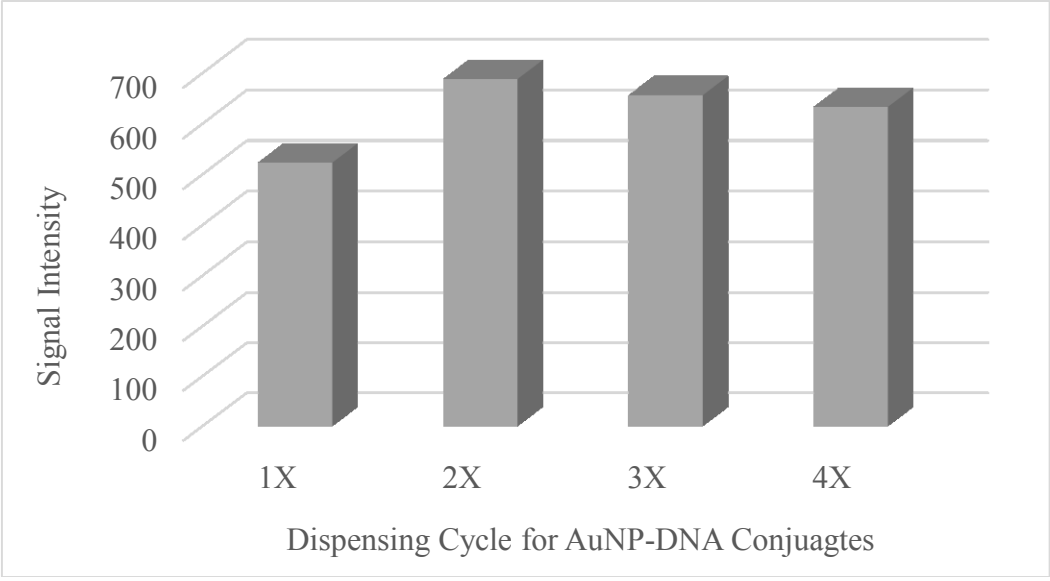

Figure 11: Graph showing varying signal response with the use of different dispensing cycles of gold labeled conjugates during the lateral flow biosensor preparation for 50 nM target DNA.

After conjugate preparation, the amount of AuNP-DNA conjugates dispensed on the conjugate pad of the Lateral Flow Biosensor was optimized (graph shown in Figure 11). Different dispensing cycles with Airjet 3000 were used to obtain a conjugate pad with different amounts of AuNP-DNA conjugates. Each dispensing cycle resulted in more conjugate on the strip which further enhanced the intensity of the band. Two dispensing cycles were chosen as the optimum amount of conjugate with maximum signal response and minimum background.

Other conditions such as the type of nitrocellulose membrane (Figure 12), and the amount of test and control probe on the membrane were optimized. Two types of membranes HFB18004 (3 min) and HFB 24004 (4 min) were used in the preparation of the Lateral Flow Biosensor. Better sensitivity was obtained with the 4 min membrane due the slower movement of the solution, allowing more complete DNA hybridization.

The test and control zones were prepared using different dispensing cycles for the streptavidin-biotin probes on the membrane (Figure 13). DNA probes with final concentrations of 50 nmol were dispensed twice on the nitrocellulose membrane when preparing test/control zones to achieve maximum signal response without interference from free AuNP probes.

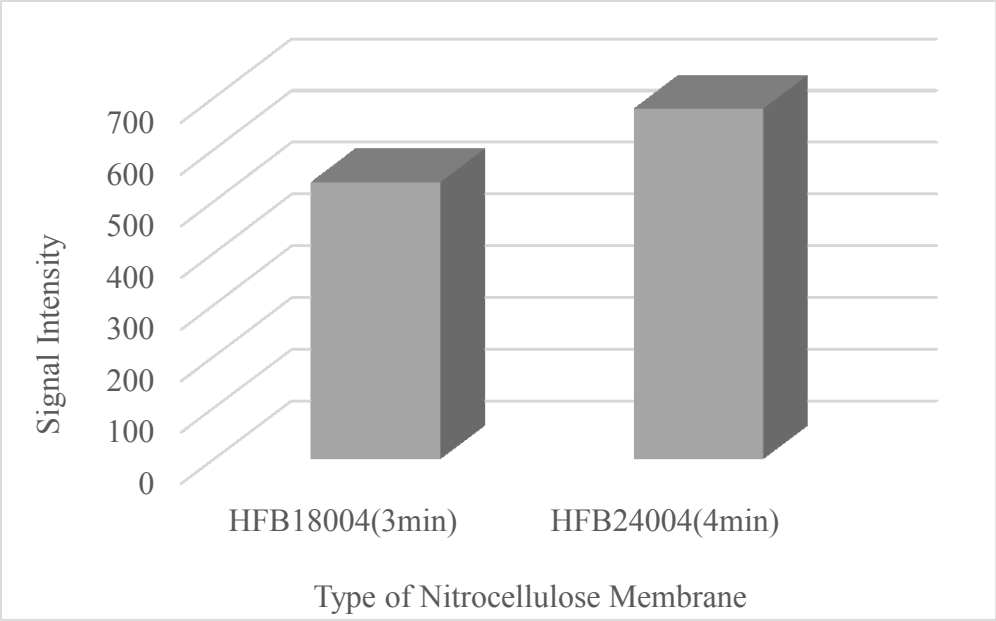

Figure 12: Graph comparing the response from use of two different membrane types used in the preparation of lateral flow biosensor.

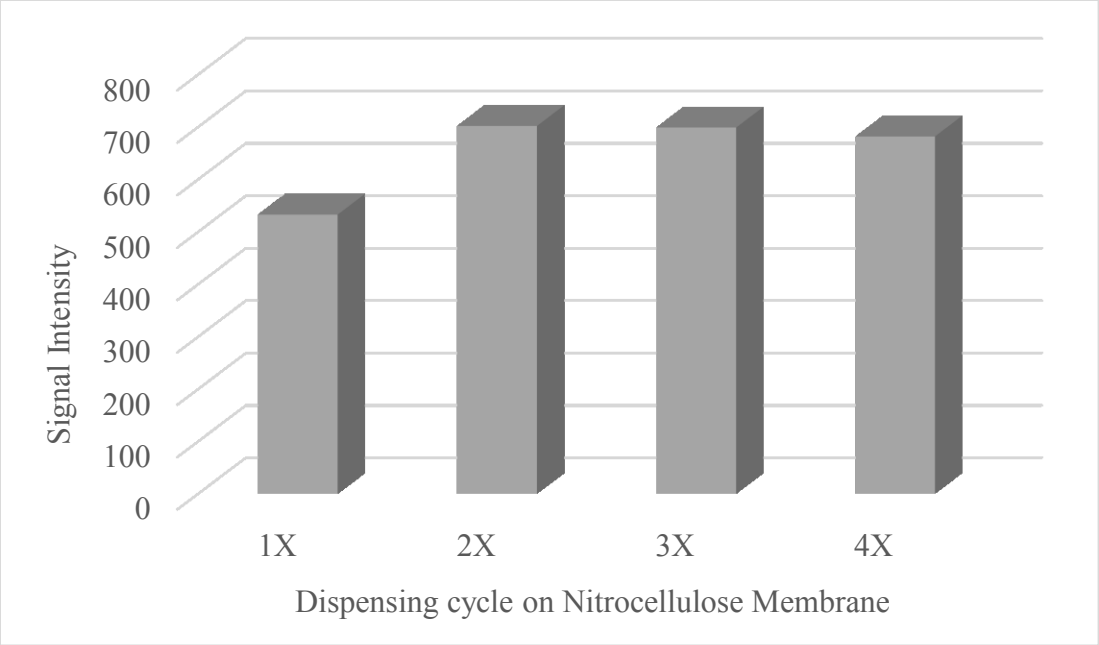

Figure 13: Graph showing the response from the use of different number of dispensing cycles of capture/control probes on the nitrocellulose membrane of the lateral flow biosensor.

3.5. Results and Discussion

Under these optimized conditions, the Lateral Flow Biosensor was tested for the presence or absence of the target DNA as illustrated in Figure 14. The presence of target DNA was confirmed visually via the appearance of a red line on the test zone. The quantitative detection was performed by measuring the intensity of the red band due to the accumulation of the AuNP conjugates on a portable strip reader. Figure 14 shows a typical Lateral Flow Biosensor image recorded from a portable strip reader camera. The test was performed in the absence (A) and the presence of target 1 (B), target 2 (C), and both targets (D) simultaneously. The response indicates that the presence of either or both targets do not interfere with the accurate working of the Lateral Flow Biosensor.

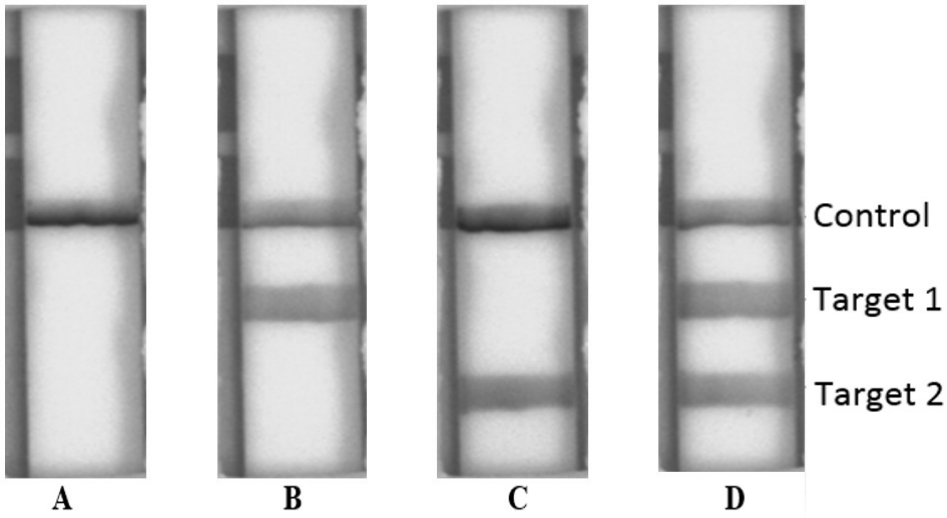

Figure 14: Shows the photographic signal response from (A) absence of nucleic acid in test solution, (B) presence of 50 nM Target 1 in 100 µl sample solution, (C) presence of 50 nM Target 2 in 100 µl sample solution and (D) presence of both targets in 100 µl solution.

Different concentrations of target DNA (0 nM, 0.1 nM, 0.5 nM, 1.0 nM, 5.0 nM, 10 nM, 50 nM, 100 nM and 200 nM) were tested thrice each. A red band in the control zone appeared in the presence or the absence of any or both DNA targets, validating the function of the Lateral

Flow Biosensor. The intensity of the red line on the test zone increased with increasing concentration of the target in the sample solution. Figure 15 shows the combined response and Figure 16 shows the calibration curve in the presence of different concentrations of each target DNA. The increasing concentrations of the target DNA shows increase in signal intensity for a linear response till 200 nM, and a minimum detection limit of 0.1 nM for both targets.

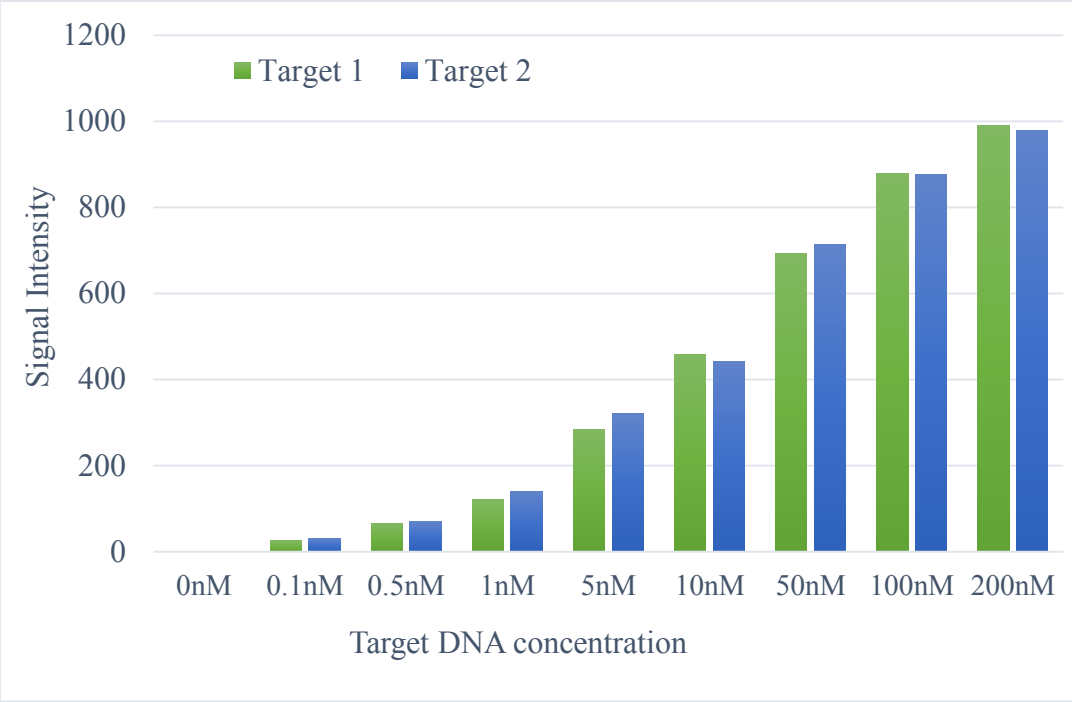

Figure 15: Signal Intensity recorded using Portable Strip Reader in the presence of two targets at varying concentration.

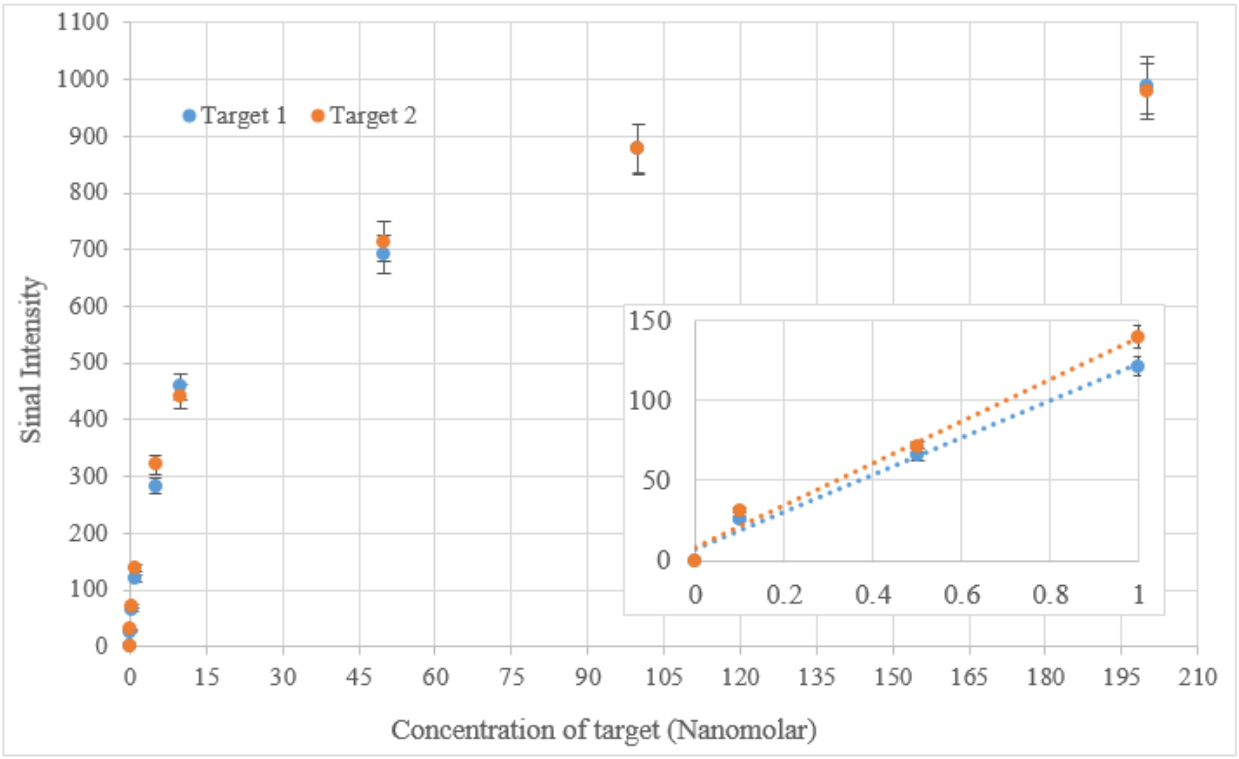

Figure 16: The calibration curve for target 1 and 2 with a detection limit of 0.1 nM for each target nucleic acid. Inset shows the linear curve for lower concentrations (0 nM to 1.0 nM)

The Lateral Flow Biosensor was also tested for interference from other biological components like non-complementary oligonucleotides (randomly designed) and proteins (IgG, IgM, thrombin, PDGF). These tests were performed with non-complementary DNA or protein in the sample solution with and without target DNA. The results indicate that the biosensor showed no decrease in sensitivity and proved to be very selective for the desired target(s) only. The reproducibility of the biosensor system was analyzed by repeat testing of six strips with different target concentrations. The Relative Standard Deviation obtained was 5.5%.

3.6. Conclusion

In this chapter, a platform for simultaneous detection of multiple nucleic acid sequences in a sample mixture was developed. The DNA targets could be easily acknowledged by observing the appearance of the red band and quantified using the portable strip reader instrument. The Lateral Flow Biosensor, as optimized, allows for selective detection with a limit of 0.1 nM for each synthesized oligonucleotide. Because of high sensitivity, this approach is capable of recognizing and detecting nucleic acids in about 20 minutes visually. The work is further improved by altering the methodology in order to lower the detection limit, yet maintaining the biosensor's selectivity to the chosen target analyte.

CHAPTER 4. SIGNAL AMPLIFICATION

This chapter introduces a novel application of a new method for the preparation of gold nanoparticle (AuNP) DNA conjugates introduced by Hsing et al. (2009). The method developed in this work requires fewer reagents and time to provide a highly sensitive method to detect DNA on a Lateral Flow Biosensor. This section further outlines an introduction to various methods used to conjugate AuNPs to oligonucleotides over the last decade, and its advantage over other amplification strategies used to detect single-stranded nucleic acids.

4.1. Introduction

Gold nanoparticles have exhibited various interesting physical and chemical properties which make them interesting candidates for visual detection and an integral part of research (Burda et al., 2005). In addition to their remarkable optical properties, AuNPs can be stabilized using a wide variety of molecules including the most commonly used thiol-modified oligonucleotides (Love et al., 2005). DNA-functionalized AuNPs play an important role in the fields of bio-sensing and nanotechnology. More recently, the advantages offered by functionalized AuNPs have been used for the detection of proteins (Thanh & Rosenzweig, 2002), carbohydrates (Otsuka et al., 2001), metal ions (Kim et al., 2001; Liu & Lu, 2003) and nucleic acids (Liu et al., 2009). Sensitive DNA detection is extremely important and great efforts have been made to develop new strategies to improve selectivity and sensitivity of these assays (He et al., 2000; Wang et al., 2002; Makrigiorgos et al., 2002). Such techniques offer optical detection via DNA hybridization and base-coupled reactions in conjugation with AuNP labeled with oligonucleotides (Weiss, 1999; Fang & Tan, 1999). The absorption frequency from metal nanoparticle aggregation depends on the size and distance between two metal particles; hence,

detection methods that use color change (red to purple) are also widely used (Elghanian et al., 1997; Mirkin et al., 1996; Storhoff et al., 2000).

4.2. Brief Literature Survey

The striking advantages offered by gold nanoparticles loaded with multiple oligonucleotides has enabled their applications in molecular diagnostics (Xu et al., 2007; Lee et al., 2007; Nam et al., 2003; Park et al., 2002; Elghanian et al., 1997), nanofabrication (Alivisatos et al., 1996; Mirkin et al., 1996; Park et al., 2008; Nykypanchuk et al., 2008), cell imaging (Seferos et al., 2007), molecular nano-electronics (Cohen et al., 2005; Cohen et al., 2006; Ullien et al., 2007), and gene regulation (Rosi et al., 2006). Since DNA hybridization offers great advantage in detection assays, labeling techniques like a florescent dye or an enzyme molecule (e.g. HRP) is often used to increase sensitivity of detection systems. Using fluorophores such as organic dyes on DNA probes lowers the detection limit, but suffers many disadvantages as well: the challenge is the poor photo-stability of many fluorophores and the limit to the number of molecules labeled on each DNA conjugated on AuNP. Use of organic dyes also results in serious photo–bleaching, and hence, irreproducible signals. Other nanomaterials used are quantum dots, silica and metal nanoparticles (Chan et al., 1998; Cao et al., 2002; Maxwell et al.,. 2002; Li et al., 2002; Taton et al., 2000; Santra et al., 2001). Among them, AuNPs still remains the most widely used, with relatively high sensitivity and stability. There are many strategies and methods used for preparing AuNPs conjugated with DNA probes, which include direct adsorption of alkylthiol or disulfide-labeled oligonucleotides on the surface of AuNPs (Mirkin et al., 1996). Covalent binding of oligonucleotides to pre-activated surfaces of metal nanoparticles (Pathak et al., 2001) and adsorption of biotinylated probes on the particles, coated with surface avidin (Alivisators et al., 1996; Niemeyer et al., 1998) are among other methods used inconjugation. The most widely-

used method employed for conjugation of oligonucleotide on the metal nanoparticle surface is the functionalization of 5' or 3' end of the nucleic acid with an akylthiol group (Mirkin et al., 1996). The easy modification of such groups at nucleic acid termini and the strong affinity between AuNP and thiol groups makes them the most stable conjugates among other commonly-used procedures. With all of these possible procedures, it is necessary to understand the importance of the coverage of the DNA on the nanoparticle. Usually, better results are obtained with higher loading efficiency to increase the magnitute of detection. Another important factor to be considered is the time consumed in the preparation of these conjugates, and the stability of each particle to provide fast and reproducible results.

The most commonly-used method of self-assembly of thiol modified DNA strands on AuNP was developed by Mirkin and coworkers in 1998. This approach follows a two-day incubation process to directly link thiol-terminated oligonucleotides to citrate-stabilized AuNPs. The process also requires further incubation of 40 hours to complete the aging process after the addition of NaCl to achieve high loading efficiency and stable conjugation. The procedure was eventually improved by Brust et al (2003) who used vacuum centrifugation to speed the process and eliminated the aging step which saved about 20 hours. Another conjugation method was reported by Alivisatos et al (2005, 2007) to minimize the salt effect by coating AuNPs with bis-(p-sulfonatophenyl) phenylphosphine dehydrate dipotassium salt before the immobilization of thiol labeled oligonucleotides on AuNPs. The process reduced the preparation time to 12 hours. The process of conjugation still needed improvement until the work done by Hsing et al. in 2009: the group introduced a new conjugation method that enabled rapid immobilization of thiolated DNA probes on a stable AuNP in the presence of salt. This novel method relied on fast and reversible binding of mononucleotide on the surface of AuNPs. Compared with conventional

methods that require overnight incubations and delicate control of ionic strength; this method enabled synthesis within 4 hours at high ionic strength. The stable conjugates with about 80 DNA strands per particle were prepared in 0.1 M salt solution at 60°C without need of special equipment.

This novel approach of conjugation was applied in this work to enhance the labeling efficiency of DNA on AuNP, thereby increasing the sensitivity of the system. The approach was applied for the detection of single-stranded nucleic acid targets and its application for the detection of human genomic DNA. The high sensitivity of this system comes from the direct amplification of the signal due to the number of DNA strands that can be loaded onto a single AuNP. The amount of DNA on each AuNP directly correlates to amplification, and therefore a lower detection limit is achieved. Further details covering the procedures and methods used for the assay are discussed in upcoming sections.

4.3. AuNP-DNA Conjugate Preparation

Preparation of AuNP-DNA conjugates was according to the method described by Hsing et al. (2009) with slight modifications. To a fivefold concentrated solution of gold nanoparticle colloid, which was prepared according to the procedure described in Chapter 2, approximately 7 μM dATP was added. The mixture was incubated at room temperature for 15 minutes before addition of salts for aging. Slowly, the mixture was brought to a concentration of 0.015% SDS and 0.1 M NaCl before addition of thiolated oligonucleotide (0.25 O.D. / 8.25 μg/ml). The AuNP solution containing DNA probes is incubated for 3 hours in a water bath at 60 °C. The solution is centrifuged at 12000 RPM at room temperature followed by three washing cycles with PBS 0.1M solution. The AuNP pellet is re-suspended in 1 ml Eluent Buffer (20 mM Na_3PO_4, 5%BSA, 0.25% Tween 20 and 10% sucrose) and stored at 4 °C until use. This method was

predicted to load more DNA strands on each nanoparticle compared to the older preparation method described in Section 2.2, as illustrated in Figure 17.

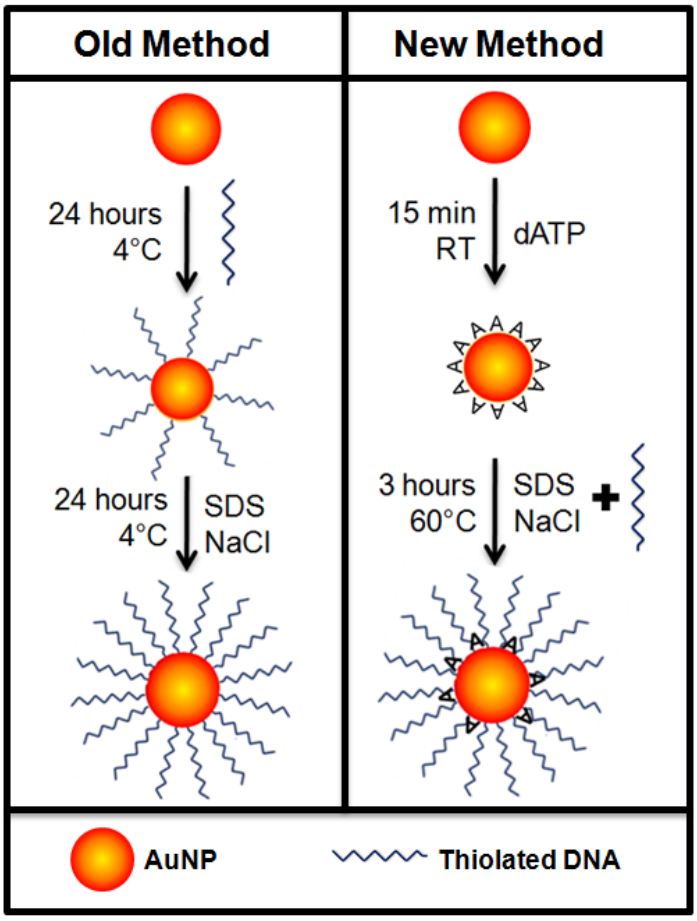

Figure 17: Illustrates the comparison between Standard method used by Liu et al (2009) (Old Method); common method for preparation of Gold-Nanoparticle-DNA conjugates and the Altered Hsing et al. (2009) (New Method); the procedure used in this research.

4.4. Lateral Flow Biosensor

The Lateral Flow Biosensor for the detection of a single nucleic acid target was prepared in the manner described in Chapter 2. The nitrocellulose membrane was prepared with one test line and one control line. The streptavidin-biotin DNA conjugates were prepared using two oligonucleotide sequences, shown below:

Target 1

5’-ATGAAGAAGGCCTTCGGGTTTGTGTGGGAAGGGAGTAAAGTTAATACC-3’

Test Probe 1 (Test line for Target 1)

5’-Biotin/GTCAATGAGCAAAGGTATTAACTTTACTCCCTTCC-3’

Thio Probe 1 (Target 1)

5’-ThioMC6-D/CTGAAAGTACTTTACAACCCGAAGGCCTTCTTCAT-3’

The target sequence chosen here was same as in the work for multiple target DNA detection (Chapter 3). The sequence of the target is a part of human genomic DNA, and therefore the recognition probes used on the biosensor can also be used for the detection of real samples (i.e. human genomic DNA).

The biosensor prepared for this work also compared the new conjugates (Section 4.3) with the traditional method of conjugate preparation (Section 2.2). Hence, two different sets of strips were prepared that varied in the type of conjugate used.

4.4.1. Test procedure

The sample testing was performed by diluting desired amount of target to a final volume of 100 µl using ¼ SSC + 4% BSA buffer. The sample was pipetted slowly onto the sample pad. After 10 minutes, 20 µl of the same buffer was added and the biosensor was kept at room temperature for another 20 minutes. The intensity of the signal (red line) was recorded using the portable strip reader DT1030.

Human blood serum was spiked with target DNA to mimic real sample conditions. 10 µl of undiluted human serum was successfully detected on the biosensor without any interference from the components of the mixture. Tests were performed by diluting sample in running buffer

to a volume of 100 µl and pipetting on the strip. Signal intensities were observed visually and recorded via a portable strip reader for quantitative response.

Human genomic DNA (obtained from Sigma Aldrich, USA) was also tested on the biosensor. The desired concentration of the double-stranded human genomic DNA was incubated in boiling water for 10 minutes to denature the double helix, then cooled in ice water for another 10 minutes to avoid re-annealing of the single strands. A total volume of 100 µl (diluted with ¼ SSC + 4% BSA) was pipetted on the sample pad. The biosensor was observed for a red band at the test zone and the control zone of the strip. Appearance at both zones indicated the successful detection of Human genomic DNA.

4.4.2. Working principle

Since this work is a further improvement of the detection of single DNA targets on a Lateral Flow Biosensor, the principle behind the detection remains the same as described by Liu et al. (2009). Briefly, the thiolated probe DNA which was attached on AuNP was designed to be complementary to a part of the target DNA. The other half of the target DNA was complementary to the DNA probe which was dispensed onto nitrocellulose membrane as the test line via streptavidin-biotin linkage. The addition of target DNA on the sample pad moves the solution via capillary forces towards the conjugate pad and the target DNA hybridizes with the DNA labeled on AuNPs. This complex moves further towards the nitrocellulose membrane and forms a sandwich complex with the immobilized streptavidin-biotinylated DNA probe. This leads to the accumulation of gold nanoparticles on the test line creating a macroscopic red band. The AuNP-DNA conjugates that did not form the complex moved along the nitrocellulose membrane and formed a double stranded complex with the control DNA probe dispensed at the control zone of the membrane. These red bands were characteristic of the amount of target

present in the sample solution. The intensity of the red band at the test zone increased with the increase in concentration of the target DNA, whereas the intensity at the control zone solely depended on the amount of conjugate used on the biosensor. In the case of no target DNA in the sample solution (the Control Test), there was no red band observed on the test line. The control zone always gave a red band after an assay to confirm the function of the biosensor.

4.5. Optimizations of Different Parameters

Various factors were taken into consideration for the detection of the target DNA. Liu et al. (2009) reported a sensitive and easy technique for the detection of DNA using gold nanoparticles. But the procedure for the preparation of AuNP-DNA conjugate was complicated and time consuming: it required 2 days and a higher concentration of the thiolated DNA probe (1 O.D.). This work describes the same detection method with a lower detection limit and an easier and faster technique for preparing conjugates. During the preparation of the AuNP-DNA conjugates, multiple conditions were changed and optimized. Figure 18 shows the different concentrations of dATP used in the preparation of the conjugates vs. the signal intensity for a precise concentration of target DNA. Compared with the different amounts used, 7.05 μM dATP gave the best results. More dATP saturated the signal intensity without any significant increase in response from target.

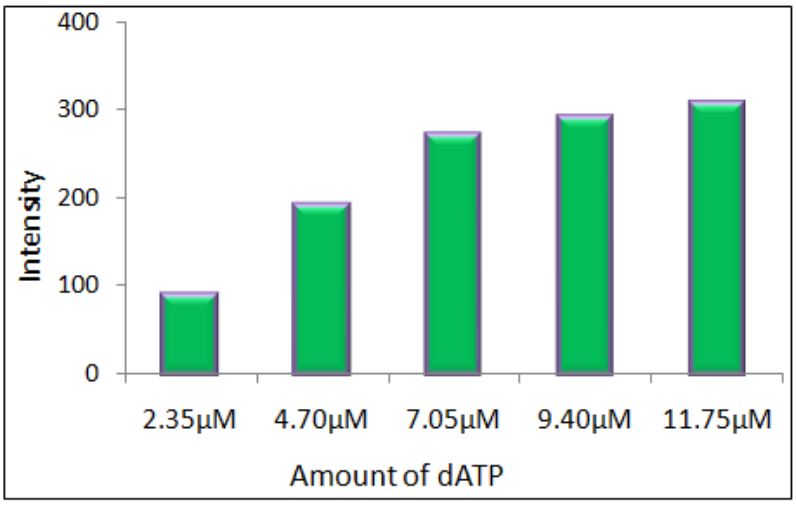

Figure 18: The graph shows the different amount of dATP used in the preparation of AuNP-DNA Conjugates.

The next optimization was the amount of DNA (OD) used for preparation the conjugates, shown in Figure 19. Of the different concentrations tested, 0.25 OD was the optimum amount of DNA. Addition of more DNA was presumed to maximize the oligonucleotides' density on the AuNP, giving maximum signal intensity for the test zone detection.

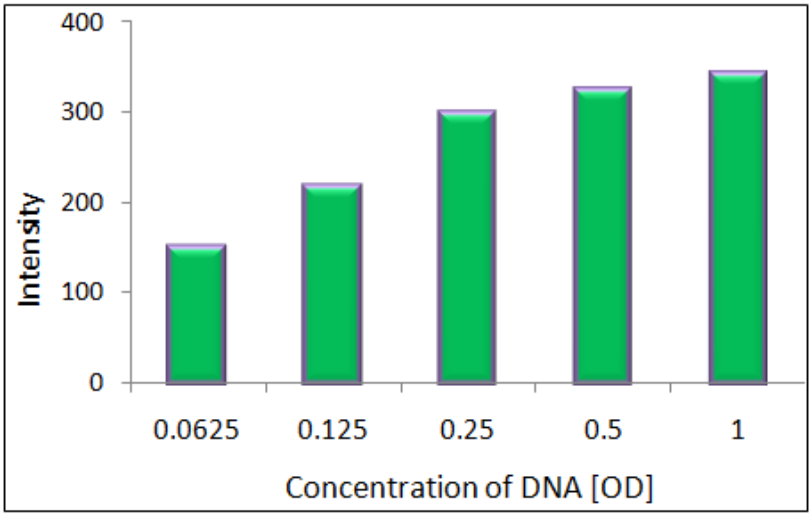

Figure 19: The graph shows the signal intensity recorded by the use of different concentration of DNA in conjugate preparation.

Figure 20 shows the comparison of the percentage of SDS in 1 ml AuNP. A final percentage of 0.015% gave the best results during the conjugate preparation method. It was theorized that a higher percentage of SDS is expected to stabilize the AuNP from aggregating, but the signal saturated at 0.015%. Lesser amounts of SDS changed the color of the conjugates to dark purple, which is a sign for the aggregation of the particles, and hence the decrease in signal intensity.

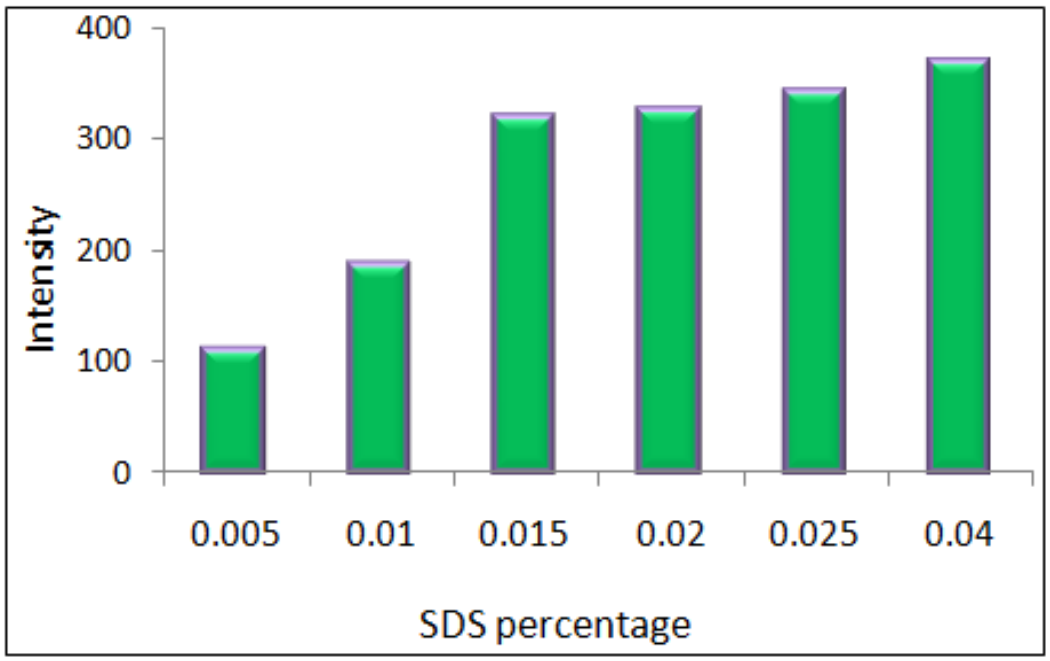

Figure 20: Graph showing the comparison of signal intensity in the presence of different amount of SDS used during conjugate preparation.

More optimizations were done with the amount of conjugates on the conjugate pad. The optimum volume was determined by optimizing the number of dispensing cycles used for the conjugates during the preparation of the biosensor. Figure 21 shows the signal response from biosensors prepared containing different volumes of conjugates on the conjugate pad. Dispensing three cycles of conjugates provided the best results. Less conjugate resulted in less signal intensity, as there was less AuNP-DNA available for hybridizing with the target, and hence the intensity of the red band changed. The signal intensity was expected to increase with more than

three cycles, but it resulted in high background color on the nitrocellulose membrane because of the presence of excess AuNP-DNA which remained un-hybridized.

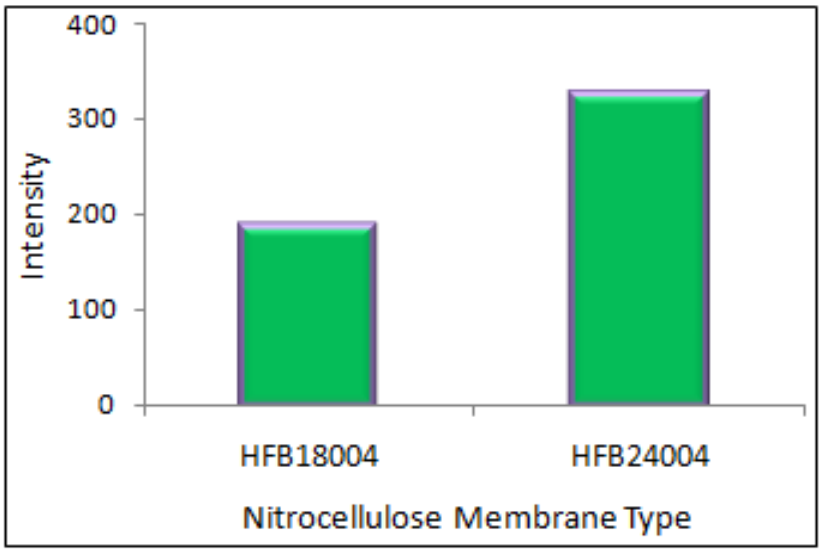

Figure 21: Shows the response from test on a lateral flow biosensor with different amounts of conjugates dispensed.

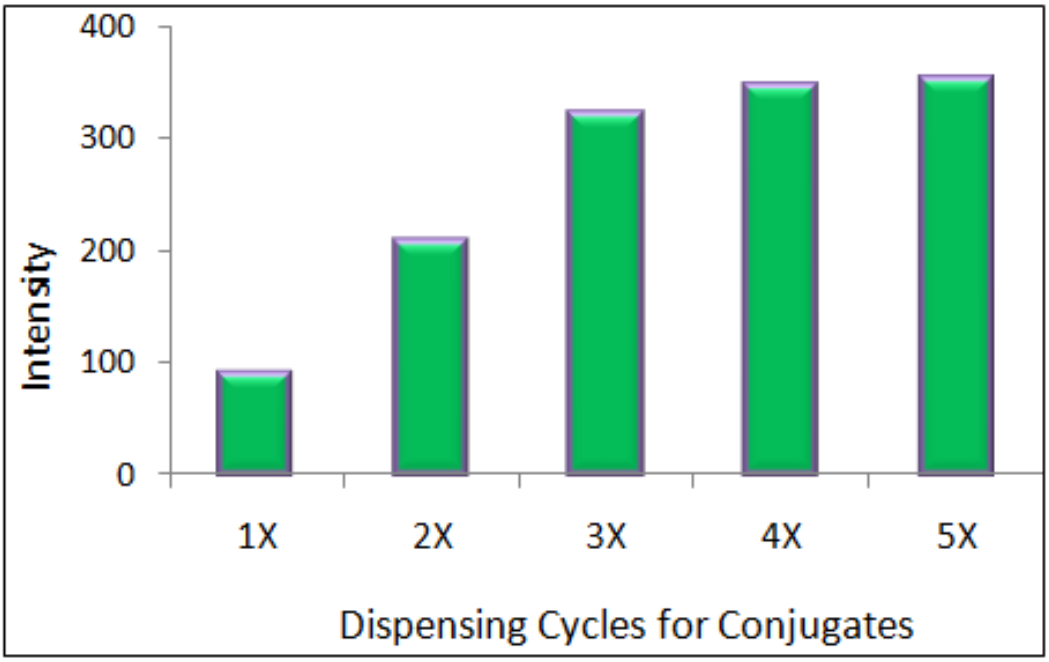

Figure 22: Illustrates the comparison between the signal intensity from the use of different nitrocellulose membrane on the Lateral Flow Biosensor.

After the optimization of the preparation of conjugate and their amounts on the biosensor, other components of the strip were also adjusted. Figure 22 indicates the type of nitrocellulose membrane used in the preparation of the biosensor. Out of the two available types of nitrocellulose, HFB18004(3min) and HFB 24004(4min), the latter was used because the time taken by sample solution to travel through the membrane was about 4 minutes, providing extra time for the DNA probes to properly hybridize, providing better results.

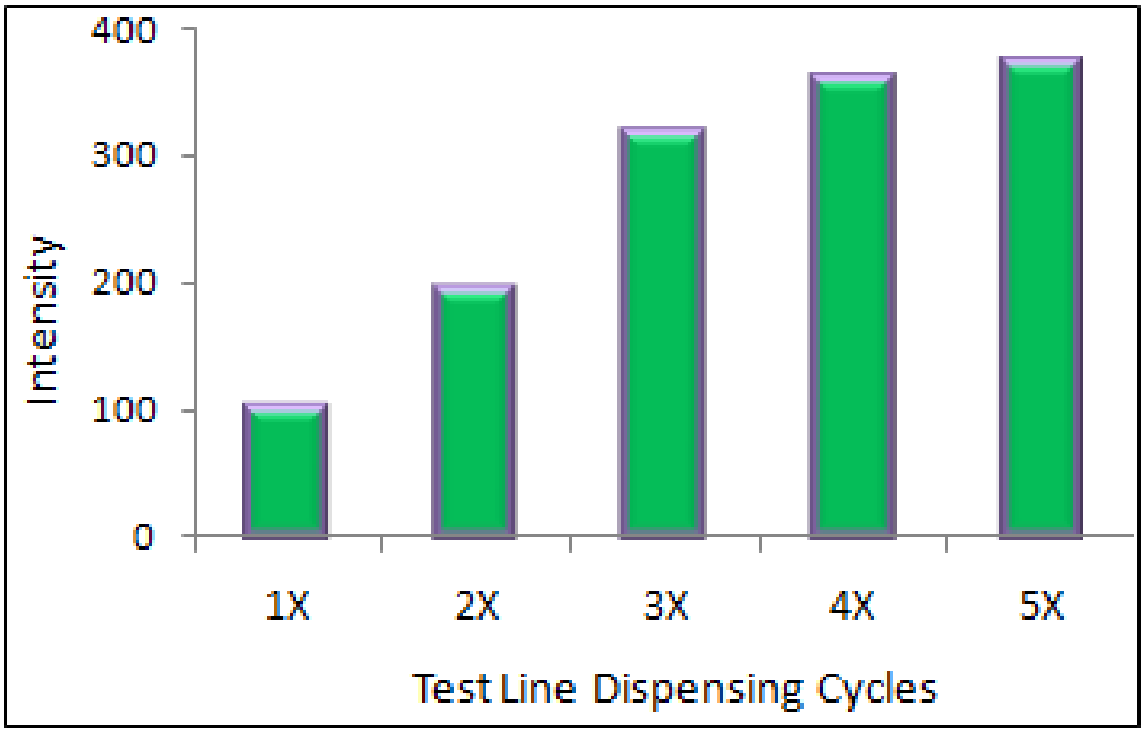

Figure 23: Compares the different number of times the test line was dispensed on the biosensor vs the response recorded via the portable reader.

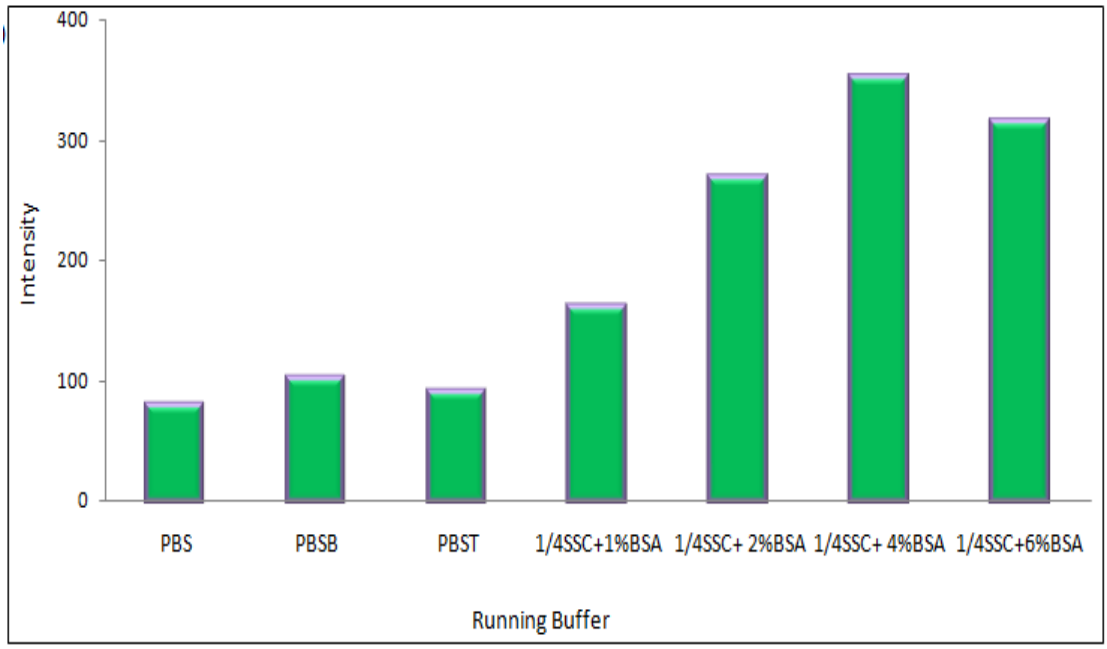

Figure 24: Comparison of signal intensity vs the running buffer used on the biosensor

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In addition, Figure 23 shows the comparison between the number of dispensing cycles for the test line on the nitrocellulose membrane. The results indicated that dispensing the streptavidin-biotin DNA conjugates three times gave the best results.

Finally, different types of running buffers were also tested under the optimized conditions. The target DNA was diluted in different buffers, shown in Figure 24. Signal response was recorded and ¼ SSC + 4% BSA was selected as the best running buffer with least background and high sensitivity for target DNA.

4.6. Results and Discussion

A new method was used for the preparation of gold nanoparticle-DNA conjugates, requiring less time and fewer reagents. Tests were performed to compare the response from the use of conjugates prepared with the traditional method vs. the new method using dATP. The *old method* was used by Liu et al. (2009); the *new method* required only about 0.25OD of DNA

probe and 3 hours preparation time, yet yielded far better results. Figure 25 represents the difference in the signal intensity for 0.5 nM concentration of the target DNA, (a) using the conjugates prepared by the *old method* and (b) using conjugates prepared by the *new method*. The variation in the signal shows the increase in sensitivity of the biosensor just by using different AuNP-DNA conjugates. The conjugates using the old method gave a signal intensity of 16 (test line) as compared to the signal of 221 (test line) recorded for the conjugates prepared using the new method. The experimental parameters were kept identical for both assays. The bands on the image represents test line (left) and control line (right). The signals recorded using the portable strip reader are also shown.

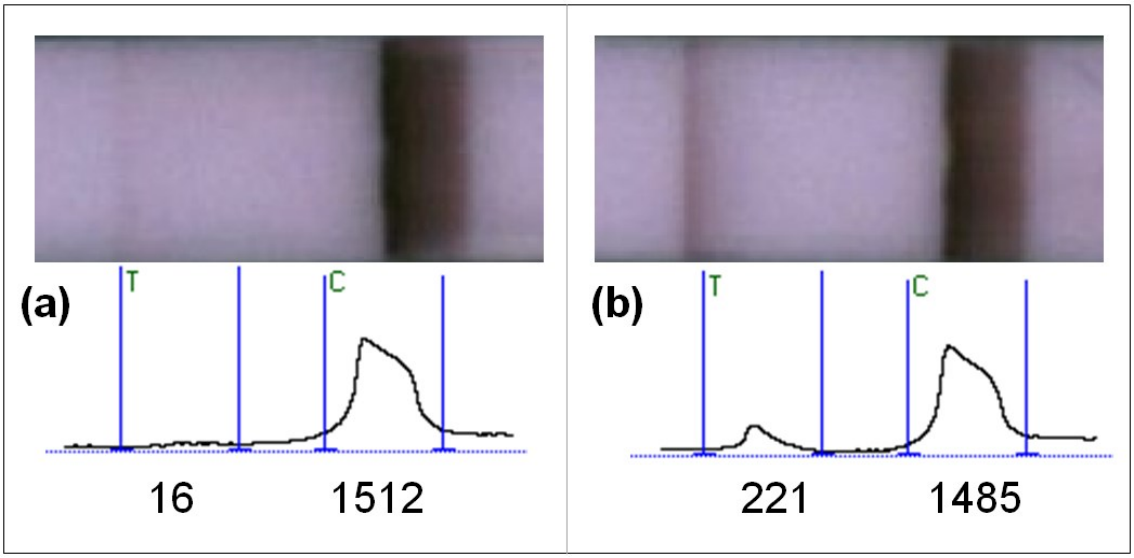

Figure 25: Shows the comparison between the signals for 0.5 nM DNA concentration using the Gold-Nanoparticle-DNA conjugates prepared by (a) the Old Method and (b) the New Method.

The lateral flow biosensor with newly developed conjugates was also put to test with different concentrations of target DNA. As expected, the signal intensity on the biosensor increased with increasing amounts of target DNA. Figure 26 shows the image (visual) response captured via camera for varied target amounts. The control line appearance confirmed the

accurate function of the biosensor. The quantitative response was recorded and the resulting peaks were generated from the portable strip reader. The detection limit of 0.01 nM with a linear range of 0.01 nM – 100 nM was obtained for a 30 minute assay. This biosensor is capable of analyzing different concentrations of target DNA without any interference from the presence of other non-complementary DNA strands.

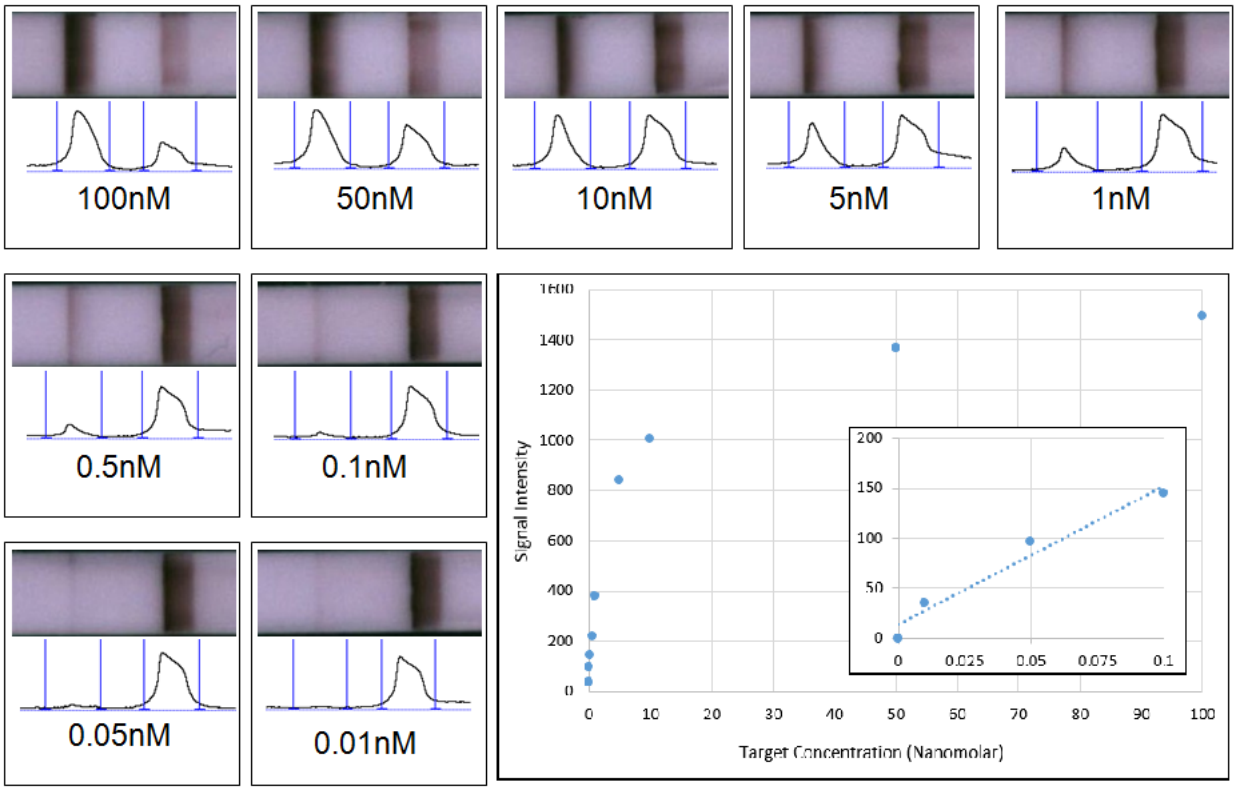

Figure 26: Illustrates the biosensor response for different concentrations of target DNA and their respective signals obtained from the portable strip reader. The calibration curve for the intensity or signal obtained vs. the DNA concentration. Inset shows the linear response obtained at lower concentrations ranging from 0.01 nM to 0.1 nM.

The basis for this research was to make this biosensor more sensitive to small amounts of target DNA so that it can be used widely and for on-site detection or analysis. This biosensor, compared to work of Liu et al. (2009), is about 50 times more sensitive using simpler preparation techniques. If compared to other techniques used for detecting DNA samples, this biosensor is a

fast, easy and quite sensitive method. This procedure without amplification can provide good sensitivity for detecting 0.01 nM of target DNA in 100 µl of sample solution within half an hour, and a high reproducibility was achieved with a Relative Standard Deviation value of 4.08%. The major improvement done to this work compared to any other method of nucleotide detection was to the time and money required for diagnostics, as it does not require any kind of expensive instrumentation or reagents which could add to the cost of the biosensor.

Interference from human serum was also tested on the lateral flow biosensor. DNA target samples spiked with human serum showed equivalent responses compared to target in buffered solution. The results obtained were plotted on a dot plot (Figure 27(a)), and showed linear with respect of log (concentration) response with increasing concentrations of target.

The target DNA sequence selected was a part of human genomic DNA. A sample of human genomic DNA was also detected by the biosensor, but since the genomic DNA is a long, double-stranded sequence, it took relatively longer assay time of about 45 minutes. The lowest amount of genomic DNA that can be detected on the biosensor is 0.25 ng/ml (~7.69×10^{-15}M). Figure 27(b) shows the calibration curve for different concentrations of signal intensity vs. genomic DNA. This promising biosensor showed great results for human genomic DNA detection, which implies that it can be used for real-world applications.

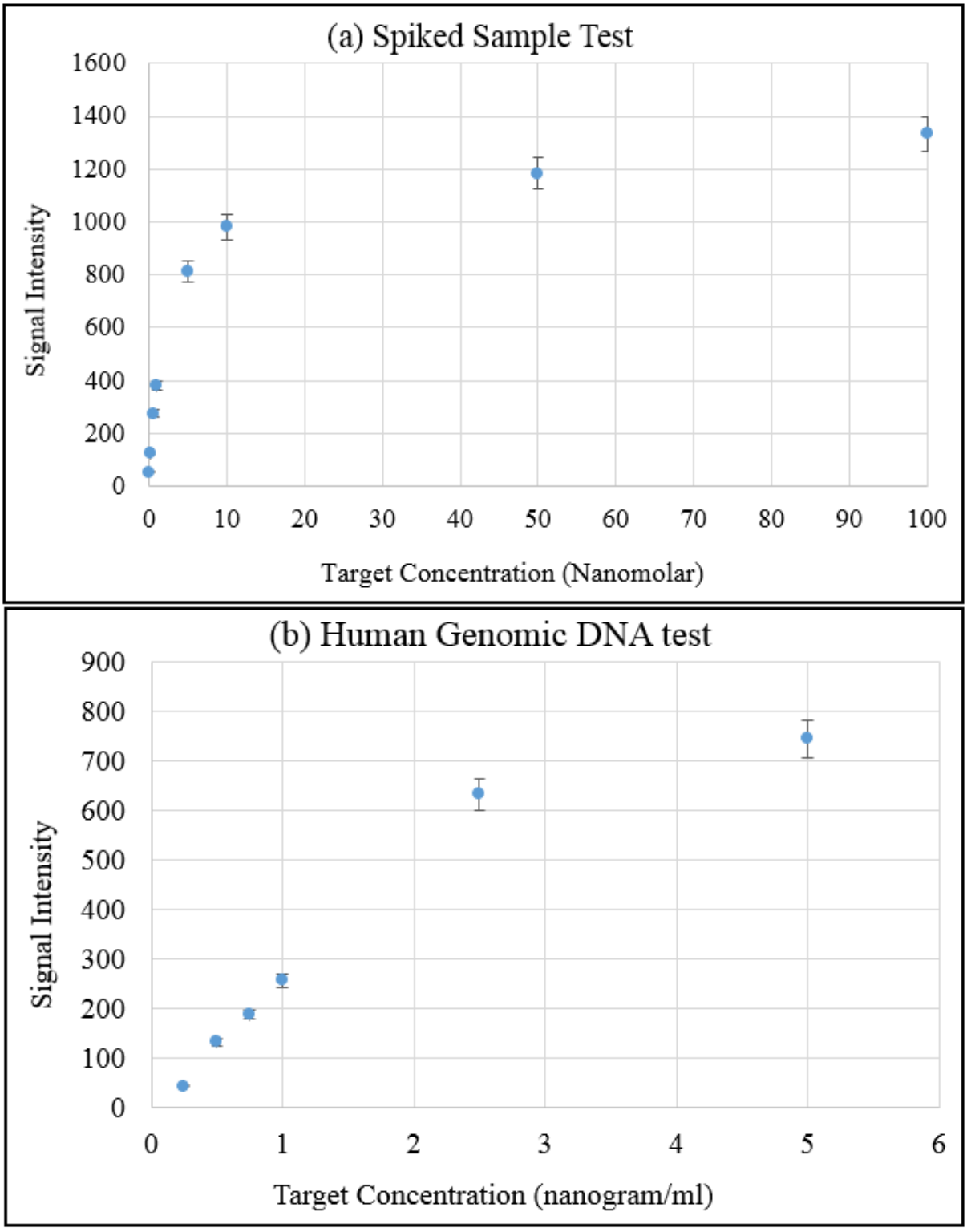

Figure 27: Shows calibration curve for (a) different concentrations of Target DNA spiked with 10 µl of undiluted human serum for every 100 µl of sample solution and (b) different concentrations of human genomic DNA as a target.

4.7. Conclusion

This work successfully developed a biosensor which is sensitive and fast for the detection of nucleic acid samples. This biosensor involved a conjugate preparation method which required less time and reagents as compared to the biosensor (DNAB) already introduced using the same principle of detection. Without the need for an amplification strategy, the technique provided a detection limit 50 times lower compared to the previously-known method. This fast, simple, reproducible, sensitive, low cost, and disposable biosensor based on sandwich hybridization immunoassay has a detection limit of 0.01 nM. The biosensor can also be used for real-sample detection in serum with a detection limit of 0.05 nM, and human genomic DNA with a detection limit of 0.25 ng/ml. The biosensor can be used for point of care and practical applications for DNA analysis.

CHAPTER 5. DETECTION OF *C. TRACHOMATIS* AND *N. GONORRHOEAE*

This chapter reports a DNA-AuNP based lateral flow nucleic acid biosensor for the sensitive detection of multiple STI targets based on sandwich hybridization reaction. The accumulation of the AuNP conjugates on the test zones enabled visual detection of multiple nucleic acids simultaneously. This proof of concept is demonstrated with the simultaneous assay for the detection of DNA from *Chlamydia trachomatis* and *Neisseria gonorrhoeae* strain samples. This study provides a simple, rapid, specific and low cost approach for the detection of multiple targets in a sample without interference. The work also shows great potential and promise for parallel point-of-care (POC) diagnosis and clinical applications.

5.1. Introduction

In the United States, *Chlamydia trachomatis* is the most common bacterial STI. It is also a major cause of pelvic inflammatory disease (PID) that leads to infertility and ectopic pregnancy. There are estimated to be three million new cases recorded every year. CT is an obligate, intracellular gram-negative bacterium. Mostly found in young women and men, this infection, if left undetected or untreated, can cause inflammation and scarring in both male and female reproductive systems. The second most prevalent disease in the USA is infection by *Neisseria gonorrhoeae.* NG is a pathogen responsible for causing 87.5 million new infections worldwide every year. Although gonococcal infection can be treated with use of antibiotics, no vaccines are available. More importantly, NG has also been associated with the transmission of other sexually transmitted diseases, including human immunodeficiency virus (HIV) (Chen et al., 2000; Jang et al., 1998; Pare et al., 1998).

Current screening practices differ around the world. Culture analysis of swab specimens was considered the diagnostic gold standard traditionally. Improvements were made by

developing antigen detection tests, enzyme immunoassay and nucleic acid hybridization. Lately, amplified assays, ligase chain reaction (LCR), strand displacement and transcription mediated assays have been used. Although culture tests are 100% accurate, theydo not provide high sensitivity to the desired target. Non-culture methods also suffer from disadvantages like antibody cross reaction and false positives. Nucleic acid amplification tests (NAATs) have become very popular assay for both CT and NG. These assays amplify and detect nucleic acid sequences unique to either CT and/or NG. These assays offer high sensitivity and selectivity and do not require a viable micro-organism. They offer a lot of advantages including high performanceand reliability results without interference from specimens such as those contained in urine or swabs. Despite of the ease of the assay, the NAATs' disadvantages are their cost, reduced performance in the presence of inhibitors and the need for sampling pre-treatment.

5.2. Brief Literature Survey

C. trachomatis and *N. gonorrhoeae* are of the two most prevalent bacterial sexually transmitted infections reported to the Center for Disease Control and Prevention (CDC), accounting for over 1.6 million reported infections in 2010 (Guaschino et al., 2000). The CDC estimates that these STIs have high rates of infection among female sex workers in developing countries, and cost the global health care system $1.5 billion annually (Castriciano et al., 2002). Since these infections, especially CT, are most often asymptomatic, the CDC recommends yearly screening in all sexually active women ages 16-25 years of age (Banoo et al., 2006). Traditional laboratory diagnosis of these infections is done by culture for *N. gonorrhoeae* and cell culture or antigen detection for *C. trachomatis* (Vasoo et al., 2009). Recently, NAATs have become widely accepted and are now recommended by the CDC as the test of choice; however, current NAATs are classified as high or moderate in complexity, and may take 1-2 days for results (Mills et al.,

2010; Okele et al., 2011). Also, molecular amplification techniques have been demonstrated to have improved sensitivity compared to culturing (Babo et al., 1991; McKiernan et al., 2010) and other diagnostic assays for the detection of chlamydia infection in urine, endocervix (Boman et al., 1997), throat and urethral swabs (Maass et al., 1994), sputa (Quinn et al., 1994), broncho alveolar lavage fluids (Tong et al., 1993), and eye secretions (Toye et al., 1996). New assays and new platforms which provide results at the time of patient visits are urgently needed, since many patients do not return for their results when laboratory-based tests that require several days to complete are performed. For this reason, lateral flow immuno-chromatographic tests or Rapid-Diagnostic-Tests (RDTs) provide emerging tools to screen infectious diseases even in resource-limited settings or remote locations where conventional approaches (e.g. clinical examination, standard lab-tests and microscopy) are limited (Zhu et al., 2012).

Today, with the achievements of nanotechnology, nanoparticle-based biosensors have aroused great interest, especially because nanoparticles can take advantage of signal amplification to achieve high sensitivity and selectivity for target analysis (Dong et al., 2013; Su et al., 2013; Zhang et al., 2013). A kind of lateral flow strip biosensor that combines nanoparticles with conventional immunoassay has attracted significant attention in biological analysis and clinical diagnosis in past years (Singer and Plotz, 1956; Zhang et al., 2006). Lateral flow strip biosensors are considered one of the most promising diagnostic technologies, owing to their simplicity, rapid analysis, low costs, high sensitivity and specificity (Zhang et al., 2006). In addition, they show less interference and more long-term stability over a wide range of climates (Cho et al., 2005).To date, several lateral flow strip biosensors have been developed to detect many objects such as DNA, mRNA, proteins (Mao et al., 2009; Xu et al., 2008), biological agents (Fisher et al., 2009; Liu et al., 2009; Nakasone et al., 2007; O'Keeffe et al., 2003; Xia et

al., 2009) and chemical contaminants (He et al., 2011; Li et al., 2007). Nevertheless, there are very few reports on multiplex DNA analysis using lateral flow strip biosensors. As a result, it is highly desirable to develop a facile, rapid and economical approach for the accurate detection of multiple DNA targets with a real-time application.

Herein is reported a lateral flow nucleic acid biosensor (LFNAB) for specific and sensitive detection of multiple nucleic acid sequences simultaneously. Under optimal conditions the platform was also applied to the successful detection of DNA extracted from *C. trachomatis* and *N. gonorrhoeae* cell strains with PCR amplification. The amplified product of the two STIs was tested directly on the LFB-CT/NG for visual response without the need for sample processing and washing steps. The promising properties of the approach are reported in the following sections.

5.3. Preparation of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* Sample

DNA from chlamydia and gonorrhoeae cultures were prepared by the Chelex method (Walsh et al., 1991). Mixtures of 50 ml of specimen and 200 ml of a 5% suspension of chelating resin (Chelex 100; Sigma, St. Louis, Mo.) in Tris HCl buffer (0.01 M, pH 8.0) were incubated at 56 °C for 15 to 30 minutes. The chelating resin was held in suspension by continuous stirring with a magnetic bar while it was added to the specimens. After incubation, preparations were mixed gently and heated at 100 °C for 8 to 10 minutes. After being mixed again, preparations were stored at 270 °C. Immediately before testing, preparations were centrifuged for 30 seconds in a micro centrifuge and 10 ml of the supernatant was used for PCR. A three-plex Takara SpeedSTAR PCR mixture was made containing forward- and biotin-labeled reverse primers (Gaydos et al. 2000) for CT (400 nM) and NG (400 nM).

Primers used for C. *trachomatis* (CT) -Product (316bp) and N. *gonorrhoeae* (NG) - Product (132bp):

Forward (CT): 5`- GGCGATATTTGGGCATCCGAGTAACG -3`

Reverse (CT): 5`- Biotin/-TCAAATCCAGCGGGTATTAACCGCCT -3`

Forward (NG): 5`- CGGTTTCCGTGCGTTACGA -3`

Reverse (NG): 5`- Biotin/-CTGGTTTCATCTGATTACTTTCCA -3`

This mix was split into four different aliquots. To each aliquot, additional template was added:

1. No additional template
2. CT template
3. NG template
4. CT and NG template

These mixes were then split into PCR tubes (four copies each) of 50 µl each and were run in a commercial thermocycler (iCycler) with one cycle at 95 °C for1 minute and 40 cycles at 95 °C for10 seconds and 55 °C for 30 seconds). The end product is a sequence with the 5' end labeled with biotin on one of the strands of the double-stranded product. On denaturation of the product, two sequences are obtained with half of the single strands labeled with biotin.

5.4. Preparation of Lateral Flow Strip for *C. trachomatis* and *N. gonorrhoeae*

The following sequences were designed for Lateral Flow Biosensor.

Oligonucleotides used for LFS-CT/NG were as follows:

CT-Probe 1 (LFS-CT-Test Line): 5'-CCTTTACCCCACCAACTAGC-3'/Biotin/-3′

CT-Probe 2 (LFS-CT-Conjugate): 5′-ThioMC6-D/CCAATCTCTCAATCCGCCTA-3′

NG-Probe 1 (LFS-NG-Test Line): 5'-ACTCGGAACAAATTGAATGC-3'/Biotin/-3′

NG-Probe 2 (LFS-NG-Conjugate): 5′-ThioMC6-D/GCGTGAAAGTAGCAGGCGTA-3′

CT/NG Probe 3 (LFS-CT/NG-Control): 5′-Biotin/TACGCCTGCTACTTTCACGC-3’

The LFS-CT/NG was prepared according to the procedure described in Chapter 2 with slight modifications. Briefly, the sample application pad was saturated with a buffer (pH 8.0) containing 0.25% Triton X-100, 0.05 M Tris-HCl, 0.15 M NaCl for one hour; dried and soaked again in ½ SSC and 10% Tween for 10 minutes. The conjugate pad was dispensed with a desired volume of AuNP-DNA conjugate solution onto the glass fiber pad. Nitrocellulose membrane was dispensed with DNA probes (CT probe 1, NG probe 2 and CT/NG Probe 3) at different locations to form test zones and the control zone. To facilitate their immobilization on the nitrocellulose membrane, streptavidin was used to react with the biotin-labeled DNA probes to form the streptavidin-biotin DNA conjugates. Finally, the sample pad, conjugate pad, nitrocellulose membrane, and absorption pad were assembled on a plastic adhesive backing. Each part overlaps 2 mm to ensure the solution is migrating through the strip during the assay. Strips with a 3 mm width were cut by using the Guillotine cutting module CM 4000. The sample pad of the cut strip was soaked in 0.025 mg/ml streptavidin and dried before use.

5.5. Assay Procedure and Working Principle for CT/NG Assay

The LFS-CT/NG was used to detect the PCR product, Figure 28. The sample was prepared by diluting PCR product in water followed by heat denature. The sample (100µl) was heated in boiling water bath for 5 minutes and the LFS-CT/NG was inserted in the tube immediately following the heat step, such that the samples were still warm. The double-stranded PCR product denatures into single strands on heating and the streptavidin on the sample pad binds to the biotin-labeled product produced from the use of reverse biotin primer. The streptavidin allows the other complementary half of the PCR product to move along the

biosensor and hybridize to the DNA labeled on AuNPs. The conjugate-target complex migrates along the membrane and forms a sandwich complex with the probes on the test zones. The excess conjugates form a double-stranded complex on the conjugate zone giving a red band at the end of the test run. The bands were visualized within 15 minutes as illustrated in Figure 29. For quantitative measurements, the strip was inserted into the strip reader DT1030, the optical intensities of the test lines and the control line were recorded simultaneously by using the "GoldBio strip reader" software. LFS-CT/NGs were stored at 4 °C until ready for use and were then brought to room temperature before applying samples.

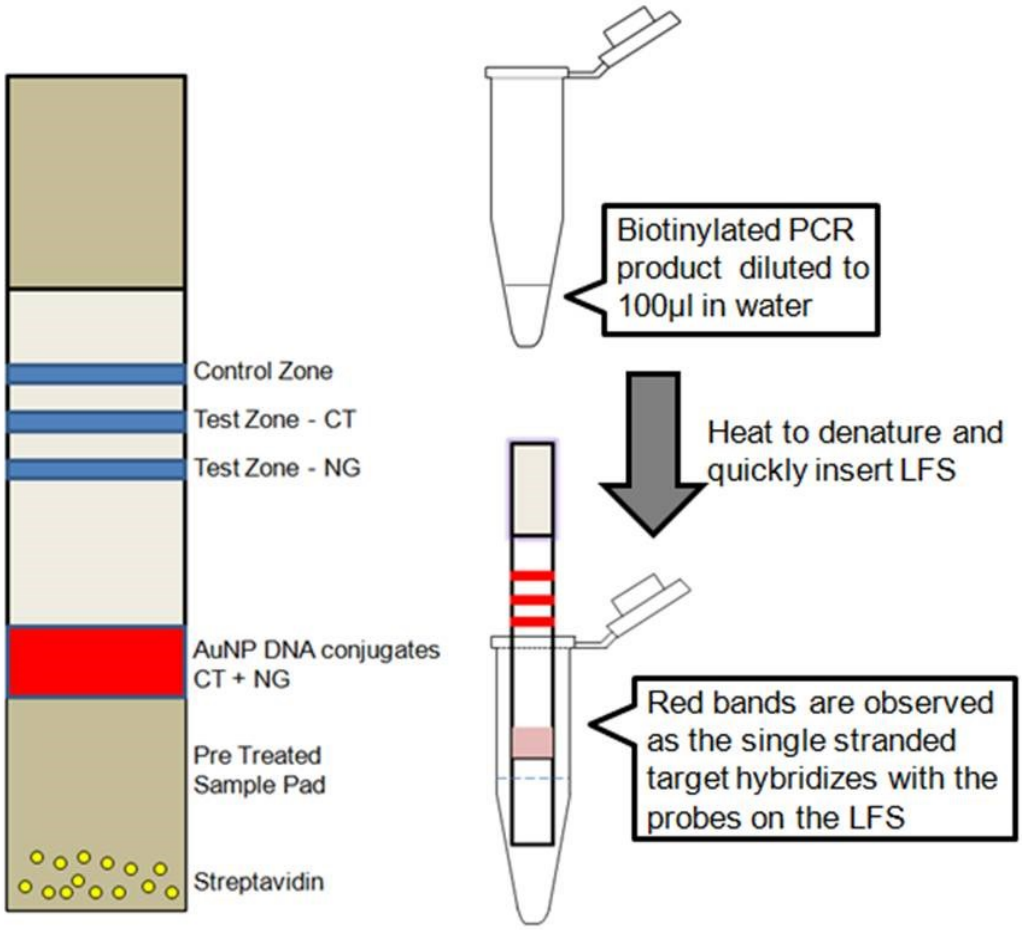

Figure 28: Typical analytical procedure to detect PCR-amplified DNA with the LFS-CT/NG

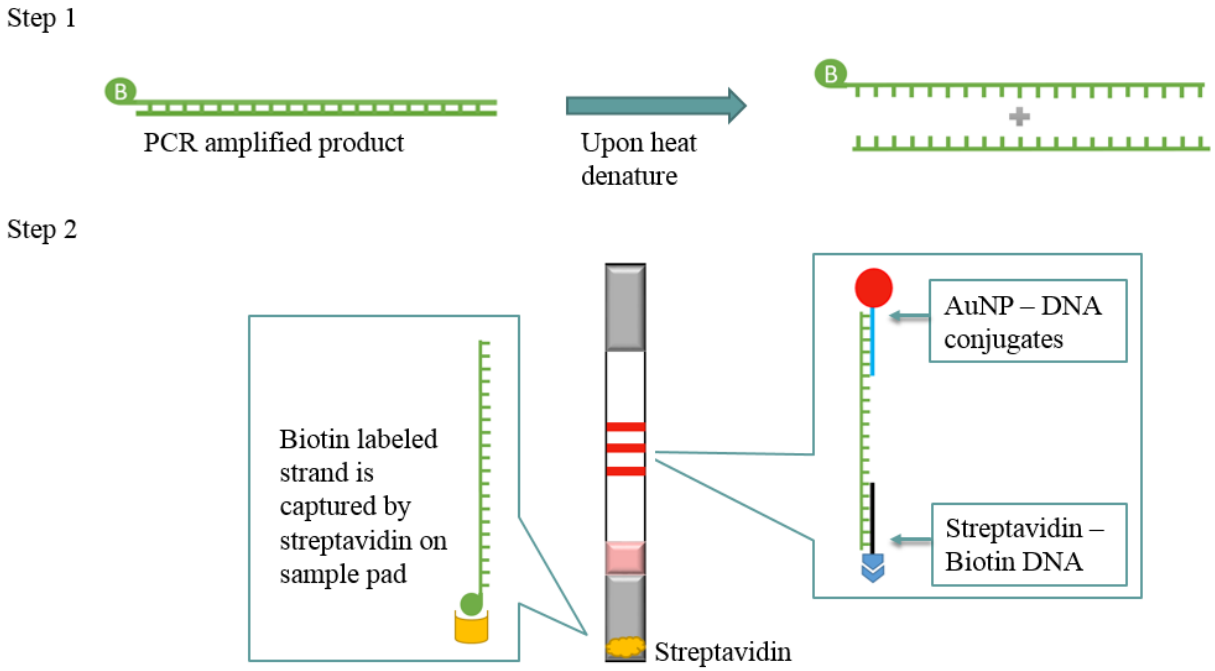

Figure 29: Shows the detailed mechanism of working principle on the lateral flow biosensor upon application of the test sample.

5.6. Optimizations of Different Parameters

Various conditions were optimized during the development of an assay for the simultaneous detection of CT and NG. The first optimization was performed on the sample pad preparation. Since this work eliminates the use of running buffer, using water for testing instead, the sample pad was tested with components of running buffer to initiate more sensitive detection of samples. Figure 30 shows the signal response from the use of different buffers. The sample pad was soaked in the listed buffers for 30 minutes each. Testing was done with 5 µl PCR diluted to 100 µl using distilled water. The results indicated that the use of half diluted 20XSSC in combination with Tween-20 increased the signal response immensely without any false responses without target present. Therefore, 1/2SSC + 10% Tween was selected for additional treatment of the sample pad for 30 minutes. The soaking time was also optimized during the work. It was observed that soak time affected the response greatly under the sample working

conditions. Based on the results obtained in Figure 31, 10 minutes soaking time was identified as the best condition. Increasing time increased the signal, but the background noise increased as well. This led to false adsorption of conjugates on the nitrocellulose membrane. More optimizations were performed during the preparation of the LFS-CT/NG. Different types of nitrocellulose membrane were compared. Unlike the usual trend from previous projects, the membrane HFB24004 showed a lesser response compared to HFB18004 (Figure 32). It is hypothesized that since HFB24004 membrane forces solution to travel slowly through the membrane due to the pore size, this possibly results in the re-hybridization of the target strand to the complementary strand from the PCR product. The membrane that allows faster movement limits the re-hybridization of denatured PCR product, thereby permitting the target strand to move along the biosensor.

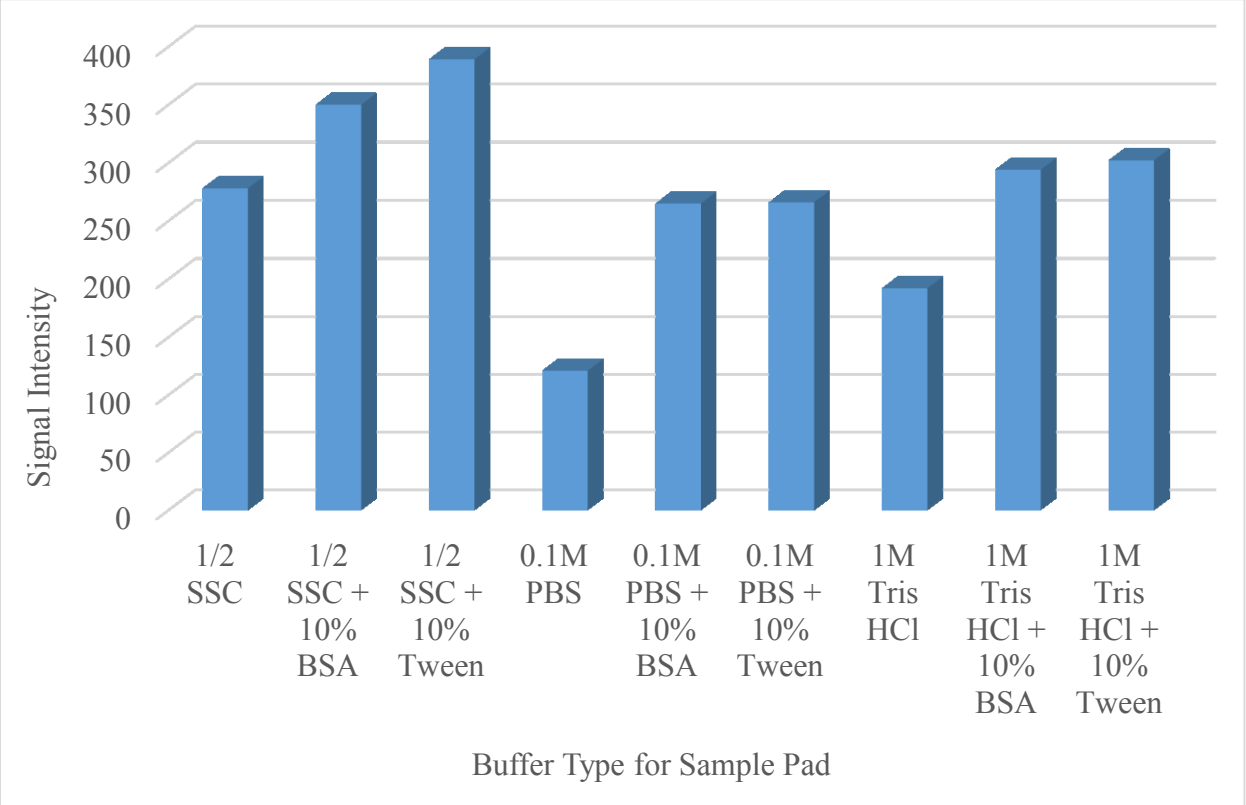

Figure 30: Comparison of signal intensity vs type of buffer used to treat sample pad before test.

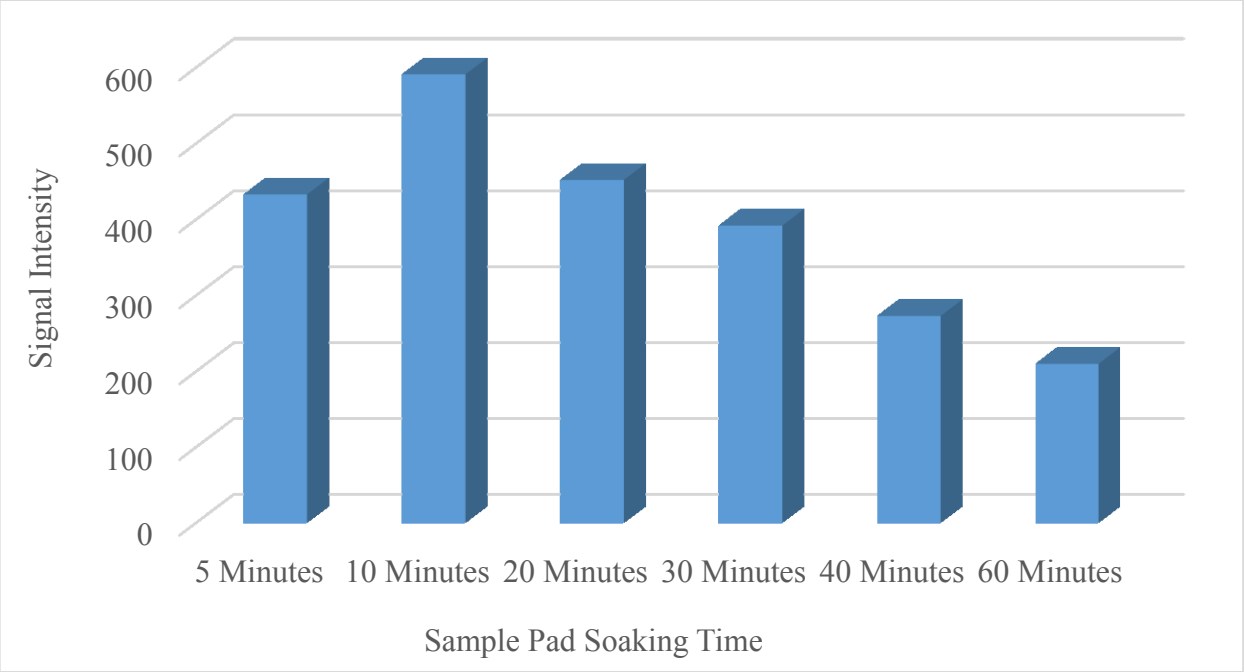

Figure 31: Amount of soak time for buffer vs signal intensity obtained for 5 µl product test.

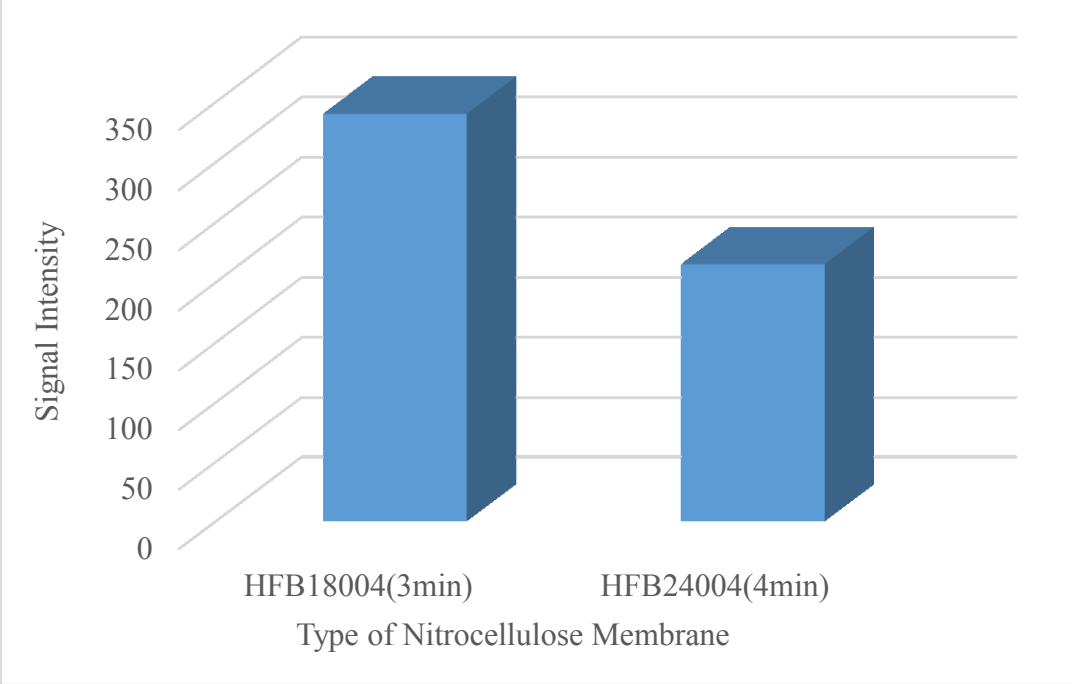

Figure 32: Graph comparing the signal response from the use of different types of nitrocellulose membrane during the preparation of LFS-CT/NG.

The amount of capture probe on the test zones was also optimized. 100 nmol liquid was dispensed multiple times on the test zone. Two test lines and one control line were dispensed with specific probes conjugated with streptavidin for immobilized dispensing. Figure 33 compares the signal response from different number of cycles used. Higher concentration of test line cycles increased false signal in the presence of a non-complementary target. The LFS-CT/NG was further improved by analyzing more conditions and optimizing the amount of conjugate loaded on the conjugate pad of the biosensor. As shown in Figure 34, best results were obtained with 2 dispensing cycles. The conjugate pad was prepared by mixing the two conjugates (AuNP-CT-DNA and AuNP-NG-DNA) in equal volumes followed by dispensing different number of times.

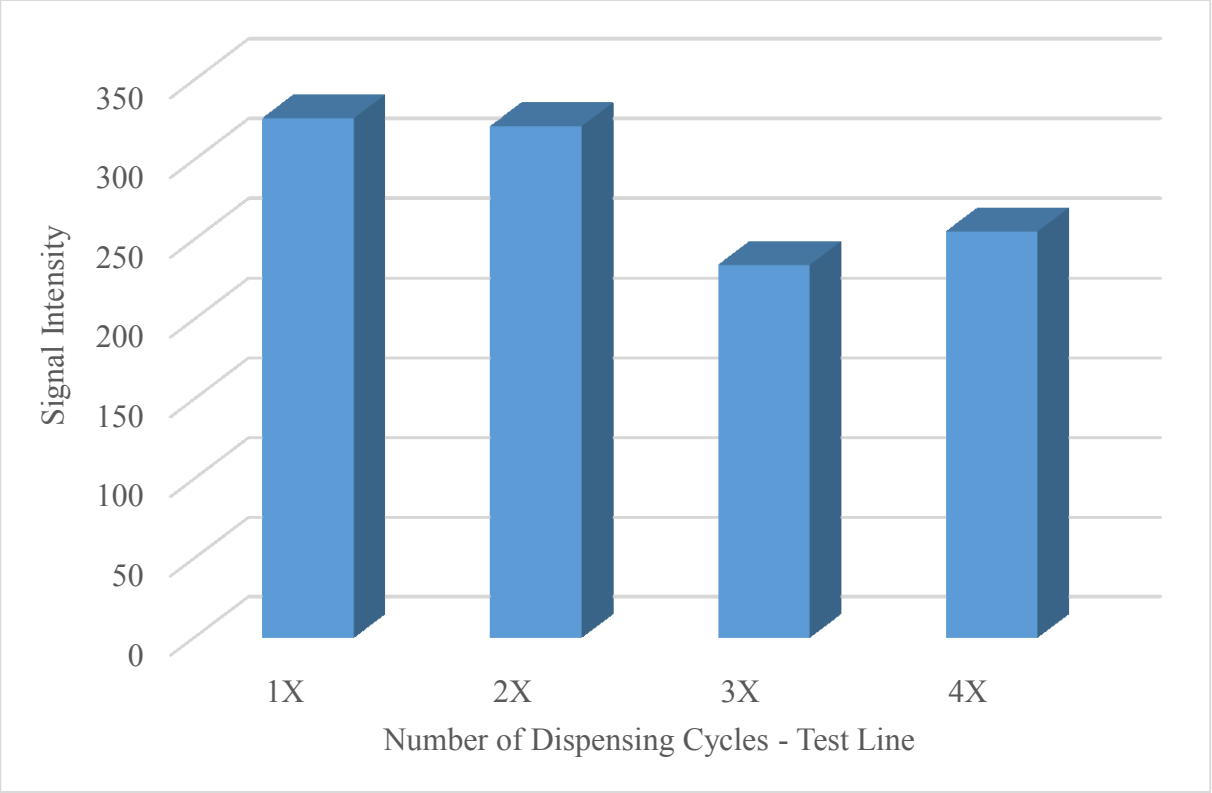

Figure 33: Shows the difference in signal obtained with increasing concentration of Test Line amount dispensed on the LFS-CT/NG.

After the assembly of all components of the LFS-CT/NG, the biosensor was cut into 3 mm wide pieces. Finally, on the edge of each sample pad, streptavidin solution was loaded with a pipette. 10 µl solution with different concentration of streptavidin was dropped and air dried. Figure 35 compared the response from different amounts of protein used per strip. Above 0.025 mg/ml, there was not a significant increase in the signal response. Hence, 0.025 mg/ml was chosen as the optimum amount of streptavidin per strip. The LFS-CT/NG was stored at 4 °C until further use.

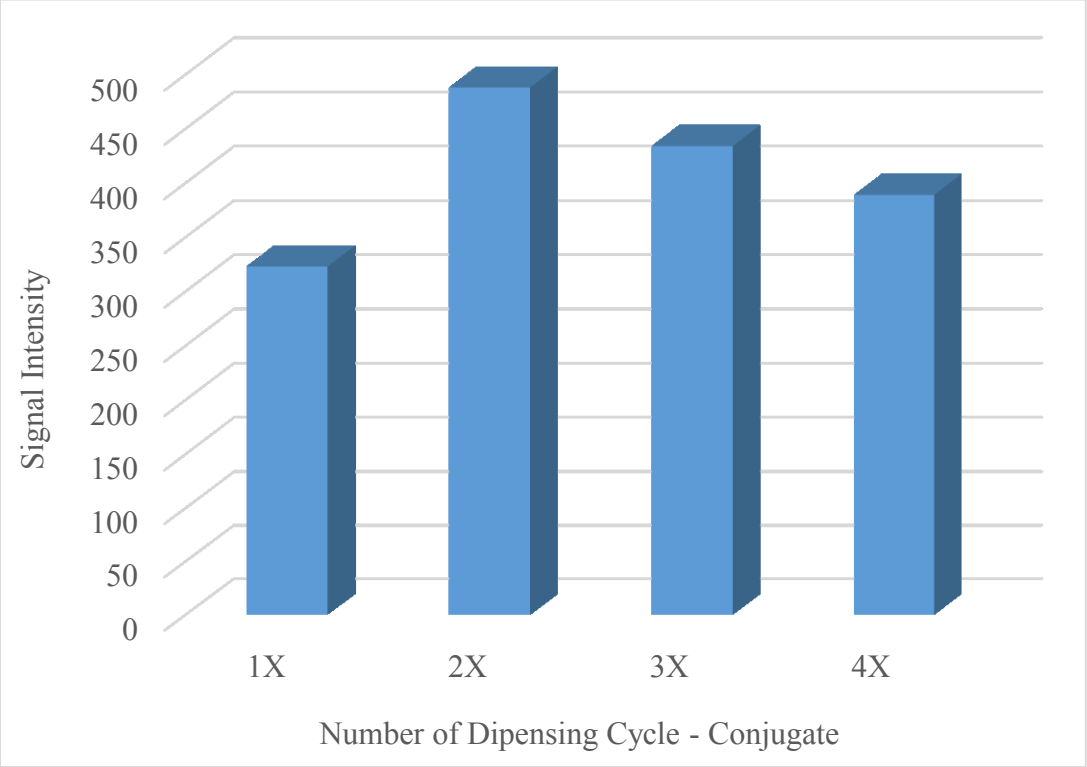

Figure 34: Shows the response recorded with increase in concentration of AuNP-DNA conjugates mixtures dispensed on the LFS-CT/NG

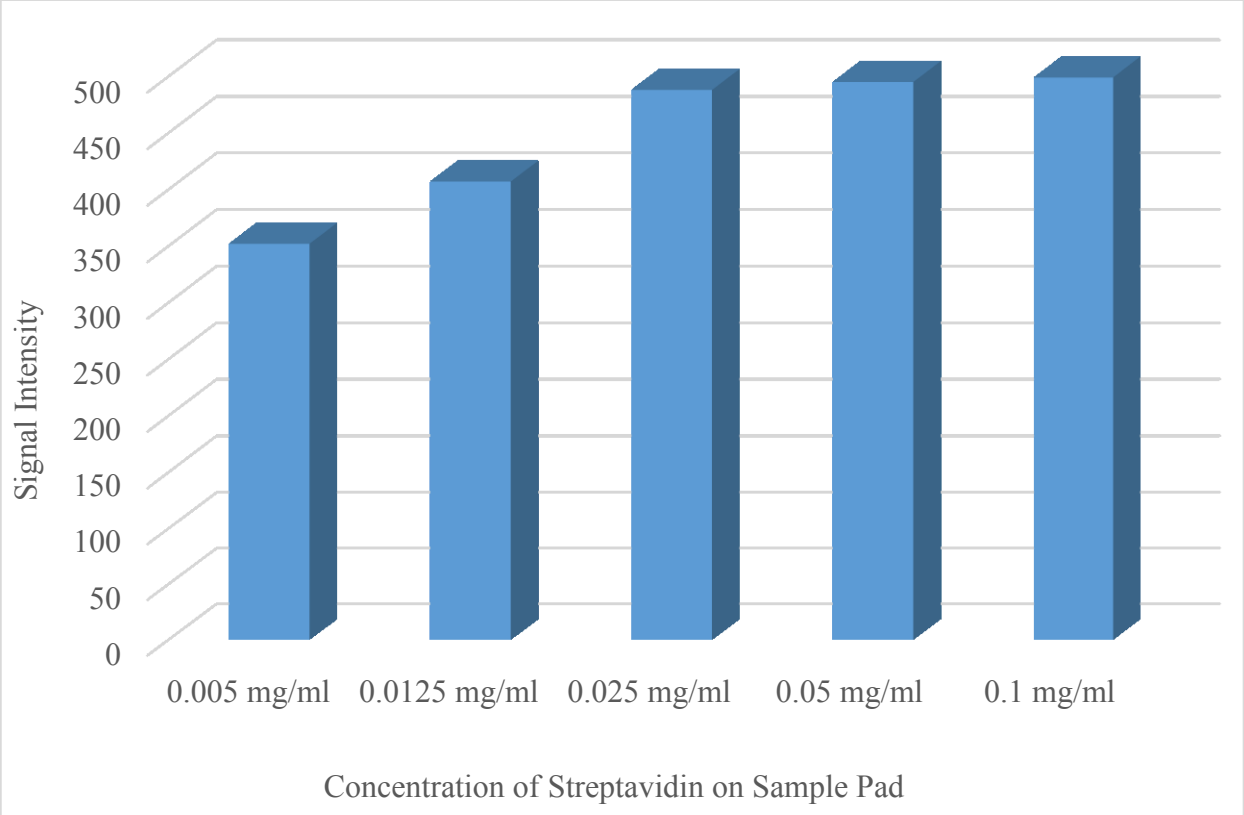

Figure 35: Graph showing the amount of Streptavidin loaded on the sample pad after the assembly of LFS-CT/NG vs the signal obtained.

5.7. Results and Discussion

Upon completion of PCR for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, the amplified product was validated via gel electrophoresis. Figure 36 shows the gel image obtained after the samples were run on 3% agarose gel. The 50bp dsDNA ladder was run in the first lane to the left (lane 1) with a non-template control (lane 2), CT as template (lane 3), NG as template (lane 4) and CT+NG templates (lane 5). The position of the bands on the gel indicate that the samples (CT and NG) amplified as 316bp and 132bp products, respectively. Two bands were observed in lane 5 of the gel indicating that both templates were amplified without interference from each other. Smaller bands were observed at the bottom of all lanes indicating the presence of excess primers in the reaction mixture. The biosensor was tested in the presence of both/none

target products (Figure 37). The LFS (1) and (2) shows the control response in the presence of water (without target) validating the function of the biosensor. The LFS (3), (4) and (5) gives the response in the presence of 10 µl CT, 10 µl NG and 20 µl CT+NG, respectively. For quantitative measurements, intensities of the test zones were recorded with the portable strip reader. The reproducibility of the system was also confirmed by testing replicates of the diluted sample. Figure 38 shows the LFS-CT/NG image taken from a camera: the response from control (left) and four sample strips containing CT and NG PCR products diluted in water. Same concentration was tested for both targets but the difference in the intensity of two test lines is due to the difference in concentration of the PCR product, because of the length of the amplified product. The LFS-CT/NG successfully detected both targets simultaneously and without interference, sans any complex purification or separation procedures, suggesting potential clinical applications of the LFS-CT/NG in biomedical diagnosis.

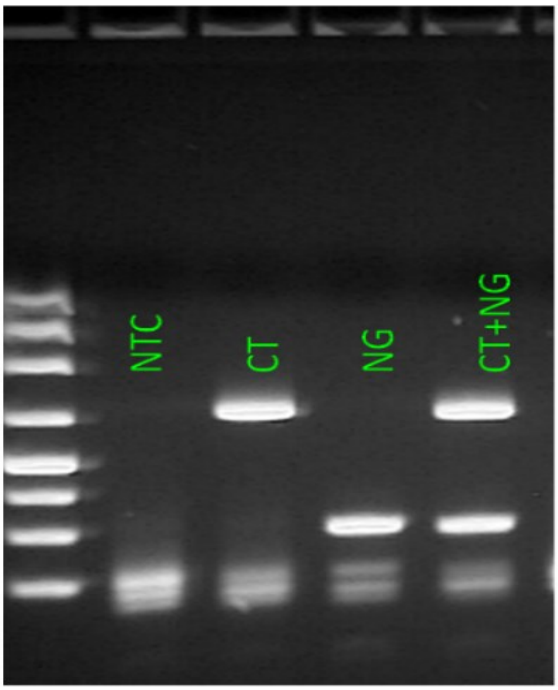

Figure 36: Illustrates the gel electrophoresis images containing (from left to right) Ladder, non-template control (NTC), CT, NG and CT+NG PCR products.

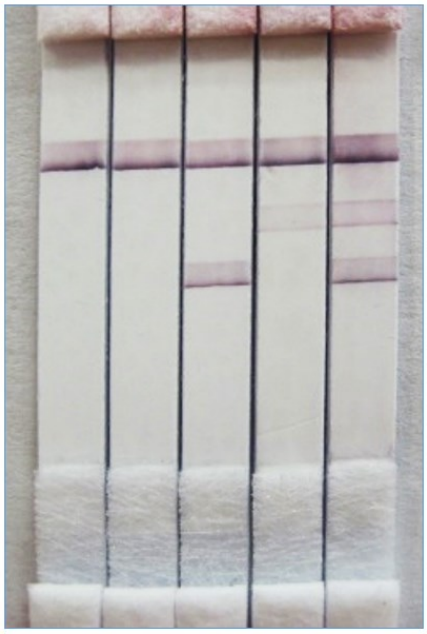

Figure 37: Typical photo images of (1) and (2) represent control conditions with 10 µl NTC + 90 µl Water. (3) and (4) show the presence of 10 µl NG target and CT target respectively. Strip (5) is the presence of both targets in equal volumes. Assay time: 20 minutes

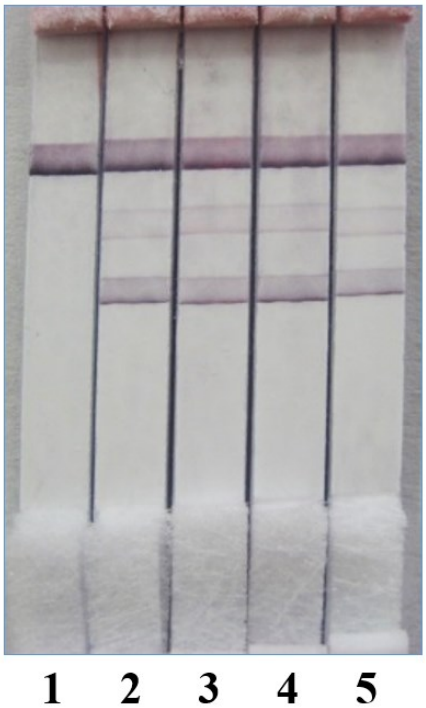

Figure 38: Typical images showing the reproducibility of the LFS-CT/NGs in presence of two targets. Strip on the extreme left is Control (NTC in water).

5.8. Conclusion

In this work, the lateral flow biosensor was applied to the successful, simultaneous detection of *Chlamydia trachomatis*- and *Neisseria gonorrhoeae*-amplified DNA samples. Compared to traditional methods, this approach provides a simple, fast, selective and sensitive method for simultaneous detection of nucleic acids without any complex sample treatment nor expensive instrumentation. This study opens new opportunities for the detection of multiple nucleic acids in a sample matrix without interference. This work can be further improved by introducing more test zones for multiplex detection of different STIs. Due to its reliability and accuracy, this research's biosensor shows great promise for the development of point of care detection of multiple STIs.

CHAPTER 6. DETECTION OF HPV-16 IN PATIENT SAMPLES

This chapter discusses a lateral flow biosensor designed specifically to detect samples of Human Papilloma Virus Type 16 from patient samples. This work employed the biosensor discussed previously for the detection of multiple nucleic acids with high selectivity and sensitivity achieved by various experiments and optimizations. This section further details the work to create a successful platform for the detection HPV-16.

6.1. Introduction

HPV is amongst the most common STIs among people who are sexually active. The CDC estimated that 20 million people are infected with HPV-16 in the United States, and about 5.5 million new cases are reported annually. The prevalence of HPV-16 is two times higher in women as compared to men. HPV-16 is the most common cause of cervical cancer. It also causes vulvar, anal, vaginal and penile cancers as well as other diseases which result in tumor formation (Cogliano et al., 2005).

Human Papilloma viruses are small, double-stranded DNA viruses known to infect epithelium. To date, there are more than 100 types of HPV, classified by the genetic sequence of their outer capsid protein. Usually HPVs are known to cause common warts. But about 40 types infect the mucosal epithelium that is associated with cervical cancer. The non-oncogenic types, known as the low risk HPVs, can cause benign or low-grade cervical cell abnormalities, but the oncogenic (high risk) tend to be carcinogenic. High risk HPVs includes types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69, 73 and 82. These high risk HPVs are associated with about 99% of cervical cancers. Of these, type 16 is found in 50% of these cancers (Kreimer et al., 2005; D'Souza et al., 2007). For diagnostic purposes, HPV has not yet been isolated from cultures; the infection can be identified in clinical samples only. Currently, the only technique

approved by the Food and Drug Administration is the Hybrid Capture 2 (hc2) High-Risk HPV DNA Test used for clinical samples. But the widely-used approach does not offer specific results. This technique, which is only approved for use in women, can only provide positive or negative results, but does not differentiate the type of HPV present (Parkin 2006; Koutsky et al., 1999; Weinstock et al., 2000).

6.2. Brief Literature Survey

The importance of detecting HPV and related biomarkers has led to development of several methods that can be employed to discriminate among lesions with high risk progression to cervical cancers. Since HPV cannot be propagated in tissue cultures, molecular biology techniques are used. The presence of HPV in cervical samples can be identified from morphological, clinical and serological findings (Villa & Denny, 2006; Porras et al., 2010). However, molecular biology techniques provide much better diagnosis for HPV. Nucleic acid hybridization assays use *in situ* hybridization, southern blotting and dot plot hybridization to detect HPV. As elaborated by Villa & Denny (2006), these techniques generate high quality information, but suffer from disadvantages such as low sensitivity and hence the need for large amounts of purified DNA. Signal amplification assays like Cervista HPV and Hybrid Capture 2 provide quantitative results with high sensitivity to genotyping. Yet, these techniques fail to detect HPV types selectively and are not designed to genotype individual samples (Hwang & Shroyer, 2011; Wright & Schiffman, 2003; Frazer et al., 2011; Otero-Motta et al., 2001; Johnson et al., 2008; Einstein et al., 2010). Another commonly-used method involves nucleic acid amplification tests (NAATs). This method includes various techniques developed in recent years to increase the sensitivity of the detection along with multiplex analysis of samples. Some of the common NAAT methods include microarray analysis (Rahman et al., 1996), PapilloCheck for

multiplex assay (Pista et al., 2011; Bryant et al., 2011), PCR and real time PCR (Zaravinos et al., 2009; Camargo et al., 2011; Coser et al., 2011; Carvalho et al., 2010; Nogara et al., 2012; Maver et al., 2010; Naqvi et al., 2004; Satiago et al., 2006), COBAS 4800 HPV test (Heideman et al., 2011; Mateos et al., 2011), HPV genome sequencing (Sanger et al., 1992; Smith et al., 1986; Lee et al., 1992; Novais et al., 2011), CLART human papillomavirus 2 (Pista et al., 2011), INNO-LiPA (Van Hamont et al., 2006; Sukasem et al., 2011; Lenselink et al., 2009; Schmeink et al., 2011), Linear array (Dobec et al., 2011) and more. Some of these techniques provide specific results and determine high risk vs low risk HPVs. An additional risk with these tests: the signal response from HPV genotypes can contaminate the previously amplified material and lead to false positive responses. Since such techniques are expensive and fail to distinguish between HPV-16 and HPV-18—which are responsible for roughly 70% of cervical cancers (source)—this work introduces a lateral flow biosensor that can distinguish HPV-16 from HPV-18 PCR amplified product. The samples are applied on the biosensor and can be watched for the presence of a red band at the test zone indicating the presence of the target HPV-16. The following sections include the details of the work done to achieve this goal.

6.3. Lateral Flow Biosensor

The biosensor was developed on a backing layer with assembly of a sample pad, conjugate pad, nitrocellulose membrane and absorption pad. The detailed procedure is described in Chapter 2. A few modifications were made to the biosensor for its specificity to the HPV-16 target. The sample pad was prepared by soaking the cellulose fiber membrane in a buffer (pH 8.0) containing 0.25% Triton X-100, 0.05 M Tris-HCl, and 0.15 M NaCl for 1 hour at room temperature (RT). The dried sample pad was soaked in ½ SSC + 10% BSA solution for 10 minutes. After drying completely, the sample pad was assembled with the rest of the components

of the biosensor. The conjugate pad was prepared by labeling a thio-labeled probe to 15 nm gold nanoparticles (AuNPs) using the dATP method introduced in Section 4.3. The sequence used for conjugation was 5'-/5ThioMC6-D/ATG ATC TGC AAC AAG ACA TAC ATC GAC CGG-3'. The Nitrocellulose membrane was designed with a test zone and a control zone. Two different streptavidin-biotin labeled DNA conjugates were prepared using 5'-/BIO-/TGT ACG CAC AAC CGA AGC GTA GAG TCA CAC-3' and 5'-/BIO-/CCG GTC GAT GTA TGT CTT GTT GCA GAT CAT-3' for the test and control zones respectively. The biosensor's four components were assembled and cut in 3 mm wide pieces before testing as shown in Figure 39. The target DNA was extracted from cell lines and amplified with PCR for testing. The expected amplified product after the PCR was used to design the probe sequence for conjugates, control and test lines.

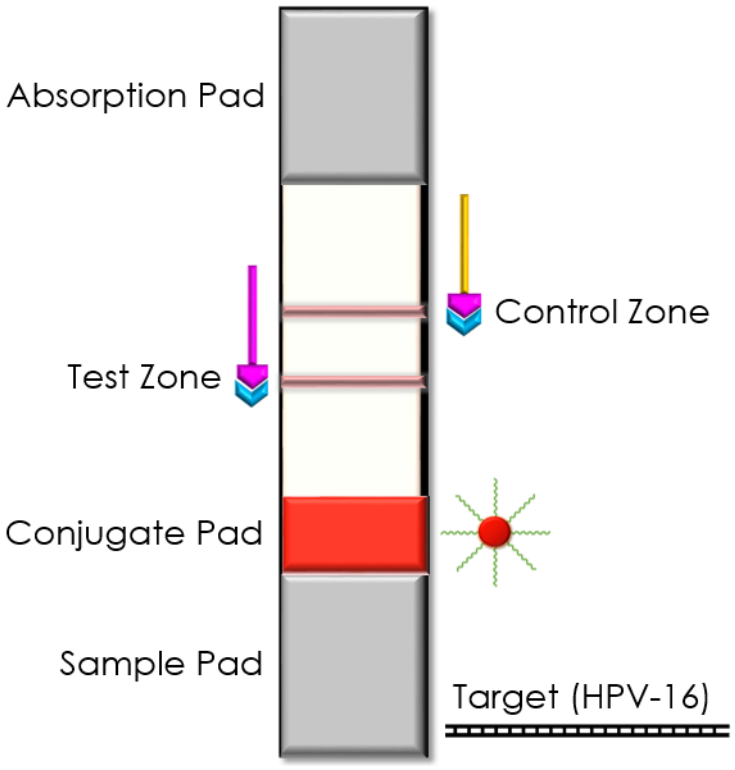

Figure 39: The schematic representation of the lateral flow biosensor for the detection of HPV 16 double stranded PCR product.

6.4. Sample Preparation

Multiple cell lines were obtained from ATCC, Manassas, VA and cultured. The cell lines included ATCC CRL-1550 (Ca Ski), an adherent, epithelial cell line isolated from the cervix of a 40 year-old female with epidomoid carcinoma. The CRL 1550, used as the target cell line, is reported to contain HPV-16 genome (about 600 copies per cell). Other cell lines included ATCC CCL-2 (HeLa) adherent, epithelial cell line isolated from a 31 year-old female's cervix with adenocarcinoma. Since CCL 2 is reported to contain HPV-18 sequences, this cell line was used as a control cell line for selectivity against other high risk HPVs. Two more cell lines that were randomly selected were also used as controls: ATCC CCL-119 (CCRF-CEM) and ATCC HTB-22 (MCF7) cell lines which do not contain any HPV sequences. The cells were cultured in conditions according to the procedures and protocols stated by ATCC.

The whole cells from individual cultures were collected in PBS and different dilutions were used to extract total genomic DNA using the Quick-gDNA MicroPrep obtained from Zymo Research, CA. The total extracted DNA from each cell line was amplified for HPV-16 sequence. The primers selected (Forward Primer: 5'-CTGTCAAAAGCCACTGTGTCCTG-3' Reverse Primer: 5'-GTGTGCCCATTAACAGGTCTTCCA-3') allowed us to attain a double-stranded DNA sequence of about 400 base pairs. The resulting 400 bp was used as a target sequence for the lateral flow biosensor.

The PCR amplification was carried out by using the standard protocol provided by Gaiffe et al. (2012) with slight modifications. The E6 HPV-16 gene was amplified after a hot reaction started at 94 °C for 5 minutes. The reaction was amplified for 35 cycles for 30 seconds at 94°C, 30 seconds at annealing temperature (52°C) and 20 seconds at 72°C followed by a 5 minute extension at 72°C. The amplified product was run on a 3% agarose gel for confirmation of the

desired product. For testing the sample on the lateral flow biosensor, the PCR product was denatured by heating the mixture in boiling water bath for 5 minutes and the biosensor was inserted in the 1.5 ml micro-centrifuge tube containing the sample solution to be tested (Figure 40). The biosensor was added immediately to avoid cooling of the sample. The lateral flow biosensor was observed after 20 minutes for the appearance of one or two red bands on the nitrocellulose membrane. The intensity of the red bands was quantitatively measured using the portable strip reader.

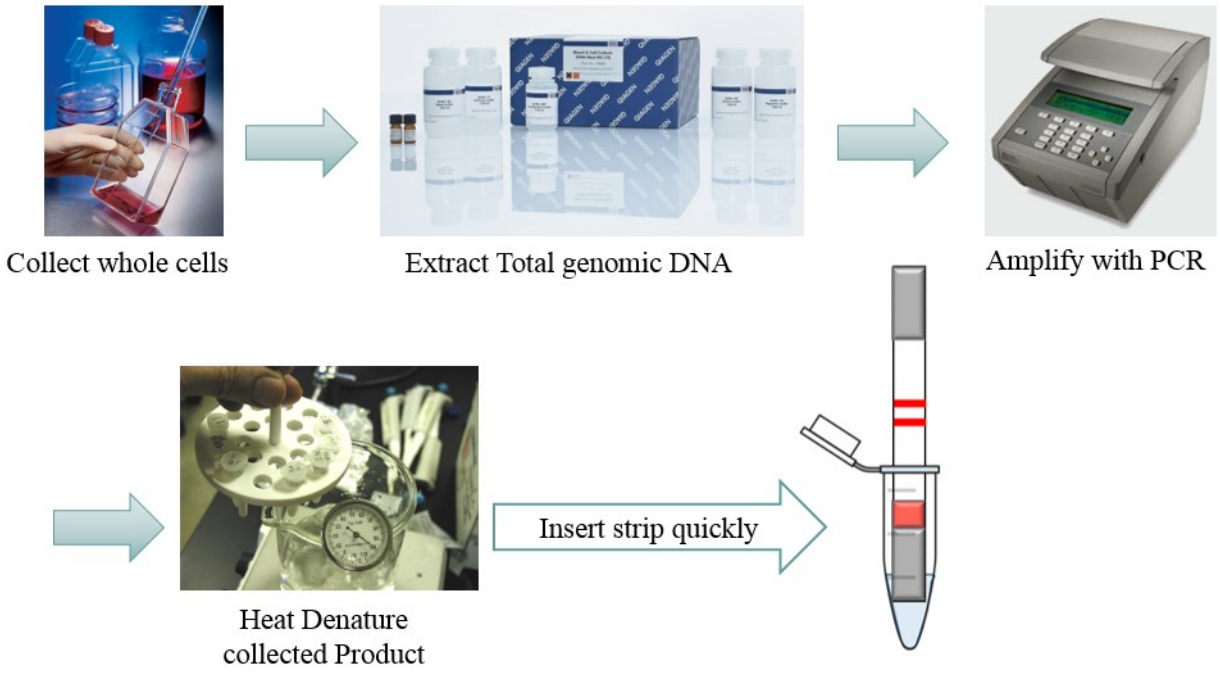

Figure 40: Shows the stepwise procedure for testing HPV-16 extracted from cell samples on a lateral flow biosensor.

6.5. Optimizations of Different Parameters

During the development of a biosensor for specific detection of HPV-16, various factors were considered and optimized for best results. The following experiments allowed us to maximize the signal response from the target cell line and minimize the background or control

signal from buffer or other cell lines. Figure 41 compares the use of different nitrocellulose membranes employed in the preparation of the lateral flow biosensor. Use of 3 minute (HFB18004) type showed higher signal response for target solution compared to the 4 minute nitrocellulose membrane. It was reasoned that the longer time on the membrane was thought to cool the sample solution, hence re-hybridizing the denatured strands from the PCR product. The amount of streptavidin-biotin labeled DNA probes dispensed on the nitrocellulose membrane was also altered during this work. Figure 42 shows the number of dispensing cycles using Biojet 3000 for both test and control lines. The bars represent the signal response from the target solution in presence of increasing amount of test line probe. The test line dispensed twice with DNA probes showed the maximum signal intensity in the presence of target. Higher concentrations increased the response but the response from control cell lines increased as well, giving false positives. The optimum amount of two dispensation cycles was chosen to prepare the strips.

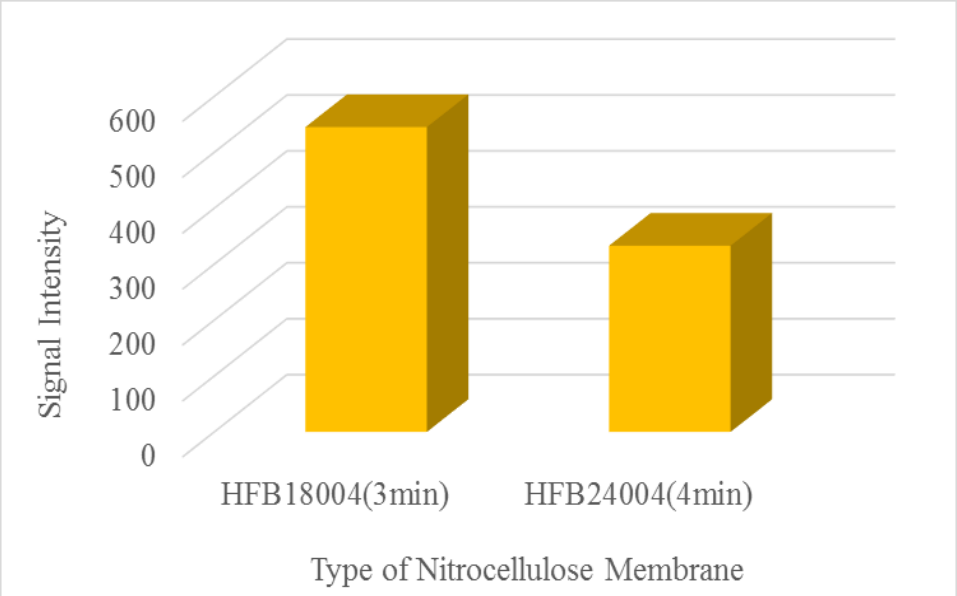

Figure 41: Shows the comparison of different nitrocellulose membrane used in the Lateral Flow Biosensor preparation.

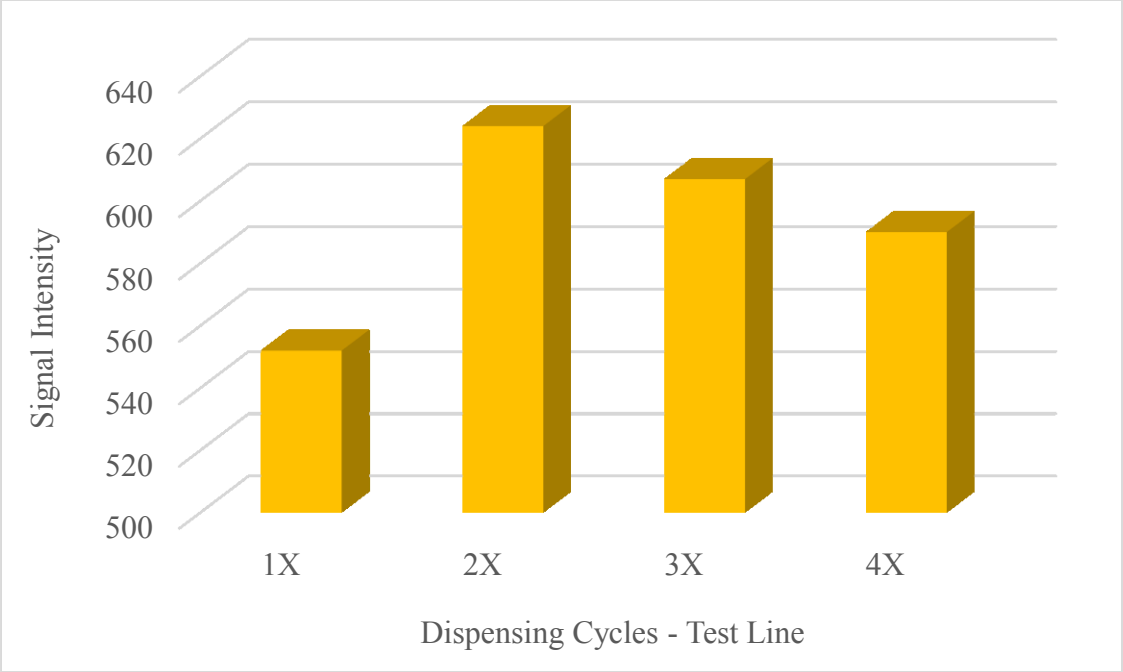

Figure 42: Bar graph showing the comparison between the signal intensity obtained at the test zone with increasing concentration of test line probe.

Other optimizations included the type of buffer used to soak the sample pad and the time of the soak. This was done to replace the use of running buffer in testing sample. The elimination of running buffer allows a researcher to test the amplified product directly without any dilutions or sample treatments. Figure 43 compares the signal obtained from the use of different buffers soaked for 30 minutes on the sample pad. The signal intensity was improved when the soaking time for the buffer was reduced to 10 minutes as shown in Figure 44. These results conclude that presence of SSC buffer on the sample pad increased the hybridization efficiency immensely.

Figure 43: Bar graph comparing the signal response on the lateral flow device prepared with sample pad soaked in buffer for varying times.

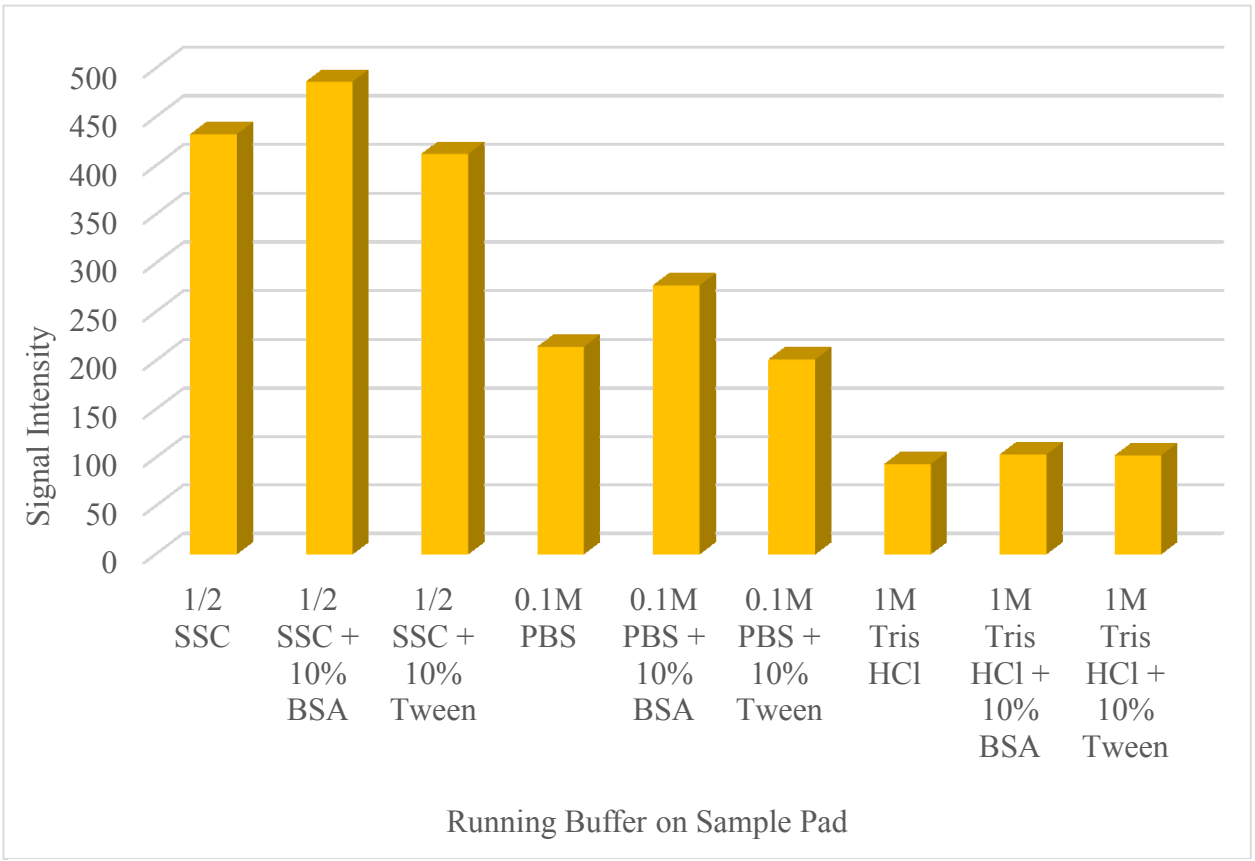

Figure 44: The response recorded from the use of different buffer components for Sample Pad vs. the signal intensity at the test zone.

As expected, BSA increased the sensitivity of the system by increasing the signal response compared to the use of Tween. Other buffer types showed no significant response. The amount of time the buffer was soaked on sample pad affected the signal intensity greatly. Even with the use of the optimum buffer type, if the soaking time was over 10 minutes there was a substantial decrease in the signal intensity as the concentration became too high, which affected the movement of the solution on the strip. It was observed that for higher soaking time the sample solution did not even move past the conjugate pad, hence even a control line was not observed. This led to the protocol of 10 minute soak time for a pre-treated sample pad.

Optimizations were also performed for the amount of conjugate dispensed onto the conjugate pad. Each cycle of dispensation added more conjugates, increasing the concentration of AuNPs labeled with DNA probes. It was expected that increased amounts of AuNP-DNA would increase the signal intensity at the test line and the excess conjugate would hybridize at the control zone. As illustrated in Figure 45, after three dispensing cycles, there was not a significant signal increase observed for the test line. Therefore, three cycles for AuNP-DNA conjugates were dispensed on the conjugate pad for assembly onto the lateral flow biosensor.

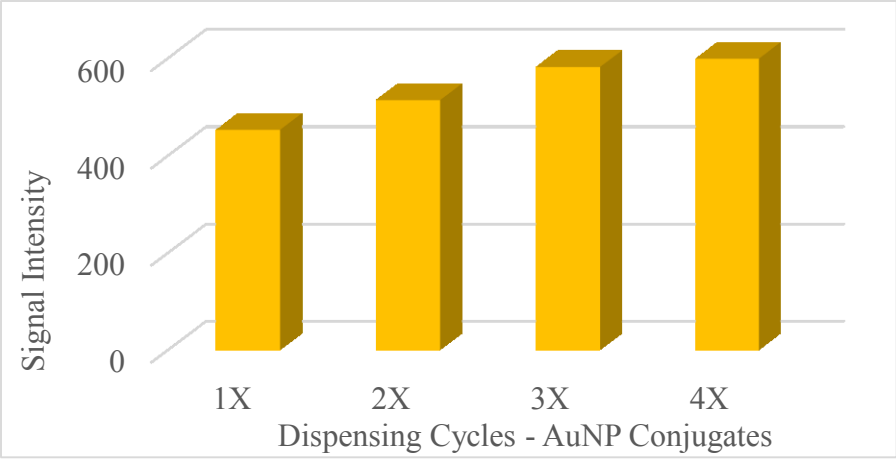

Figure 45: Compares the response on lateral flow biosensor prepared with different number of dispensing cycles for AuNP-DNA conjugates.

6.6. Results and Discussion

After optimizations of different parameters, the samples were amplified and run on an agarose gel to confirm the product formation before testing on the lateral flow biosensor. This chapter includes different experiments and tests performed to successfully detect HPV-16 without interference from other high risk HPVs.

Significant experiments were conducted to increase the concentration of the amplified product after PCR to increase the sensitivity of the system. The PCR conditions were altered to increase the amount of target DNA. It was evident that the use of primers in equal concentrations would give a double-stranded product of 400 base pairs in length. The amplified product was confirmed by running samples on a 3% agarose gel. Figure 46(a) shows the amount of dsDNA product after PCR. In the gel image, lane 1 and lane 6 contained 10 µl solution of 100bp ladder. Lane 2 was run with 10 µl PCR amplified product from the genomic DNA of HPV-16 (sample target). Lane 3, 4 and 5 were control lanes containing 10 µl PCR product amplified using genomic DNA from CCL-2 (HPV-18), CCL-119 (CCRF-CEM) and HTB-22 (MCF-7) respectively. It was observed that no product was formed in control cell lines. The product seen in lane 2 specific for CRL-1550 (target cell line) was obtained corresponding to the 400 bp band of the ladder, confirming the desired amplification. Figure 46(b) shows a gel image of a 3% agarose gel run with samples obtained after a PCR using altered working conditions. The samples for amplification were run with varying concentrations of primers. The forward and reverse primers had a final concentration of 0.25 µM and 0.05 µM respectively in the mixture before run. The use of reverse primer in smaller amounts led to increased formation of single-stranded DNA in the product. The presence of increased ssDNA in the final product was expected to increase the response on the lateral flow biosensor because the sequence of the

ssDNA was complementary to the chosen probes, incorporated on the biosensor for the specific detection of HPV-16. In the gel image, Lane 1 contains 100bp ladder (15 µl), Lane 2, 3, 4 and 5 contains 10 µl PCR amplified product obtained after amplification of genomic DNA obtained from HPV-16, HPV-18, CCRF-CEM and MCF-7 respectively. Lane 2 showed two bands for the target cell line, HPV-16, as expected. The band at 400bp position corresponding to the ladder is the dsDNA product formed from the use of two primers specific for the target. The band at approximate position of 225bp was believed to be the band formed through the presence of excess forward primer in the PCR mixture. To confirm the validity of this hypothesis, the two samples obtained from two PCR runs were tested on the later flow biosensor. The results obtained from the sample run in gel (B) were better than product obtained from the use of primers in equal concentrations.

The PCR product obtained from the use of primers with varying concentrations of both primers was thereby used to perform further experiments to detect HPV-16 samples on strips. Figure 47 shows the image captured from a camera after the test completion. Different samples obtained after PCR were diluted ten times using distilled water and tested on the lateral flow biosensor. Strip A contained only water; therefore, only one red band was observed in both strips (duplicates). The band observed corresponds to the control zone on the membrane, verifying the proper function of the biosensor. Strips B, C and D were tested with PCR product from control cell lines. Since the PCR mixture contained primers to amplify a specific region of the HPV-16 capsid genome, there was no amplification for control cell lines, and no response was observed on the test zone for the three. Two red bands were observed in Strip E. The band at this strip's test zone indicated the presence of desired target DNA obtained after amplification through PCR.

This showed that the biosensor is specific only to HPV-16, and can distinguish between other cell lines, including that with HPV-18.

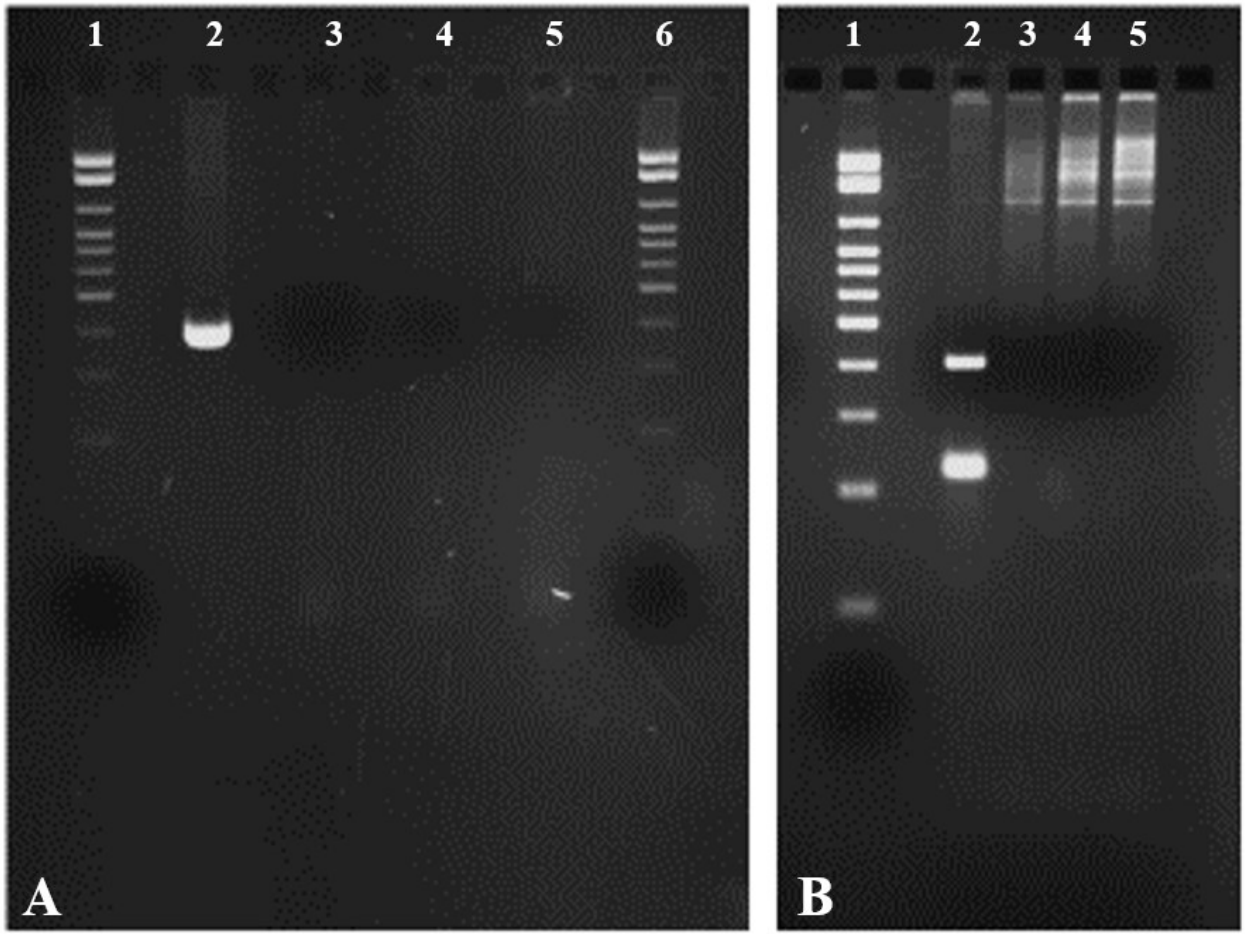

Figure 46: Gel image after running 10µl of PCR amplified product on a 3% agarose gel. (A) Shows the products obtained after PCR run from the use of Forward and Reverse Primers in equal concentrations. (B) Shows the products from differing amounts of the two primers. Each image corresponds to Lane 1 (and/or Lane 6): Ladder, Lane 2: HPV 16, Lane 3: HPV 18, Lane 4: CCRF-CEM and Lane 5: MCF-7.

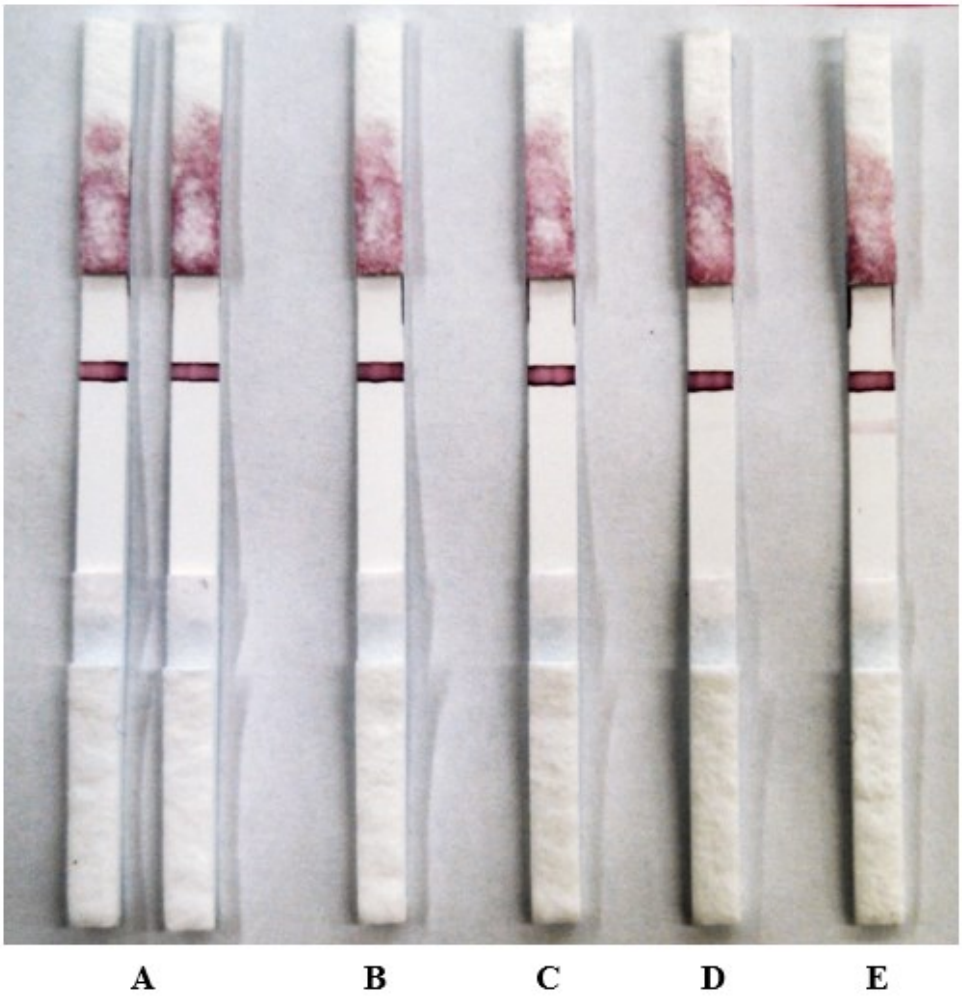

Figure 47: Shows the image response obtained on the lateral flow biosensor after completion of a test. (A) 100 µl water + 0µl PCR Product, (B) 90 µl water + 10 µl PCR Product from MCF-7, (C) 90 µl water + 10 µl PCR Product from CCRF-CEM, (D) 90 µl water + 10 µl PCR Product from HPV-18 and (E) 90 µl water + 10 µl PCR Product from HPV-16 were tested after heat denature in boiling water.

Another experiment was performed to evaluate the sensitivity of the biosensor. About 1 million cells were isolated from each cell line. The whole cells were subjected to serial dilutions corresponding to different numbers of cells. Each sample constituting a different number of cells was used to isolate total genomic DNA. The isolated DNA was then used as a template for PCR. The amplified products from each cell line were run on an agarose gel to visualize presence of any bands that would indicate amplification. No bands were obtained for control cell lines for

any number of cells. Amplification was only expected in the target cell line (HPV-16), which showed bands on a 3% agarose gel. Figure 48 shows the gel image with the bands obtained after PCR amplification of HPV-16 genomic DNA. The image also shows the base pair position for the ladder used. The PCR completion resulted in two bands as expected because of the use of primers with different starting concentrations.

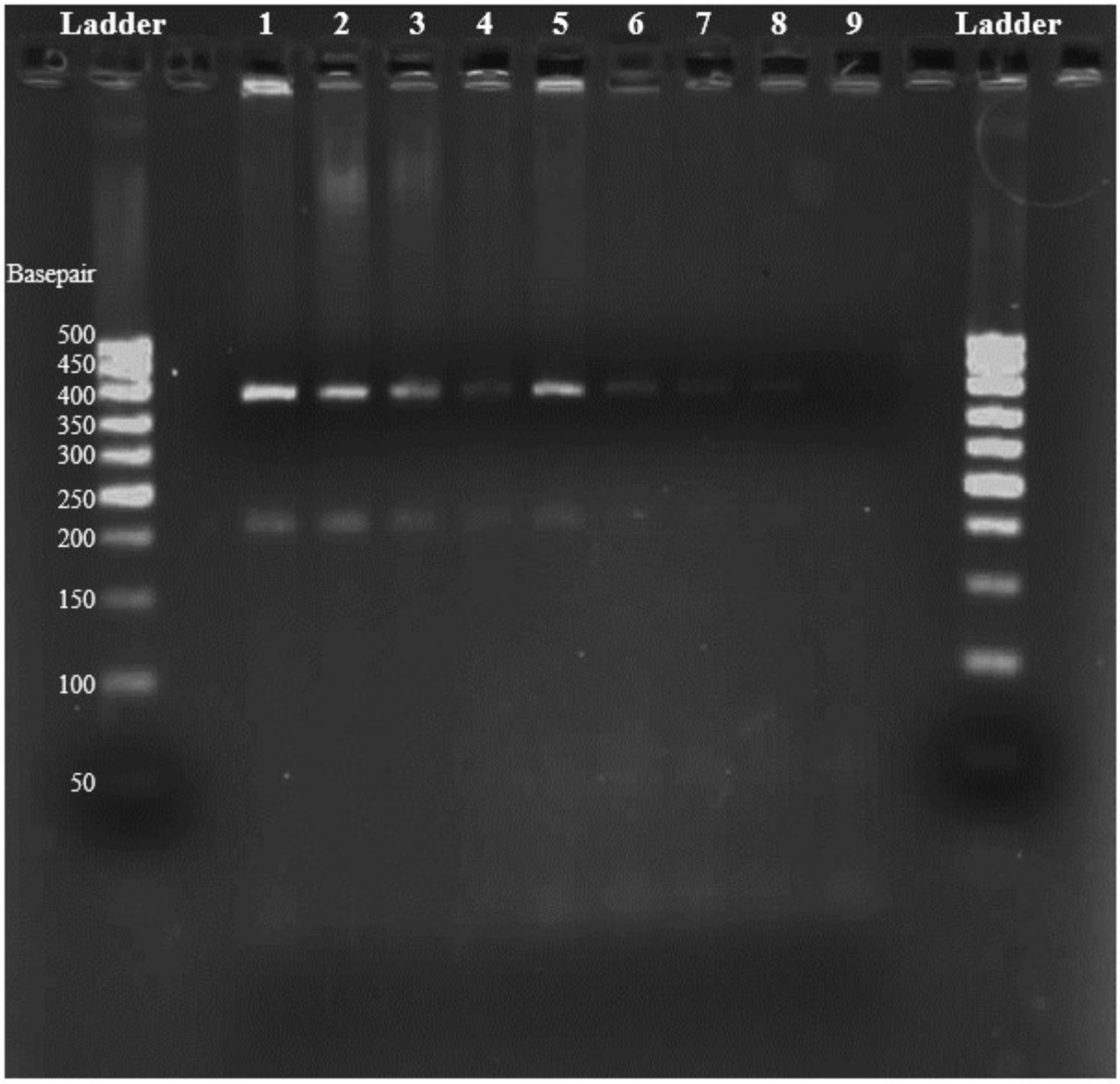

Figure 48: Gel Image showing the PCR product upon amplification. Lane 1 to 9 corresponds to different number of HPV-16 cells used. Lanes represent PCR product diluted to correspond with 100000, 10000, 1000, 100, 10, 1, 0.1, 0.01 and 0.001 cells respectively

The gel image response indicates that the band intensity decreased corresponding to the number of cells used for the extraction of genomic DNA, which henceforth were used as template for PCR amplification. The amplified product (approximately 100 µl) was also tested directly on the biosensor without dilutions or treatments. The amplified product was heat denatured and biosensor was added to the tube. Signal was obtained after 30 minutes. Figure 49 shows the image captured using a camera after test completion. Three strips were tested for control only. Water was used as the sample solution without any PCR product. Two lines are observed for strips 1 to 9 corresponding to test and control line. The varying intensity of the test line was the result from the use of different amounts of amplified product. Lane 1 through 9 parallels the PCR product. Serial dilutions were made of the PCR product to correspond to 100000, 10000, 1000, 100, 10, 1, 0.1, 0.01 and 0.001 cells respectively.

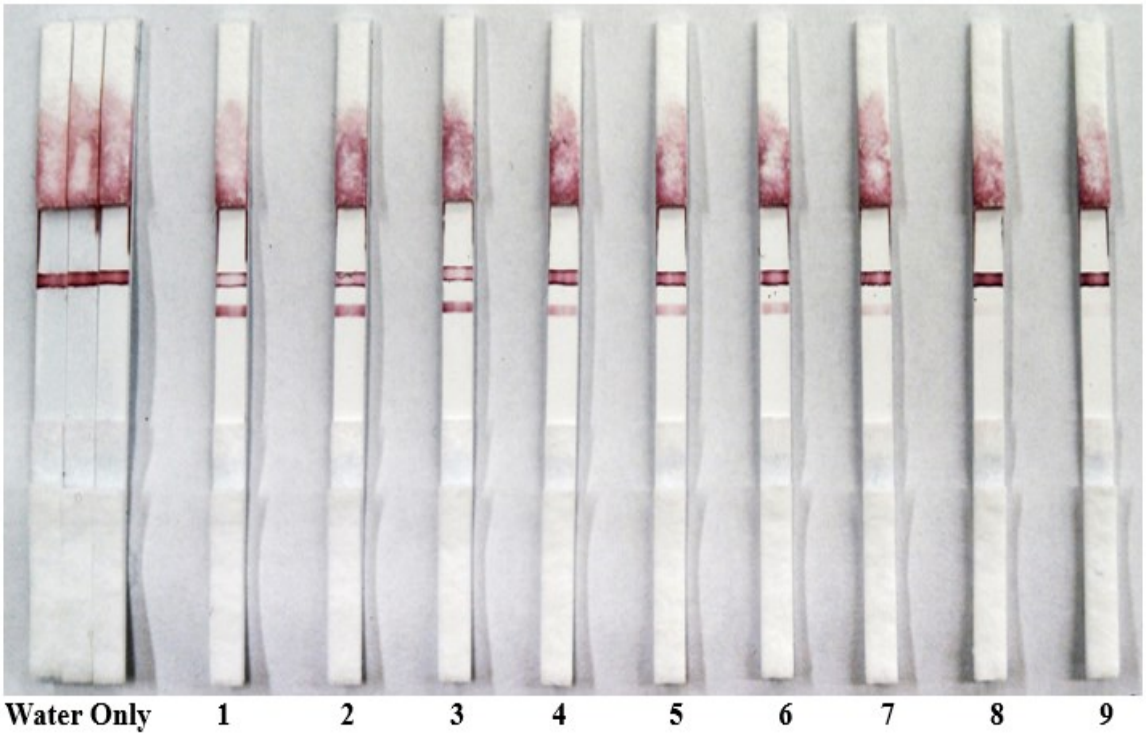

Figure 49: Image showing the biosensor after completion of a test. Water only strips contained no PCR product. Strips 1 to 9 were tested with 100 µl of PCR product obtained from decreasing number of cells' genomic DNA.

The lateral flow biosensor was also tested for real-time application for HPV-16. Clinical samples were obtained from Dicarta, CA, containing positive cervical swab samples of people infected with HPV-16. The five samples were comprised of one negative and four positive samples for HPV-16. Each swab was centrifuged down and used for extraction of genomic DNA. The extracted product was used a template for PCR. The amplified dsDNA was denatured and tested on the lateral flow biosensor. Figure 50 shows the results obtained from the clinical samples captured via a camera. Strip A showed no response for test line, whereas strips B through E showed varied response on both test and control zones, confirming that A was the negative sample and B through E were samples positive for HPV-16.

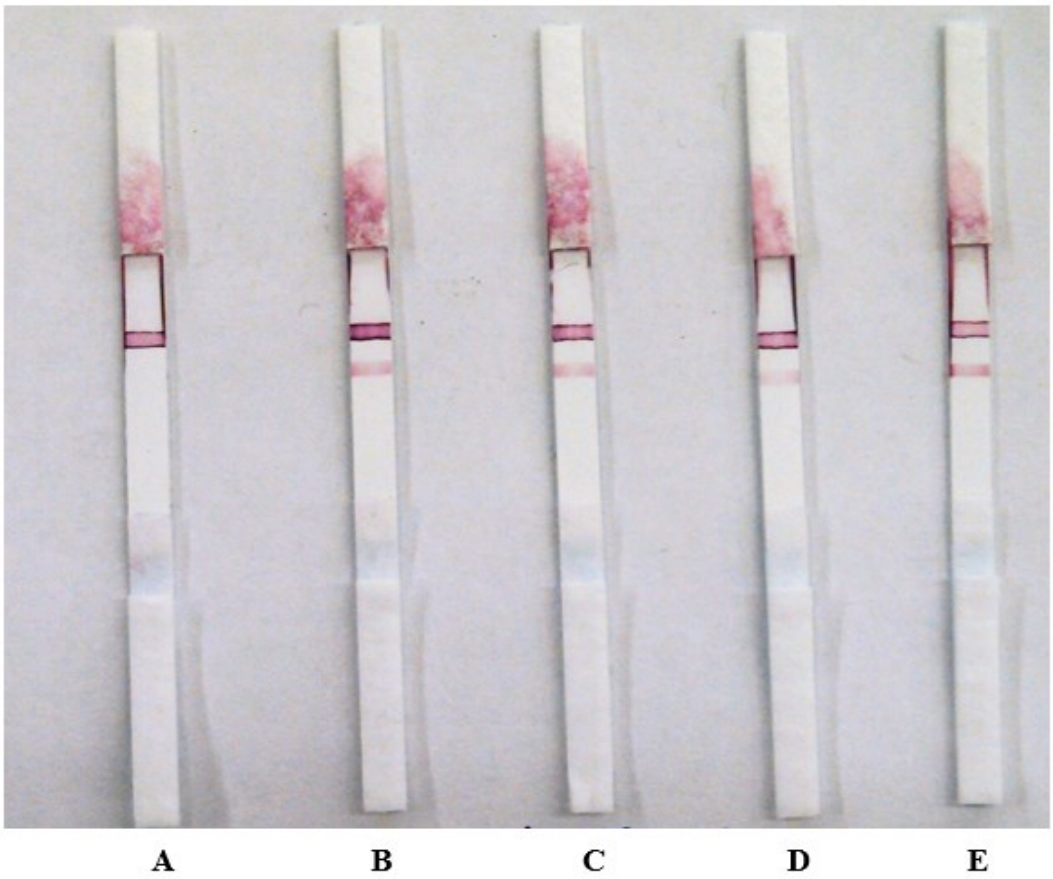

Figure 50: Real sample detection of negative (A) and positive (B-E) for HPV-16 in cervical swab.

6.7. Conclusion

This work successfully developed a platform for the detection of HPV-16 in patient samples. Clinical cervix swabs were obtained, and DNA extracted from the sample was amplified via PCR. The amplified product was used to design complementary probes to capture the target for detection. The heat-denatured product was directly applied on the lateral flow biosensor to deliver a visual response within 30 minutes. The biosensor designed herein is capable of successfully differentiating HPV-16 from other cell lines, including high risk HPV-18. This effort provides a sensitive approach to identify HPV in real samples, and demonstrate the biosensor's capability to be used as a point of care diagnostic tool.

CHAPTER 7. SUMMARY

DNA detection is of crucial importance in the field of point of care diagnostics. The Lateral Flow Biosensor developed in this work offered great sensitivity and selectivity against a wide range of nucleic acid targets. Short oligonucleotides specific to diseases or infections can be easily detected via the lateral flow platform introduced in this dissertation. The simultaneous and sequence-specific detection was achieved by introducing gold nanoparticles (AuNPs) functionalized with short oligonucleotides specific to target nucleic acids. DNA hybridization reactions between the target and AuNPs labeled with oligonucleotides were captured on different test zones of the biosensor designed specifically for individual targets. This sensitive approach provided visual results within 20 minutes and quantitative responses were obtained via portable strip reader in 30 minutes. This work allowed the successful identification of specific single-stranded nucleic acid targets of about 40-60 bases in length from a mixture of non-complementary sequences and other analytes.

Despite the low detection limit of 0.5 nanomolar for each target detected simultaneously, the biosensor needed more improvements to increase sensitivity to allow for its use in real-sample detection. It was proposed that altering the methods used in the preparation of the biosensor might improve the quality of the detection method. A new approach was employed for the conjugation of oligonucleotides on AuNPs that used dATP (deoxyadenosine triphosphate) as a surface blocker. The new preparation method allowed easy exchange of dATP with thio-modified DNA at high temperatures. The 3 hour incubation created stable AuNPs functionalized with a high number of DNA on the surface. The new preparation method was applied to the detection of nucleic acids and, upon comparison with old methods of conjugate preparation, lowered the detection limit to 0.01 nanomolar. Real-sample analysis was successfully performed

using human genomic DNA with a detection limit of 0.25 ng/ml (~$7.69*10^{-15}$ M). Direct detection of human genomic DNA at varying concentration proved the potential of the biosensor for its use in point of care diagnostic applications.

The sensitive approach developed in this work was put to test for the detection of other targets related to sexually transmitted infections. The work was improved by simplifying the test procedure for selective and sensitive detection of DNA isolated from STIs. The biosensor was modified to allow direct detection of samples without the need for buffers or instrumentation for analysis of the response. A test solution of single-stranded nucleic acid can be applied directly to the biosensor for a visual response within 20 minutes. Since the target concentration in DNA isolated from STIs was very low, the nucleic acids to be detected were amplified using PCR. Since PCR provided a high increase in the number of target copies, a successful detection platform was established that selectively detected STIs from a sample mixture. The biosensor technology was also applied to the detection of HPV16 DNA from patient samples obtained from clinics. Various cell lines (HeLa, CaSki etc.) were used to isolate DNA and amplified target was directly detected on the lateral flow biosensor.

Due to the low copy number of target DNA in CaSki cells, the biosensor was unable to detect the target sequence directly without amplification. Since the biosensor developed in this research can distinguish between small oligonucleotides, STIs, and even allow for specific detection of HPV-16 without interference from other HPV high risk types (e.g. HPV-18), it was concluded that further work in the field of amplification for the biosensor can allow us to build a platform to diagnose samples with yet more simplicity and sensitivity.

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