DEVELOPMENT OF A NUCLEIC ACID-BASED LATERAL FLOW ASSAY (NABLFA) FOR EASY DETECTION OF BREAST CANCER

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor Philosophiae in the Department of Life and Consumer Sciences, College of Agriculture and Environmental Sciences, University of South Africa.

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ABSTRACT

Women in Africa currently have the lowest registered cases of breast cancer. However, they have the highest mortality rates. In South Africa (SA), women are at a greater risk of been diagnosed with breast cancer due to changes in behavioural and genetical risk factors. Such high mortality rates can be attributed to the late diagnosis of the disease due to inaccessibilityand the high cost of current diagnostic tools. Breast cancer is asymptomatic, at early stages, which is the best time to detect it and intervene to prevent high mortality rates. Proper risk assessment, campaigns and access to adequate healthcare need to be prioritised among patients. Early detection of breast cancer can significantly improve the survival rate of breast cancer patients since therapeutic strategies are more effective at this stage. Early detection can be achieved by developing devices that are simple, sensitive, low-cost, and employed at point-of-care (POC) especially, in low-income countries (LIC). Nucleic acid-based lateral flow assays (NABLFA) methods that combine molecular detection with the immunochemical visualisation principles have recently emerged as tools to use for disease diagnosis even at low biomarker concentrations. Developing a NABLFA offers a rapid, simple, sensitive and inexpensive tool disease detection at POC.

In this study, three known breast cancer target genes: tumour suppressor and transcription factor *(p53)*, phosphatase and tensin homolog *(PTEN)* and breast cancer gene *(BRCA1)* were identified through literature mining as molecular targets. The expression of these genes in various human cell lines was validated through bioinformatics and real-time polymerase chain reaction (R -PCR). DNA was extracted from MCF7, T4D7, MDA231, MCF12A,BHK-21, A375 and Me-180 cell lines using Wizard® Genomic DNA purification kit and amplified by conventional PCR using two sets gene-specific primers; with one set tagged with biotin (forward primer) and digoxigenin (dig, reverse). The NABLFA was developed by immobilising dig antibody (anti-dig) on the test line and biotin on the control line on a nitrocellulose membrane. The 14nm citrate-capped gold nanoparticles (AuNPs) were used as a colorimetric label. AuNPs were synthesised using citrate reduction method and characterised by UV-Vis absorption

spectroscopy and transmission electron microscopy (TEM). Then conjugated with streptavidin using biotin-streptavidin chemistry and used as detection probe in the NABLFA. The binding and sensitivity of the analyte (biotin-tagged PCR product) to anti-dig was accessed by wet testing. The LFA strip was dipped in a tube containing biotin-tagged PCR product and AuNP-streptavidin conjugate. The binding of analyte to anti-dig was visually monitored after 15mins incubation with a show of red lines on both control and test lines.

In silico analysis demonstrated that *p53* and *BRCA1* are highly expressed in breast cancer than normal tissues whereas *PTEN* is highly expressed in normal breast tissues than cancerous. This was further confirmed by conventional and real-time quantitative PCR (RT-PCR). The developed NABLFA successfully detected the PCR amplicons of *p53* and *PTEN* validated by the appearance of two red lines both the control and the test line indicating the presence of the test biomarker. A rapid AuNPs-based NABLFA was successfully developed with a turn-around time of 10mins. The assay had a limit of detection of 0.06ng/ml and 0.125ng/ml for *p53* and *PTEN* respectively*.* The AuNP-based NABLFA could serve as a screening tool for various diseases in resource-limited and LIC. Furthermore, in resource-limited areas, the assay can be developed into loop-mediated isothermal amplification (LAMP) diagnostic test to eliminate the use of conventional PCR in POC settings.

LIST OF PUBLICATIONS AND PRESENTATIONS

Publications:

1. **B. Dyan** et al. A Review of the Nucleic Acid-Based Lateral Flow Assay for Detection of Breast Cancer from Circulating Biomarkers at a Point-of-Care in Low Income Countries: A review. *Diagnostics* **2022**, *12*(8), 1973; https://doi.org/10.3390/diagnostics12081973.

2. **B. Dyan** et al. Development of a Nucleic Acid- Based Lateral Flow assay (NABLFA) for Detection of Cancer Biomarkers at Point-of-Care: Manuscript in preparation.

Presentations:

B.Dyan. Development of a Nucleic Acid-Based Lateral Flow Assay (NABLFA) for easy detection of breast cancer. Oral Presentation. NIC 10th year celebration, 14-16 October 2018. CSIR International Conventional Centre.

DECLARATION

I, Busiswa Dyan, declare that **Development of a Nucleic Acid-based Lateral Flow Assay (NABLFA) for Easy Detection of Breast Cancer** is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Signed

Date: **November 2022**

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Dedication

I dedicate this thesis to my sister **Andiswa Faith Dyan** for her support and love during my studies.

When a person really desires something, all the Universe conspires to help that person to realize her dream."

— Paulo Coelho, the Alchemist

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ART	Antiretroviral therapy
AIDS	Acquired immunodeficiency syndrome
AuNPs	Gold nanoparticles
AOL	Adjuvant online
BRCA1	Breast cancer gene
CANSA	Cancer Association of South Africa
ctDNA	Cell-free circulating tumour DNA
CRC	Colorectal cancer
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DCN	Diagnostic consulting network
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assays
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug administration
FBS	Fetal bovine serum
GEPIA	Gene Expression Profiling Interactive Analysis
HPRT	Hypoxanthine phosphoribosyl transferase
FISH	Fluorescence hybridization
HIC	High-income countries
HIV	Human immunodeficiency virus
IHC	Immunohistochemistry

KRAS	Kirsten rat sarcoma viral oncogene homologue
LFA	Lateral flow assay
LFB	Lateral flow biosensors
LMIC	Low- middle-income countries
MirDIP	microRNA data integration portal
MP	MammaPrint
MRI	Magnetic resonance imaging
NABLFA	Nucleic acid-based lateral flow assay
NCR	National cancer registry
NEAR	Nicking enzyme amplification reaction
NIC	Nanotechnology innovation centre
PCR	Polymerase chain reaction
PEG-SH	Polyethylene glycol-thiol
POC	Point-of-care
PTEN	Phosphatase and tensin homolog
QCM	Quartz crystal measurement
RIA	Radioimmunoassay
RBCs	Red blood cells
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SANBs	South African National blood service
SARS-Cov-2	Acute respiratory syndrome coronavirus 2

SA	South Africa
STRING	Search tool for retrieval of interacting genes
SNPs	Single nucleotide polymorphisms
SPR	Surface Plasmon resonance
SSA	Sub-Saharan Africa
TEM	Transmission electron microscopy
TiGER	Tissue-specific gene expression and regulation
Tp53	Tumour suppressor and transcription factor
UMC	Upper-middle-income
UICC	Union for International Cancer Control
USA	United States of America
WBCs	White blood cells
WHO	World Health Organization

CHAPTER 1   INTRODUCTION

1.1   BACKGROUND

Breast cancer is known to be the second largest/common cancer that affects women after cervical cancer globally. The reported number of diagnosed cases has increased rapidly, with 2.3 million cases annually[1]. The majority of breast cancer cases, 90-95%, are attributed to lifestyle, while 5% -10% of these cases are due to heredity[2] as shown in Figure 1.1. Strategies and campaigns have been developed to bring awareness and encourage women to screen and test for breast cancer. However, these are inaccessible to women in low and middle-income countries (LMIC) and as a result, they are still diagnosed at an advanced stage of the disease. Consequently, more than 685 000 mortality rates are reported globally each year due to delayed diagnosis of breast cancer[3].
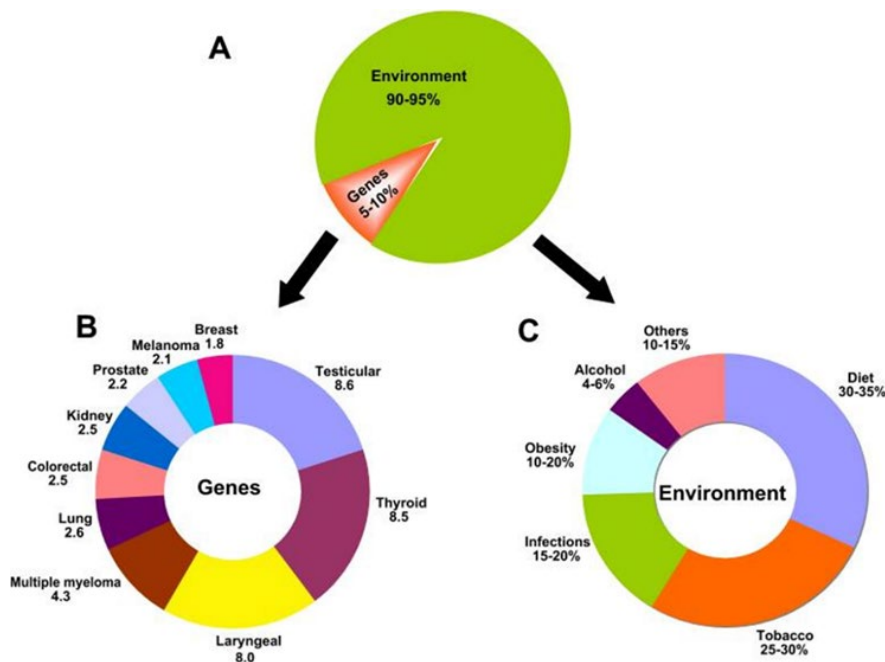
Figure 1.1: The percentage distribution of heredity and environmental factors. Reprinted with permission[2], Copyright Springer Science 2008.

Breast cancer survival rate can be improved by early detection and therapeutic intervention. Currently, mammography, magnetic resonance imaging (MRI), X-ray imaging, ultrasound, computerised tomography (CT) scan, and tissue biopsies are the standard approved methods

used for detection[4]. Despite their effectiveness, these methods have limitations that hinder their usage in countries with low socioeconomic status, such high medical cost , qualified person(s) to analyse data and invasiveness obtain a sample (biopsy) [5,6]. Countries with weak health systems and limited screening or prevention programs continue to be affected by high mortality rate[7].

There is a need to develop low-cost diagnostic tools that will still provide high sensitivity and specificity. As mentioned above, it is clear that breast cancer diagnosis at early stages is a crucial part of treating and management of the disease. Literature is replete with reports that disease-specific biomarkers are key to identifying individuals at risk of developing a particular disease. Monitoring these biomarkers provides an effective way to follow the progress of the disease[8]. Biomarkers are involved in various cellular processes essential for human life; any alteration in the biomarker level and expression indicates a disease development. These can be in a form of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, metabolites[9] and changes in their expression levels can be exploited to differentiate between healthy and disease conditions[5].

Over the years, standard methods have been developed and used to detect or measure biomarkers and, these include; enzyme-linked immunosorbent assays (ELISA)[10], radioimmunoassay (RIA)[11], and electrophoretic immunoassays[12]. These standard methods are known to give precise results, but their shortfall is that they require specialised equipment, multiple washing steps and long turn-around time[13]. Therefore, developing lateral flow biosensors (LFBs) for use at the point-of-care (POC) has emerged as a feasible strategy for LMIC. Most importantly LFBs allow for the detection of biomarkers circulating in various biological fluids such as saliva, blood, urine, serum and plasma. The elevated levels of cell-free nucleic acids in the bloodstream of cancer patients are amongst the biomarkers used to detect breast cancer[14] by ELISA and RT-PCR. However, these techniques are time-consuming and require costly instruments for detection, which becomes a limitation for use in Low Income Countries (LIC), hence recent research focuses on the use of designed nucleic acid-based systems have shown potential in reducing the turn-around time. Although the NABLFA has the potential for improving testing accessibility and clinical outcomes, the

6

technology is novel and has limitations, especially for the LICs. One major hurdle is that the NABLFA system still requires sample preparation and PCR amplification of the target genes before the samples can be applied on the LFA. For example, Warren et al. developed a POC diagnostic test using synthetic urinary biomarkers for non-communicable disease such as stroke, heart disease and cancer. The method used synthetic biomarkers that were conjugated to iron nanoparticles, the synthetic biomarker was made up of peptide substrate and a reporter. The conjugated nanoparticles are injected into a patient, upon delivery the nanoparticles passively target diseases sites such as solid tumours or blood clots. The upregulated protease will cleave the peptide substrates and release the reporters that are then cleared into the urine. The urine is collected and the reporters are then detected by a lateral flow assay (LFA) as shown in Figure 1.2.[15]
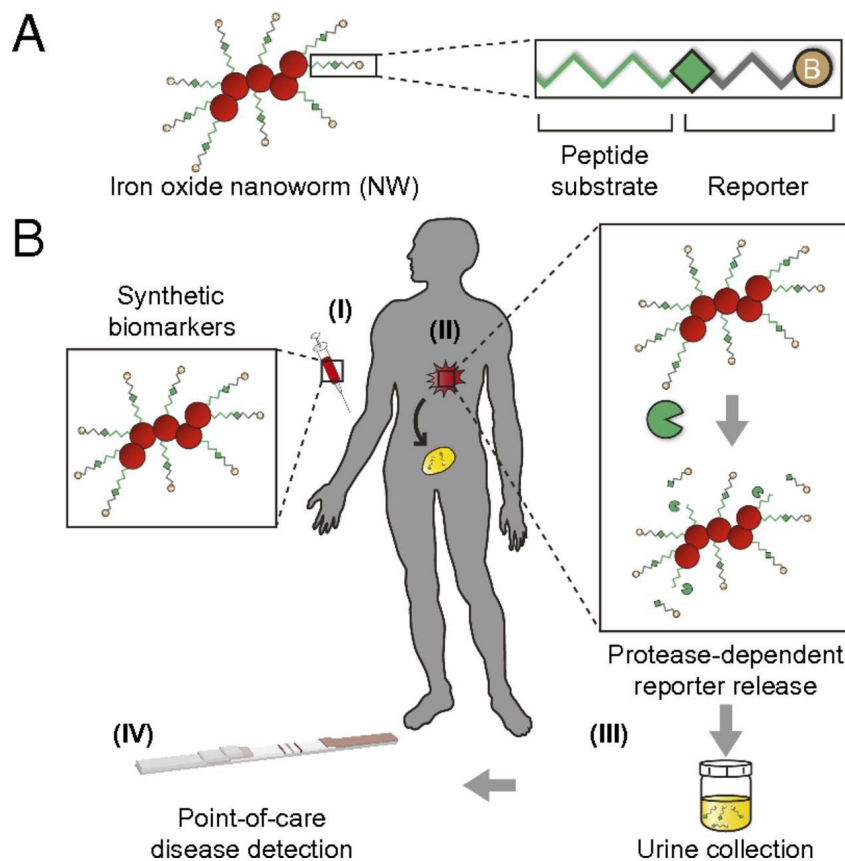
Figure 1.2: Detection of synthetic biomarkers conjugated to iron (II) oxide Nano worm in urine using LFA. Reprinted with permission[15], Copyright PNAS 2014.

1.2 AIM OF THE STUDY

1.2.1 AIM:

The main aim of the study was to develop AuNPs-based NABLFA for the detection of breast cancer-specific biomarkers.

1.2.2 SPECIFIC OBJECTIVES:

   i.   To identify differentially expressed breast cancer genes using literature mining.
   i.   To design probe labelled target gene-specific primers.
   ii. To evaluate gene expression from human cell lines using *in silico* analysis and quantitative polymerase chain reaction (qPCR).
   iii. To amplify the genes from DNA by PCR as test samples for NABLFA.
   iv. To synthesise and characterise 14nm AuNPs.
   v.   To develop a detection probe by conjugating biotin-streptavidin to the AuNPs surface.
   vi. To develop AuNPs-based NABLFA.

CHAPTER 2   LITERATURE REVIEW

2.1   INTRODUCTION

Cancer occurs when cells grow uncontrollably, forming a tumour; and is reported to be the second major cause of death worldwide after heart-related diseases[16]. The most common cancers are breast, lung, prostate, cervix, brain, colon, thyroid, and pancreatic cancers[17]. Cervical cancer is the number one killer among women, with 85% of women diagnosed with cervical cancer and 88% of deaths reported[18]. Approximately 14% of these cases were reported for women in Sub-Saharan Africa (SSA)[19], with mortality rate varying from 27.6 per 100,000 in Southern (46.2), Western (37.3), Eastern (29.9), and Central Africa (27.9) compared to <2 per 100 000 in western Asia, Australia, New Zealand, and Europe[20],[18]. The numbers are also rapidly increasing in SSA for breast cancer cases, an increase in incident rates of breast cancer from 19.7 per 100 000 to 36.9 per 100 000 was reported from 2000 to 2015. In South Africa (SA) alone, breast cancer accounts for 22% of all malignancies[20] and lifetime risk of developing breast cancer in SA is estimated to be 1 in 25 women[21].

Breast cancer survival rates vary globally, with high-income countries (HIC) having better survival than LMIC. When comparing survival rates with HIC, the United States of America (USA) has a survival rate of 89.9% compared to 52.3% in SSA[22]. The low survival rates in SSA are due to breast cancer diagnosis occurring at an advanced clinical stage, comorbidities, race and aggressive pathological characteristics of breast cancer[23]. Globally, guidelines were developed to support planning, implementation, monitoring, and early detection programs for breast cancer. The programs were found to be successful when focused more on communities[18]. However, these programs are deficient in LMIC. Furthermore, SSA countries are also burdened by other infectious diseases, which results in lower survival rates. Human immunodeficiency virus (HIV)/ acquired immunodeficiency syndrome (AIDS) is one of the dominant infectious diseases in SSA; SA carries 20% of the global HIV burden, with 15% of new infections and 11% of AIDS-related death[24]. In 2018, about 7.52 million South Africans were reported to be living with HIV/AIDS, with 62% of them on antiretroviral therapy (ART)[24]. Many studies have been conducted to understand whether the prevalence of HIV/AIDS in SA

contributes to the low survival rates of breast cancer. Even though HIV is not an oncogenic disease, it can indirectly cause infected patients to develop other malignancies by suppressing T-cell function[25]. An estimated 30-40% of HIV patients are expected to have cancer in their lifetime[26].

Studies have shown that HIV positive patients are usually found with advanced stages (III/IV) of breast cancer at diagnosis compared to HIV-negative patients [21]. The survival rate of HIV/AIDS patients diagnosed with breast cancer is low[27]. In SA, private care patients are mostly funded by medical aid schemes, which offer voluntary health care insurance to 15% of the population. While the majority of South Africans depend on public health care[28]. The low socio-economic status of the country has a considerable contribution to low survival rates. The majority of South Africans travel distances to access healthcare at hospitals and may even be required to visit more than two healthcare facilities for treatment and diagnosis. This is one of the contributors to breast cancer diagnosed at advanced stages and the associated low survival rates[29].

The figures and facts mentioned above demonstrate the importance of early diagnosis as a vital part of treating and managing breast cancer to ensure the survival of patients. Mammography, ultrasound imaging, and magnetic resonance imaging are commonly used for breast cancer diagnosis. These methods are image-based methods, which work by localising the cancer tissue[30]. Other methods such as immunoblotting, immunohistochemistry (IHC), Enzyme-linked immunosorbent assay (ELISA), and in-situ fluorescence hybridisation (FISH), are used to detect breast cancer biomarkers[31]. The advancement in research and technology has enabled researchers to come up with improved methods for diagnosis. For instance, electrochemical technologies, electrochemical biosensors, nano transistors, photonic crystals, and microfluidics-based technologies are emerging as new technologies used for diagnosis. Although all these technologies provide improved diagnostic methods, they are still limited in LMIC as they require high-cost equipment, sample pre-treatment, long-time analysis and trained personnel.

Genomic profiling tools (MammaPrint, MP) in conjunction with online tools (adjuvant online (AOL) and Predict (www.predict.NHS.UK) have been used for molecular subtyping of breast cancer[8]. MP has been available in SA since 2006 and can screen about 70 genes at one time[32]. Although MP is recommended for HIC such as USA and Japan, however, the assay cost limits its use in LMIC. Also considering the impact of the severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) pandemic, it is, therefore, essential to find ways to develop cost-effective diagnostic tools that can improve the diagnosis of patients in SA[33]. The emergence of nanotechnology has led to the development of novel and sensitive diagnostic tools using nanomaterials such as metal nanoparticles, nanowires, and nanotubes for effective diagnosis of diseases. Recently, a group in Greece showed the effectiveness of AuNPs-based NABLFA for the detection of Kirsten rat sarcoma viral oncogene homologue (KRAS) mutations in cell free circulating (ctDNA) obtained from colorectal cancer (CRC) cells and blood samples[34]. The proposed diagnostic test is user-friendly and offers rapid detection of target biomarkers. The AuNPs-based NABLFA can be compatible with the current screening or diagnostic tests of various disease biomarkers and present a cost-effective tool or solution for LMIC.

2.2 BREAST CANCER IN AFRICA

Women of African origin have the lowest registered cases of breast cancer worldwide[35]. However, the number of women diagnosed with breast cancer are now increasing due to behavioural and biological risk factors. Some of the risk factors are age, breastfeeding duration, weight gain during menopause and adopting a westernised lifestyle (alcohol intake, smoking frequency)[23]. Even though the registered breast cancer incidence rates are still low in Africa, the mortality rates were reported to be higher than in HIC due to late diagnosis[35]. The difference in survival rates between HIC and LMIC is pronounced. The low survival numbers in Africa are primarily due to many reasons, which include lack of medical cost, distance to care centres, available of health practitioner, illiteracy, health beliefs, health system, and policy constraints, and social-cultural factors. These factors act as a barrier to care for patients with breast cancer in Africa[36]. More worrying is that the proportion of breast cancer cases and death are higher in premenopausal ages in Africa than in HIC. This shows that breast cancer affects women in their prime in Africa, which may have economic

consequences. The management of breast cancer in LMIC is poor compared to HIC, which might be attributed to limited resources, high cost, late presentation of disease and low awareness[37]. All these causes delays between first symptoms, diagnosis, and treatment; the awareness campaigns, patient advocacy and education of healthcare workers can help women spot cancer at its early stages and save more lives[38].

The socioeconomic differences affect the health status and outcomes of patient care. Although SA is an upper middle-income country (UMIC) in Africa as rated by the World Bank, it still has a high record of inequality among its citizens[39]. The five-year survival rate from diagnosis to death/reoccurrence is below 57% in most African countries. For example, Kenya, Uganda, Malawi, and Nigeria had a 51.1% survival rate compared to New Zealand with a 90% survival rate[40]. Even though SA has multiple tertiary hospitals and oncology centres furnished with state-of-the-art cancer facilities[27] and standard national pathology laboratories that support histopathological examination tests, the number of surviving of patients is still low [41]. Women treated in the private health care system are likely to be treated by specialists with better oncological outcomes, while women in the public care system will only receive palliative care instead of actual treatment.

Thus, women in the public sector are diagnosed at a later stage when they present disease symptoms not because they lack awareness but due to the inaccessibility of proper health care, as most SA public health care is burdened with high numbers of patients. For example, the Chris Hani Baragwanath Academic Hospital (CHBAH) in Soweto, Johannesburg, is SA's most prominent government hospital that provides tertiary care to 3-4 million population of Soweto and surrounding areas in Gauteng province[42]. Further to that, patients must get referrals from a health centre before they can receive medical and radiation oncology treatment at a nearby hospital, the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). In the Gauteng province, the diagnosis and treatment are made at CHBAH and CMJAH for the public citizens, respectively. In 2001 CHBAH started a breast cancer clinic that provides surgical treatment and follow-up to patients[43]. When a patient suspects symptoms of breast cancer, getting a diagnosis involves a lot of steps. The patient must first obtain a referral to be attended at CHBAH breast cancer clinic. The diagnosis of the patient involves clinical examination and staging, imaging with mammography and ultrasonography, an

image-guided core needle biopsy for histological diagnostic confirmation and tumour grade and receptor subtyping which is performed by the National Health Laboratory Service (NHLS)[42]. For patients to obtain their results, it takes two to three weeks or even longer in some cases. The long waiting times have been found in some cases to discourage the patients from returning for their results as this may involve costs for patient further delaying diagnosis and treatment. Therefore, a screening device for breast cancer has the potential to save lives and lessen the burden on the South Africa's health care system[44].

2.3   WHAT IS BREAST CANCER?

Breast cancer is the type of cancer that starts in the breast. Breast cancer mainly occurs in women, with a small percentage of men affected[45]. The breast cancer cells form a tumour, or a lump around the breast that can be felt or visualised through an x-ray. Like other cancers, breast cancer can invade and spread to other tissues surrounding the breast and other parts of the body and form new tumours[5]. When breast cancer spreads, the cancerous cells will often appear in the bones, liver, lungs, or brain[46]. Breast cancer can be classified as invasive and non-invasive. Wherein an invasive breast cancer, the cancerous cells spread to the ducts and possibly to the lymph nodes, while non-invasive cancer is confined to ducts or lobules where the cancer originated[47].

2.3.1   SYMPTOMS OF BREAST CANCER

Early breast cancer usually does not cause pain or show any noticeable symptoms and can go unnoticed for years. And as cancer progresses, early signs and symptoms shown in Figure 2.1 can be physically observed: a lump or thickening in or near the breast, a change in the size or shape of the breast, nipple discharge, tenderness or retraction (turning inward), skin irritation, dimpling or scanlines[48]. These changes are not breast cancer-specific and can occur as part of different conditions such as fibrocystic[49] and mastitis cystic breast disease[50]. However, having one or more of these symptoms can raise health concerns about breast cancer[46].
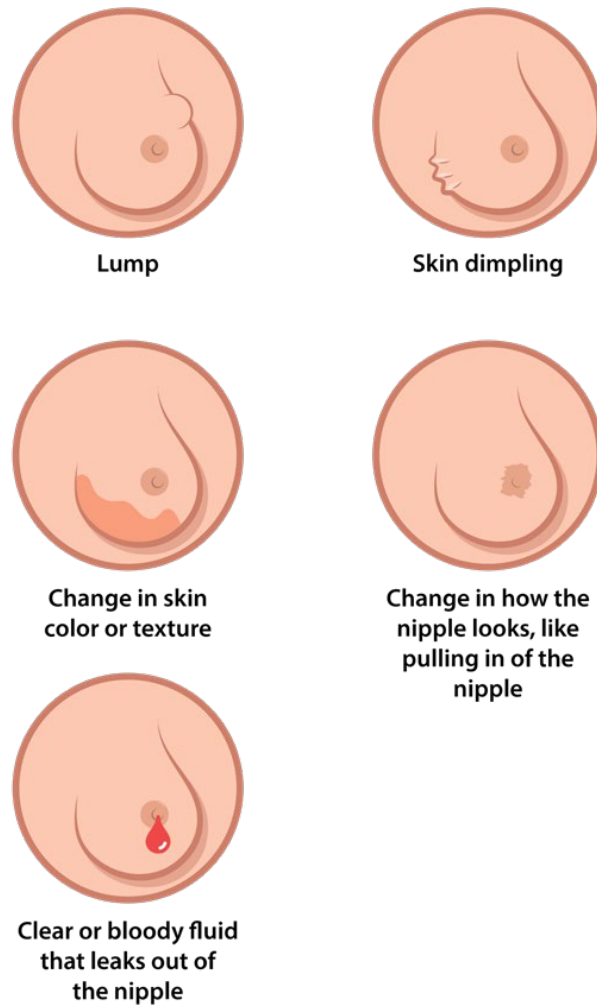
Figure 2.1: Symptoms and signs of breast cancer. Reprinted with permission[51], Copyright Wikimedia commons 2020.

2.3.2 TYPES OF BREAST CANCERS:

Breast cancer is classified based on the area of origin, the most common being ductal and lobular breast cancer as shown in Figure 2.2. Ductal carcinoma in situ: This cancer is when the cancer is in the precancerous stage or earliest stages (Figure 2.2(a)). The cancer is usually limited to the milk ducts and has not invaded any nearby tissues. This cancer is always curable but if goes untreated it can become invasive. Invasive ductal carcinoma is the most common type of breast cancer and accounts for 80% of cases. It starts in the milk duct of the breast (Ductal carcinoma in situ) and then spreads through the wall of the duct and invades the

surrounding tissue in the breast[52]. Invasive lobular carcinoma is the second subtype that begins in the lobules of the breast where milk is produced as shown in Figure 2.2(b). This cancer accounts for 10 -15% of breast cancer, and it is usually more difficult to diagnose with mammograms[53]. Lobular carcinoma *in situ* is not cancer per se but is used as a marker for high risk of developing breast cancer at a later stage. It is therefore important for women with lobular carcinoma *in situ* to have regular clinical breast examinations and mammograms[54].
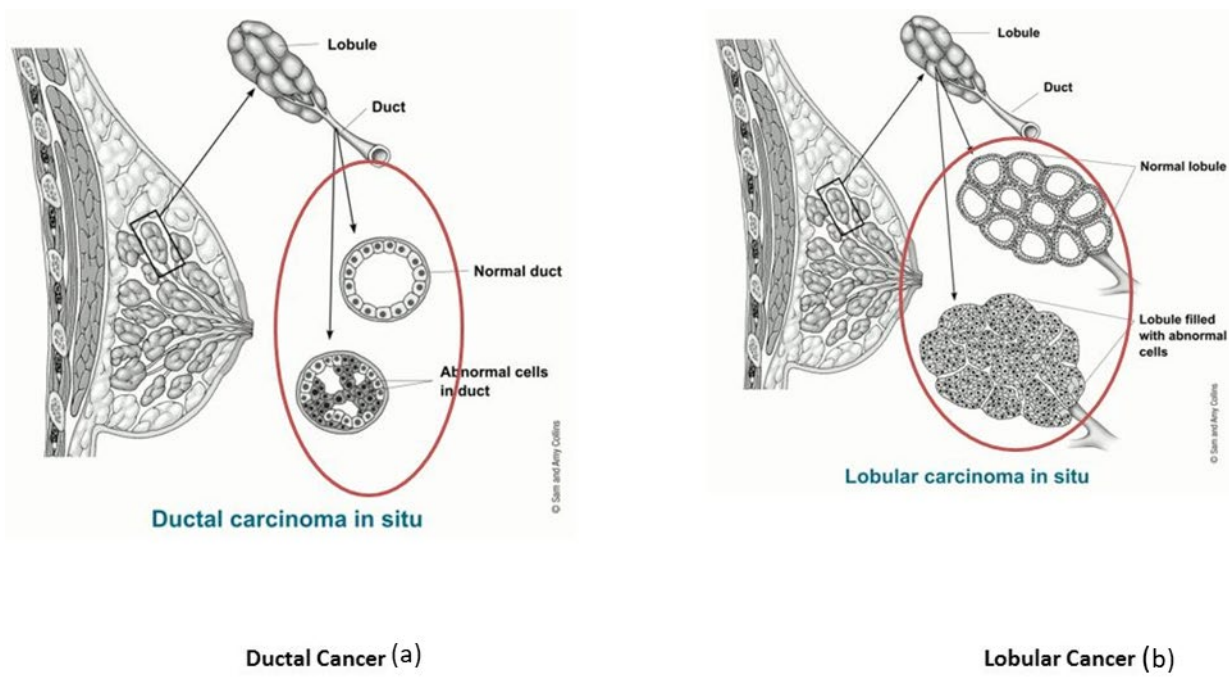
Figure 2.2: Types and characteristics of breast cancer; the ductal carcinoma and lobular carcinoma. Reprinted with permission[55], copyright American cancer society 2021.

There are some uncommon types of breast cancer that originate in other parts of the breast other than the lobes and ducts. These include angiosarcoma, Paget disease of the breast, and phyllodes tumours. Angiosarcoma starts in the cells that make up the lining of blood or lymph vessels. This type of cancer can start either in the breast tissue or breast skin. It is a rare type of breast cancer, it's caused by the obstruction of cancer cells in the skin's lymph vessels[56]. Paget disease of the breast, this type of cancer affects the skin of the nipple and the skin around the nipple (areola). Phyllodes tumours are very rare and most of the masses are not cancerous. The tumours of this type of cancer are found in connective tissue or stroma[52].

2.3.3 STAGES OF BREAST CANCER

There are two types of staging systems for breast cancer, anatomic staging and prognostic staging. The anatomic stage involves the use of physical characteristics of cancer focusing on the tumour, lymph nodes and metastasis statuses to classify breast cancer into four stages[57]. At stage 0, the disease is still confined to the milk ducts (ductal carcinoma *in situ*), stage I, the tumour is smaller than 2cm across and hasn't spread to other parts of the body including lymph nodes. A patient presenting Stage II breast cancer has one of the following; the cancer has spread to under arm lymph nodes, the lump is between 2 and 5cm whether it has spread to other parts of the body including the lymph nodes or the lump is larger than 5cm but has not spread to lymph nodes. At Stage III the tumour has locally advanced it can be any size and has spread to the skin, chest wall or internal mammary lymph nodes which are located under the breast and inside the chest. Stage IV the cancer has spread to areas of the body such as bones, lungs, liver and brain[54,57]. The prognostic stage is used to assess a patient who has already been diagnosed with breast cancer and undergoing surgery for treatment excluding those in chemotherapy. In addition to anatomical stage assessment, prognostic evaluates the expression of certain biomarkers described in section 2.3.4.[57].

2.3.4 BREAST CANCER SUSCEPTIBILITY GENES

Breast cancer occurs as a result of genetic changes or mutations in normal breast cancer cells. DNA mutations can be passed from generation to generation. Some mutations significantly increase the risks of certain cancers. DNA mutations linked to breast cancer are hereditary while some are acquired. Acquired mutations can happen during a person's life rather than being inherited and can mutate over time[58]. Hereditary breast cancers usually occur earlier in life than acquired (sporadic) cases and are more likely to involve both breasts[59]. It is estimated that 5%-10% of all breast cancers are hereditary and are due to known breast cancer susceptibility genes[60]. These genes are divided into low, moderate, and high-risk breast cancer susceptibility genes (Table 2.1). The high-risk breast cancer susceptibility genes include

BRCA1, BRCA2, PTEN, P53, LKB1/STK11, and CDHI. The CHEK2, TGFβ1, CASP8, and *ATM* genes belong to the low to moderate-risk breast cancer susceptibility genes[59].

Table 2.1: List of known breast cancer genes.

Low-risk genes	Moderate risk genes	High-risk genes
FGFR2	*CHEK2*	*BRCA1*
TOX3	*PALB2*	*BRCA2*
MAP3KI	*BRIPI*	*PTEN*
FAM84B/C-MYC	*ATM*	*TP53*
LSPI		*LKB1/STK11*
NEK10/SLC4A7		*CDHI*
COX11		
CASP8(D302H)		
TNP/IGFBP5/IGFBP2/TNSI		
NOTCH2/FCGRIB		
RAD5ILI		
MRPS30/FCGRIB		
ESRI		

P53, PTEN and BRCA1 genes were selected to be the genes of interest for this study. The *p53* is a known gene identified in 20-35% of breast cancer tumours, while the *PTEN* gene is also found in 4% of women. *BRCA1* and BRCA2 are high-risk genes with 59-87% and 35-80% risk of mutations, respectively. *BRCA1* and *BRCA2* mutations are responsible for more deadly tumours. The *BRCA1* and *BRCA2* mutations are located on chromosome 17 and chromosome 13, respectively. has 300 mutations and *BRAC2* has 1600 mutations that cause cancer[61]. Some

of the mutations include 185delAG, 185delAG, 6174delT, 6174delT, 5382ins, CS1832P, T2766I, N2781I, and K2860T, K3083E or 9475A>G. The mutations are found to be more common among certain geographic or ethnic groups. For example, *BRAC1* (3036del4) and *BRAC2* mutations are high in Jewish women from Ashkenazi (Eastern Europe)[62]. Asian, Hispanic and Native American women, however, have a lower risk of carrying breast cancer susceptibility genes[63] while in SA, *BRCA1, PALB2* and *RAD5IC* genes are responsible for breast cancer diagnosis[64]. Women who have inherited some mutations in these genes have a high risk of developing breast cancer, ovarian cancer, colon cancer and other types of cancer during their lifetime[60]. Men with *BRCA1* mutations (3232A>G) also have an increased risk of developing breast cancer[65]. *BRCA1* mutations are also associated with an increased risk of other cancers, for example, pancreatic cancer, prostate cancer and ovarian cancer[66]. Mutations in the *BRCA1* gene are associated with an increased chance of developing male breast cancer and cancers of the prostate and pancreas. An aggressive form of skin cancer called melanoma is also more common amongst people who have *BRCA2* mutations[67].

2.3.5 DIAGNOSIS OF BREAST CANCER

Breast cancer screening and diagnosis starts with self-examination as shown in Figure 2.3. Self-examination is done lying down or standing, placing the right arm behind the head. The left hand is used to feel for lumps using an over-lapping dime-sized circular motion of the finger to touch the breast for any lumps[68].
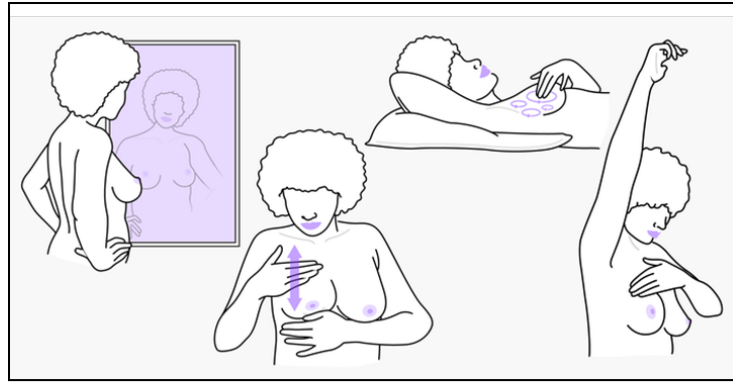
Self-examination

Figure 2.3: Breast cancer screening through self-examination method. Reprinted with permission[69] , copyright Insider Inc. 2022.

The screening can be followed by clinical validation. The current diagnostic methods for breast cancer are shown in Figure 2.4. These methods include biopsy, endoscopy, diagnostic imaging methods and mammography. In biopsies a small tissue sample is surgically removed from the suspicious growth area of the breast and examined under a microscope for the presence of cancer cells, this procedure can be done by a surgeon or a radiologist[70]. In endoscopy, a flexible plastic tube with a tiny camera on the end is inserted through the nipple through breast ducts deep into the breast. This tube allows a physician to view the lining of the lactiferous ducts looking for abnormal tissue[68]. Diagnostic imaging methods such as X-rays, Computerised Axial Tomography (CAT) scans, Magnetic Resonance Imaging (MRI), and ultrasound are used to produce an internal picture of the breast and its structures. These systems study the anatomy of the breast and can identify any possible abnormalities[38].
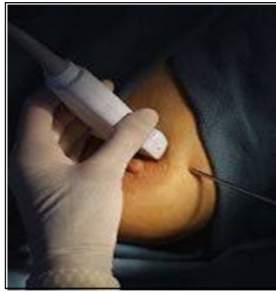
Core needle breast biopsy

Breast cancer MRI

Mammography

Breast cancer ultrasound

Figure 2.4: Clinical diagnostic methods for breast cancer core needle biopsy[71], MRI[72], mammography[73] ultrasound[74] methods used to diagnose breast cancer. Reprinted with permission, copyright American cancer society 2021.

Ultrasound uses sound waves to produce an image of breast tissues. The image generated will show the entire breast, including the hidden areas close to the chest wall. It is used to determine whether the breast is filled with fluid (a cyst) or solid objects (tumours). Mammography uses X-rays images of breast tissue, to examine for the signs of cancer[75]. Table 2.2 compares the different techniques used to detect breast cancer. Most of these methods apart from the self-examination method mentioned above are invasive, require skilled personnel, expensive equipment, time-consuming, and long waiting periods to obtain test results, and most importantly, they can be costly. There is an urgent need for a quick breast cancer diagnostic method or technique that will be minimally invasive and less expensive[68].

Table 2.2: Advantages and disadvantages of different techniques for the diagnosis of breast cancer

Technique	Advantage	Disadvantage	*Cost per consultation
Biopsy	The results provide all the characteristics of the cancer cells.	Require surgery to get a sample. Women who may not have breast cancer will have the surgery just to clear them.	R11,000–R26,000
Endoscopy	More details about the cancer cells (colour, texture). Short operation time. Can see cancer in hidden areas.	Requires surgery and may leave a scar.	R1,000-R4,000
Diagnostic Imaging (CAT-Scans, X-rays Breast MRI)	Screening of high-risk women gives more information about the suspicious area. Detect the spreading of cancer to other parts of the body and Monitor reoccurrence after treatment.	A contrast solution (dye) is intravenously injected into your arm. This dye can affect your kidneys, a test for kidneys must be performed before it's injected. The procedure is invasive and requires too many tests.	R6,000-R12,000
Breast self-exam	Detect tumour at an early stage.	Validation must be followed-up with molecular tests.	Free

*Note: The costs for consultations in South Africa are adapted from Med clinic tariffs, 2021[76].

2.4   CIRCULATING BIOMARKERS FOR BREAST CANCER DIAGNOSIS

Body fluids such as blood, urine, and cerebrospinal fluid contain all the information that would reflect the health status of an individual. Blood has been used over the years to diagnose various diseases. An average human adult has about 5 litres of continuously circulating blood that delivers nutrients and transports metabolic waste throughout the body[77]. Blood content is made up of 54.3% plasma, 45% red blood cells (RBCs), and 0.7% white blood cells (WBCs) by volume[78]. Plasma, the fluid part of the blood consists of proteins, nucleic acids, nutrients, and waste products. It also maintains electrolyte balance and protects the body from infection and blood disorders[79]. The serum is produced when blood-clotting factors are removed from the plasma[68], which is then used to detect any biological molecules that are present in the blood.

Tumours are known to release molecular biomarkers into the blood during their growth and progression[80]. Biomarkers may be a source of biological material that can be used to detect cancer. Biomarkers can be used to classify cancer or its prognosis, which can choose better therapeutic strategies[81]. Nucleic acids are carriers of genetic information secreted into the bloodstream in a form of circulating nucleic acids. They are found in small amounts in healthy individuals in the human serum[80], elevated levels in the serum usually suggest epigenetic alterations of a primary tumour[82]. It has been reported that the circulating nucleic acids are released into the bloodstream by proliferating or dying (both necrotic and apoptotic) cells[83]. The circulating DNA and micro ribonucleic acid (miRNA) found in serum are known to contain tumour-specific mutations that enable the detection of cancer from blood[84]. For example, Warkiani et al. developed a microfluidic technology that uses a drop of blood to detect nucleic acid biomarkers[85]. This technology combines fluid handling and silver reduction in a microfluidic chip (m-chip). The technology required only 1µl of blood, however, the technology had low sensitivity, semi-quantitative results, and low throughput[85].

2.4.1 NUCLEIC ACIDS IN BREAST CANCER DIAGNOSIS

DNA in the human bloodstream was first reported in 1948 by Mandel and Meta[86]. Despite the ground-breaking nature of their work, little attention was drawn to their findings until 1966, when Tan et al. demonstrated the presence of DNA in serum from the plasma of patients suffering from systemic *lupus* erythematosus[87]. Since then, DNA could also be detected in other diseases associated with tissue destruction (hepatitis, metastatic carcinoma, and military tuberculosis), the authors proposed that DNA molecules found in serum may originate from endogenous tissue breakdown[87]. Several years later, Leon et al. also reported the presence of DNA in the serum of cancer patients[88]. DNA amounts were obtained using sensitive radioimmunoassay, which was based on anti-DNA antibodies obtained from patients with *lupus erythematosus*. The results showed that 50% of cancer patients had high levels of DNA in their serum compared to healthy patients[88]. ctDNA can be detected in various malignancies not limited to cancer. Stroun et al. reported that the increased ctDNA content exhibited genetically identical characteristics to tumour DNA[84]. Two groups confirmed the presence of tumour-associated oncogene, BCR-ABL[89] and CA19.9[90] for leukaemia and pancreatic cancer, respectively.

The discovery of DNA in the plasma led researchers to find other types of nucleic acids circulating in plasma. RNA was also found to be one of the nucleic acids secreted in plasma in the form of microRNAs. A study done by Kodahl et al. in breast cancer confirmed that elevated levels of microRNAs (miRNA) in the blood are also associated with tumour development and progression[91]. The authors collected blood samples from patients and used qPCR to compare circulating concentrations of microRNAs particularly miR-10, miR-34a and miR-155, found in the blood of patients with primary breast cancer and patients with no cancer [91]. Their results showed elevated concentrations of miRNAs (miR-155, miR-34, miR-10b, and miR-141) in samples of patients with primary breast cancer. Furthermore, Heneghan et al. also reported elevated levels of other miRNAs (miR-885-5p, miR-1, miR-95 and miR-929) in blood from patients with breast cancer[92]. Lorio et al. also identified 13 miRNAs, which included miR-21, miR-125a, miR-205, miR-335, and miR-126 that were secreted into the bloodstream in

patients with breast cancer and this revealed valuable biological information about the tumour[93].

All these reports provided evidence that tumour cells do release cancer-related nucleic acids (DNA and miRNAs) into the bloodstream. A lot of work has been done in identifying biomarkers that are secreted by breast cancer cells. Most of the techniques used to detect these biomarkers are still molecular-based techniques such as PCR, enzyme-linked immunosorbent assay (ELISA), and mass spectrometry (MS)[94], immunoblotting, immunohistochemistry (IHC), and in-situ fluorescence hybridisation (FISH)[31].

2.4.2 MOLECULAR DIAGNOSTIC METHODS USED FOR DETECTION OF NUCLEIC ACIDS

Currently, PCR, ELISA, and MS are technologies that are used for molecular diagnosis of diseases from blood samples[81]. However, these methods are limiting as they require tedious sample preparation, purification and sophisticated instruments as summarised in Table 2.3. They are time and labour intensive, expensive and require highly trained personnel. Therefore, there is still a need to develop less invasive, faster and less expensive diagnostic methods that can lead to quicker detection of cancer[68]. Advancements in research and technology have enabled researchers to come up with improved molecular methods for diagnosis. The latest addition to breast cancer diagnostics is the Prosigna assay, which was approved by the Food and Drug Administration (FDA) to determine the risk of recurrence in breast cancer patients after surgery. The assay studies changes in mRNA expression of a panel of 50 genes associated with various molecular subtypes of breast cancer, collectively known as the Prediction Analysis of Microarray 50 (PAM50)[95–97]. In addition to these technologies, NABLFA devices offer a more straightforward option to detect or identify PCR products using the naked eye without additional equipment and skilled personnel. NABLFA are favourable devices to be developed due to their ease of use and require no specialised or costly equipment[98].

Table 2.3: Clinical and pre-clinical molecular methods for detection of nucleic acids

Method	Advantage	Disadvantage	Ref
Microarrays	Analysis of thousands of genes in a single test to create molecular tumour profiles	Require long hybridisation times Prolonged wash steps that can take up to 24 h	[99]
RT-PCR	DNA amplification increases sensitivity Test multiple samples simultaneously	Requires a series of temperature changes Tedious sample preparation Equipment	[100]
Nano pore sensor	Label-free. Small sample size Amplification free Distinguish single-nucleotide differences	No reproducibility or adaptability of biological system	[101]
Micro-fluid devices	Rapid purification of nucleic acids	Challenging to integrate blood pre-treatment steps	[102]
A three-mode electrochemical sensor (HPD-SENS)	Detect low concentrations of miRNA 10 aM to 1 mM range Multiple miRNAs on a single electrode. Exhibits high selectivity and specificity.	Detection of low of copy number of sample of DNA/RNA in samples for early onset of a disease	[103]

2.5   LATERAL FLOW ASSAY (LFA)

LFA is a technique that is based on detecting analyte/antigens and has emerged as a good technique for diagnosing diseases using body fluids. It is based on the interaction between antibodies and antigens. It can be performed at the POC without requiring sophisticated equipment[104]. There are two types of lateral flow formats, namely, sandwich and competitive formats. These are in the form of a test strip that is housed in a cassette. The sandwich is usually used to detect antigens with multiple epitopes, for example, those used to detect infectious diseases. In contrast, the competitive format is used to test antigens with a single epitope, such as those used for drug abuse tests. The LFA shown in Figure 2.5 comprises a sample pad, conjugate pad, nitrocellulose membrane, and wick pad, which are mounted on a backing card. The nitrocellulose membrane is dispensed with antibodies as test lines and control lines and fitted with a conjugate pad sprayed with antibodies conjugated to a label (usually gold nanoparticles, blue latex beads, or silver nanoparticles). These labels can be seen or visualised at low concentrations[105].
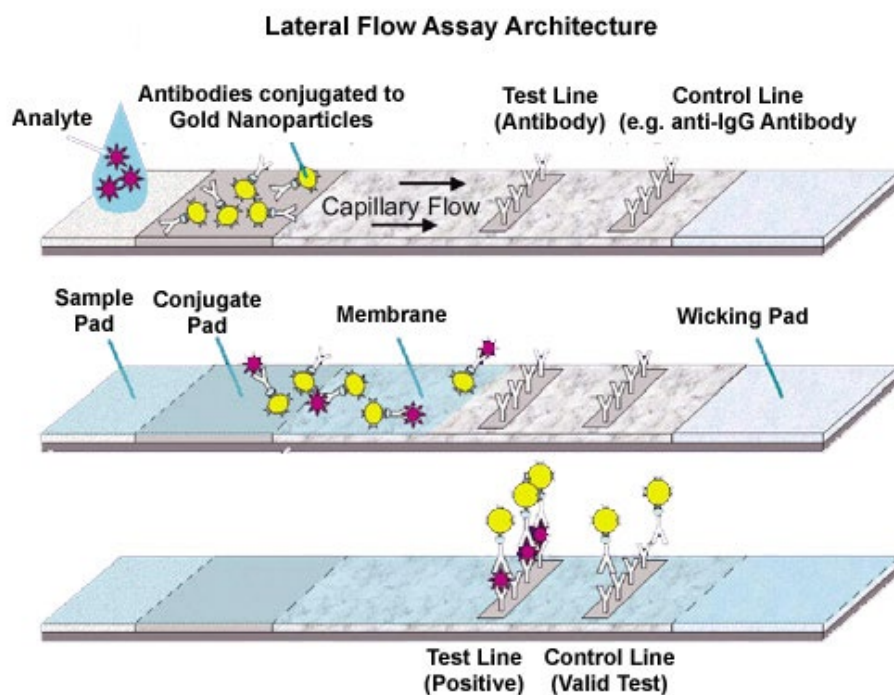
Figure 2.5: Lateral flow immunochromatography assay for detection of analytes. Reprinted with permission[106], copyright Elsevier 2020.

The test strip is run by adding a sample, for example, a drop of blood, into a model well. This is then followed by adding a few drops of running buffer. The liquid then runs along the test strip allowing the labelled antibodies to migrate through the test strip. When a sample contains the target antigen, the antigen will either bind to the labelled antibodies. This will be indicated by the presence of red colour developing at the test line as the labelled antibodies bind to the test line. The test takes 10-15mins to run and obtain results, which is the most important advantage over other molecular diagnostic tests. LFAs have recently been used to detect nucleic acids in biosensors to detect pathogens in food, infectious diseases, and cancer biomarkers[104].

2.5.1 DETECTION LABELS USED IN LFA

There is a long list of detection labels that can be used for LFA, which include nanoparticles (gold, carbon, magnetic, silver, selenium, quantum dots), dyed latex beads (blue, red latex beads), up converting phosphorus, enzymes (horseradish peroxidase), organic fluorophores, fluorescence labelled liposomes etc.[107]. However, the most commonly used labels are gold, silver, dyed latex beads and carbon black particles[98]. Choosing a label is based on the advantages of the label, most importantly the label should be able to be detected by the target at very low concentrations and also be able to retain its properties after conjugation with a bio-recognition molecule such as an antibody. It is also expected that it will not change the features and properties of the bio-recognition probe[108]. Labels that produce a colour signal after detection of the target, are always preferable for LFA because these labels make it easy to detect and read results with the naked eye thus cutting the extra step of analysing results using software. Hence coloured labels are always preferred. Table 2.4 shows the advantages and disadvantages of commonly used detection labels [109].

Table 2.4: List of label conjugates used in NABLFA

Conjugate label	Advantage	Disadvantage
AuNPs	Easy to synthesise. Can control size and shape. Can be used in biological applications. Has optical properties. Can modify their surface. Small size but can be easily visualised because of their distinct red colour.	Optical signal not strong, toxicity effect of metal core, tumour targeting low efficiency.
Silver enhanced gold	Can trace small quantity biomolecules.	High levels of false positives. Requires multiple washes to prevent false positive but not always effective.
Blue latex beads	Very stable in solutions. Multi-coloured. Biomolecules can be covalently attached to the beads.	The beads can be too large and not have an intense colour which results in the reduction of parking density on test lines leading to low sensitivity.
Black carbon NPs	Very stable. No activation needed.	Small functional groups. Preventing possible attachment of proteins or DNA.

2.5.1.1 ADVANTAGES OF USING AuNPs IN LFA

A variety of nanoscale structures have emerged with novel properties suitable for a range of biological and biomedical applications, which include: semiconductor quantum dots, magnetic nanoparticles, polymeric particles, carbon-based nanostructures, and metallic nanoparticles[110]. Compared to other nanostructures, metallic structures, such as AuNPs, have proven to be the most flexible nanostructures. AuNPs have become of great interest in the development of LFA, this is because of their simple synthetic route and ability to control their size, shape, composition, structure, assembly, and encapsulation, as well as the resulting tunability of their optical properties[111].

AuNPs are favourably used in LFAs due to their high-affinity features towards biomolecules (DNA, peptides and antibodies), enhanced stability and ease of functionalisation[112]. The surface of AuNPs is multivalent and allows for the incorporation of bio-molecules via covalent and non-covalent conjugation on the surface[113]. The physical interaction between bio-molecules and AuNPs depends on three important facts shown in Figure 2.6: (a) Dative binding between the Au conducting electrons and amino acid sulphur atoms of the bio-molecule. (b) The ionic attraction between the negatively charged AuNPs and the positively charged bio-molecule. (c) The hydrophobic attraction between the bio-molecule and the Au surface[114].
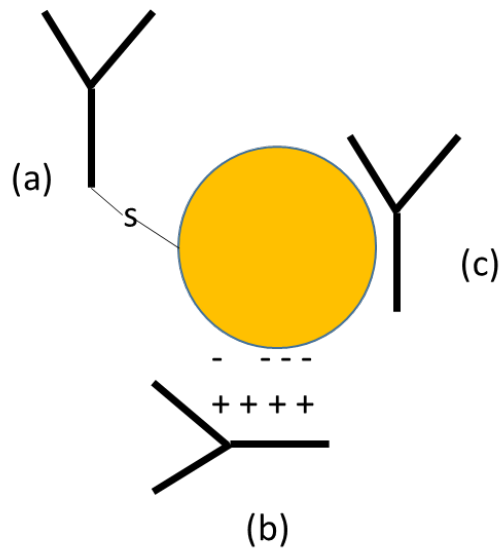
Figure 2.6: The Hydrophobic and ionic interactions between bio-molecules and AuNPs surface, (a) covalent bond is formed due to (b) ionic and (c) hydrophobic interactions. Reprinted with permission [114], copyright Elsevier 2016.

Chemical interactions can also be used to attach bio-molecules on the AuNPs surface, the interaction is achieved in the following ways shown in Figure 2.7: The first step in chemical interactions involves chemisorption via thiols, this can be followed by the use of bi-functional linkers such as adapter molecules like streptavidin and biotin[115].
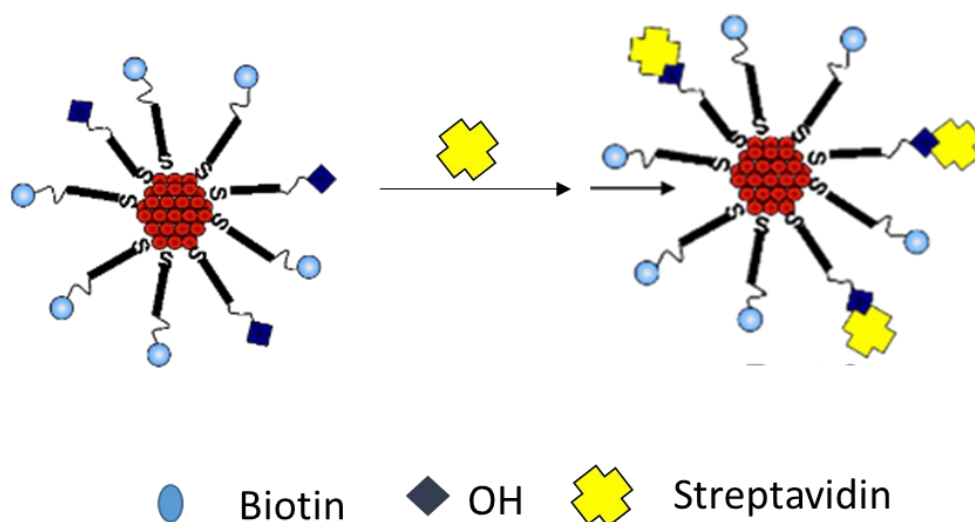
Figure 2.7: chemical interactions between bio-molecules and AuNPs surface using PEG-biotin and Streptavidin. Reprinted with permission[115], copyright MDPI 2015.

Both covalent and non-covalent conjugation are extensively used in bio-conjugation of AuNPs, this opens great opportunities for AuNPs to be used in biomedicine. Adding to all the advantages above is the ability to control the size of AuNPs, making them easier to flow through the nitrocellulose membrane used in the development of the LFA. The flow rate is dependent on the pore size of the membrane and particle size, thus increasing the sensitivity of the test. Nitrocellulose membranes are manufactured with different thicknesses to allow different AuNPs conjugates of different sizes to migrate and flow from one end of the test strips to the end. For example, Unistar offers five different membranes i.e. CN95, CN110, CN180, CN140, and CN150[116] as shown in Table 2.5. Although they are made from the same material they differ in thickness and flow rate, depending on the choice of the membrane can decide on the size of AuNPs to use as a detection label.

Table 2.5: List of different membranes with different specifications.

Membrane material	Thickness µm	Capillary speed (s/mm)
CN95	240-270	65-115
CN110	185-215	90-130
CN180	255-285	90-150
CN140	120-160	135-175
CN150	240-280	90-180

The difference in specifications and characteristics of these membranes make it possible to design unique, different AuNPs conjugates for different test kits. Also, the surface area to volume ratio of AuNPs allows for the conjugation of multiple biomolecules on their surface [104]. Another advantage of AuNPs use in LFA is their distinct red colour, this enables results to be visualised with the naked eye, eliminating an extra step of using equipment to read test results. The results can be analysed by visualising a red line on the test strip. This makes AuNPs-based LFAs be one of the POC diagnostic tests to be developed for fast, easy diagnosis[117].

2.5.2 NUCLEIC ACID-BASED LATERAL FLOW ASSAY (NABLFA)

NABLFAs are under development for the detection of genetic material, for example, various genetic markers, DNA or RNA specific for infectious and chronic diseases[98]. The added advantage of using the NABLFA is test amplify nucleic acid sequence that is specific to the analysed sample using primers with two different tags[118]. NABLFA gives high sensitivity and specificity compared to the LFA based on antibodies. The advantage of using NABLFA is that DNA is very stable at high temperatures. The test can be used in low-resource areas with no infrastructure. Another significant advantage of using the NABLFA is more diminutive than immunoassays, making it possible to bind more on the AuNPs and membrane. The nucleic acids have known sequences and can be easily modified at each end to achieve specific binding[119].

Nucleic acid tests are essential in diagnosing and treating genetic diseases. Several NABLFAs have been developed to detect DNA, mRNA, proteins, and other biological agents[120]. Developing these nucleic acid-based tests for fundamental research and clinical applications has become widely attractive because they offer better simplicity, are less time-consuming and less labour intensive, and are being developed for POC technologies[121],[122] by incorporating LAMP technologies. Most NABLFAs are based on hapten-antibody or hapten-protein (e.g., biotin-avidin) complexes when PCR products are amplified using hapten-labelled aptamers[122]. The aptamers/primers are tagged with two different tags as shown in Table 2.6, these were specific for the detection of pathogenic bacteria from food[105].

Table 2.6: Primers used for PCR amplification of listeria monocytogenes and Listeria genetic

Primer	Sequences	Tag	Specificity
LIP1	5'GATACAGAAACATCG	5'biotin-TEG	*L. monocytogenes*
LIP2	5'GTGTAATCTTGATGC	5'digoxigenin	*L. monocytogenes*
C	5'AGGTTGGACCCTACC	5'biotin-TEG	genus *Listeria*

The detection procedure starts with the amplification of genes of interest using PCR. In the second part, the biotinylated PCR product is directly transferred to the sample pad with

running buffer, which moves the AuNPs probe along the nitrocellulose membrane by capillary action. As shown in Figure 2.8(a), a target-specific primary antibody is conjugated to a detection label (e.g., AuNPs, latex beads, etc.). A streptavidin capture is dispensed on the test line, while on the control line, a secondary antibody (anti-mouse) is dispensed. As shown in Figure 2.8(b) a running buffer is added to move the AuNPs-antibody-biotinylated aptamer through the nitrocellulose membrane. In a case of a positive sample, the virus will attach to the AuNPs-antibody-biotinylated aptamer moving along the membrane. Once it reaches the test line, the complex will attach to the test line through biotin-streptavidin interaction on a test line, and the excess complex will move right through to bind to the secondary antibody. The test strip will have two lines indicating a positive test. The test strip will show one line, on the control line when the test is negative[108].
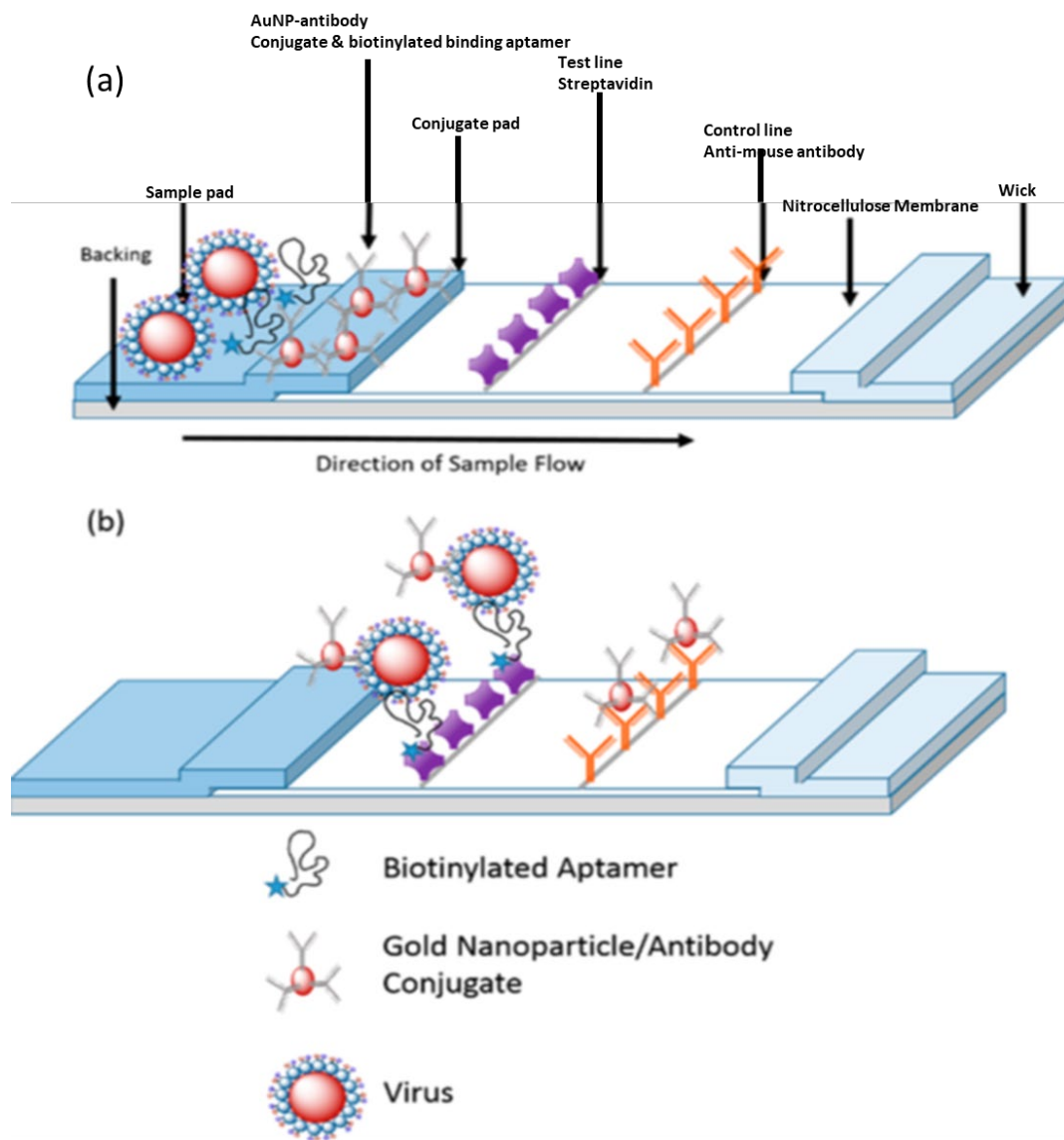
Figure 2.8: AuNPs-based NABLFA for detection of genetic materials, for example, various genetic markers, DNA or RNA. Reprinted with permission[119], copyright Elsevier 2015.

The feasibility of NABLFA, as mentioned in section 2.1, has been demonstrated in the detection of various cancers. Notably, a group in Greece developed an AuNPs-based NABLFA for the detection of KRAS mutations in DNA samples extracted from CRC cell lines and blood. The test was able to detect four single nucleotide polymorphisms (SNPs) that correspond to the normal KRAS gene and three of the most common mutations in the KRAS gene related to CRC in synthetic DNA samples, cancer cell lines, and ctDNA isolated from blood samples of CRC patients[34]. KRAS gene is used for cancer prognosis, response to chemotherapy and resistance to anti-EGFR therapy[123]. The AuNPs-based NABLFA as shown in Figure 2.9, was able

to differentiate single KRAS mutations in ctDNA from cells and blood. These systems demonstrate that existing biomarkers present in low amounts can be detected for various diseases including breast cancer.
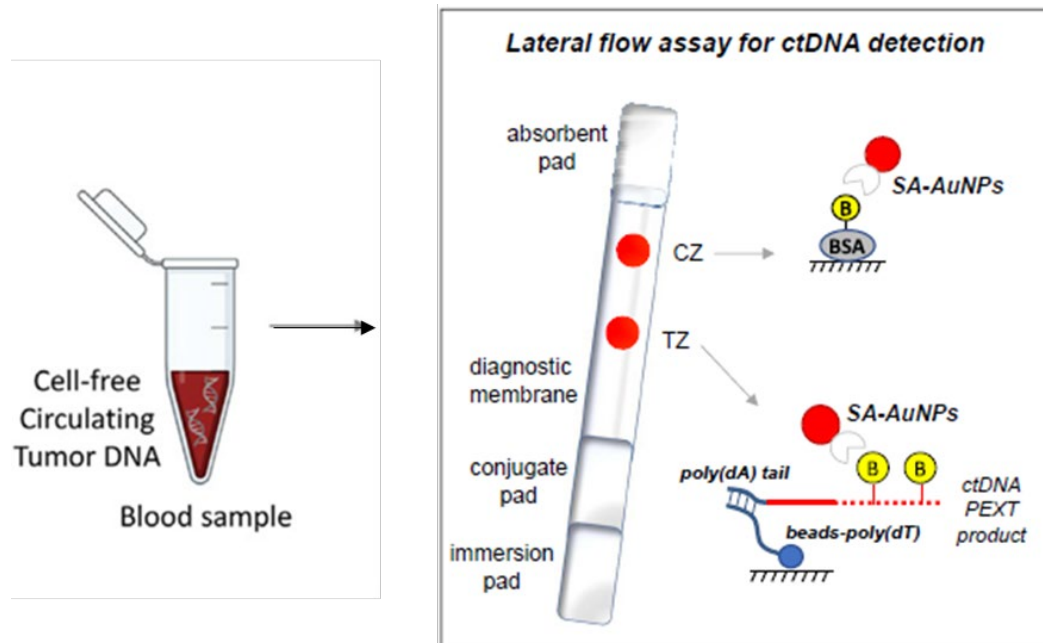
Figure 2.9: AuNPs-based NABLFA for rapid detection of ctDNA in blood samples. Reprinted with permission[34], copyright Elsevier 2021.

CHAPTER 3   MATERIALS AND METHODS

3.1   REAGENTS, MATERIALS AND SUPPLIERS

3.1.1   CELL CULTURE

The cell lines used in this study were purchased from the American Type Culture Collection (ATTC, Manassas, USA), and a gift from the University of Western Cape (UWC, Western Cape) and University of Johannesburg (UJ, Gauteng) as shown in Table 3.1, and the media were purchased from Separations (Pty) Ltd, Johannesburg, SA.

Table 3.1: List of cell lines used for DNA and RNA extraction.

Cell type	Origin	Disease	Species	Supplier	Media
MCF7	Breast cancer	adenocarcinoma	*Homo sapiens*	ATTC	DMEM
T47D	Breast cancer	adenocarcinoma	*Homo sapiens*	ATTC	DMEM
MDA-231	Breast cancer	adenocarcinoma	*Homo sapiens*	UWC	DMEM
MCF12A	Breast	normal	*Homo sapiens*	UWC	DMEM
A375	Skin cancer	adenocarcinoma	*Homo sapiens*	UJ	DMEM
Me-180	Ovarian cancer	adenocarcinoma	*Homo sapiens*	UJ	McCoy's
BHK-21	Hamster	normal	*Hamster*	ATTC	DMEM

3.1.2   AGAROSE GEL ELECTROPHORESIS

Agarose, Tris Base Boric Acid, Ethylenediamineteraacetic acid (EDTA) were purchased from Sigma Aldrich, St Louis, USA. GelRed® Nucleic Acid Gel Stain was purchased from Biotium, Fremont, USA. Casting tray and a comb are both from Bio-Rad, California, USA.

3.1.3   AuNPs SYNTHESIS AND CONJUGATION MATERIALS

Hydrogen tetrachlorourate trihydrate, disodium citrate, streptavidin and anti-digoxigenin antibody were purchased from Sigma Aldrich, St Louis, USA. PEG-Biotin and PEG-OH were purchased from ProChimia, Gdynia, Poland.

3.1.4   ASSEMBLY OF LATERAL FLOW STRIP

Nitrocellulose membrane, conjugate pad, wick/absorbent pad, backing card and plastic cassette housing were all purchased from Diagnostic Consulting Network (DCN), California, USA.

3.1.5   PCR AND qPCR REAGENTS

OnePCR^{TM} Hotstar PCR mix was purchased from Anatech, SA. Wizard® Genomic DNA purification kit, ReliaPrep^{TM} RNA cell Miniprep system kit and Goscript^{TM} reverse transcription kits were purchased from Promega, Wisconsin, USA. Untagged primers were synthesised from Inqaba Biotech, Pretoria, SA. The Tagged primers synthesised from Integrated DNA technologies (IDT), Iowa, USA. SensiFAST SBR green mix non-ROX kit was purchased from Meridian bioscience, Tennessee, USA.

3.2   PREPARATION OF SOLUTIONS AND REAGENTS

3.2.1   CELL CULTURE

Media preparation

The media were prepared by mixing and adding 10% heat-inactivated foetal bovine serum (FBS), and 1% penicillin-streptomycin (both were purchased from Sigma Aldrich, St Louis, USA) into DMEM and McCoy's media.

Trypsinisation

Phosphate-buffered saline (PBS), (between pH 7.5-8.5) was purchased from Cytiva, Massachusetts, USA and trypsin-EDTA (0.05% trypsin) was from Sigma Aldrich, St Louis, USA and were used as provided by manufacturers.

3.2.2 PCR REACTIONS

The PCR and qPCR reactions were prepared in a 0.2 ml Eppendorf tube pre-chilled on ice as shown in Tables 3.2 and 3.3, respectively.

Table 3.2: Preparation of PCR reaction in 0.2ml Eppendorf tube.

Reagents	Stock Concentration	Volume	Final Concentration
DNA	50ng/µl	1µl	1ng/µl
Forward primer	0.5µM	1 µl	0.01µM
Reverse primer	0.5µM	1 µl	0.01µM
OnePCR™ Hotstar PCR mix	2x	25 µl	1x
Water	-	22 µl	-
Total Volume		50 µl	

Table 3.3: Preparation of qPCR reaction per reaction in 0.2ml Eppendorf tube.

Reagents	Stock Concentration	Volume	Final Concentration
cDNA Template	20ng/µl	5µl	5ng/µl
primers	3.2µM	2.5µl	0.4µM
SBR green mix	2x	10ul	1x
Nuclease water	-	2.5µl	-
Total Volume		20µl	

3.2.3 PREPARATION OF 1X TBE- BUFFER

The buffer was prepared by dissolving 10.8g of tris and 5.5g of boric acid in 800ml deionised water then 40ml of 0.5M EDTA (pH 8.0) was added to the solution. The buffer volume was adjusted to 1 litre with distilled water.

Preparation of stock of 0.5 M EDTA (pH 8.0) in 500ml

A 9.305g of EDTA was dissolved in 200ml distilled water, O.1M sodium hydroxide was used to adjust the pH of the solution to pH 8.0. The volume was then adjusted to 500ml using distilled water.

3.2.4   1% AGAROSE GEL PREPARATION

Agarose (1g) was dissolved in a 100ml 1xTBE buffer. The solution was heated until it dissolved. Once dissolved, it was cooled down to room temperature (24°C) for a few minutes. Gelred™ nucleic acid stain (10µl) solution was added to the agarose solution, and stirred for complete distribution. The gel was poured into the casting tray and a comb was inserted into the gel to create wells. When the gel sets, the comb was taken out; the gel and 1 litre of 1XTBE buffer was added to the tank.

3.2.5   BUFFERS USED FOR LFA

The recipes of the following buffers i.e. conjugate blocking, membrane blocking, conjugate pad blocking and conjugate diluent buffers were filed as trade secrets with Mintek IP Office, and therefore their composition cannot be disclosed. The Intellectual Property (IP) number is CR2015-004.

3.3   RESEARCH METHODOLOGY

3.3.1   BIOMARKER IDENTIFICATION AND THE PRIMER DESIGN

Three cancer genes (*p53, PTEN, and BRCA1)* were identified from literature[124], [125],[126] and used as targets for NABLFA. Regions within each gene were selected for designing the primers as shown in Figure 3.1, each target gene had one to three different sets of primers, represented by A, B, and C. The designed primers were synthesised by Inqaba Biotechnical Industries (Pty) Ltd from Pretoria, SA. The primer sequences are highlighted in Tables 3.4, 3.5 and 3.6.

P53 5139bp Acc No. AH007667.2

1503bp ⟶ 1520bp

P53 B — TGTTCACTTGTGCCCTGACT — GGAGGGCCACTGACAACCA —

1973bp ⟵ 1993bp

PTEN 47152bp Acc No. AH005966.2

980bp ⟶ 999bp

PTEN A — GCAGCTTCTGCCATCTCTCT — TCACCACACACAGGTAACGG —

1767bp ⟵ 1786bp

PTEN 47152bp Acc No. AH001304717.2

1300bp ⟶ 1320bp

PTEN B — TTGAAGACCATAACCCACCAC — CCTTGTCATTATCTGCAC —

1952bp ⟵ 1971bp
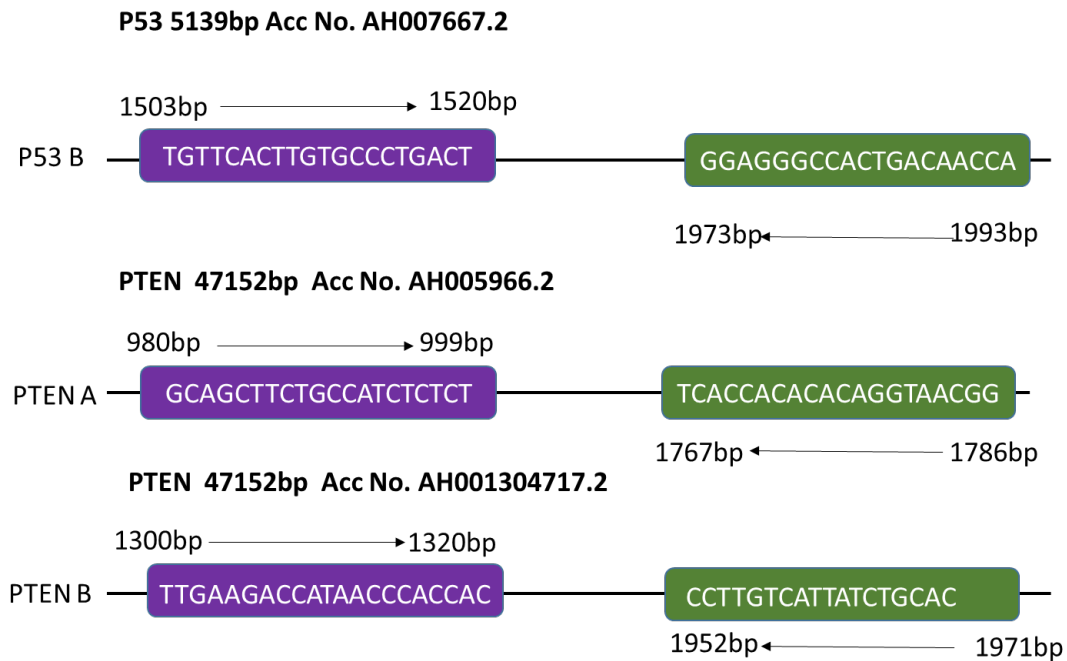
Figure 3.1: Forward and reverse primer sequences and target areas for the selected genes.

The primers used for NABLFA were designed following the principle shown in Figure 3.2, biotin was used as a control line, and the forward primers were tagged with biotin on the 5' end and tagged with digoxin on the 5' of the reverse primer (Table 3.7.) These tagged primers were synthesised from Integrated DNA Technology (IDT) from Cape Town, SA.
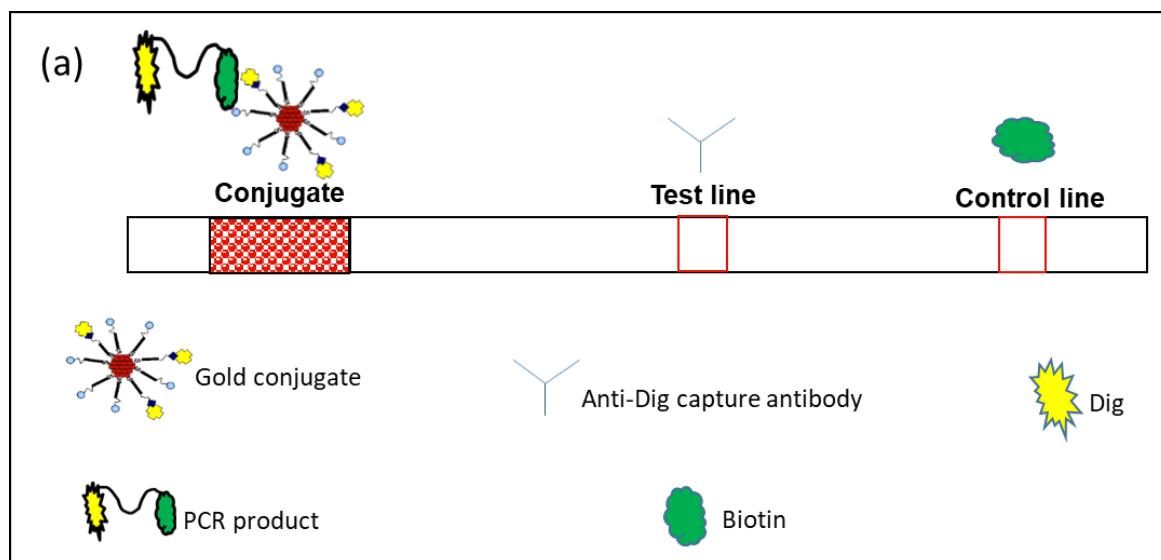
Figure 3.2: The proposed design of AuNPs-based NABLFA for detection of target genes.

Table 3.4: Primers designed for the *p53* gene.

Gene P53	Primer Sequence
p53 A Forward	5'CAC TTG TGC CCT GAC TTT-3'
p53 A Reverse	5'CCT GGG ACC CTG GGC AA-3'
p53 B Forward	5'TGT TCA CTT GTG CCC TGA CT-3'
p53 B Reverse	5'GGA GGG CCA CTG ACA ACC A-3'
p53 C Forward	5'GAC AAG GGT GGT TGG GAG TAG ATG-3'
p53 C Reverse	'5GCA AGG AAA GGT GAT AAA AGT GGA A-3'

Table 3.5: Primers design for the *PTEN* gene.

Gene TPEN	Primers
PTEN A Forward	5'GCA GCT TCT GCC ATC TCT CT-3'
PTEN A Reverse	5'TCA CCA CAC ACA GGT AAC GG-3'
PTEN B Forward	5'TTG AAG ACC ATA ACC CAC CAC-3'
PTEN B Reverse	5'CCT TGT CAT TAT CTG CAC GC-3'
PTEN C Forward	5'CGA CGG GAA GAC AAG TTC AT-3'
PTEN C Reverse	5'TAA AAC GGG AAA GTG CCA TC-3'

Table 3.6: Primers design for the *BRAC1* gene.

Gene BRAC 1	Primers
BRCA1 A Forward	5'ACC CCA ACA TTG ATT CCT TTC-3'
BRCA1 A Reverse	5'CAC AGG GAG AAA GTC TGC AAG-3'
BRCA1 B Forward	5'GGG AGC TGA GAA AGC AGC CAG C-3'
BRCA 1 B Reverse	5'TCG GCA GGA ATC CAT GTG CAG C-3'
BRCA 1 C Forward	5'AGC AGA AGA ACG TGC TCT TTT CAC GG-3'
BRCA1 C Reverse	5'ACA GTC TTC AAT GTG GAG GCA GTA GGG-3'

Table 3.7: The tagged primers used for the NABLFA.

Gene	Tagged Primers
p53 B Forward	5'-/5Biotin/'TGT TCA CTT GTG CCC TGA CT-3'
p53 B Reverse	5'-/5Digoxin/GGA GGG CCA CTG ACA ACC A-3'
PTEN B Forward	5'-/5Biotin/TTG AAG ACC ATA ACC CAC CAC-3'
PTEN B Reverse	5'-/5Digoxin/CCT TGT CAT TAT CTG CAC GC-3'
PTEN A Forward	5'-/Biotin/GCA GCT TCT GCC ATC TCT CT-3'
PTEN A Reverse	5-/5Digoxin/'TCA CCA CAC ACA GGT AAC GG-3'

3.3.2 CELL CULTURE AND MAINTENANCE

The cell lines were regularly cultured in T25 cm^2 culture flasks, purchased from Sigma Aldrich, at 37°C in a 5% CO_2 humidified incubator in their respective media. The media were supplemented with 10% heat-inactivated foetal bovine serum (FBS), 1% penicillin-streptomycin. The media was changed and replaced with fresh complete media every three days. The procedure was performed in the Biological Safety Cabinet (Laminar flow) using aseptic techniques, the cells were counted using TC20^{TM} Countess Automated cell counter from Bio-Rad.

3.3.2.1 CELL COUNT/VIABILITY: TRYPAN BLUE EXCLUSION ASSAY

The Cell viability was assessed following trypan blue exclusion assay using the TC20^{TM} Countess Automated Cell Counter from Bio-Rad according to manufacturer's instructions. Equal volumes of trypsinised cell suspension and 0.4% trypan blue dye were mixed by pipetting up and down and 20μl of the cell-dye mixture was loaded into the Countess Chamber slide. The slide was then inserted onto the Countess chamber of the TC20^{TM} countess automated cell counter and the data readout was given as total cells/ml; live cells/ml, dead cells/ml and %viability.

3.3.2.2 CELL SPLITING AND TRYPSINISATION

The trypsin and media were pre-warmed in a water bath at 37°C. The cells were split when they had reached ≥70% confluence. The media was removed from the flask using a 10ml serological pipette into a waste beaker. The cells were then rinsed gently with 5ml PBS, and

5ml of trypsin was added to the flask with cells. The cells were put back into the incubator for 2-5mins at 37°C. The flask was tapped to assist in the detachment of cells. The solution was then transferred to a 15ml tube and centrifuged using a Hettich Mikro200/200R from Sigma Aldrich at 15000 x g for 5mins. The supernatant was decanted, and 10ml of fresh media was added to the cells.

3.3.2.3 PRESERVATION OF CELLS

The cells were stored in the cryo-storage once reached 90% confluence. The total number of cells and percentage viability was determined using Trypan Blue exclusion assay, cell count was done on TC20^{TM} Countess Automated Cell Counter as described in section 3.3.2.1. The cell suspension was centrifuged at 15000 x g for 5mins. The cell pellet was re-suspended in cold freezing medium (complete growth media + 10% DMSO) at 1×10^6 cell/ml cell density. The cell suspension was dispensed in aliquots into cryogenic storage vials. The cells were frozen at a controlled rate using the CoolCell® alcohol-free cell freezing container from AEC Amersham, decreasing the temperature approximately 1°C per minute. The frozen cells were then transferred to a liquid nitrogen tank for long-term storage.

3.3.3 EVALUATION OF TARGET GENE EXPRESSION

3.3.3.1 IN SILICO ANALYSIS OF TARGET GENES EXPRESSION

Expression Analysis using GEPIA Database

The analysis of *p53, PTEN and BRCA1* transcription levels in various tumours was performed by GEPIA database[127], a web-based cancer microarray and Integrated Data-Mining Platform. The mRNA expression levels of cancer-related genes were compared between normal and cancer samples as described by Fadaka et al[128]. The threshold search criteria were set at p-value $< 1 \times 10^{-8}$, a fold change > 2, and a gene rank in the top 1% to obtain the most significant STAT3 probes. The expression profiles of the individual genes in different types of tissues were represented as bar charts in Figure 4.1 and 4.2.

Survival Analysis of target genes in some selected tumours

The Kaplan–Meier plotter is a web-based tool accessed at http://kmplot.com/analysis/index.php?pbackground.The aim of this tool is meta-analysis-based discovery and validation of survival biomarkers. In this study, the correlations between *p53, PTEN and BRAC1* expression and patient survival in breast cancer were analysed using the Kaplan–Meier plotter. According to various quantiles of biomarker expression, the tool divides patient samples into pairs of groups to analyse the prognostic value of a particular gene. The hazard ratio (HR) with 95% confidence intervals (CI) and log-rank *p*-value was also computed[128].

3.3.3.2 EXTRACTION OF RNA

The cells were cultured in T25 cm^2 culture flasks as described in section 3.3.2, when they reached 70-90% confluence RNA was extracted using ReliaPrep^{TM} RNA cell Miniprep system kit following manufacturer's instructions. The cells were trypinised (section 3.3.2.2) and collected in a sterile tube by centrifuging at 300 x *g* for 5mins. The resulting cell pellet was washed with ice-cold, sterile 1X PBS (Sigma Aldrich) then centrifuged at 300 x *g* for 5mins to collect the cells. The supernatant was carefully removed and discarded. The cell pellet was dispersed and mixed in 500µl of BL+TG buffer, then 170µl Isopropanol was added to the solution and the whole solution was mixed by vortexing for 5secs. A collection tube was placed in a mini-column tube (all the tubes used were provided with the kit). The lysed cells were transferred into a mini-column and centrifuge at 14 000 x *g* for 30secs at 25°C.

The mini-column was removed and the liquid in the collection tube was discarded, 500µl of RNA wash solution was added to the mini-column with a collection tube and centrifuged at 14 000 x *g* for 30secs. The collection was emptied as before and a 30µl DNase I incubation solution was added and incubated for 15mins at room temperature (+25°C). After the incubation 200µl of column wash solution was added and centrifuged at 14 000 x *g* for 15secs followed by a second wash with 500µl of RNA wash. The mini-column was placed into a new

column tube and 300µl of RNA solution was added and centrifuged at 14 000 x *g* for 2mins. The mini-column was placed on an elution tube and 50µl of nuclease-free water added and centrifuged at 14 000 x *g* for 1min. The purified RNA was stored at -70°C until further analysis. The concentration and purity of RNA were quantified using NanoDrop™ 2000/2000c spectrophotometer (ThermoFisher Scientific).

3.3.3.3   cDNA SYNTHESIS

RNA from the various cell lines was converted to cDNA using the Goscript™ reverse transcription kit as described by the manufacturer. Briefly, a 5µl reaction containing 5µg RNA samples, 0.5µg random primer, 0.5µg of oligo (dT)$_{15}$ and nuclease-free water (final volume to 5µl) was placed into a preheated 70°C heat block for 5mins and immediately chilled on ice-water for 5mins. The reverse transcription reaction was prepared by adding 4.0µl of GoScript™, 2µl of MgCl$_2$, 1µl PCR nucleotide mix, 0.12µl, 1µl GoScript™ reverse transcriptase and 6.8µl nuclease-free water. The 15µl of reverse transcription reaction mix was added to the 5µl of RNA and primer mix. A QuantStudio™ 5 Real-Time PCR System (ThermoFisher Scientific) was used to convert RNA to cDNA under the following conditions: Annealing at 25°C for 5mins, Extension at 42°C for 1hr and inactivation at 70°C for 15mins. The cDNA was stored at -70°C until further analysis. The concentration and purity of cDNA were quantified by NanoDrop™ 2000/2000c spectrophotometer.

3.3.3.4   qPCR ANALYSIS

The qPCR reaction mixture was prepared in a final volume of 20µl as summarised in Table 3.3, the mixture contained 400nM of untagged *p53*, *PTEN* and Hypoxanthine phosphoribosyl transferase (HPRT) primers, 1x SensiFAST SYBR® No-ROX kit (Meridian life science) master mix and 5ng/ml cDNA template. PCR reaction was performed on QuantStudio™ 5 Real-Time PCR System with the following settings: 95°C 2min (polymerase activation), 95°C 5s (denaturation), 65°C 15s (annealing), and 72°C 10s (extension) for 40 cycles. The results were analysed using QuantStudio™ Design & Analysis Software.

3.3.4 NABLFA SAMPLE PREPARATION

3.3.4.1 EXTRACTION OF TOTAL GENOMIC DNA

Genomic DNA was extracted from cultured cell lines following the instructions from the Wizard® Genomic DNA purification kit. Briefly, the cells were trypsinised and centrifuged at 15000 x *g* for 5mins, the pellet was washed with 1x PBS and centrifuged again for 10secs. Then 3μl of RNase solution was added to the pellet, and the solution was incubated for 30mins at 37°C. Then 200μl protein precipitation solution was added, vortexed and chilled on ice for 5mins and centrifuged at 15000 x *g* for 5mins. The supernatant was then transferred to an empty tube with 600μl Isopropanol at room temperature, the solution was mixed gently by inversion and centrifuged at 15000 x *g* for 1min. The resulting supernatant was removed and 600μl of 70% ethanol at room temperature was added. The solution was centrifuged at 800 x *g* for 1min, then the ethanol was aspirated and the pellet was air-dried for 15mins. After 15mins, the pellet was rehydrated overnight at 4°C in 100μl of DNA rehydration solution. The concentration of the DNA was determined using NanoDrop™ 2000/2000c spectrophotometer.

3.3.4.2 AMPLIFICATION OF TARGET GENES BY PCR

The genes were amplified using untagged and tagged primers obtained from Inqaba Biotechnical Industries (Pty) and IDT, respectively. The untagged primers were used to confirm amplification of the target genes from the genomic DNA, the tagged primers were used to generate biotin/dig-DNA samples for NABLFA. The PCR reaction was prepared in a 0.2ml Eppendorf tube pre-chilled on ice. The 25μl of the PCR reaction contained 50ng/μl DNA, 25μl OnePCR™ Hotstar PCR mix, 1μl of forwarded and reverse primers each; the volume was adjusted with nuclease-free water to 50μl. The whole mixture was mixed by using vortex and PCR performed using Eppendorf Master Cycler Gradient under the following conditions for 30 cycles: 94°C 5mins (Initial Denaturation), 94°C 40secs (Denaturation step) 62°C-68°C 1min (Annealing step), 72°C 30sec (Extension step), 72°C 5mins (Final extension step). The PCR products were analysed on a 1% agarose gel electrophoresis containing Gel Red™ to verify

and confirm the amplification of the target DNA. The gels were viewed and imaged on the Bio-Rad Image Lab 4.1 and 1kb and 100bp DNA ladders from Promega were used to estimate the sizes of the PCR products.

3.4   DEVELOPMENT OF AuNPs-BASED NABLFA

3.4.1   SYNTHESIS OF AuNPs

The AuNPs were synthesised using a standard method devised by Turkevish[129] as modified by Sosibo et al[115]. Gold chloride (1.14 g) was dissolved into 100ml of distilled water. Trisodium Citrate (1g) was dissolved into 100ml of distilled water (dH$_2$O). Gold chloride solution was filtered into a clean beaker using a 0.2μm filter. Gold chloride solution (2.5ml) was poured into an Erlenmeyer flask containing 250ml of distilled water. The heat setting on the heat/stir plate was adjusted to 250°C and stirred at 74 x *g* and the solution in the flask was brought to boil. Once the boiling had begun, sodium citrate (7.5ml) was added quickly to the centre of the flask. The heat was reduced to room temperature (20°C to 24°C) after it changes colour. The heat was then switched off when red colour developed and continued to stir for 3hrs then the sample was cooled to room temperature.

3.4.1.1   CHARACTERISATION OF AuNPs AND CONJUGATES

The AuNPs were characterised by using UV-Vis absorption spectroscopy (Multiska NGO, ThermoFischer Scientific), from 470-600nm to determine the absorbance and peak wavelength of the AuNPs. The transmission electron microscopy (TEM) was performed on a Joel JEM TEM, the operating voltage range was 20–200kV with a resolution of 2.4Å. Then the core size distribution was measured by using Image J Software (National Institutes of Health, USA). The surface charge or zeta potential of the AuNPs was analysed on a DTS1070 cuvettes by Malvern Zetasizer Nano series (Malvern Panalytical, UK) and further characterisation was done by performing 1% agarose gel electrophoresis, Unconjugated AuNPs, AuNPs-PEG-biotin and Au-PEG-biotin-streptavidin were mixed with 1% glycerol when loaded to the gel. The gel was prepared and run as explained in section 3.2.4.

3.4.1.2 *CONJUGATION OF 14nm AuNPs*

Preparation of AuNPs

Before the conjugation of AuNPs, a pH titration was done to find the optimum pH to conjugate the AuNPs and also the optimum concentration of the biotin to be conjugated. AuNPs (100μl) were added to a 96-well plate and on each well 2μg - 10μg of biotin was added. The solution was then incubated for 15mins at room temperature. To check optimum pH for conjugation, AuNPs were pH from pH 5 to 9, then 50μl (50% v/v) of 0.2M sodium chloride was added to the solutions. If the solution turned blue then the concentration of the biotin or the pH was not optimum thus the aggregation of AuNPs but when there was no colour change this meant that the pH and concentration of biotin were ideal for the reaction.

Conjugation of AuNPs with PEG-biotin and PEG-OH

Conjugation was done following a previous protocol[115]. PEG-biotin 1% (0.02 mg) and PEG-OH 99% (1.98 mg) were dissolved in 7% methanol, 164μl of PEG-biotin and 27.1 μl were thoroughly mixed in a 2ml Eppendorf tube. The mixture was then added to a 14nm AuNPs (10 ml) and the reaction was then stirred for 3hrs at room temperature. The resulting PEG-Biotin/OH-AuNPs conjugate was centrifuged at 13000 x *g*, for 20mins at 22°C. The supernatant was decanted and the AuNPs conjugate was re-suspended in 800μl 1x PBS.

Functionalisation of biotinylated AuNPs with streptavidin

After the conjugation of 14nm AuNPs with PEG-biotin, the next step was to attach streptavidin to the biotin. A solution of streptavidin (1.80mg) was added to the concentrated solution of AuNPs-PEG-biotin (800μl). The reaction mixture was swirled and stored at 4°C for 48hrs. The resulting solution was centrifuged at 13000 x *g* for 25mins at 4°C. The pellet was re-solubilised into 1ml 1x PBS.

3.5   DEVELOPMENT OF NABLFA

The LFA is based on the capture of target antigens/ antibodies from a sample (blood, urine or saliva) using either monoclonal or polyclonal antibodies raised against the targets[130]. LFA are simple devices intended to detect the presence (or absence) of a target analyte in the sample (matrix) without the need for specialised and costly equipment. At the development stage, the LFA is assembled manually. All the materials to assemble the LFA were purchased from Diagnostic Consulting Network (DCN, USA), and assembled as shown in Figure 3.3. These were put together with specific dimensions which include length, width and position of the test and control line.
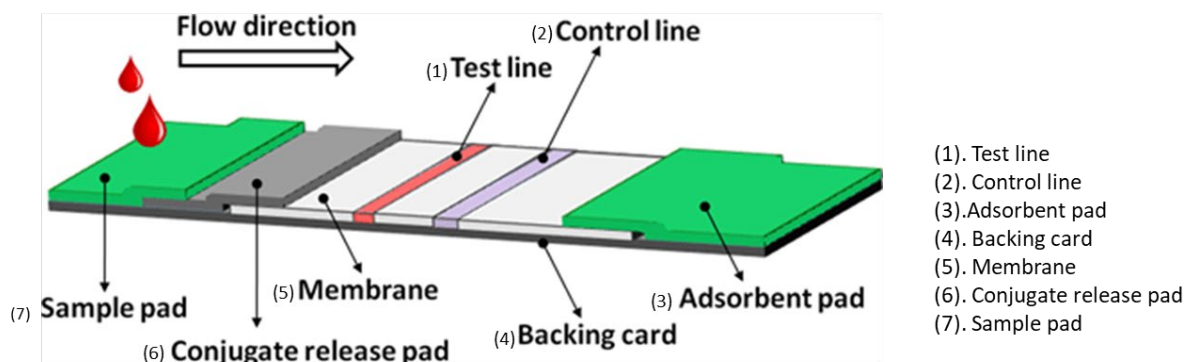
Figure 3.3: The diagrammatic representative of LFA system and its components. Reprinted with permission[131], copyright Portland press 2016.

3.5.1   TEST AND CONTROL LINE IMMOBILISATION

The test line was set at 10mm from the bottom edge of the nitrocellulose membrane and the control line at 15mm from the bottom edge of the nitrocellulose membrane. The Biojet BJQ 3000 dispenser from Biodot LTD, Irvine, USA was primed together with the test and control lines to fill up the lines to avoid the formation of air bubbles. Both the test and control lines were sprayed and the membrane was dried in an incubator set at 37˚C. The test line consisted of 2.5mg/ml of anti-dig and the control line contained 0.5mg/ml biotin.

3.5.2 BLOCKING OF THE NITROCELLULOSE MEMBRANE

The membrane blocking buffer (200ml) was poured into a Pyrex container. The membrane was blocked by slowly (avoiding air bubbles) immersing it into the blocking buffer while it is attached to the backing card for approximately 1-2mins. The membrane was then placed on the drying rack and put in the oven for 30mins at 37°C to dry. The membrane was stored in a foil pouch with desiccant (1g per pad) at room temperature.

3.5.3 BLOCKING OF THE CONJUGATE PAD

The conjugate pad was blocked in the blocking buffer, which cannot be disclosed for IP purposes. The blocking buffer (200ml) was poured into Pyrex container, the conjugate pad was blocked by slowly immersing the conjugate pad into the buffer for approximately 2mins. Once blocked the conjugate pad was taken out and put on a drying rack. The drying rack was put in the oven for 30-45mins at 37°C. The dry conjugate pad was stored in a foil pouch with desiccant (1g per pad) until its ready for use or was used immediately.

3.5.4 ASSEMBLY OF THE NABLFA

The conjugate, absorbent (wick) pads and nitrocellulose membrane were laminated onto the adhesive backing card. The two pads were laminated in a way that they overlap onto the membrane so that there is a continuous flow path from the sample. Once all the pads were laminated on the backing card, the whole card was cut into 5mm individual strips using the guillotine cutting module CM 4000 Biodot LTD and the 5mm strips were ready to be tested. The development and test of LFA is summarised in Figure 3.4.

Assembly of LFA

Membrane System

↓

Spray test line and control

↓

Dry at 37°C

↓

Block membrane

↓

Assemble membrane, conjugate pad and absorbant pad onto backing card

↓

Cut cards in strips

↓
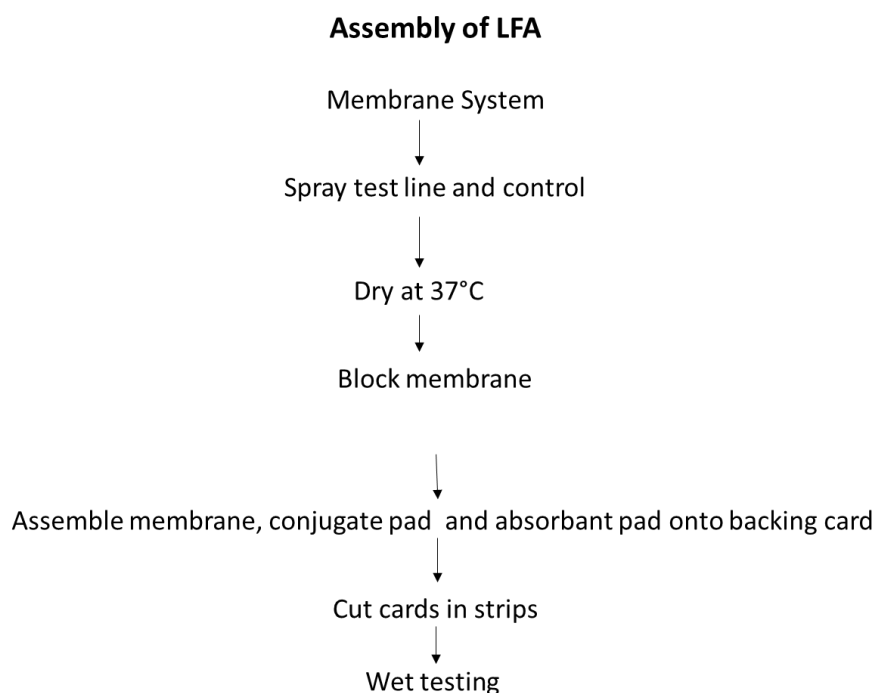
Wet testing

Figure 3.4: Schematic representation of LFA development and testing.

3.5.5   WET TESTING OF LFA

The LFA was optimised by wet testing in the early stages of development. AuNPs conjugate and test samples (PCR product) were prepared in a test tube, the test strip was immersed in the tube. The test sample was prepared as follows: 10µl AuNPs conjugate, 80µl of running buffer, and 2µl of PCR product were mixed in a 2ml Eppendorf tube. Then a 5mm test strip was inserted into the Eppendorf tube, and the solution was allowed to flow through the strip for 15mins. An example of how the wet test was performed is shown in Figure 3.5.

Figure 3.5: An example of LFA wet testing conditions.

CHAPTER 4   RESULTS AND DISCUSSION

4.1   *IN SILICO* EXPRESSION OF TARGET GENES

In silico or computer-based studies have gained a lot of attention in recent years, as a quicker way to prioritise disease diagnostic and therapeutic[132]. The normal process of identifying disease targets and developing effective therapeutic strategies can take a period of 12-15 years[133], with the emergence of infectious diseases like Ebola, Covid-19, this might not be conducive. *In silico* approach to biomarker validation could be a more effective strategy for biomarker development, which is typically fast and cost-effective process compared to the traditional methods of biomarker validation[134].

Biomarkers associated with different types of cancers, such as genes, proteins and miRNAs, have been identified in past decades using *in silico* approachs[135]. There is a range of *in silico* tools such as microRNA Data Integration Portal (MirDIP), Tissue-specific Gene Expression and Regulation (TiGER), Database for Annotation, Visualization, and Integrated Discovery (DAVID), Gene Expression Profiling Interactive Analysis (GEPIA), Prognoscan, firebrowse and search tool for retrieval of interacting genes (STRING); that are used for identification of disease biomarkers, these technologies are able to facilitate biomarker and drug discovery[136–139].

To validate the diagnostic, prognostic and/or predictive value of the candidate genes, GEPIA and Prognoscan databases were used. Most of the genes were confirmed as having good prospects as cancer biomarkers, based on their expressions on cancer cells and tumours relative normal tissues. The gene expression was evaluated by both *in silico* and qPCR analysis.

4.1.1 mRNA EXPRESSION LEVELS OF TARGET GENES

GEPIA is a web tool that compares the expression of various genes in 9736 human cancer and 8587 normal samples, analysed by real time gene expression[132]. mRNA plays a major role in the expression of targeted genes[140], because of its involvement in various pathological or physiological processes such as cell migration, invasion, proliferation and differentiation[141]. Abnormal expression of mRNA can differentiate breast cancer specimens against normal tissues[142].

The transcription levels of *p53, PTEN, and* BRCA1 were studied using the GEPIA database, the study compared their expression between normal tissues and tumours. Figure 4.1, shows that *p53* gene is over-expressed in most cancer types except in Kidney Chromophobe and pancreatic tissues (Figure 4.1a). Similarly, the *BRCA1* expression was also higher in cancer tissues (Figure 4.1c). Even though *BRCA1* is presumed to be a breast cancer biomarker[143], it's expressed by other cancers such as Oesophageal, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma and Testicular Germ Cell tumours. This rules out *BRCA1* as a breast cancer-specific biomarker. In contrast, the mRNA levels of *PTEN* were higher in normal when compared to cancer tissues (Figure 4.1b).

The GEPIA database also confirmed the transcript expression of *p53, PTEN, and BRCA1* in various solid tumours. The results showed in Figure 4.2 that *p53* was highly over-expressed in colorectal, gastric cancer, breast cancer and ovarian cancer (Figure 4.2a) while *PTEN* showed high expression in normal tissues than in cancer except in lung cancer where it was higher (Figure 4.2b) and *BRCA1* was highly expressed in all cancers except with lung cancer where its expression was lower than normal.
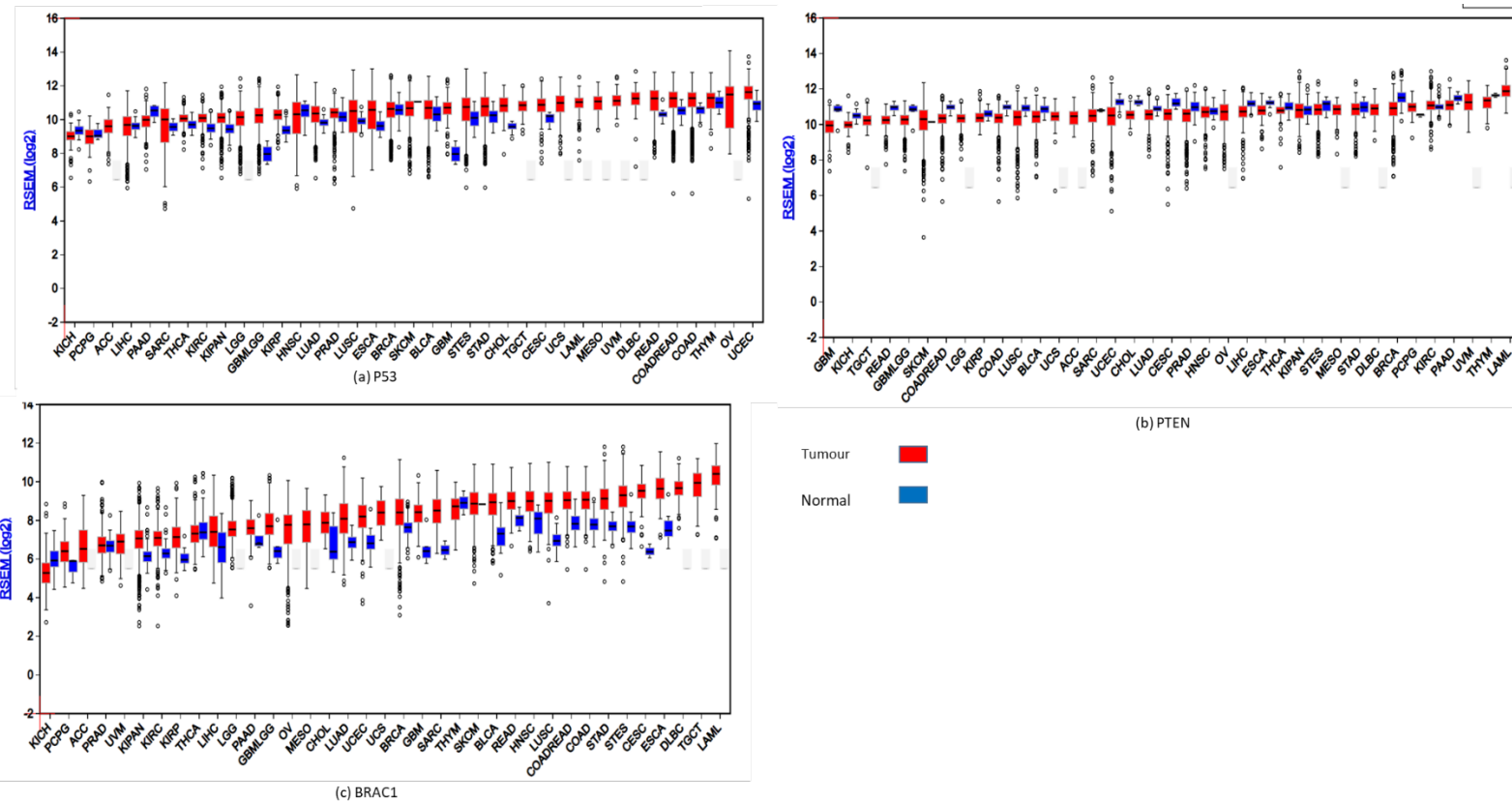
Figure 4.1: The expression levels of *p53, PTEN, and BRCA1* in cancer and non-cancer tissues. mRNA over-expression (red) and down-regulation (blue).
Abbreviations; ACC-Adrenocortical carcinoma, BLCA- Bladder urothelial carcinoma, BRCA- Breast invasive carcinoma, CESC- Cervical and endocervical cancers, CHOL-Cholangiocarcinoma, COAD-Colon adenocarcinoma, COADREAD-Colorectal adenocarcinoma, DLBC- Lymphoid Neoplasm Diffuse Large B-cell Lymphoma, ESCA-Esophageal carcinoma, FPPP- FFPE Pilot Phase II, GBM- Glioblastoma multiforme, GBMLGG- Glioma, HNSC-Head and Neck squamous cell carcinoma, KICH-Kidney Chromophobe, KIPAN-Pan-kidney cohort (KICH+KIRC+KIRP),PAAD-Pancreatic adenocarcinoma, PCPG-Pheochromocytoma and Paraganglioma, PRAD-Prostate adenocarcinoma, READ- Rectum adenocarcinoma, SARC- Sarcoma, SKCM- Skin Cutaneous Melanoma, STAD-Stomach adenocarcinoma, STES- Stomach and Esophageal carcinoma, TGCT-Testicular Germ Cell Tumors, THCA- Thyroid carcinoma, THYM- Thymoma, UCEC- Uterine Corpus Endometrial Carcinoma, UCS- Uterine Carcinosarcoma,   UVM- Uveal Melanom

(a) P53

(b) PTEN

(c) BRAC1
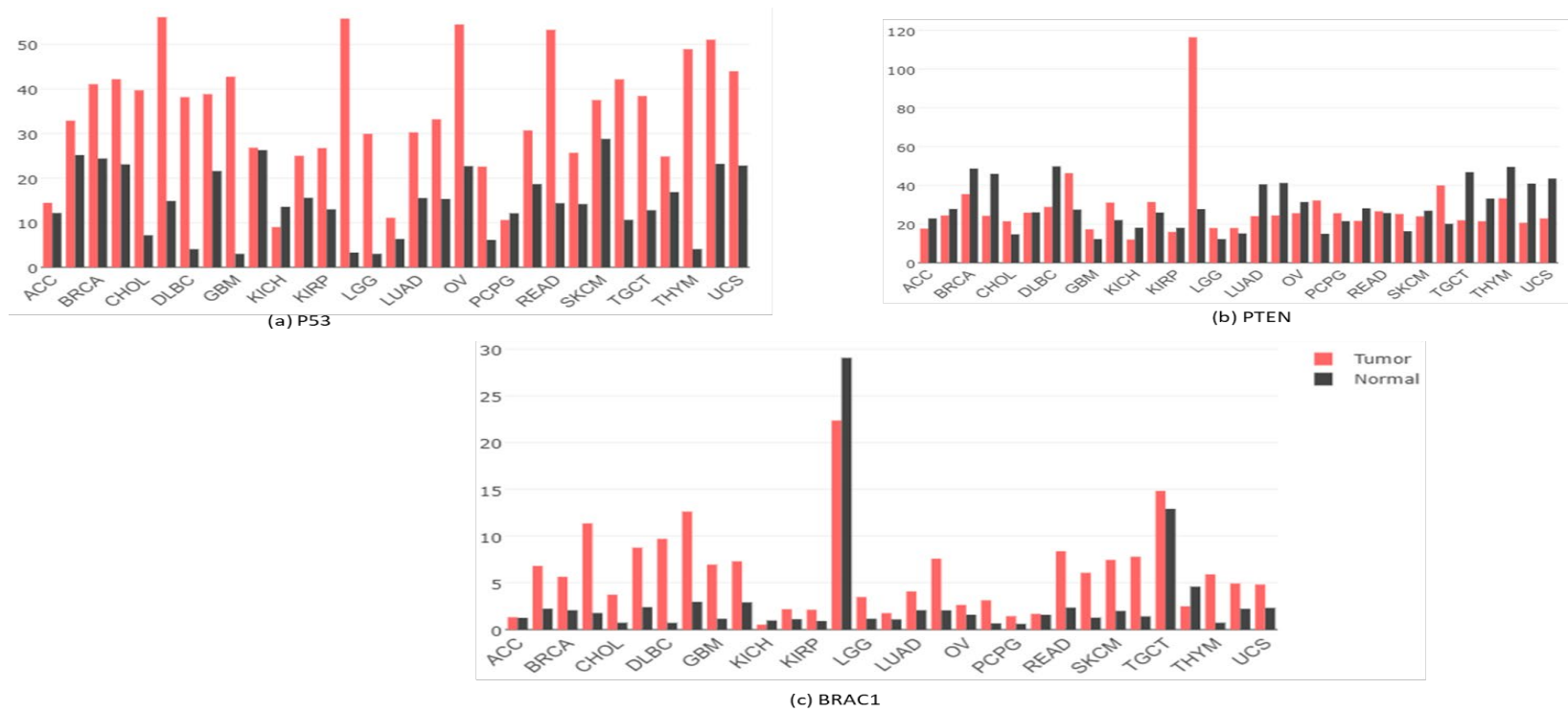
Figure 4.2: Analysis of *p53, PTEN, and BRCA 1* in solid tumours (red) and normal (black).

Abbreviations;ACC-Adrenocortical carcinoma, BRCA- Breast invasive carcinoma,CHOL- Cholangiocarcinoma, , DLBC- Lymphoid Neoplasm Diffuse Large B-cell Lymphoma, GBM- Glioblastoma multiforme, , KICH-Kidney Chromophobe, LGG-PCPG-Pheochromocytoma, and Paraganglioma, PRAD-Prostate adenocarcinoma, READ- Rectum adenocarcinoma, SARC- Sarcoma, SKCM- Skin Cutaneous Melanoma, STAD-Stomach adenocarcinoma, STES- Stomach and Esophageal carcinoma, TGCT-Testicular Germ Cell Tumors, THCA- Thyroid carcinoma, THYM- Thymoma, UCEC- Uterine Corpus Endometrial Carcinoma, UCS- Uterine Carcinosarcoma, UVM- Uveal Melanom

4.1.2 EXPRESSION LEVELS IN BREAST TISSUES

Furthermore the expression of *p53, PTEN and BRCA1* was investigated in breast cancer tissues versus normal tissues. As shown in Figure 4.3(a-c), *p53* and *BRAC1* were overexpressed compared to *PTEN* which was overexpressed in normal breast tissues compared to cancerous tissue.
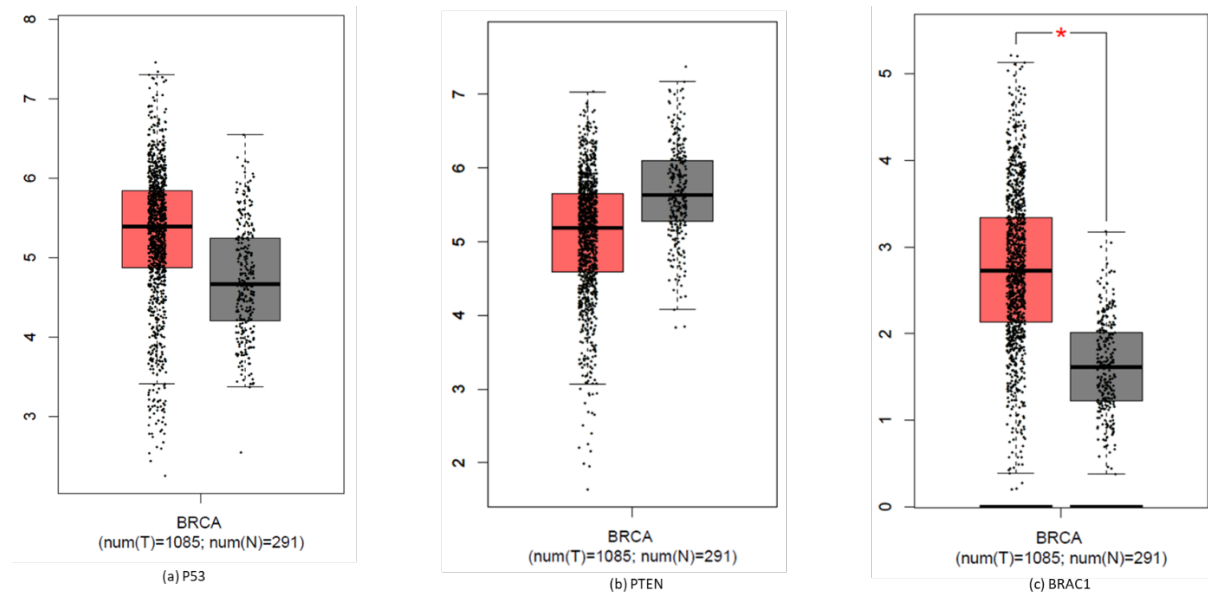
Figure 4.3: Transcription levels of *p53, PTEN,* and *BRCA1* in normal versus cancer breast tissues.

4.1.3 SURVIVAL ANALYSIS IN BREAST CANCER TISSUES

The relationship between the gene expression and patient prognosis across cancer microarray database was evaluated using Prognoscan. The estimation of the prognostic value and overall survival of breast cancer patients with alterations in the expression of *p53, PTEN,* and *BRCA1* was investigated. The results in Figure 4.4, showed that patients with high expression of *p53* and *PTEN* are associated with poor survival rates while patients with *BRCA1* showed that whether the expression is low or high the survival rate is poor.
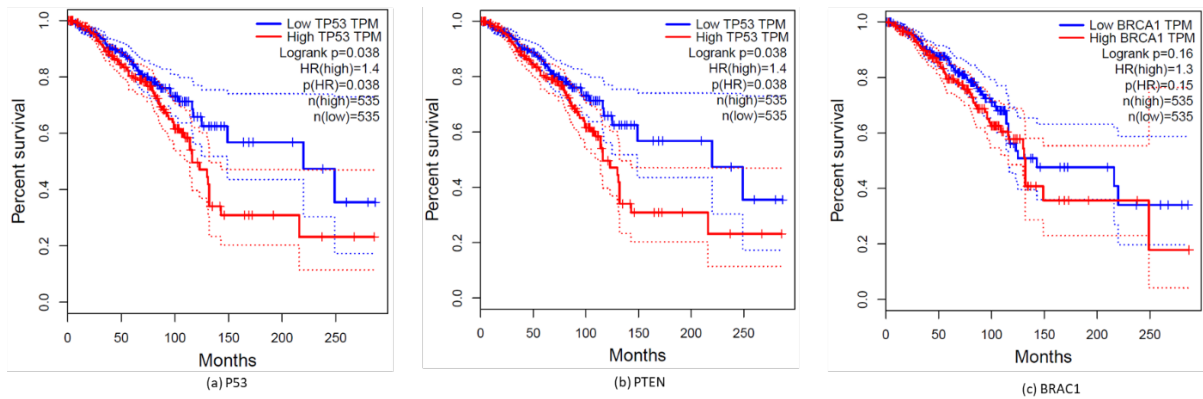
Figure 4.4: Overall survival analysis of *p53, PTEN, and BRCA1* in breast cancer patients investigated by PrognoScan database.

The overall results from GEPIA and PrognoScan database demonstrated that *p53* and *BRCA1*genes are over-expressed in various types of cancerous tumours compared to *PTEN.* This is because *p53* is the most commonly altered gene with mutations in various cancers. Various authors reported that *p53* can be used as a biomarker in the diagnosis and prognosis of cancer because tumours of highly malignant potential and poor prognosis show higher expression of *p53* gene[144]. *p53* is a key suppressor gene and is involved in various signalling pathways such as the ability to induce cell cycle arrest, DNA repair, senescence, and apoptosis[145]. More importantly in certain cancers, *p53* is mutated late in the tumorigenesis process and plays a significant role in advanced stages, which leads to a more aggressive and invasive tumour[146]. *BRCA1* accounts for 3-5% hereditary for breast and ovarian cancers, however, it's found in other cancer types such as prostate cancer, pancreatic cancer, and melanoma[147]. Its name **BR**east **CA**ncer gene 1 originated based on the fact that it was more highly expressed in breast cancer than in other cancer types.

4.2   ANALYSIS OF GENE EXPRESSION USING CONVENTIONAL PCR

4.2.1   EXTRACTION OF GENOMIC DNA

The T47D, MDA231, MCF7, MCF12A, BHK-21, A375 and Me-180 cells were cultured using conventional methods as reported in section 3.2.1. The T47D, MDA231, MCF7 and MCF12A cells are of breast origin, MCF12A is a normal breast cell line while others are cancerous[148].

The Me-180 cells are ovarian cancer cell lines, A375 cells are skin cancer cell lines and BHK-21 are hamster kidney normal cell lines. Figure 4.5 shows the morphology of MCF7, T47D, A375 and Me-180 cells viewed under the light microscope. The MCF7 cell lines were found to have an epithelial-like shape which is a polygonal shape with regular dimensions as shown in Figure 4.5 (a)[149], while T4D7 cell lines in Figure 4.5 (b) were found to have a lymphoblast shape which was similar to the shapes reported in the literature[150,151]. Figure 4.5(c-d) Me-180 and A375 both exhibited an epithelial shape[152].

(a)	(b)
(c)	(d)
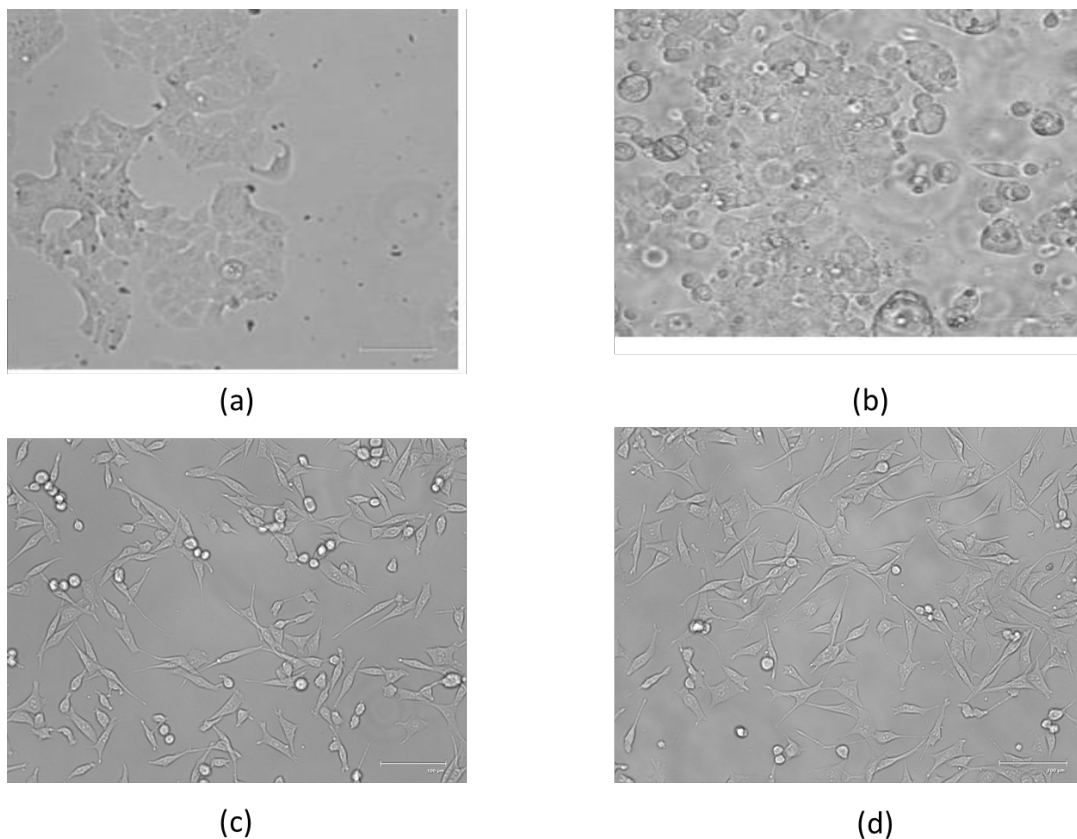
Figure 4.5: Light microscopy images of T47D (a), MCF 7 (b), A375(c) and Me-180(d) cell lines, the cell morphologies images were captured at 20X magnification.

The concentrations of genomic DNA extracted from the cells were measured using a NanoDrop™ 2000 /2000c spectrophotometer and the concentrations were as follows as shown in Table 4.1.

Table 4.1: Concentrations of genomic DNA extracted from cells

Cell type	Concentration	260/280 ratios
MCF7	465.9ng/µl	~ 1.8
T47D	394.9ng/µl	~1.84
MDA-231	578.3ng/µl	~1.97
MCF12A	142.3ng/µl	~1.83
A375	319.1ng/µl	~1.80
Me-180	226.0ng/µl	~1.84
BHK-21	365.5ng/µl	~1.83

The 260/280 ratio was also used as a secondary measure of DNA purity, the ratio is expected to be between 1.8 and 2.2. On average the ratios for DNA were between 1.8 and 1.9, this further confirmed the purity of the DNA. The extracted DNA from the cell lines (50ng/µl) was also electrophoresed on a 1% agarose gel to determine the integrity of the DNA as shown in Figure 4.6, with size that >15000bp.
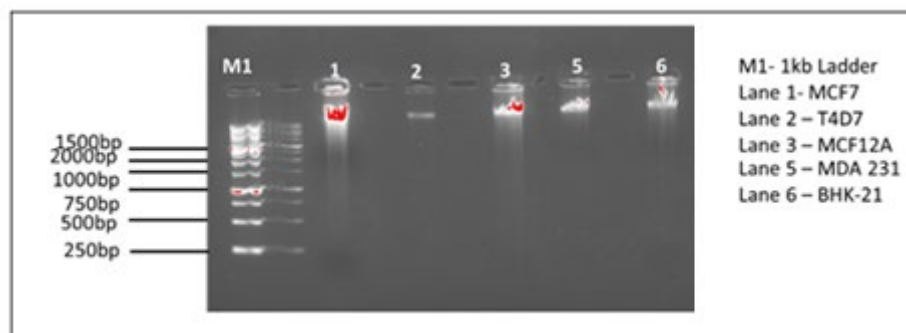
Figure 4.6: Agarose gel electrophoresis of genomic DNA extracted from MCF7, T4D7, MCF12A, MDA231 and BHK-21 cell lines.

4.2.2   PCR AMPLIFICATION OF TARGET GENES FROM GENOMIC DNA

4.2.2.1   GENE EXPRESSION IN CANCEROUS CELLS

The genomic DNA extracted from T47D, MCF7, and MDA231 cell lines was amplified using PCR to determine the expression or presence of cancer genes, namely *p53, PTEN, and BRCA1*. The genes were amplified using three sets of gene-specific primers per gene target. Figure 4.7 show 1% agarose gel electrophoresis of the PCR products obtained from the amplified genomic DNA from the three different genes (*p53, PTEN, and BRCA1*). The genes that were successfully amplified were *p53 and PTEN,* with only *p53 B, PTEN A, and PTEN B* primers. The expected sizes of the amplicons were 490bp for *p53 B*, 806bp *PTEN A*, and for *PTEN B* 652bp, which corresponded with the sizes shown in Figure 4.7. It was observed that for *p53 A*, *p53 C*, *PTEN C and BRCA1 A* primers were not able to amplify the targeted genes on the template DNA as shown in Figure 4.7a-c. The *p53* primer sequences were adapted from the study by Bharaj et al. and all three primer sets were able to amplify *p53* gene in MCF7 and T47D cell lines[124]. However, *p53 A* and *p53 B* primers failed to amplify the genes on the cell lines used in this study. Similarly, no amplification was observed with the *PTEN C* primers. This led to speculation that the target mutations may not be present in the cell lines or they require different annealing temperature.

The *BRCA1A* and *BRCA1C* primer sequences used in the study were also obtained from literature, and shown by Ozcelik et al. to amplify the genomic DNA obtained from familial breast cancer patients[125]. The specific primers were targeted at various mutations in the *BRAC* gene[125], these mutations may not be available in the cell lines used in the current study hence no amplification occurred. *BRCA1B* had multiple amplicons at 200 to 750bp in all cell lines, while the length of the amplicon in genomic DNA from familial breast cancer patients was 848bp[125].
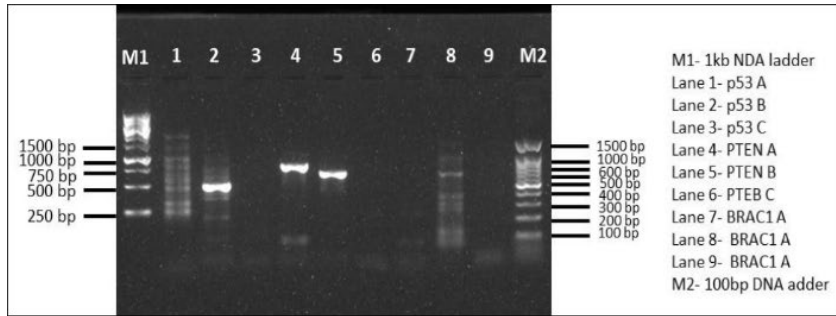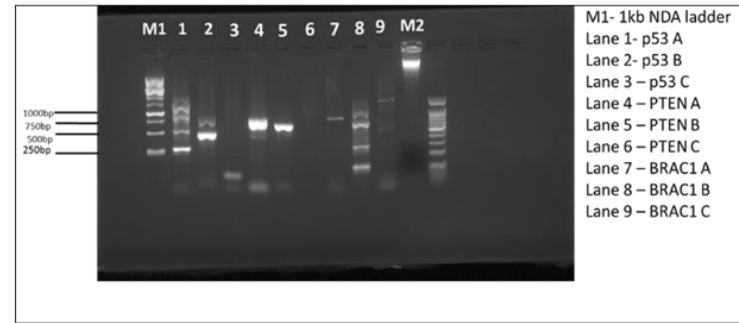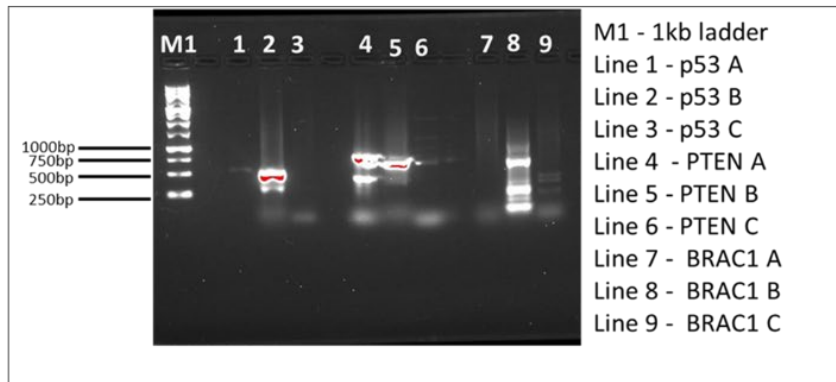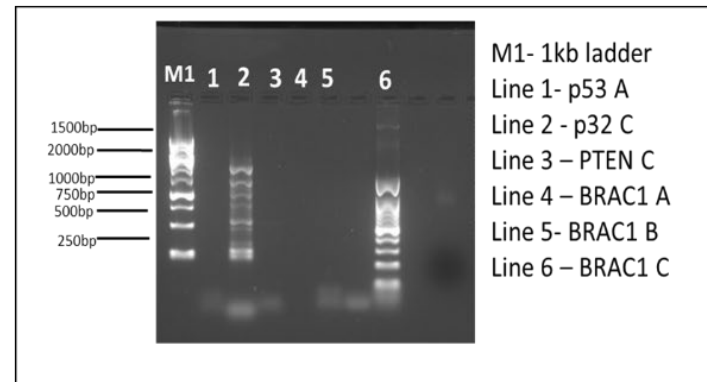
Figure 4.7: Agarose gel electrophoresis of *p53A, p53C, PTEN C, BRCA1 A, BRAC1*B and *BRAC1 C*. M1-1kb as a ladder. (a) MCF7, (b) T47D, (c) MDA231 and (d) MCF7.

Following these results mentioned above, further optimisation of *p53 A, p53 C, PTEN C, BRCA1A, BRCA1B and BRCA1C* was done with the following annealing temperatures in separate PCR reaction: 62°C; 64°C; 66°C and 68°C to obtain an optimum temperature that will amplify the target gene. However, the same results were obtained at these temperatures (Figure 4.7 (d)). This suggested that the problem might be with the primers, new primers would have to be redesigned, synthesised, and amplified following the same procedures. A way forward was to continue with the three primers *p53 B, PTEN A* and *PTEN B* from the nine primers for the preparation of the sample for NABLFA. Furthermore, an expression of these genes was investigated in non-cancerous cells to determine the specificity of these genes between cancerous and non-cancerous cell lines.

4.2.2.2   GENE EXPRESSION IN NON-CANCEROUS CELLS

Amplification of cancer genes (*p53, PTEN and BRCA1*) from genomic DNA from normal cells was evaluated using PCR. As shown in Figures 4.8 and 4.9, MCF12A and BHK-21 PCR products showed that the genes are also expressed in non-cancerous cells. Similar to PCR products of the breast cancer genomic DNA, the same size amplicons (*p53 B* at 490bp, *PTEN A* at 806bp and *PTEN B* at 653bp) were obtained. This indicated that these genes are expressed in both normal and cancer cells. However, the *p53* gene is expressed in low levels in normal cells as per *In Silico* studies. The gene is dormant until there is a trigger or expressed as a response to cellular stress. DNA damage can activate transcription of *p53* such as DNA damage by irradiation or chemotherapeutic drugs or activation of oncogenes. Cellular damage can result in a rapid increase in the level of *p53* and activation of *p53* as a transcription factor[153]. These findings were in agreement with *in silico* studies reported in section 4.1, that *p53* gene is found to be expressed in various cancers, at low levels in normal tissues and high levels in cancerous tissues. Similar to *p53*, *PTEN* gene was also found in various cancers (breast, skin, prostate and brain)[154], but with high levels in normal tissues compared to cancerous tissues. These results were validated by performing qPCR.
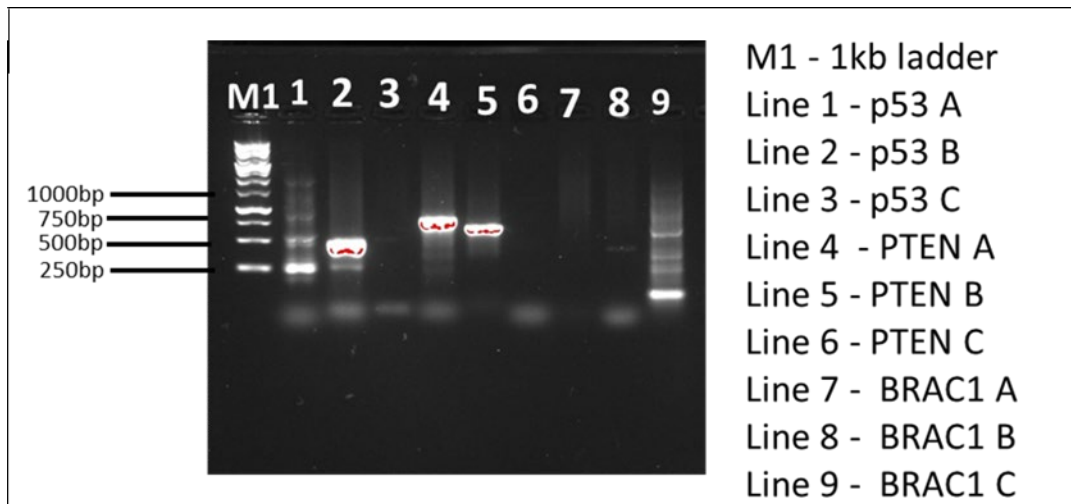
Figure 4.8 : The *p53 B, PTEN A and BRAC1 C* were amplified by PCR from MFC12A genomic DNA.
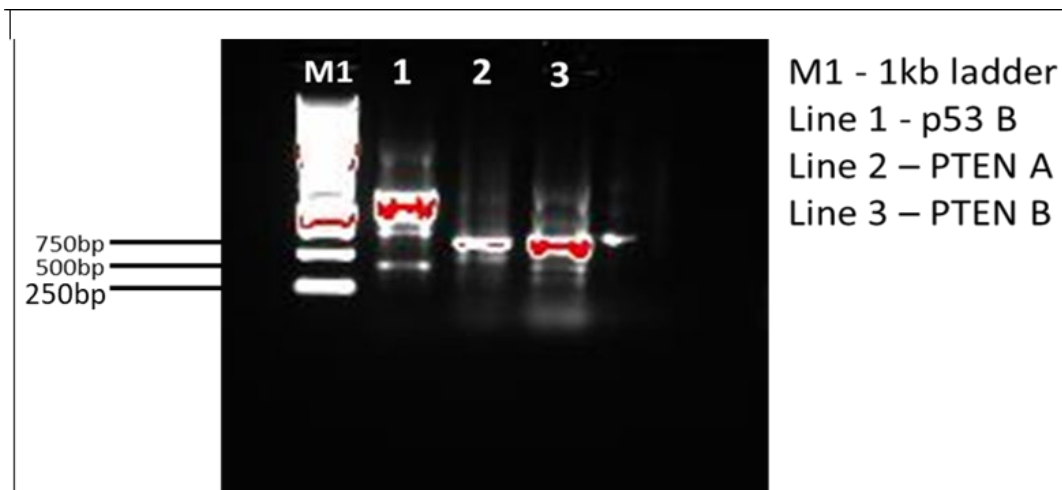
Figure 4.9: The *p53 B, PTEN A and PTEN B* were amplified by PCR from BHK-21 genomic DNA.

4.3   VALIDATION OF GENE EXPRESSION BY qPCR

P53 and *PTEN* gene expression on breast cancer (MCF7), normal breast (MCF12A) and ovarian (Me-180) cell lines was further validated by qPCR. HPRT was used as a housekeeping gene, and is one of the cellular maintenance genes that regulate basic and ubiquitous cellular functions[155]. qPCR is based on RNA quantification and will provide more specific and sensitive,[156] information on gene expression, gene loss or amplification[157]. The qPCR amplification plots shown in Figure 4.10 shows that *p53* and *PTEN* genes are expressed in the

selected cell lines. The ΔCt values for each gene were obtained from triplicate reactions, *PTEN* gene had higher ΔCt (32.1) in all cell lines meaning that *PTEN* expression was lower compared to *p53* which had a lower ΔCt value of 28.3. Zhang et al. also reported that the expression of *PTEN* is lower in cancerous tumours compared to normal tissues[158], the low expression of *PTEN* correlates with the size, grade, lymph node metastases and steroid receptor status of the tumour[159]. The current study also confirmed the presence of the genes under study through conventional PCR, *in silico* and qPCR studies; further confirming that these genes are present in the cells.

The results were in agreement with the *in silico* studies reported in section 4.1, that *p53* gene was highly expressed in cancerous tumours compared to *PTEN*. *p53* gene was reported to be highly expressed in different grades (I to III) of breast cancer tissues compared to normal tissues[160]. Furthermore, Archer et al. also reported the high expression levels of *p53* in advanced breast cancer in 92 patients with 57% having high stage tumours[161]. This shows that *p53* gene can be used as a diagnostic maker as it showed an abnormal expression in breast cancer-graded tissues. Patients with high levels of *p53* were shown to have worse prognosis, therefore *p53* can also be used to predict prognosis[144].
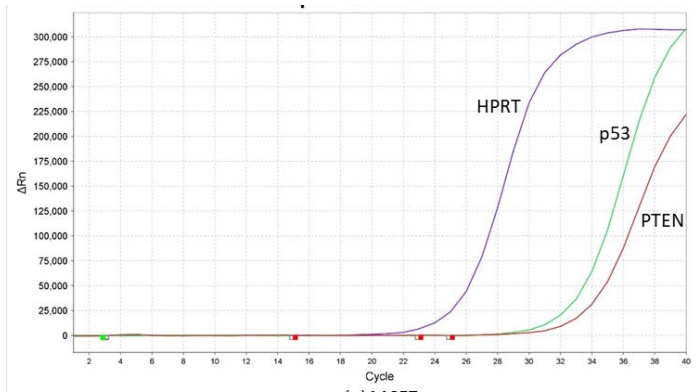
(a) MCF7
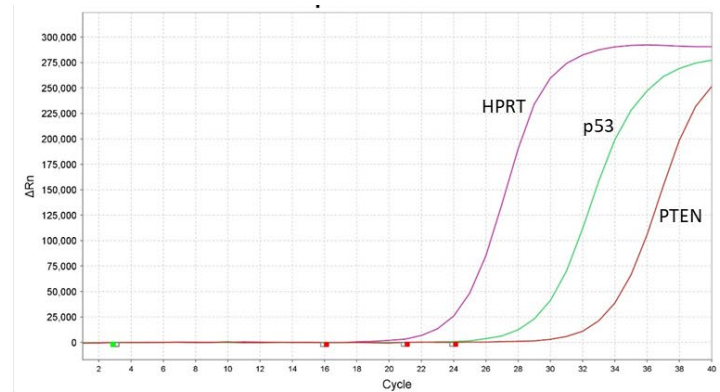
(b) MCF12A
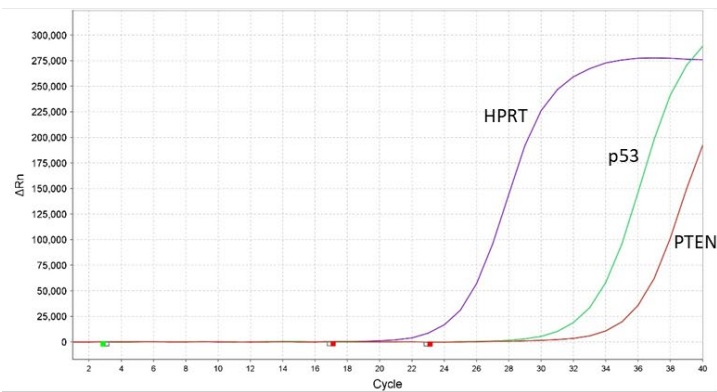
(c) Me-180

Figure 4.10: qPCR amplification curves of *p53* and *PTEN* from (a) MCF7, (b) MCF12A and (c) Me-180. HPRT was used as a housekeeping gene.

4.4 SAMPLE PREPARATION FOR NABLFA

4.4.1 AMPLIFICATION OF TARGET GENES USING TAGGED AND UNTAGGED PRIMERS

Out of nine primers, *p53 B, PTEN A and PTEN B* were able to amplify genomic DNA obtained from cancer and non-cancerous cells. The three primers were selected for further analysis, the primers were tagged with biotin on the 5' end of the forward primer and with dig on the 5' end of the reverse primer. These tags were used to assist with the detection of the analyte during the NABLFA testing. PCR product amplified by the tagged primers showed improved specificity when compared to untagged primers as shown in Figure 4.11. Although the target genes were amplified from MCF7 and T47D cells genomic DNA by both primers, the untagged primers produced multiple bands which could be due to non-specific binding. The amplicons produced by tagged primers were of correct sizes; *p53 B* appearing at 490bp, *PTEN A* at 806 bp and *PTEN B* at 652bp.

Figure 4.11: *p53, PTEN A and PTEN B* were amplified by PCR using tagged and untagged primers from different cell lines with M1-1kb as a ladder.

4.4.2 SEQUENCING OF THE TAGGED PCR PRODUCT

The PCR amplicons obtained from Figure 4.11 (above) were sent to Inqaba Biotechnical Industries for DNA sequencing data to confirm gene identity. The raw data as shown in Figure 4.12, were analysed by GeneDoc and Bio Edit programmes. The resulting DNA sequence shown in Figure 4.13(a) was then blasted on NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify if the gene sequence of the PCR product matched that of the selected genes. The results in Figure 4.13(b) showed a match of 97% with the *p53* gene, thus confirming the amplification of the *p53* gene from MCF7 cells with *p53 B* primers. Unfortunately, *PTEN A* and *B* were not sequenced as it was reported that the DNA degraded.
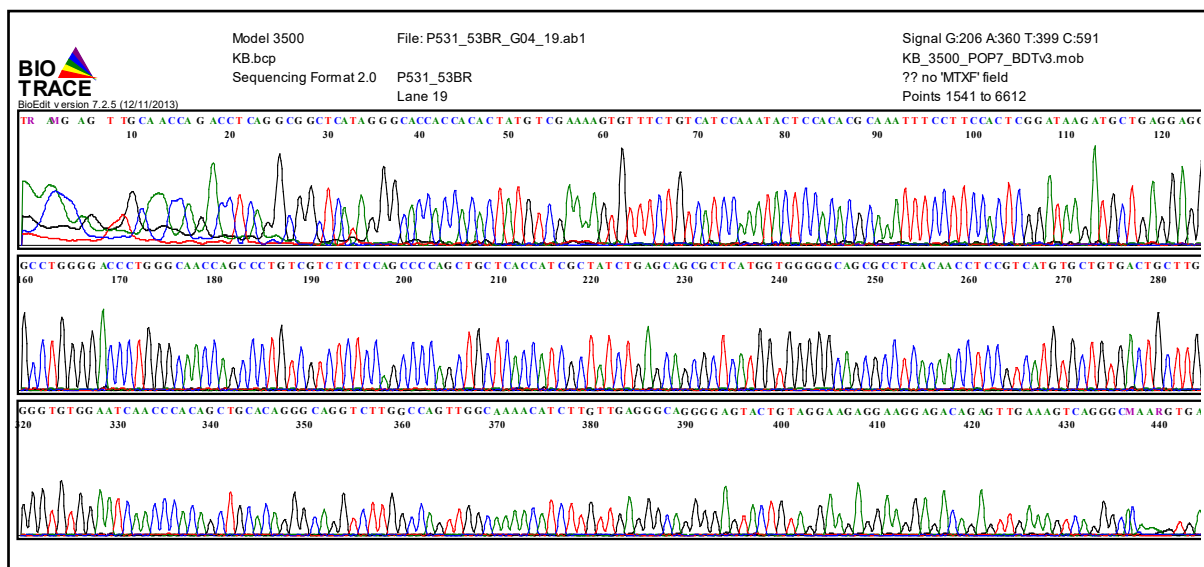
Figure 4.12: Raw data of *p53 B* PCR product sequenced by Inqaba Biotechnical Industries, the raw data was then edited and analysed using GeneDoc and Bio Edit programmes, and the resulting sequence was then blasted on NCBI blast.

GTGWCCKCATCAAGAYGTWWTGCCAACTGGCCA-GACCTGSCCTGTGSAGMTGTGGGTWG

ATTCCACACCCCCGCCCGGSACCCGCGTCCGCSCCATGGSCWTCTWCTARYGGTCACAGC

ACRTGACGGAGGYTGYGAGGCGCTACCCCCACCATGAGCGCTGCTCAGATAGCGATGGTG

AGCAGCTGGGGCTGGARAGACGACAGGGCTGGKTGCCCAGGGTCCCCAGGCCTCTGATTC

CTCACTGATTGCTCTTAGRTCTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATT

TGCGTGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATG

AGCCGCCTGAGGTCTGGTTTGCAA-CTGGGGTCTCTGGGAGGAGGGGTTAAGGGTGGTTG

TCAKGGGCCCTCCSAAA------
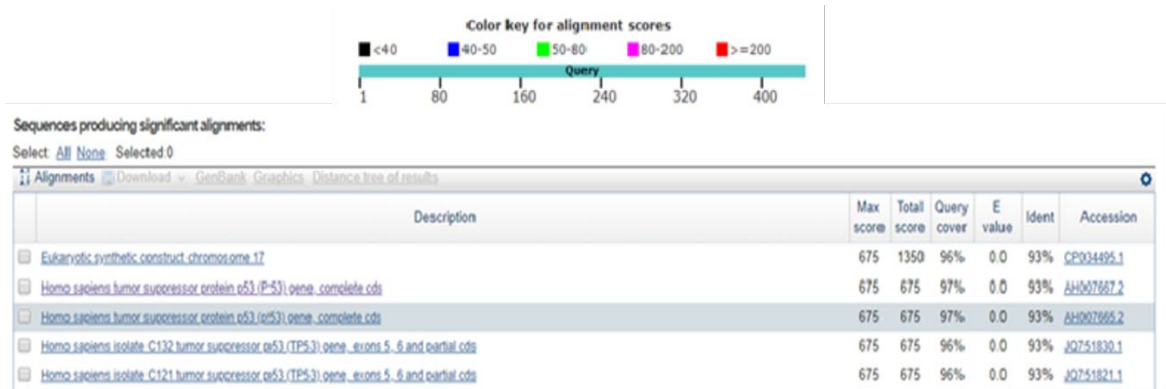
Figure 4.13: *p53 B* DNA sequence that resulted from raw data from Inqaba Biotechnical Industries (a) *p53* PCR product sequence was matching with *p53 B* gene on NCBI Blast (b).

CHAPTER 5   SYNTHESIS, CHARACTERISATION AND CONJUGATION OF AuNPs

5.1   SYNTHESIS AND CHARACTERISATION OF 14nm AuNPs

5.1.1   SYNTHESIS OF 14nm AuNPs

The AuNPs were synthesised using a citrate reduction method devised by Turkevich method[129]. Citrate was used as a reducing agent, which facilitated the reduction of gold into a lower oxidation state, thus resulting in the formation of AuNPs characterised by red colour. Initially, when citrate was added to the gold solution a colour changed from yellow to a clear colourless solution in 2mins, then to dark blue, a phenomenon attributable to the formation of gold nanowires through the nucleation of gold atoms[162]. Three minutes later, these nanowires destabilise and increase in size (depending on the desired size) the final size of the particles depends on the stoichiometric ratios of gold salt and the citrate[163]. This was followed by fragmentation and cleavage thereby forming spherical citrate-capped AuNPs, the whole process is shown in Figure 5.1. The average time to form the AuNPs was reported to be 2-5mins.

Synthesis of 14nm AuNPs

Au solutions   Addition of citrate   Nucleation of Au atoms   14nmAuNPS
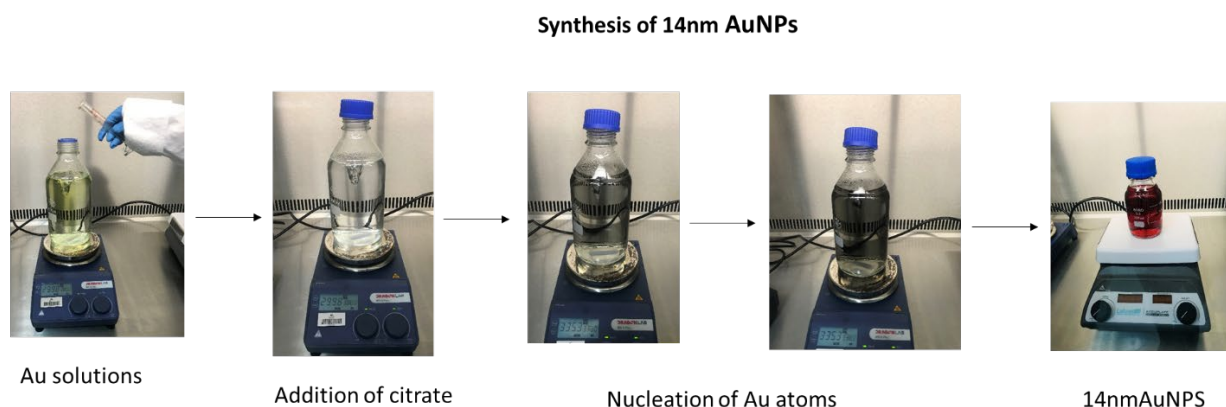
Figure 5.1: Synthesis process of 14nm AuNPs using the citrate method.

5.1.2 CHARACTERISATION OF 14nm AuNPs

5.1.2.1 *UV-VIS CHARACTERISATION*

The optical properties of AuNPs were characterised by UV-Vis spectroscopy which measures the surface plasmon resonance (SPR) of AuNPs. The SPR is an oscillation of electrons in the conduction band of the nanoparticles. The oscillation of electrons on the surface of the particle determines the SPR[164]. Figure 5.2 shows the UV-Vis spectrum of 14nm AuNPs synthesised using the citrate method.The SPR of 14nm AuNPs was observed at 519nm, which was similar to the SPR reported for 14nm AuNPs synthesised by Sosibo et al.[115] and Haiss et al.[165].
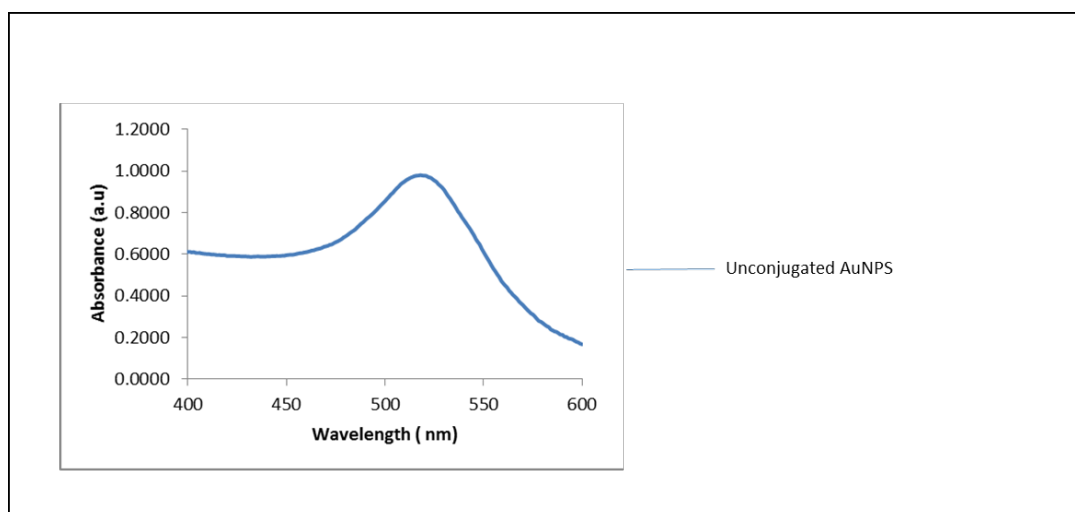
Figure 5.2: Optical absorption spectra used to measure the SPR of 14nm AuNPs.

5.1.2.2 *TEM ANALYSIS OF 14nm AuNPs*

The morphological properties of 14nm AuNPs were characterised using Jeol JEM Transmission Electron Microscopy (TEM) and core size was analysed by imageJ. As shown in Figure 5.3, the TEM micrograph shows the dominance of spherical AuNPs formed with an average core size diameter of 14nm. The AuNPs were monodisperse with 90% uniformity of size distribution.

(a)   (b)
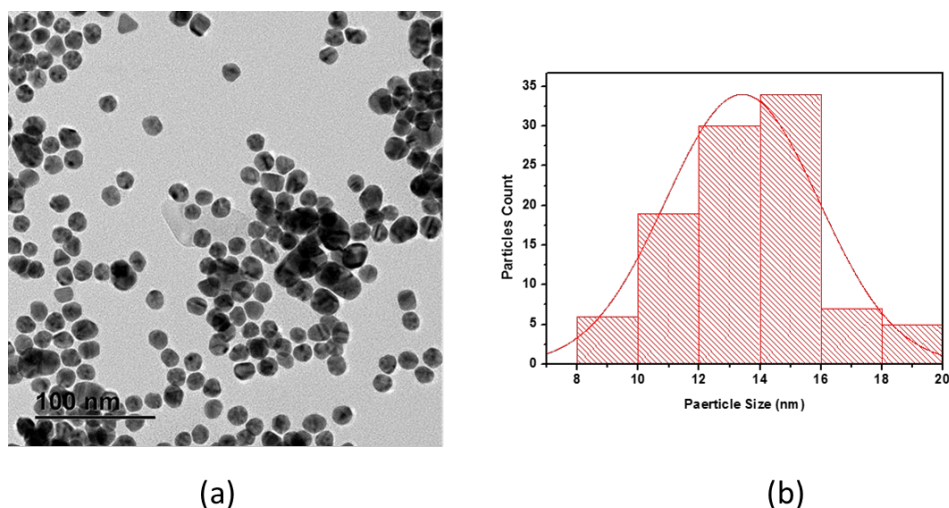
Figure 5.3: (a) TEM image of 14nm AuNPS and the (b) size distribution graph.

5.2   FUNCTIONALISATION WITH PEG-BIOTIN AND STREPTAVIDIN

5.2.1   FUNCTIONALISATION OF AuNPs WITH PEG-BIOTIN AND PEG-OH

Citrate-capped AuNPs are negatively charged, this helps in attaching biological molecules for example proteins to them[117]. This capability increases the use of AuNPs in biological applications such as diagnostics and targeted drug delivery. Attaching biomolecules to the AuNPs will improve their stability and solubility. It has also been reported that the high affinity that Au has for sulphur also aids in the use of AuNPs for biological applications. It was demonstrated that the sulphur atoms or thiolated biomolecules have high affinity and bind to the Au surface[166]. This phenomenon was reported by Otsuka et al.[167], using thiolated PEG ligands (PEG-SH) to functionalise AuNPs. The desired functional groups can be OH, COOH, and biotin which help in the bio-functionalisation of AuNPs for downstream applications.

The 14nm AuNPs in this study were functionalised with thiolated PEG-OH and biotin. The PEG ligands are known to improve the stability, solubility and bio-compatibility of AuNPs, for use in biological applications[167]. The schematic diagram showing the functionalisation of 14nm AuNPs with PEG-biotin and PEG-OH is shown in Figure 5.4. PEG-OH was used for stabilisation of the AuNPs, the PEG-biotin was added to explore biotin-streptavidin chemistry in the NABLFA development.
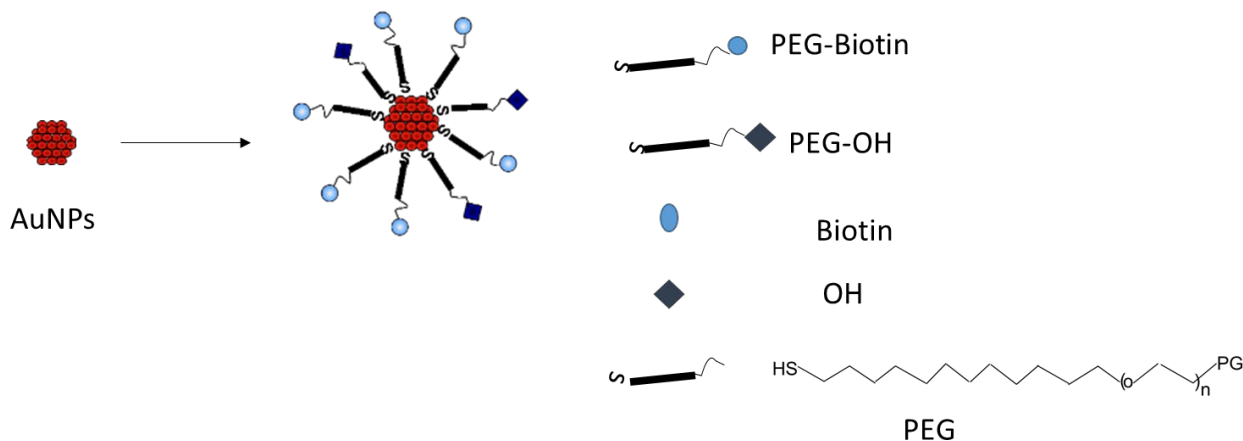
Figure 5.4: The functionalisation and stabilisation of AuNPs with PEG-biotin and PEG-OH[115].

5.2.2 CONJUGATION OF STREPTAVIDIN TO THE AuNPs

Streptavidin was attached to the AuNPs-PEG-biotin to produce AuNPs-PEG-biotin-streptavidin conjugate. Streptavidin is known to have a high affinity for biotin, it binds four molecules of biotin per one molecule of streptavidin[168]. Due to this high affinity of biotin to streptavidin, the reaction was expected to yield AuNPs conjugates shown in Figure 5.5. The excess or unbound streptavidin was removed by centrifugation.
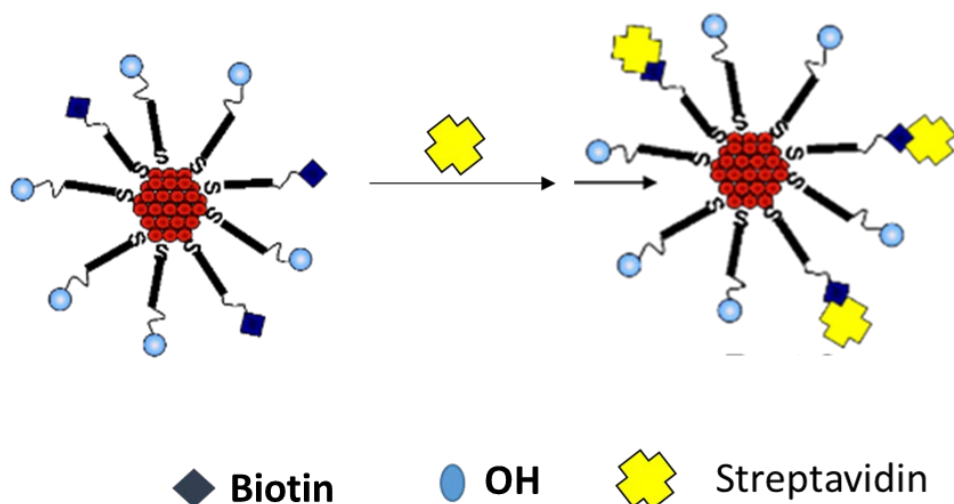
Figure 5.5: Functionalisation 14nm AuNPs using streptavidin- biotin chemistry[115].

5.3 CHARACTERISATION OF FUNCTIONALISED AuNPs

5.3.1 UV-Vis CHARACTERISATION OF FUNCTIONALISED AND UNFUNCTIONALISED AUNPS

The conjugation of PEG-biotin and streptavidin to the AuNPs was confirmed by comparing the UV-Vis spectra of unconjugated AuNPs and AuNPs-PEG-biotin-streptavidin conjugate. Figure 5.6 shows the shift in SPR from 519nm for unconjugated AuNPs to 523nm for the AuNPs-PEG-biotin-streptavidin conjugate. The change in SPR was due to the binding of biotin and streptavidin on the surface of the AuNPs. Changes in SPR were also reported as an indication of successful conjugation bio-molecules on AuNPs by other studies[115,165].
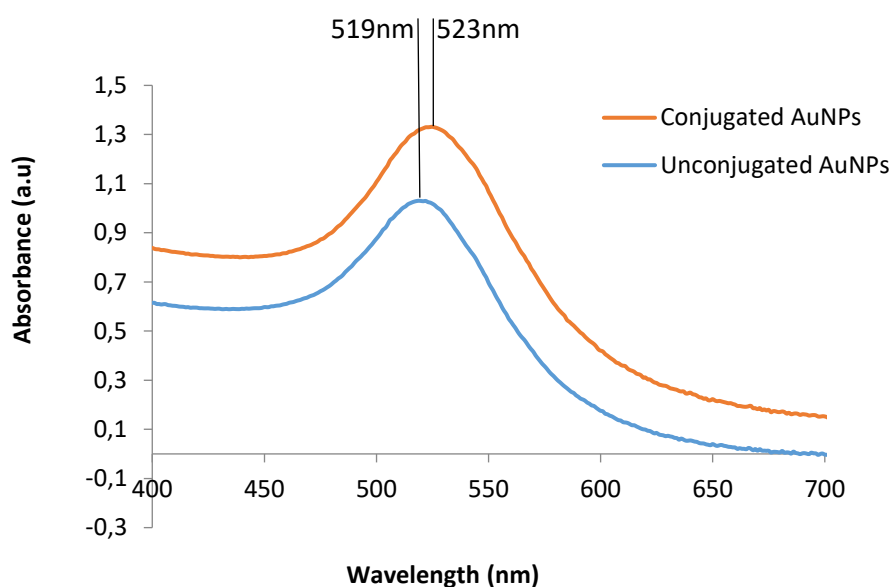
Figure 5.6: Optical absorption spectra for AuNPs and after conjugation with PEG-biotin and streptavidin.

5.3.2   TEM ANALYSIS OF AuNPs-PEG-BIOTIN-STREPTAVIDIN CONJUGATE

The 14nm AuNPs-PEG-biotin-streptavidin conjugate was characterised using Jeol JEM TEM and ImageJ to determine the core size. The image showed no aggregation of AuNPs after the bio conjugates were introduced, the TEM analysis confirmed the core size and shape of the AuNPs were not affected by conjugation. The core size remained at 14nm and the shape of the AuNPs-PEG-biotin-streptavidin conjugate was still monodispersed and spherical as shown Figure 5.7.

(a)   (b)
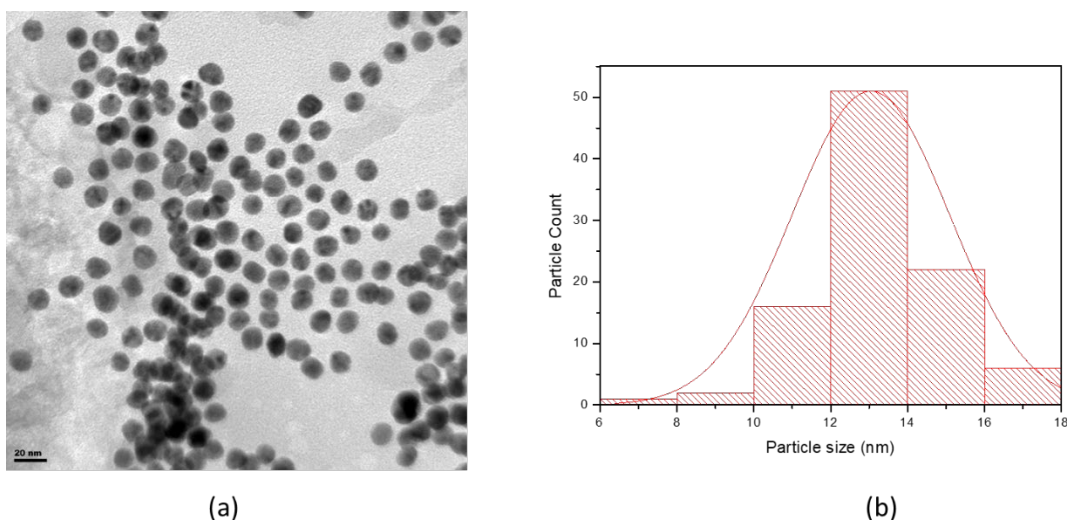
Figure 5.7: TEM image(a) of 14nm AuNPS functionalised with biotin and streptavidin and the (b) size distribution graph.

5.3.3   ZETA POTENTIAL OF FUNCTIONALISED AND UNFUNCTIONALISED OF AuNPs

Zeta Potential or surface charge of AuNPs in solution was measured as described by Anuary et al. [169]. Figure 5.8 Figure 5.8 displays different layers that form when a particle is introduced in a solution. During conjugation, a double layer of ions that forms electrical potential at the borderline (slipping plane) will affect the zeta potential of the nanoparticles. The   zeta potential values range between +100mV to -100Mv [169], with molecules between +30Mv and -30Mv considered to be stable.
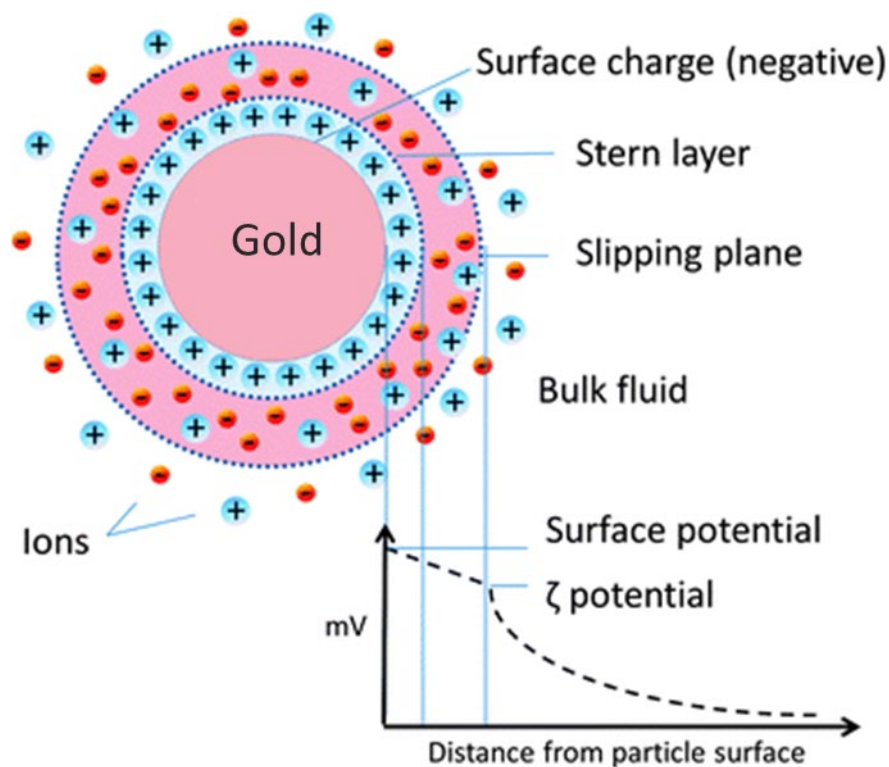
Figure 5.8: illustration of AuNPs in solution, explaining the different layers that form the charge of the particle. Reprinted with permission[170], copyright Signpost 2018 .

 The zeta potential analysis for unconjugated AuNPs, AuNPs-PEG-biotin and AuNPs-PEG-biotin-streptavidin conjugates was measured by Malvern zetasizer nano series instrument. This was done to monitor the surface charge changes as the particles were being functionalised. The zeta potential of unconjugated AuNPs was -47.9mV ± 7.76mV as shown in Figure 5.9(a), the zeta potential changed to -28.3mV ±11.9mV after conjugation of PEG-biotin Figure 5.9(b) and to -25.1mV ±5.27mV for AuNPs-PEG-biotin-streptavidin (Figure 5.9(c)). This showed that upon functionalisation of AuNPs the surface charge increased as an indication of changes in the surface of the citrate capped AuNPs.
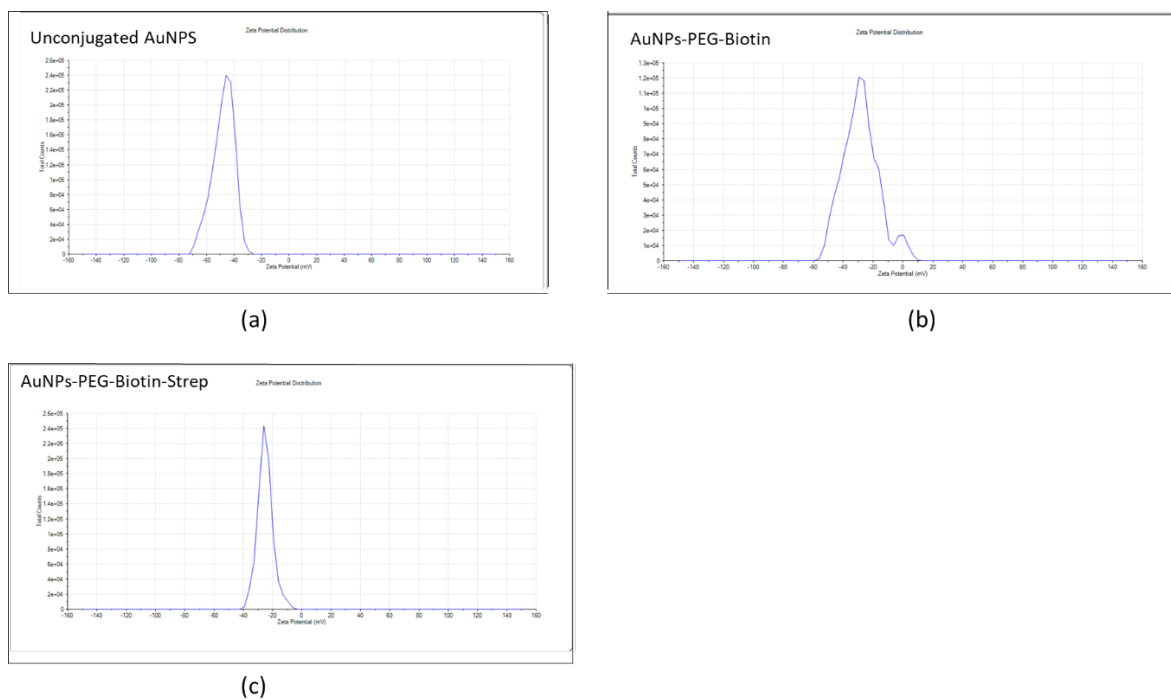
Figure 5.9: Zeta potential analysis measuring the surface charge of the AuNPs.

5.3.4   AGAROSE GEL ELECTROPHORESIS (AGE) ANALYSIS

Further characterisation was performed on unconjugated AuNPs, AuNPs-PEG-biotin and AuNPs-biotin-streptavidin AuNPs. The AuNPs conjugates were analysed by 1% agarose gel electrophoresis. Gel electrophoresis has mainly been used to separate DNA and proteins[171]. The mobility of molecules on the gel electrophoresis is dependent on three factors, namely, size, shape and charge. The bigger the molecule size, the slower the migration. The direction and distance of migration depend on the charge (positive or negative) and the nature of the surface ligand that bears the charged functionality. Gel electrophoresis enables the distinction between unconjugated AuNPs and those bearing surface functionalities due to their difference in migration patterns as a result of the impediments introduced by varying degrees of surface coverage[172].

The same technique was used in this study to analyse the effects of surface modifications on AuNPs, by monitoring their migration patterns on an agarose gel. Figure 5.10(a) shows the agarose gel loaded with AuNPs conjugates before the migration and Figure 5.10(b) shows the conjugates after the migration. The AuNPs-PEG-biotin (lane 2) and AuNPs-PEG-biotin-streptavidin conjugate (lane 3) migrated towards the anode while the unconjugated remained stationary on the well. The unconjugated AuNPs on Figure 5.10 (lane 1), showed a sign of agglomeration by changing colour to blue after electrophoresis. The agglomeration can be attributed to the high ionic strength of the AuNPs. The difference in mobility also suggested a difference in the composition of AuNPs conjugates as reflection size and charge difference.

(a)   (b)

Lane 1- Unconjugated AuNPs
Lane 2- AuNPs-PEG-Biotin
Lane 3- AuNPS—PEG-Biotin- Streptavidin
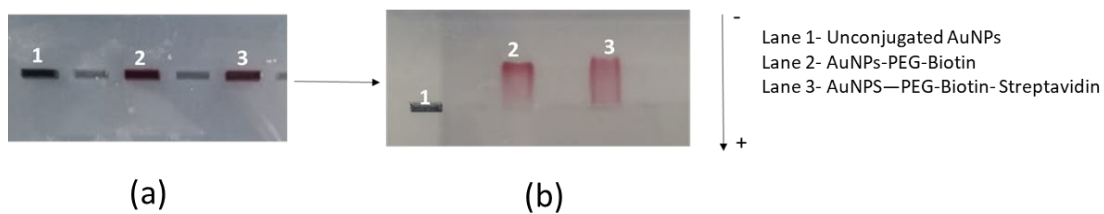
Figure 5.10: Analysis of AuNPs vs conjugates migration by agarose gel electrophoresis. AuNPs loaded on 1% agarose gel before (a) and after migration (b).

CHAPTER 6   THE NABLFA DEVELOPMENT AND TESTING

6.1   THE PRINCIPLE OF NABLFA

The principle of NABLFA is based on the binding of AuNPs-PEG-biotin-streptavidin conjugate (prepared in section 5.2.2) to the PCR product (prepared in section 4.4.1). As illustrated in Figure 6.1, the AuNPs on the AuNPs-PEG-biotin-streptavidin conjugate was used as a reporter probe while the PEG-biotin-streptavidin served as a detection probe for the analyte. The analytes used were the *p53 B* and *PTEN A and B* PCR products. As previously discussed in chapter 4, the *p53* and *PTEN* PCR products were amplified primers tagged with biotin and dig at 5'end and 3'end, respectively. Anti-dig IgG antibody was dispensed as a test line on the NABLFA, and biotin on the control line.
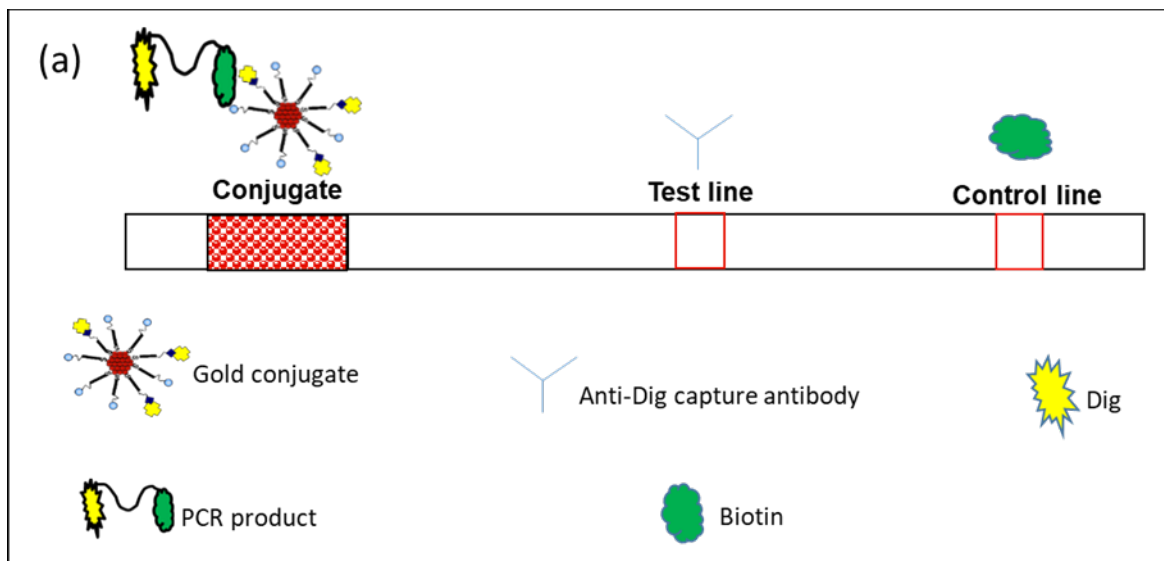
Figure 6.1: Illustration of the principle of the NABLFA used to detect PCR products.

6.1.1 THE TESTING PROCEDURE

The AuNPs-based NABLFA is a colorimetric assay that can be monitored visually following the principle of LFA. A negative test will be identified by the formation of single red line on the control line. In the absence of the target PCR product, the AuNPs-PEG-biotin-streptavidin conjugate will pass through the test line and only bind to the biotin immobilised on the control line forming red colour as shown in Figure 6.2.
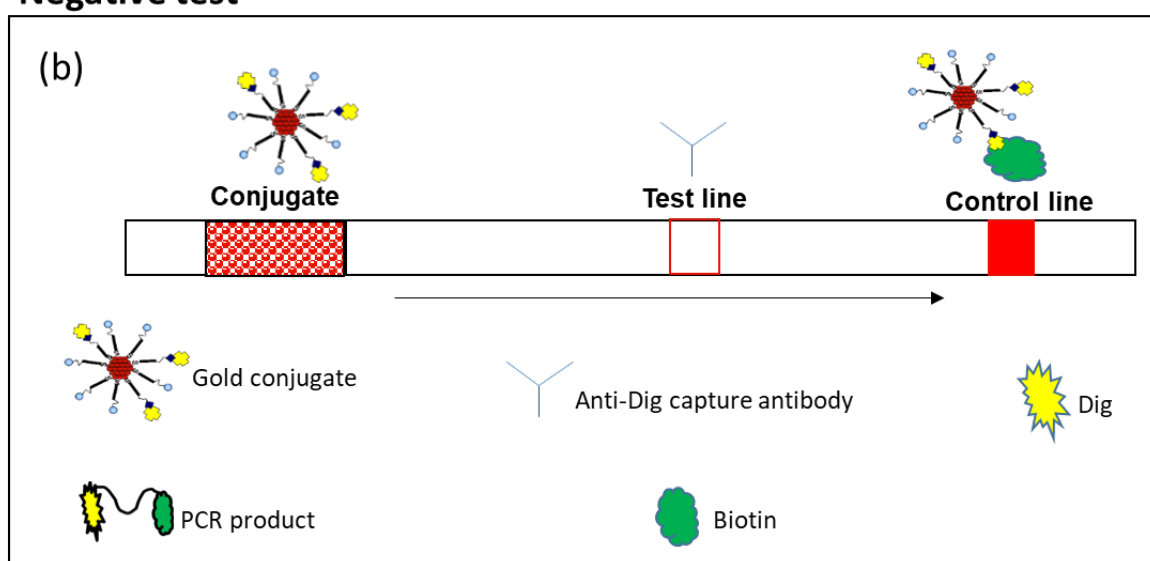
Figure 6.2: Illustration of a negative test.

A positive test will be recognised by red lines in both the test and control lines. In the presence of an analyte (target PCR product), the AuNP-PEG-biotin-streptavidin conjugate will bind to the PCR product and forms AuNPs-PEG-conjugate-biotin-streptavidin forming an AuNPs-PEG-biotin-streptavidin-biotin-PCR product complex (AuNPs-PCR complex). The AuNPs-PCR complex will flow through the nitrocellulose membrane and bind to anti-dig IgG antibody on the test line producing a red line. Then the excess AuNPs-PEG-biotin-streptavidin conjugate will migrate through the test line and binds to biotin on the control line and form another red line as illustrated in Figure 6.3. The assay reported in this study follows a similar concept reported by Ang et al.[173] used NABLFA to detect cholera genes amplified from human stools.

Positive test
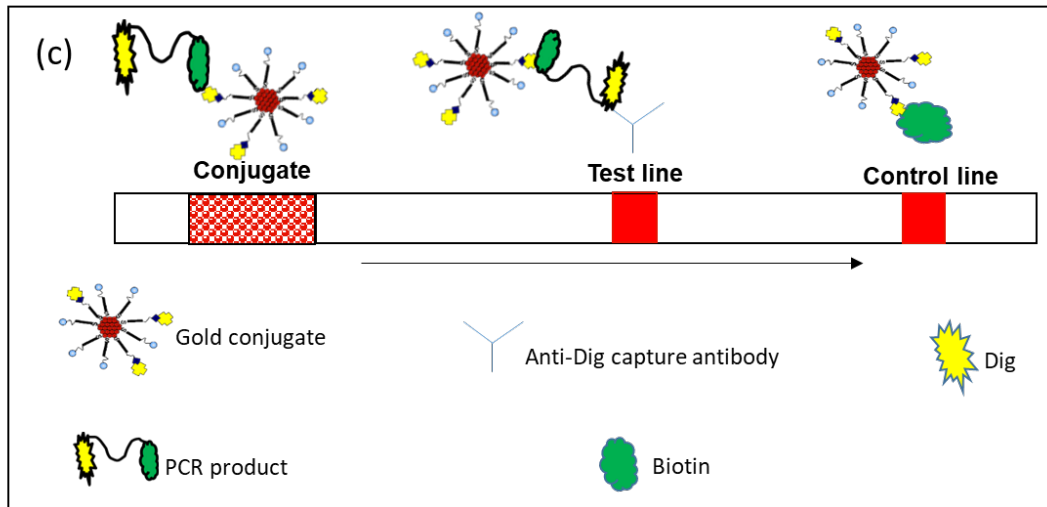
Figure 6.3: Illustration of a positive test.

6.2 EVALUATION OF BINDING ACTIVITY OF NABLFA

The performance of NABLFA was assessed on *p53* and *PTEN* PCR product samples from breast cancer cell lines (MCF7) using wet testing. The NABLFA strip was immersed into AuNPs-PEG-biotin-streptavidin conjugate solution with and without PCR product for 10-15mins. The AuNPs-PEG-biotin-streptavidin conjugate started flowing through the nitrocellulose membrane by capillary action. The test and control line on the strip were monitored for red colour development.

For a test with no PCR product the AuNPs-PEG-biotin-streptavidin conjugate migrated through the strip and bound to biotin on the control line. The AuNPs-PEG-biotin-streptavidin-biotin complex on the control line formed a red line observed in Figure 6.4. The formation of a red line in the control line represents a negative test.

Figure 6.4: The blank test: the testing was done with no PCR product, this was done to validate the test and the results expected were a negative test.

The PCR products (*p53, PTEN)* were mixed with AuNPs-PEG-biotin-streptavidin conjugate forming AuNPs-PCR complex and a 5mm NABLFA was immersed in the solution. The binding of the AuNPs-PCR complex to the test line (anti-dig) formed a red line as shown in Figure 6.5. Red lines in both test line and control lines represented an analyte positive test. The model system designed showed that nucleic acid targets can be detected visually by NABLFA.
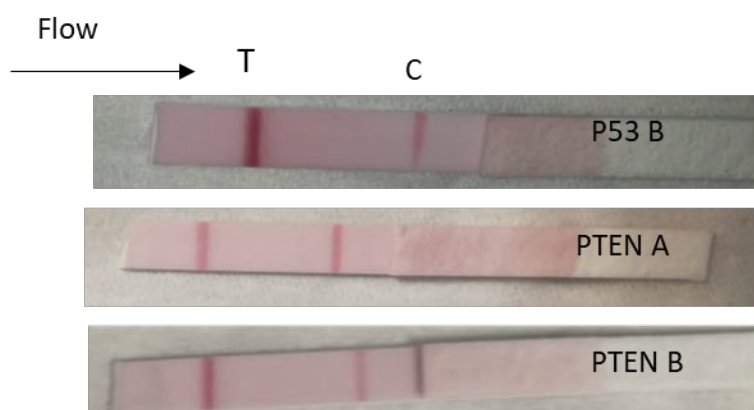
Figure 6.5: NABLFA showing the detection of MCF7 PCR product (*p53 B, PTEN A* and *PTEN B)* using NABLFA.

6.2.1 SPECIFICITY TEST

The AuNPs-based NABLFA was also able to detect *p53* and *PTEN* gene from PCR products obtained from various cell lines such as Me-180 (ovarian cancer cell line) and A375 (skin cancer cell line) as shown in Figure 6.6(a-b), respectively. The visual appearance of the AuNPs in the test strip was consistent in all test samples.

(a)   (b)

Figure 6.6: NABLFA for *p53* gene using ovarian (a) and skin cancer (b) cell lines.

Even though the purpose of this study was to develop a breast cancer NABLFA, it was discovered that these genes are not specific to breast cancer only. The selected genes are also associated with other high-risk cancers such as ovarian, skin, pancreatic, colorectal, and breast cancer[174]. As shown in Figure 6.6, the NABLFA was able to detect the genes in ovarian and skin cancer cells. These results were also in agreement with the *In silico*, conventional and qPCR analysis done in sections 4.1, 4.2, 4.2.1 and 4.2.2 that these genes are found in cancerous and normal cell lines. Apostolou et al. reported that *p53* is a penetrant gene for a variety of tumours such as sarcoma, brain cancer, adrenocortical carcinomas and early-stage breast cancer; thus it can be used to detect breast cancer at early stages. Carriers of the gene have a lifetime cancer risk that exceeds 90%[174,175] and breast cancer is the most regular malignancy among carriers of the gene, with 5% of females diagnosed before the age of 30[176]. The *PTEN* gene is also found in other cancers as well with a lifetime risk of 50% for breast cancer, 10% for thyroid cancer and 5%-10% for endometrial cancer[177].

The NABLFA developed in the study can serve as a proof-of-concept that can be used to detect various cancers as well. Having an assay that is able to detect high-risk genes can assist in screening individuals at the onset of any cancer. This will improve clinical management of the disease and provide early disease-specific treatment options. When an individual tests positive for any of the selected genes further action can be taken promptly.

Furthermore, an oncologist specialising in breast cancer can easily screen for breast cancer. The AuNPs-based NABLFA demonstrated the proof-of-concept that disease-associated nucleic acids such as DNA or RNA can be detected by LFAs to diagnose or screen for cancer. This is consistent with other studies that used AuNPs-based systems for the detection of SARS-CoV-2 genes from human RNA samples[178], and *Leishmania infantum* DNA amplified from dog blood[179]. These systems hold promise for developing a rapid and low-cost biosensor for visual detection of breast cancer genes that can be used at a POC diagnosis.

The presence of several genetic markers in various body fluids such as blood[91–93], urine[180], nipple aspirate fluid, tears, and sweat[181] (Figure 6.7) that are associated with breast cancer suggest that AuNPs-based NABLFA can be suitable for detection of the disease at a POC. This also validates the initial aim of the current study, that nucleic acids can be detected at the POC within body fluids without a need for the additional amplification step.
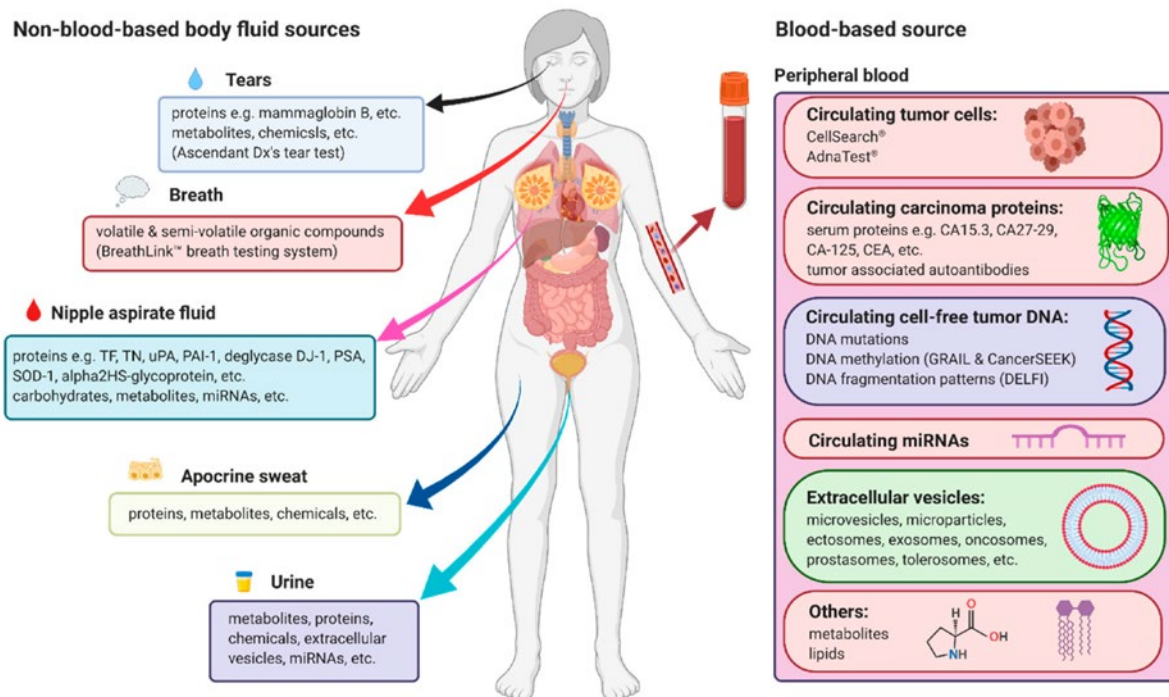
Figure 6.7: Non-invasive sampling of biomarkers that can be used for early detection of breast cancer in human samples. Reprinted with permission from MDPI[181].

6.2.2   THE DETECTION LIMIT OF NABLFA (SENSITIVITY)

The sensitivity or the lowest amount of PCR product that can be detected by the NABLFA was evaluated in the MCF7 PCR product at a concentration range from 0.006ng/ml to 1.2ng/ml. As shown in Figure 6.7, the NABLFA had a detection limit of 0.06ng/ml and 0.125ng/ml for *p53* and *PTEN,* respectively. The sensitivity of the NABLFA was considerably improved when compared to that of the conventional LFAs, which ranges between 0.1 µg/ml and 0.1 mg/ml. The system demonstrated a sensitivity that was similar to signal-amplified LFAs and ELISA at the ng/ml range within 15mins[182,183]. Of the tested genes, *p53* showed higher sensitivity compared to *PTEN*, the differences between the two might be due to the differences in sizes of the amplicons (*PTEN A*-806bp, *p53 B*-490bp). Different sizes of analytes were reported to migrate at different rates on the nitrocellulose membrane[184], and might not be a reflection of one gene overexpressed in tumour cancers than the other. Nonetheless, commercially available LFAs are recommended to have 87-97.5% sensitivity and the NABFLA demonstrated better performance and proved to be suitable for the detection of cancer nucleic acid

biomarkers in solution. The sample pre-amplification step improved the sensitivity of NABLFA which can be visually detected through the colour generated by the AuNPs without any additional readout devices.
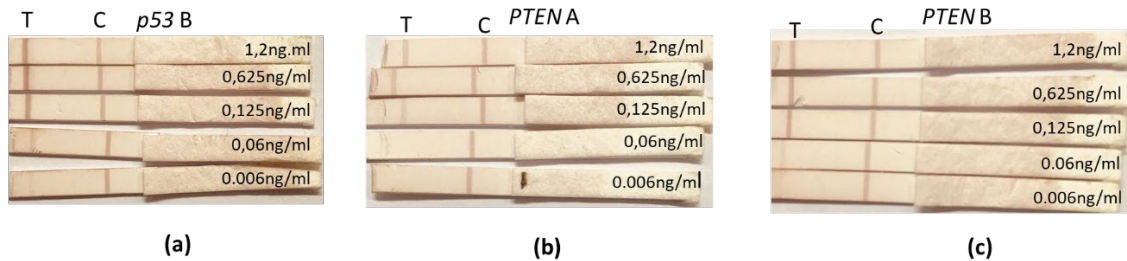
Figure 6.8: NABLFA showing detection limit test for all PCR products with different concentrations.

The NABLFA is compatible with the current screening or molecular diagnostic tests for disease biomarkers and presents a cost-effective system for LMICs. Thus, the NABLFA is of clinical value and displays features that are desirable for POC testing even in resource-limited settings. Although the NABLFA has the potential for improving testing accessibility and clinical outcomes, the technology is novel and has limitations, especially for the LICs. One major hurdle is that the NABLFA system still requires sample preparation and PCR amplification of the target genes before the samples can be applied on the LFA. Thus, POC testing at or near the place of patient care might not be possible within minutes and due to additional sample preparation steps might take hours instead. Moreover, the diagnostic system described here will require the use of a laboratory, PCR machine, and skilled personnel which further discredits the POC testing disclaimer[183].

The lack of clinical NABLFAs or LFAs for the diagnosis of cancer does not discredit their clinical value; in fact, they could tap into the same success and market as the LFAs for infectious diseases [185]and their convenience can encourage patients to do regular check-ups. The LFAs are currently being integrated for cancer diagnostics; this was brought to light by the collaborative effort between SCIENION (Berlin, Germany) and the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (North Rhine-Westphalia, Germany) to develop a bladder cancer LFA. The multiplex LFA will be used to detect ten bladder cancer biomarkers in urine samples at a POC[186] further validating the usefulness of these systems in LICs[187].

6.2.3 RESEARCH LIMITATIONS

The study aimed to develop an AuNPs-based NABLFA for the detection of breast cancer in LIC areas at POC. Although NABLFA has maintained a significant role in disease diagnosis, the systems used are not yet suitable for POC testing, since nucleic extraction and the amplification steps are crucial for successfully developing a NABLFA with robust sensitivity that is suitable for POC testing. This challenge can be overcome by using technologies that incorporate the extraction and amplification processes as a single step, which ultimately requires limited use of instrumentation. This is true of isothermal techniques such as the RT-LAMP, which require no thermo-cycler. Thus, RT-LAMP can be combined with an LFA[188]; LAMP is able to amplify DNA under isothermal conditions, providing the same specificity and sensitivity of conventional PCR[189,190].

Combining the LAMP and LFA technologies will incorporate all steps in one assay as shown in Figure 6.9, i.e. DNA isolation (from blood, saliva and urine samples), amplification and detection of nucleic acids by LFA at a POC[191]. LAMP is considered superior to the new generation of PCR technologies (such as continuous flow PCR, droplet PCR, digital PCR, ultrafast photonic PCR and insulated isothermal PCR)[192], and is better constructed for in-field use[193]. LAMP is inhibitor-tolerant, that is, amplification is not suppressed by biologically-borne inhibitors found in blood, urine and saliva, amplification can be readily achieved from unprocessed raw samples such as swabs and whole blood, prolonged storage time of reagents that have been preserved by lyophilisation[193,194]. The LAMP technology shown in Figure 6.9 was used to detect HIV from saliva samples, with the sensitivity of 10^5 HIV virions/ml[191].
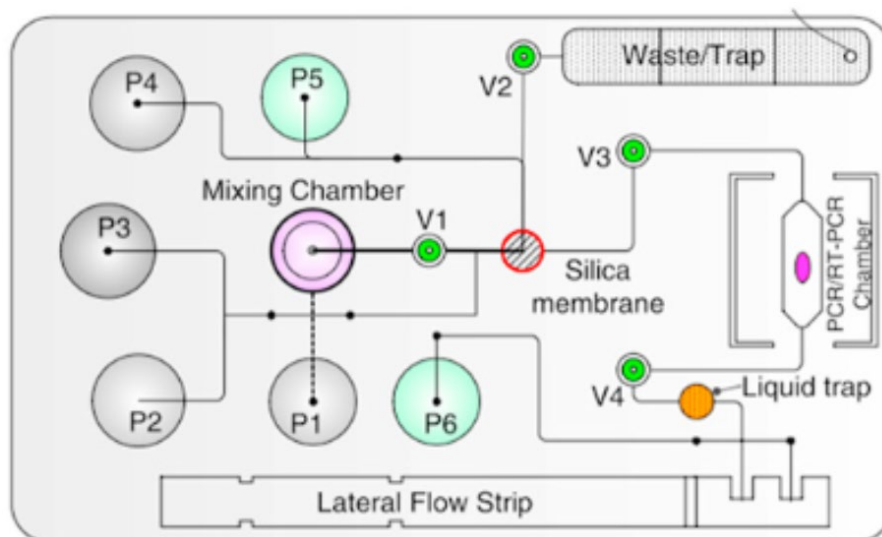
Figure 6.9: LAMP based microfluidic LFA for detection of nucleic acid markers at POC. Reprinted with permission[100],copyright American chemical society 2012.

The results presented in this thesis can be applied in developing a LAMP system making it possible to detect breast cancer biomarkers in remote areas. Successfully developing a screening tool for breast cancer and other cancers will definitely change survival and mortality rates. The procedure for diagnosis involves a lot of steps and results that take two to three weeks to obtain and months in some regions. This lengthy waiting period discourages the patients to return for their results as this will invariably involve a cost and management of numbers in government hospitals. The aforementioned reasons could be some of the reasons that women of African origin have the lowest registered cases of breast cancer[35]. Though contradictorily, the number of women diagnosed with breast cancer is now increasing due to behavioural and biological risk factors[23]. The sooner individuals know their status, the earlier therapeutic strategies can be implemented. Further studies are underway to identify breast cancer-specific biomarkers that can be non-invasively detected by the AuNPs-based NABLFA from body fluids[183,195].

CHAPTER 7   CONCLUSION AND FUTURE WORK

7.1   CONCLUSION

In the last few years, NABLFA has been adopted for the detection of nucleic acid markers with the naked eye. These devices are becoming more popular because they offer a simple analysis of analyte based on change of colour which results in rapid diagnosis of diseases[130]. Nucleic acid tests are essential in the diagnosis of genetic diseases, several NABLFAs have been successfully developed to detect DNA, mRNA, proteins, and other biological agents[120]. Developing these nucleic acid-based tests for fundamental research and clinical applications has become widely attractive because they offer simplicity, and are less time-consuming and labour intensive compared to conventional PCR methods[121,122]. The NABFLA follows the same principles as LFAs, except that, the nucleic-acid detection starts with the amplification of genes of interest using PCR and use of the PCR product (amplicons) as a test sample[108]. These systems could play an essential role in LIC areas, they are not only cost-effective but are also reliable and sensitive[196]. The NABLFA in this study served as a proof of concept that nucleic acid can be tested using LFA in liquid samples. LFA allows for the detection of circulating nucleic acid in various biological fluids, such as saliva, blood, urine, serum, and plasma

The proposed NABLFA was capable of detecting the *p53* and *PTEN* genes. The study demonstrated the detection of cancer genes using AuNPs-based NABLFA even though these genes were found to be expressed by other cancers such as ovarian, skin and brain cancer. The NABLFA developed does not limit detection to breast cancer but can also be applied to other cancers. The AuNPs were used as colorimetric labels and considered favourable nanomaterials to be used in LFAs because of their good mobility through nitrocellulose membrane and ease of surface functionalisation[197]. Colorimetric detection is also an integral part of NABLFA which offers a more straightforward option to detect or identify PCR products using the naked eye without additional equipment and skilled personnel[98]. The *In silico* and qPCR analysis proved that *p53* and *BRAC1* are highly expressed in breast cancer than in normal tissues whereas *PTEN* is more in normal breast tissues than in cancer. Therefore, the aforementioned genes (*p53 and BRAC1*) can be used as biomarkers for cancer diagnosis, and

can predict the prognosis [144]. The current study has shown that nucleic acid markers can be detected using an LFA offering an alternative method for screening and diagnosis of various diseases, including breast cancer in LICs. Despite the availability of various quantification approaches such as northern blot, DNA microarrays and qPCR these methods suffer from costly instrumental requirements and skilled technicians, which limit their usefulness in LIC. Therefore NABLFAs provide an easy approach in detecting biomarkers as a liquid biopsy since they can be isolated from a variety of body fluids (blood, urine and saliva)[104]. It is also well-known that there are some technical bottlenecks preventing the use of PCR-based methods at POC settings. To mitigate this problem other applications of PCR such as digital PCR, continuous flow PCR and ultrafast photonic PCR were developed[198,199]. However, these technologies are only suitable for analysis when the prior knowledge about the mutation is available and controlling their tempeture[200] to resolve the on-site detection issues, microfluidic-based (LAMP) techniques have been integrated with PCR.

7.2 FUTURE WORK

The discovery of nucleic acid circulating in different blood fluids such as urine, blood and saliva has opened opportunities to design and develop a diagnostic tool for a wide range of diseases including breast cancer. Nucleic acids offer additional advantages as a type of biomarker over antigens, antibodies, and metabolites. They are carriers of genetic information and can be secreted into the bloodstream in the form of circulating nucleic acids. They are detectable in small amounts in the sera of healthy individuals[80], which means elevated levels would suggest epigenetic alterations of a primary tumour[82].

In the current study the nucleic acid (DNA) was extracted from cells and tested in solution. The next step will be to test the AuNPs-based NABLFA in body fluids, especially blood. The team is busy procuring blood samples of patients with cancer from laboratories, companies and South African National blood service (SANBs). Successfully detecting these genes directly from blood will be a win for breast cancer patients. Furthermore, the AuNPs-based NABLFA will offer a less invasive, simple diagnostic test that does not require skilled personnel or a controlled environment to detect breast cancer and other cancers. The NABLFA market is

predicted to reach 12.6 billion USD in 2026, demonstrating the importance and uptake of these technologies by clinical practitioners[185]. Various companies have introduced various NABLFA for POC testing for cancer biomarkers[201].

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2014-05-15

Ref. Nr.: 2014/CAES/109

To:
Applicant: Prof Z Dlamini
Department of Life and Consumer Sciences
College of Agriculture and Environmental Sciences

Dear Prof Dlamini

Request for Ethical approval for the following research project:

Class application: Translational Genomics and Drug Discovery Research Group

The application for ethical clearance in respect of the above mentioned research has been reviewed by the Research Ethics Review Committee of the College of Agriculture and Environmental Sciences, Unisa. Ethics clearance for the above mentioned project (Ref. Nr.: 2014/CAES/109) is given as a class approval for the duration of the research project.

Please be advised that should any part of the research methodology change in any way as outlined in the Ethics application (Ref. Nr.: 2014/CAES/109), it is the responsibility of the researcher to inform the CAES Ethics Committee. In this instance a memo should be submitted to the Ethics Committee in which the changes are identified and fully explained.

The Ethics Committee wishes you all the best with this research undertaking.

Kind regards,

Prof E Kempen,
CAES Ethics Review Committee Chair

Prof MJ Linington
Executive Dean: College of Agriculture and Environmental Sciences

University of South Africa
Preller Street, Muckleneuk Ridge, City of Tshwane

111