Evaluation of laboratory based molecular technologies for the detection of human papillomavirus

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, School of Pathology, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

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Declaration

I, Nosipho Zwane, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine (Molecular Medicine and Haematology) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Nosipho Zwane

05th of October 2025 in Johannesburg, South Africa

Presentations arising from this study

Conference: MBRT Research Symposium

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Abstract

Human papillomaviruses (HPV) are associated with over 95% of cervical cancer (CC) incidence. Cytology screening using the Papanicolaou test (Pap smear) to detect potentially cancerous cells is a current 'gold standard' for identifying CC risk. There is growing evidence that molecular assays detecting high-risk HPV genotypes offer a relatively more sensitive and less invasive CC screening alternative to cytology. However, some of these available HPV DNA testing platforms have little to no published validation data and others have high costs per test. More analytical evaluations of HPV tests and optimization of front-end processing variables, e.g., nucleic acid extraction, are required to establish feasible workflows for high-throughput processing. Thus, this study sought to compare the performance of three extraction protocols on residual liquid-based cytology (LBC) and to evaluate singleplex and duplex laboratory developed tests (LDT) to determine an optimal workflow for HPV detection.

Spin column, heat lysis and magnetic bead-based extraction were performed on 46 residual LBC specimens and the extracted DNA for each was tested on singleplex HPV16 and HPV18 real-time PCR assays to determine the most suitable extraction method. After the best-performing extraction method was determined, it was applied to processing additional residual specimens that were tested on singleplex (n=61) and duplex PCR (n=175) assays targeting HPV genotypes 16, 18, 31, 35, 45, 52 and 58. The PCR results were compared to the BD Onclarity™ HPV assay.

Automated magnetic bead-based extraction had higher average agreement (92.4%) compared to spin column extraction (90.3%) and heat lysis (90.2%) across both HPV16 and HPV18. Singleplex PCR had 70.0 – 95.0% overall agreement across targeted genotypes while the duplex PCR had 93.6% overall agreement when compared to the BD Onclarity™ HPV assay. The duplex assay had good intra- (%CV less than 3%) and inter-run performance (%CV less the 7%), comparable to commercial tests.

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Finally, a special thank you to my family and friends. To my mom Portia Zwane and my sister Thandeka Zwane, your support and words of encouragement have meant the world to me, thank you.

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List of Abbreviations

°C	Celsius
%CV	Percentage coefficient of variation
AARMS	Academic Affairs and Research Management System
AGC	Atypical glandular cells
AIS	Adenocarcinoma in situ
ASC-H	Atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion
ASC-US	Atypical squamous cells of undetermined significance
BGLT3	Human beta globin locus transcript 3
CC	Cervical Cancer
CIN	Cervical intraepithelial neoplasia
Ct	Cycle threshold
CV	Coefficient of variation
Delta Rn	Change in fluorescence
DNA	Deoxyribonucleic acid
EIA	Enzyme immunoassay
FAM™	6-carboxyfluorescein
FDA	Food and Drug Administration
GLOBOCAN	Global Cancer Observatory
GP5+/GP6+	General primer 5 and 6
HC2	Hybrid capture 2
HPV	Human papillomavirus
HR-HPV	High-risk Human papillomavirus
HSIL	High-grade squamous intraepithelial lesions
LBC	Liquid-based cytology
LMICs	Low- and middle-income countries
LR-HPV	Low-risk Human papillomavirus
LSIL	Low-grade squamous intraepithelial lesions
MGB	Minor groove binder
µL	Microlitre

mL	Millilitre
mRNA	Messenger ribonucleic acid
NHLS	National Health Laboratory Service
nm	nanometer
nM	Nanomolar
NILM	Negative for intraepithelial lesion or malignancy
NFQ	Non-fluorescent quencher
NPA	Negative percentage agreement
Pap smear	Papanicolaou test
PCR	Polymerase chain reaction
PPA	Positive percentage agreement
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcription
s	Seconds
SSA	Sub-Saharan Africa
Tm	Melting temperature
UNG	Uracil-N-glycosylase
VALGENT	VALidation of HPV GENotyping tests
VIC™	Asymmetric xanthene dye
WHO	World Health Organization

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1. Introduction

1.1 HPV and cervical cancer

Human papillomaviruses (HPV) are non-enveloped, double-stranded deoxyribonucleic acid (DNA) viruses associated with 5% of human cancers (1) including cervical, vaginal, oropharyngeal, penile, vulvar and anal cancers. The virus is transmitted through mucosal or skin contact, manifesting as a papilloma, or benign outward-growing lump, with the potential of being a direct carcinogen in infected intraepithelial cells (1). In 2018, approximately 80% of HPV-attributable cancers were cervical cancer (2). Over 95% of cervical cancer incidence is linked to HPV, with the burden of mortality mainly in developing countries (3). Statistics reported by the World Health Organization (WHO) show that there were 660 000 new cases, and 350 000 deaths linked to cervical cancer in 2022, and incidence was highest in low- and middle-income countries (LMICs) in sub-Saharan Africa (SSA), Central America and South-East Asia (3). Figure 1.1 shows this pattern in the geographic distribution of estimates of age-standardised cervical cancer incidence rates using The Global Cancer Observatory (GLOBOCAN) 2022 database (4).

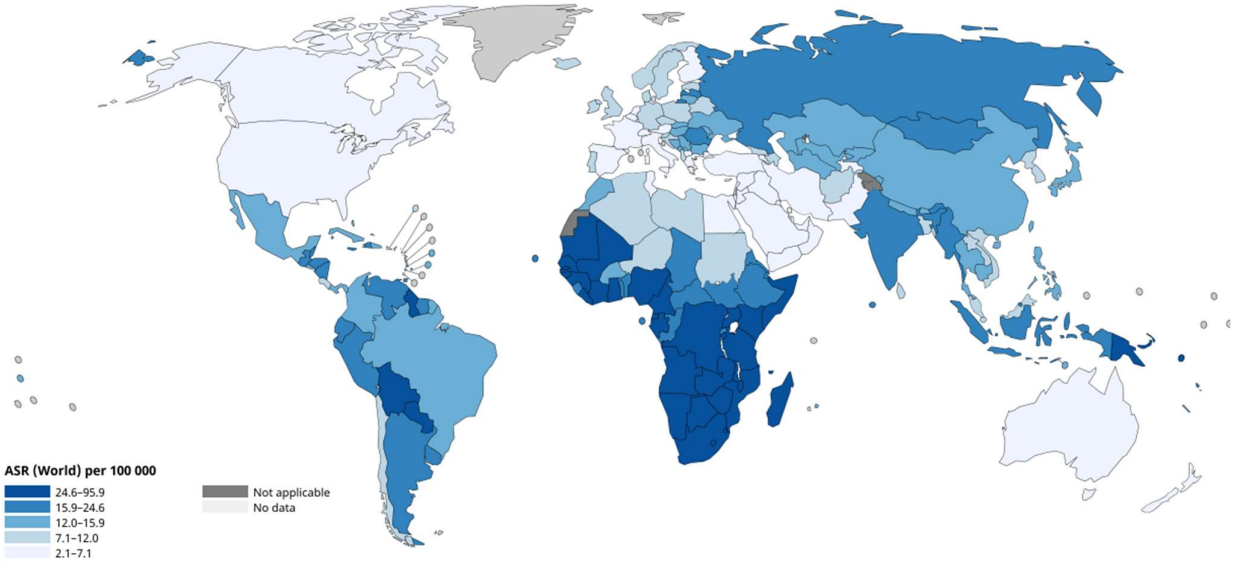

Figure 1.1: **Global estimates of age-standardised incidence rates for cervical cancer calculated per 100 000**. The incidence burden is concentrated in Africa, Asia and Latin America (Data and figure from Global Cancer Observatory 2022 database (4))

There are over 150 HPV types that have been identified and divided into 5 genera; *alphapapillomavirus*, *betapapillomavirus*, *gammapapillomavirus*, *mupapillomavirus* and *nupapillomavirus*. Pathogenic HPV types are part of the *alphapapillomavirus* genus (5). The alpha genus of HPV is further divided into species, each comprised of multiple genotypes. *Alphapapillomavirus 9* and *alphapapillomavirus 7* are the most common

viral causes of invasive CC. The species group *alphapapillomavirus 9* includes HPV16, 31, 33, 35, 52, 58 and 67 (6) and reportedly accounts for over 70% of cervical cancer globally while 15% of cervical cancer is linked to the species *alphapapillomavirus 7* (7) which includes the HPV18, 39, 45, 56, 59, 66, 68, 70 and 85 genotypes (6).

Additionally, the HPV types in the *alphapapillomavirus* genus are further distinguished into mucosal and cutaneous types based on viral tropism. The mucosal types are classified as either low-risk HPV (LR-HPV), which rarely causes disease, and high-risk HPV types (HR-HPV) (8), including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, which can cause cancer (9).

1.2 Local and global distribution of HPV genotypes

According to aggregated data on the global prevalence of HPV genotypes in invasive cervical cancer (CC), HPV16 and HPV18 are attributed to approximately three-quarters of CC across all regions (10). Although HPV16 is consistently the most prevalent in all regions globally (61.7%), the order of prevalence among other genotypes varies across different areas; notably, Africa has a relatively higher percentage of CC attributed to HPV35 compared to the rest of the world, accounting for 4-10% of cervical cancer cases compared to the global estimate of 2% (10–12). According to data for South Africa reported by the ICO/IARC Information Centre in 2023, HPV16 was the most commonly detected HPV genotype in cases of high-grade cervical lesions and cervical cancer (24.5% and 50.7% respectively), but HPV52 (17.5%) was the most predominant in low-grade cervical lesions (13).

A review of local data from research conducted across five provinces in South Africa between 1989 and 2021 indicated that the HPV16 genotype had the highest prevalence (8.80%) in the general population, followed by HPV35 (4.86%), HPV18 (4.14%), HPV58 (3.65%) and HPV52 (3.62%) (14). Stratification of the study population also showed HPV16 (6.60%) as the most prevalent genotype in women with abnormal Pap smear results, followed by HPV genotypes 35 (2.43%), 18 (2.24%), 58 (1.70%), 33 (1.62%), 45 (1.57%) and 52 (1.52%). In women with normal cytology, the main genotypes were HPV16 (2.03%), 35 (1.85%), 58 (1.58%), 45 (1.55%), 18 (1.40%), 52 (1.30%) and 68 (1.19%). In a similar study at a tertiary hospital in Gauteng, the prevalence of high-risk HPV DNA was reported to be 48.1% and highest in women < 30 years old (15).

1.3 WHO targets for cervical cancer elimination

The WHO has developed strategic targets to eliminate cervical cancer by setting the *90-70-90* goals to have "90% of girls fully vaccinated with HPV vaccine by age 15 years, 70% of women screened with a high-performance test by 35 years of age and again by 45 years of age, and 90% of women identified with cervical disease receive treatment" (16). To achieve this, more efficient methods of screening need to be well established to ensure greater screening coverage. Developing, evaluating and validating molecular testing for the detection of HPV, including HPV DNA and ribonucleic acid (RNA) testing platforms, has been identified

as pivotal to improving routine screening (17). Furthermore, genotyping tests can provide useful data on context-specific distribution and prevalence of HPV types and allow for surveillance on whether vaccine coverage of prevalent HPV types is sustained (18). For primary prevention, three Food and Drug Administration (FDA)-licensed vaccines are currently available for immunisation against HPV - Gardasil (quadrivalent vaccine) which covers HPV6, 11, 16, and 18, Gardasil-9 (nonavalent vaccine) which protects against HPV6, 11, 16, 18, 31, 33, 45, 52, and 58, and Cervarix (bi-valent vaccine) which provides coverage for HPV16 and 18 only. Cervarix is used in South Africa's HPV vaccination programme targeting adolescent girls 9 years old and older and has been transitioned from a two-dose to a single-dose schedule as of 2024, following recommendation by the WHO (19).

Given the importance of high-performance screening tests as part of the WHO's cervical cancer elimination target, this study focused on molecular diagnostic platforms including HPV DNA testing and genotyping. An overview of the current literature on the scope of available tests for screening including molecular testing and its performance relative to the current gold standard, cytology, is given in section 1.4.

1.4 Literature review

1.4.1 Cervical Cancer Screening and Diagnostic Tests

Screening for pre-cancerous cervical cells or HPV infection with HR genotypes is critical for the early detection and treatment or prevention of cervical cancer in otherwise asymptomatic women. Screening methods include cytology and, more recently, HPV DNA testing. South African guidelines for cervical cancer screening highlight both the Papanicolaou test (Pap smear) and HPV testing as suitable for screening but note that resource-limited settings might not yet be able to integrate sophisticated molecular systems (20). Following screening, triage of high-risk positive cases involves follow-up with diagnostic testing through colposcopy and/or cervical biopsy. Colposcopy involves the use of a lighted, magnifying instrument, the colposcope, to visually examine the cervix for possible abnormalities, and a cervical biopsy is a procedure where a specimen of cervical tissue or cells is removed for microscopic examination (21). Screening offers an opportunity to triage those at highest risk towards treatment and prevents unnecessary use of these invasive procedures and over-treatment while allowing early pre-cancer detection.

1.4.2 Cytology

The Pap smear is a screening procedure where cells are collected from the cervix using a cytobrush. In traditional cytology testing, the cells collected on the brush are smeared directly onto a slide for microscopic evaluation to identify dysplastic or abnormal cells. More recently, liquid-based cytology (LBC) specimens, where collected cells are suspended in preservative media, have been utilised and evaluated using staining methods in the laboratory. The Pap smear is the current gold standard for primary cervical cancer screening.

Results from the Pap smear are reported according to the Bethesda system which categorises abnormal results as either atypical squamous cells of undetermined significance (ASC-US), atypical glandular cells (AGC), low-grade squamous intraepithelial lesions (LSIL), atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H), high-grade squamous intraepithelial lesions (HSIL), adenocarcinoma in situ (AIS) or cervical cancer cell (squamous cell carcinoma or adenocarcinoma) (22).

The identification of abnormal cells triages patients for follow-up testing with colposcopy and cervical biopsy where biopsy results will indicate the degree of cervical intraepithelial neoplasia (CIN) – CIN 1, CIN2 or CIN3. A grading of CIN1+ indicates dysplasia of the epithelium, CIN2+ refers to abnormal cells observed at the basal two-thirds of the cervical lining, and CIN3+ indicates the presence of precancerous cells in greater than two-thirds of cervical lining thickness (23). The limitations of the Pap smear as a primary screening method include the invasiveness of specimen collection, the need for a trained health-care worker for collection, poor uptake of screening in low and middle-income areas, low reproducibility and sensitivity, and a lack of suitably skilled cytologists and suitable equipment in LMICs (24–26).

1.4.3 Molecular testing for HPV

Molecular assays for the detection of HPV are increasingly being investigated and integrated into primary screening programmes as an alternative to cytology. In addition, genotyping tests that give type-specific infection results have become key additions to a growing network of evidence highlighting the efficiency of molecular testing to improve outcomes related to HPV infection. A comparative study of three primary testing strategies; cytology, HPV molecular testing, or cytology and HPV co-testing, reported that for CIN2+, primary HPV molecular testing and co-testing with both cytology and molecular testing had higher sensitivity (95.9% and 98.0% respectively) compared to primary cytology's 48.0% (27). In addition, evidence from a 12-year retrospective study found that CIN2+ detection rates increased (0.2% to 0.49%) with the introduction of co-testing compared periods of cytology testing alone (28).

There are different molecular techniques applied to detecting HPV: signal amplification assays including hybrid capture 2 (HC2) systems (Qiagen, Hilden, Germany), and nucleic acid amplification assays e.g. real-time polymerase chain reaction (PCR) and genome sequencing (29) are the most prominent. In signal amplification, immobilised nucleic acid probes, e.g. RNA probes, target specific DNA sequences. RNA and DNA binding forms hybrids that can be identified through the capture of the hybrid by specific antibodies and chemiluminescent solution whose light emission (Figure 1.2) is recorded as a positive result for the presence of HPV in the specimens (30).

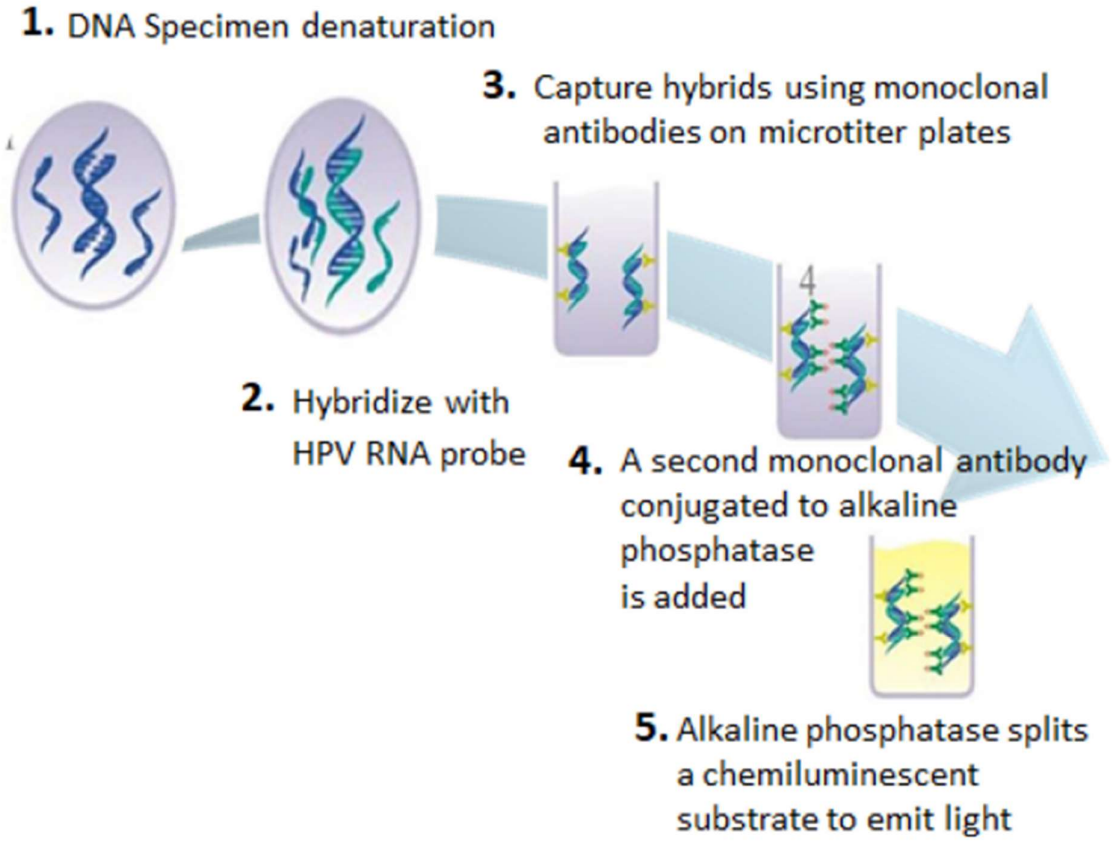

Figure 1.2: **Five-step procedure in signal amplification assays** (figure from Parmin *et al.* (31))

Nucleic acid amplification assays are PCR-based methods that detect target genes through annealing and extension of primers complementary to a region of interest, generating millions of copies of the gene. In real-time PCR systems using TaqMan probe technology (Figure 1.3), fluorescently labelled probes emit light when cleaved during each cycle, where the amount of fluorescence emitted is proportionate to the increasing number of copies amplified (32). Commercial nucleic acid amplification assays detecting HPV include the PapilloCheck® (Greiner Bio-One, Kremsmünster, Austria), Abbott RealTime High-Risk HPV assay (Abbott, Abbott Park, Illinois, U.S.A.), cobas® 4800 HPV Test (Roche, Basel, Switzerland), Xpert® HPV (Cepheid, Sunnyvale, California, USA), Allplex™ HPV HR Detection (Seegene, Seoul, South Korea), BD Onclarity™ HPV assay (Becton Dickinson, Franklin Lakes, New Jersey, USA) and the Alinity m HR HPV (Abbott, Abbott Park, Illinois, USA).

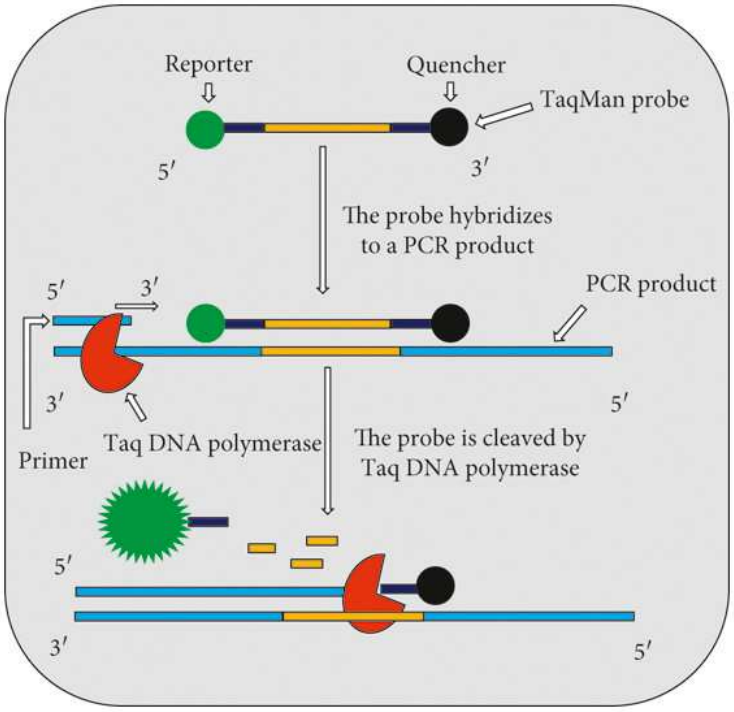

Figure 1.3: **Schematic of nucleic acid amplification using TaqMan probe technology** (figure from Reta *et al.*(33))

At the end of 2023, there were at least 264 distinct commercial HPV molecular tests (34). It has been previously reported that close to 90% of developed HPV assays have not been evaluated with consensus guidelines (35) and 82% have no published evaluation or validations (36). This shortcoming leaves a gap in data on the clinical performance of a vast number of HPV test methods. To enable more standardised methods for HPV testing, the VALidation of HPV GENotyping tests (VALGENT) framework has reported validation of several novel genotyping assays. Validated tests within this network include GP5+/6+ PCR-based enzyme immunoassay (EIA) kit HPV GP HR, BD Onclarity HPV assay and PapilloCheck® HPV-screening test, following testing of specimen panels and comparison with a known validated method (37). Other tests which have met standard validation criteria (35) include HC2, Abbott RealTime HPV assay, Alinity m HR HPV, Anyplex HPV HR, cobas® 4800 HPV test, PapilloCheck®, and Xpert® HPV (38).

According to international guidelines, assays being validated should produce 90% sensitivity and 98% specificity for ≥ CIN 2 when tested against a standard comparator test (35). For example, the Alinity m HR HPV assay, targeting 14 HR-HPV genotypes in a multiplex reaction, has a sensitivity of 98.4% for CIN2+ and 95.1% for CIN3+ and a specificity of 91.1% when compared against HC2 (39). A comparative evaluation of the cobas® 4800 HPV assay and the Linear Array HPV genotyping test (Roche, Basel, Switzerland) as a reference showed total agreement was 94.7% between the two tests (40). The Xpert® HPV assay has also

shown good concordance results when compared to the validated cobas® 4800 HPV and Qiagen HC2 tests (95.8% and 94.1% agreement, respectively) (41). The Xpert® HPV assay has the advantage of being a point-of-care test, as it can be operated outside a laboratory setting, as seen in a demonstration study that applied the assay to a single-visit screen-and-treat algorithm (42). Additionally, the test has a rapid turn-around time of 60 minutes (43). The Xpert® HPV assay has a reported sensitivity of 91.5% and 92.3% in self-collected and clinician-collected specimens respectively when cytology and histology were used as reference (44). In contrast, careHPV™ (Qiagen), a point-of-care test based on signal amplification technology, has sensitivity of 75.6% in self-collected specimens and 86.4% for clinician-collected specimens of CIN2+ lesions with cytology as a reference (44).

More recently, Bellosillo *et al.* (2024) reported on the performance of the automated multiplex real-time PCR, Vitro HPV Screening assay (Vitro S. A., Sevilla, Spain) using current guidelines for test validation for cervical cancer screening. The assay, targeting the L1 GP5+/GP6+ region and detecting 14 HR-HPV genotypes, had clinical sensitivity and specificity non-inferior to the validated comparator, cobas® 4800 HPV test. For specimens with reported CIN2+, the assay had 100% agreement with the reference test for HPV16 and 18 (45).

The Allplex assay, which builds on the technology of the antecedent Anyplex assay by providing distinction between 14 HR-HPV genotypes in a relatively shorter turn-around-time, has also recently been evaluated and found to meet clinical validation standards including non-inferior performance relative to a comparator test (HC2). The assay had relative clinical sensitivity and specificity (1.01 and 1.02 respectively) well above the required minimum values for both parameters (46).

In-house assays have also been evaluated as potential testing methods. Lee *et al.* (47) developed a multiplex PCR targeting 15 HPV types and found an agreement rate of 91.9% (kappa = 0.84) between the multiplex assay and the validated HC2 methods. Another reported in-house PCR using consensus primers GP5+/GP6+ had substantial agreement with HC2 (Cohen's kappa value 0.691, 95%CI: 0.664 - 0.718) and higher sensitivity (100% v. 73% for HC2) that was especially evident in its higher HPV detection rates in LBC specimens with low-grade cytological abnormality compared to HC2 (48).

In addition to clinical performance comparable to currently validated tests, emerging HPV molecular assays are evaluated under criteria of inter- and intra-laboratory reproducibility. For example, the AmpFire HPV Screening 16/18/HR assay (Atila Biosystems, Mountain View, CA, USA) has been shown to produce 96.4% and 95.3% reproducibility for intra-laboratory and inter-laboratory testing respectively (49), results that

recommend the test for addition into the existing list of assays validated on current internation consensus standards.

Currently, the major challenge in development and implementation of HPV testing platforms is establishing an efficient balance between clinical specificity and sensitivity, especially when reporting genotype results. Tests that report individual genotypes allow stratification of screen-positive patients according to risk based on genotype oncogenicity (50). Conversely, some evidence shows that limiting the number of HPV genotypes reported to a selected number (especially based on prevalence data) can increase clinical specificity although negatively affecting sensitivity (51). Another key challenge in fully establishing molecular testing for HPV as a primary screening tool is the complexity of test methodology and infrastructure requirements. In addition, there is a high cost-per-test for many HPV DNA assays, which is an especially significant obstacle for LMICs (52).

1.4.4 Specimen types for HPV testing

Validation of LBC specimens in molecular testing has been critical to development of testing algorithms that include HPV assays and testing workflows that will allow use of residual specimens from cytology testing in molecular platforms, thus improving efficiency through one specimen used for multiple tests. BD SurePath™ (Becton Dickinson) and ThinPrep® PreservCyt® Solution (Hologic, Marlborough, Massachusetts, USA) are common liquid-based media for cell enrichment and preservation of cervical cells for use in Pap testing. When compared, SurePath-collected and ThinPrep®-collected specimens were shown to have 93.8% overall HR-HPV concordance when paired specimens were evaluated on the cobas® 4800, with SurePath specimens producing lower Ct values and fewer false negative results (53). Furthermore, the direct application of LBC specimens to HPV testing has proven efficient by having low test invalidity rate (0.05− 0.10 %) across different specimen collection media and endogenous controls (54).

Studies on HPV assays have mainly made use of clinician-collected cervical cell specimens - routinely collected for cytological testing - for clinically evaluating molecular platforms. To improve the algorithm for testing and increase screening uptake of primary cervical cancer screening, less invasive specimen types may be more effective. In a study population of HIV-positive women in Zambia, testing for HPV on first-void urine on the Xpert® HPV had a sensitivity and specificity of 84.8% and 92.3%, respectively, and 89.80% agreement with parallel cervical specimens (55). The current challenges with testing first-void urine include achieving an adequate HPV DNA concentration and determining the ideal initial specimen volume to be collected (56).

Additionally, self-sampling using vaginal swabs has also been investigated as an alternative specimen type. Feng *et al*. (57) reported sensitivity, specificity and accuracy of 89.8%, 98.2%, and 96.3% respectively in self-

collected vaginal swabs tested using real-time PCR. Self-sampling offers the potential to reach previously missed populations in primary screening and HPV detection (58). Self-collected urine and vaginal swabs have reported concordance rates of 90.6% (k = 0.792) and 95.5% (k = 0.898) respectively for HR-HPV when compared to clinician-collected cervical specimens. In addition, high relative sensitivity to cervical specimens for CIN2+ detection has been observed for both self-collected vaginal swabs (1.00) and urine (0.95) (59).

1.4.5 Nucleic acid extraction

In addition to good quality specimens, the clinical performance of HPV testing methods relies on efficient DNA extraction and acceptable DNA quality. Variations in DNA processing methodologies, including specimen input volume for extraction, use of manual or automated extraction, and the amount of DNA used as template, can influence the performance of an assay and the results produced. Assays such as the cobas® 4800 HPV assay and Xpert® HPV use specimen-to-result processing and include nucleic acid extraction within their platforms (i.e. extraction, PCR and result reporting are conducted in a single system). Other assays focus only on the PCR, with a variety of acceptable front-end processing solutions, usually requiring more hands-on processing steps.

Comparing manual and automated extraction, a study reported that the AmpliLute Liquid Media Extraction kit (manual extraction) had higher specificity, sensitivity, positive predictive value and negative predictive value than the MagNa Pure system (automated extraction) (73.2%, 69.15%, 79.43% and 61.32% v. 67.32%, 67.02%, 76.87% and 55.75%, respectively) when compared against cytology results (60). A report on the AmpliLute (manual), QIAamp DNA Blood mini kit (manual) and NucliSENS EasyMAG (automated) found 55% HPV type-specific concordance across the three extraction methods when evaluated on the Linear Array and 75% concordance between the tested extracts on the INNO-LiPA (61). Regarding volume, Dunn *et al.* (62) reported that the larger cervical cell specimen input volume (1 mL) for extraction using the DNA Blood Mini Kit (Qiagen) produced better signal intensity compared to 250µL in the QIAamp® MinElute™ Media Kit (Qiagen) protocol when tested on the Linear Array HPV test.

Crude extraction using Proteinase K digestion has also been reported as effective on LBC specimens. An evaluation of extraction efficacy comparing the Proteinase K method to the commercial QIAmp (Qiagen) for extraction from LBC specimens with known low-grade cytological abnormalities, found both methods to have yielded similar proportions on valid specimens (96.8% and 96.4% respectively) when extraction was confirmed by PCR testing of human housekeeping gene, beta globin (63). Recently, a ‘non-extraction’ method using PharmaDirect (Pharmaline, Ankara, Türkiye), a pre-denaturation solution added directly to the PCR reaction mix allowing for direct PCR testing without prior DNA extraction, showed 11.4% HPV positivity

comparable to 11.9% obtained on cervical swab specimens extracted using Molgen PurePrep Pathogen DGX (Molgen BV, Veenendaal, The Netherlands) (64). This non-extraction method had 88.1% sensitivity and 99.0% specificity when compared to nucleic acid extraction (64). Evidently, the need for evaluation of testing platforms for HPV is still present, including the optimization of variables such as nucleic acid extraction and specimen type.

This study adds value to the current landscape of molecular diagnostic testing for HPV by presenting a cost-effective testing workflow for detection and genotyping of HR-HPV types. Development of an in-house assay with an efficient front-end processing method has the potential application to improving screening in lower-income areas and providing coverage of genotypes that match current prevalence.

1.5 Aim and Objectives

1.5.1 Aim

The aim of this study was to optimise and evaluate three nucleic acid extraction methods and two molecular assays for detection of HPV for use as standard of care, compared against a validated assay as a reference method.

1.5.2 Objectives

a) To compare the performance of spin column, heat lysis and magnetic bead-based extraction methods on singleplex HPV16 and HPV18 real-time PCR and determine an optimal extraction protocol.
b) To optimize a singleplex and duplex real-time PCR assays for detection of seven clinically relevant HR-HPV genotypes.
c) To determine concordance/correlation between the multiplex real-time PCR assay and the reference test.

2. Ethical considerations

The study was approved by the Human Research Ethics Committee (Medical) at the University of the Witwatersrand, Johannesburg (M230649). In addition, the project was registered with Academic Affairs and Research Management System (AARMS) under Prof. Pamela Michelow for the use of residual specimens from the National Health Laboratory Service (NHLS) (PR2346610).

3. Methods and Materials

3.1 Specimen selection

Residual LBC specimens in ThinPrep® (Hologic, Bedford, MA, USA) were collected from the Department of Cytology, NHLS, Braamfontein between October 2023 to March 2024, and tested using the BD Onclarity™ HPV Assay (Becton, Dickinson, New Jersey, USA). A total subset of 175 specimens was pre-selected based on their result from this assay and positive specimens were chosen to include all HPV genotypes targeted in the study. The sample set consisted of 56% positivity for HPV and, from reference cytology results, 49/175 of the specimens were HSIL, 58/175 were reported as LSIL and 68/175 were negative for intraepithelial lesion or malignancy (NILM). All identifying information was removed and each specimen was anonymized. Testing was blinded at each phase until the test was completed, and results were then made available once comparative analysis was initiated. Specimen blinding was performed by the supervisor through providing assigned specimen lists based on cytology results and reference results from the BD Onclarity™ HPV Assay, which were only provided to the student after each testing phase was completed. Specimen aliquots were transferred into sterile tubes (two 2 millilitres (mL) aliquots per specimen) and stored at 4 degrees Celsius (°C) until downstream testing was performed.

3.2 Extraction and PCR testing overview

An optimal extraction method was determined by first comparing the performance of three protocols used to extract DNA from a subset of clinical specimens that were positive for HPV16 and 18 by BD Onclarity™ HPV Assay. Extracted DNA was then tested on a real-time PCR assay to detect HPV16, 18 and an internal control gene for human beta globin locus transcript 3 (BGLT3) in singleplex reactions (as detailed in section 3.6.1 below), and results were compared against the BD Onclarity™ HPV results. The testing overview is shown in Figure 3.1. The best-performing extraction protocol was selected for downstream evaluation of an extended singleplex PCR assay targeting additional high-risk HPV genotypes (HPV31, 35, 45, 52 and 58) followed by a multiplex assay covering the same targets (Figure 3.2).

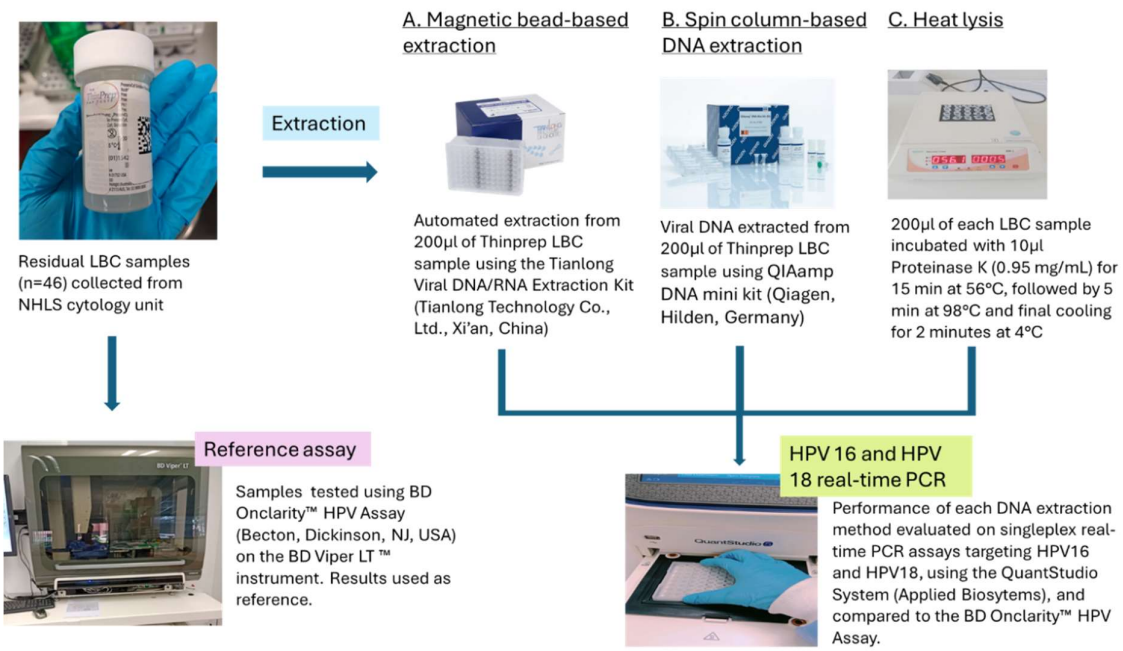

Figure 3.1: **Workflow for extraction and PCR testing**

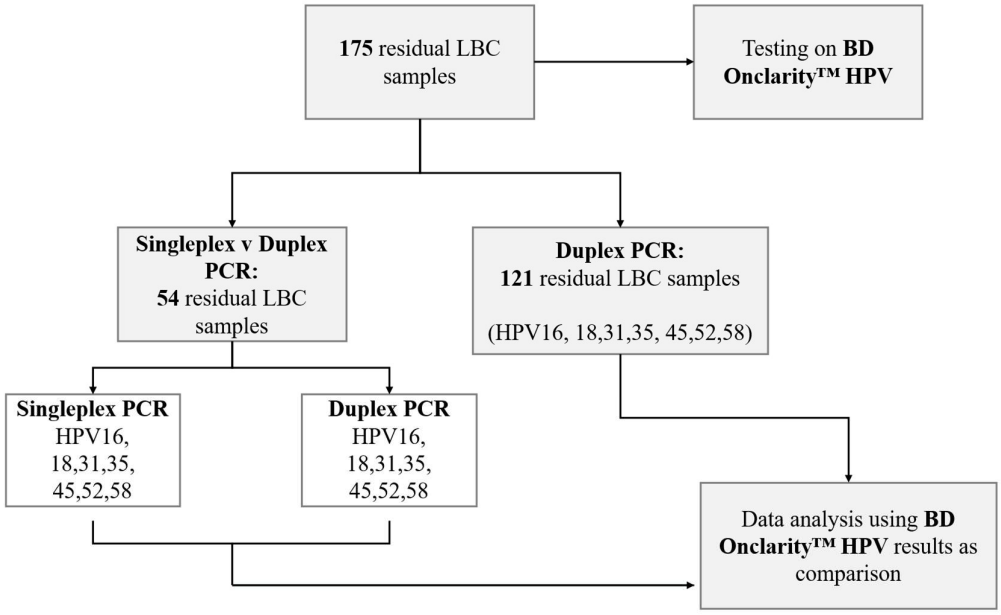

Figure 3.2: Singleplex and duplex PCR testing method. A single extraction method was selected for further application into singleplex and duplex PCR testing workflows. A subset of specimens (n=61) was tested to compare singleplex and duplex PCR, followed by PCR testing of all remaining residual LBC specimens (n=114) on the optimised duplex assay.

3.3 Reference testing: BD Onclarity™ HPV Assay on the BD Viper™ LT system

The BD Onclarity™ HPV Assay was used as a 'reference' test for HPV results. The assay is an FDA-approved HPV genotyping test that screens for 14 HR-HPV types and reports human beta-globin as an internal control (65). It detects HPV using real-time PCR and determines specimen positivity based on a pre-set cycle threshold (Ct). All specimens (n=175) were tested using the assay on the BD Viper™ LT system (Becton, Dickinson, New Jersey, USA). Before testing, LBCs were brought to room temperature and mixed by vortexing. For each specimen, 500 microlitres (µL) was transferred into a BD Onclarity™ HPV LBC Diluent Tube and mixed briefly. Specimens were placed on a BD Viper™ LT Specimen Rack and processed with one positive and negative control in each batch. Prior to use, the lyophilized BD Onclarity™ HPV negative Control and Positive Control were rehydrated by transferring diluent reagent from diluent tubes into the control tubes, followed by inversion to homogenise the control solution. After the pre-processing steps, the specimen rack containing specimens and controls was placed on the pre-warm heater of the BD Viper™ instrument for 60-minutes to ensure homogeneity of the specimen matrix. Following the pre-warm step, the specimen rack was placed into the instrument for automated extraction and PCR. The assay reported individual genotype results for HPV16, 18, 31, 45, 51 and 52, and pooled results for HPV33/58 (pool 1), HPV 56/59/66 (pool 2) and HPV35/39/68 (pool 3).

3.4 Analysis of pooled results on Allplex™ HPV28 Detection

Allplex™ HPV28 Detection (Seegene) results of a portion of specimens that were positive on channels reporting pooled results on the BD Onclarity™ HPV Assay were available for further analysis to distinguish individual genotypes. Testing was done by other laboratory personnel at the Wits Diagnostic Innovation Hub (WITS DIH). Briefly, the Allplex™ HPV28 Detection is a multiplex real time PCR assay that can detect 19 HR-HPV types and 9 LR-HPV types - with all targets reported individually (i.e. no pooled reporting). Using the Seegene STARlet-AIOS™ system, magnetic bead-based nucleic acid extraction and PCR amplification were performed in a single fully automated process (66).

3.5 DNA extraction method comparison

Three methods for DNA extraction were evaluated in the study: spin column, heat lysis, and magnetic bead-based extraction. Forty-six (n=46) specimens were selected for extraction with specimen order predetermined during blinding to ensure each run contained known negative and positive specimens by the BD Onclarity. The number of specimens extracted was determined by the available testing wells on the extraction plate for magnetic bead-based extraction which accommodates 16 specimens per plate. Due to the inclusion of two positive controls in the extraction process, only 46 residual specimens could be extracted as 3 plates were used in the procedure (16x3=48). For accurate parallel comparison of each method, this sample size was therefore

maintained for all three extraction methods. Three separate 200µL aliquots of each specimen were prepared in sterile Eppendorf tubes and a single aliquot was used for each method as shown in Figure 3.1. To validate extraction performance, the ACCURUN® 381 HPV Type 16 and 18 messenger ribonucleic acid (mRNA) Positive Controls (SeraCare, Milford, MA, USA) were also extracted on each method. The positive controls consisted of human cells in a buffered methanol solution, with HPV16 and 18 mRNA, transcribed from the E6 and E7 viral genes, integrated into the cell genome (67). Extraction and PCR runs were considered valid if positive controls were amplified.

3.5.1 Magnetic bead-based DNA extraction

Magnetic bead-based DNA extraction uses magnetic beads coated with a polymer with high affinity for DNA binding that are used to separate nucleic acids from the lysate during extraction (68). Isolation of the DNA after cell disruption is achieved by aggregating the DNA-bound magnetic beads by applying a magnetic field. In this study, the Tianlong Viral DNA/RNA Extraction Kit, T014H (Tianlong Technology Co., Ltd., Xi'an, China), an automated magnetic-bead kit, which comprises of a 96 deep well plate pre-filled with reagent and magnetic beads, was used. The automated extraction process involved cell lysis, binding of DNA to magnetic beads and, using specialised magnetic rods, adsorption and transfer of magnetic beads into reagent wells for two successive wash steps and elution of DNA. Prior to extraction, the plate was gently inverted to resuspend the magnetic beads. Each of the LBC specimens was brought to room temperature and an aliquot of 200µL was added to the relevant well of the pre-filled reagent column of the plate. The extraction plate was then loaded onto the Libex Nucleic Acid Extractor (Tianlong Technology Co., Ltd., Xi'an, China) with 16 specimens on each extraction plate. The final elution volume after extraction was 80µL.

3.5.2 Spin column DNA extraction

Extraction of LBCs using spin column-based DNA extraction was also tested. For this evaluation, the QIAamp DNA mini kit (Qiagen) was used to extract DNA from the selected specimens. The DNA Purification from Blood or Body Fluids Protocol (Spin Protocol) was followed for processing (69). An initial lysis or digestion of cell proteins was performed by applying Proteinase K to the specimen. For each specimen, 20µL of Proteinase K was added to a 1.5mL microcentrifuge tube. An aliquot of 200µL of LBC specimen was then added to the tube, followed by 200µL of lysis buffer - Buffer AL. The mixture was homogenised by pulse-vortexing for 15s and incubated at 56°C, using an HB-1 block heater (Wealtec, New Taipei City, Taiwan), for 10 minutes for cell lysis, followed by a brief centrifugation step to remove residual drops from inside the lid. To precipitate the DNA in solution, 200µL of 100% ethanol was added and mixed by pulse-vortexing for 15 seconds (s). The specimens were then briefly centrifuged and added into a QIAamp Mini spin column attached to a 2mL collection tube and centrifuged at 8000 revolutions per minute (rpm) for one minute to bind the DNA

to the silica membrane of the spin column and filter out proteins and contaminants as flow-through. The resulting filtrate in the collection tube was discarded and the spin column placed in a clean collection tube. For DNA purification and removal of residual contaminants, 500µL of wash buffer AW1 was added to the spin column followed by centrifugation at 8000 rpm for one minute. After centrifuging, the filtrate was discarded, and the spin column was placed onto a new collection tube for a second wash step using 500µL of wash buffer AW2 buffer followed by another centrifugation step at 14 000 rpm for three minutes. The spin column was transferred to a 1.5mL microcentrifuge tube and 200µL of elution buffer (Buffer AE) was added to remove DNA from the silica membrane and facilitate its elution into solution. A final centrifugation at 8000 rpm for one minute was performed to elute the DNA into the microcentrifuge tube. DNA was stored at -20°C prior to real-time PCR testing.

3.5.3 Heat lysis

Specimens were also extracted using a heat lysis method coupled with crude Proteinase K digestion as previously described by Chu *et. al.* (2020), but with some modifications: the ratio of Proteinase K to the specimen was 1:20 instead of 1:5 and the initial incubation step was at 55°C rather than 56°C. These changes were made in line with the manufacturer instructions for the Thermo Scientific Proteinase K (Thermo Scientific, Waltham, MA, USA) which recommended a concentration of 0.05-1mg/mL and outlined that optimum enzyme activity was between 50-55°C. Proteinase K is a proteolytic enzyme that digests cellular proteins, including those found in the cell membrane, allowing release of DNA from cells. Heat lysis provides thermal shock to further facilitate disruption of the cell membrane. For each specimen, 10µL of Proteinase K was added to a 2mL microcentrifuge tube at a final concentration of 0.95mg/mL and mixed with 200µL of the LBC specimen to form a homogenous solution. The tubes were then incubated for 15 minutes at 55°C, then 98°C for five minutes on the HB-1 block heater, followed by a final cooling for two minutes at 4°C by refrigeration (70). After extraction, specimens were stored at -20°C until downstream PCR was performed. Prior to the PCR, specimens were centrifuged at 900 rpm for one minute

3.6 Real-time PCR

Real-time PCR is a molecular technique that detects target regions on nucleic acids using gene-specific primers and probes that anneal and allow amplification of the nucleic acid and an increase in the number of copies during cycles of denaturation, annealing and extension. The data on change or increase in copy number is recorded in real-time at the end of each cycle. The increase in copy number is detected in real-time due to the emission of a detectable fluorescence signal emitted by the probe when it is bound to an amplified gene of interest. For this study, TaqMan® Gene Expression Assays (Applied Biosystems, Waltham, MA, USA) were used for detecting HPV genotypes and the endogenous internal control, BGLT3. Each of the type-specific and

endogenous control assays target protein-coding regions of the DNA through "predesigned and preformulated" primer and probe sets (71). Forward and reverse primers in the assay anneal to complementary target sequences of the DNA during PCR and the TaqMan® minor groove binder (MGB) probe anneals at a site between the two primers. TaqMan® MGB probes were formulated by the manufacturer to include a reporter dye, FAM™ (6-carboxyfluorescein) or VIC™ (asymmetric xanthene dye), at the 5′ end of the probe, a non-fluorescent quencher (NFQ) dye at the 3′ end, and MGB, that increases the melting temperature (Tm), at the 3′ end of the probe. FAM™ and VIC™ have emission wavelengths of 517 nm and 551 nm respectively (72). The reporter dyes were selected on the basis of potential scaling and application of the test onto alternative systems that only detect FAM™ and VIC™. Cleaving of the annealed probe during amplification of the target DNA removes the reporter dye from the probe and the quencher dye allowing it to produce detectable fluorescence, a signal for a positive specimen (71).

3.6.1 Testing of extracted eluates on singleplex HPV16 and 18 real-time PCR

A PCR reaction mix with 5µL of TaqPath™ 1-Step RT qPCR Master Mix (Applied Biosystems), 1µl TaqMan® Gene Expression Assay (HPV 16, 18 or BGLT3), 12µL of nuclease-free water and 2µL of extracted DNA was prepared for each specimen (total reaction volume = 20µL). The assay IDs for the targets were Vi07921925_s1 for HPV16, Vi07921926_s1 for HPV18 and Hs04406765_s1 for BGLT3 (Applied Biosystems). Final concentrations for the primer and probes in each reaction were 900 nanomolar (nM) and 250nM respectively. The PCR thermal cycling parameters used are shown in Table 3.1 and were set as per manufacturer instructions. The PCR was performed on the QuantStudio 5™ and QuantStudio™ 7 Flex Real-time PCR Systems (Applied Biosystems).

Table 3.1: PCR Protocol

Step	Cycles	Temperature	Time
UNG incubation	1	25°C	2 minutes
RT incubation	1	50°C	15 minutes
Enzyme activation	1	95°C	2 minutes
Amplification	40	95°C	3 seconds
		60°C	30 seconds

UNG = Uracil-N-glycosylase

RT = Reverse Transcription

Specimens were considered ‘HPV positive’ if both the tested HPV genotype and BGLT3 were detected or if only the HPV genotype was detected. Specimens were reported ‘negative’ for HPV if only the BGLT3 was amplified. Results with no amplification for both HPV and the internal control were considered ‘invalid’ and the specimens were re-tested on the PCR. This was aligned with the reporting criteria on the BD Onclarity assay (65). In addition, results where software analysis could not accurately report the target as either positive or negative were defined as ‘inconclusive’.

3.6.2 Data analysis of real-time PCR results from extraction method comparison

PCR data were analysed using the QuantStudio™ Design and Analysis 2 software (Applied Biosystems, Waltham, MA, USA) and compiled using Microsoft Excel (Microsoft, Redmond, WA, United States). PCR results of each extraction method were compared against parallel results reported by BD Onclarity™ HPV Assay. Method comparison included calculation of percentage similarity of results and Cohen's kappa statistic (k). The kappa value was interpreted as: (1) <0.20 = poor, (2) 0.21 - 0.40 = fair, (3) 0.41 – 0.60 = moderate, (4) 0.61 – 0.80 = good, and (5) 0.81 – 1.00 = very good (73). Additionally, positive percentage agreement (PPA) and negative percentage agreement (NPA) were determined. PPA was referred to as the proportion of positive results that were also positive on the comparator test (BD Onclarity™ HPV Assay) and NPA was calculated as the percentage of negative results that were also reported negative by the comparator test. According to FDA statistical guidance, PPA and NPA are determined instead of sensitivity and specificity, respectively, when a test is compared against a non-reference standard (74). PCR performance in this study was compared to BD Onclarity™ HPV Assay rather than to clinical diagnosis, and thus NPA and PPA values were used. All statistical analysis was performed using STATA version 18 (StataCorp, College Station, TX, USA). The best performing extraction-PCR workflow was selected for testing of a larger sample set with extended genotype targets.

3.6.3 Singleplex real-time PCR testing for HPV16, 18, 45, 35 31, 52 and 58 and BGLT3 (n=61)

Singleplex testing for a larger pool of targets (HPV 16,18, 45, 35 31, 52 and 58 and BGLT3) (Applied Biosystems) was performed on 61 LBC specimens using the selected extraction-PCR workflow. The expanded set of genotypes was selected based on the current most prevalent HPV genotypes in South Africa (13).

Ten positive specimens per HPV target genotype were chosen for testing, including LBC specimens with co-infection which were tested on more than one target. In addition, a set of 10 HPV negative specimens were tested on each target. Master mix preparation and PCR protocol setup for each target were performed as described in section 3.6.1.

3.6.4 Evaluation of duplex PCR HPV16, 18, 45, 35 31, 52 and 58 and BGLT3 (n=61)

Multiplex PCR allows for two or more target DNA sequences to be detected simultaneously through nucleic acid amplification. Target-specific primer pairs and fluorescently labelled probes are included in one reaction, providing more rapid detection of multiple targets in comparison to singleplex testing. For this study, duplex PCR testing (two targets per reaction) was performed, and target combinations were tested as outlined in Table 3.2. The assignment of HPV target combinations was determined by the type of reporter dye used for the genotype-specific probe. Target specific primer-probe sets labelled with FAM™ were combined with those labelled with the VIC™ reporter dye in a single reaction mix as shown in Table 3.2.

Table 3.2: Duplex PCR target combinations

	Targets
Reaction mix 1	HPV16 (FAM™), HPV58 (VIC™)
Reaction mix 2	HPV18 (FAM™), HPV52 (VIC™)
Reaction mix 3	HPV45 (FAM™), HPV31 (VIC™)
Reaction mix 4	HPV35 (FAM™), BGLT3 (VIC™)

First, the same subset of specimens (n=61) tested in 3.6.3 were evaluated on this multiplex assay to establish its performance relative to the singleplex assay. Once feasibility of the duplex was confirmed, an additional 121 residual specimens were tested for (total specimens = 175) for a larger scale evaluation.

LBC specimens were processed using the chosen extraction method and a PCR reaction mix including 5µL of TaqPath™ 1-Step RT qPCR Master Mix, 1µL TaqMan® Gene Expression Assay for target 1 and target 2 as per manufacturer recommendations, 11µL of nuclease-free water and 2µL of extracted DNA was set up for each specimen. The thermal cycling protocol, run data and statistical analysis, including method comparison parameters, also followed the procedure outlined in section 3.6.1.

3.6.5 Data analysis for singleplex and duplex assays

Analysis of PCR data was performed as described in section 3.6.2. Outcomes for singleplex and duplex assays were compared against results reported on the BD Onclarity™. Method comparison included calculation of percentage similarity of results and Cohen's kappa statistic and included: (1) Singleplex v. BD Onclarity™, (2) singleplex v. duplex and (3) duplex v. BD Onclarity™. In addition, PPA and NPA were calculated for the

final duplex PCR against the BD Onclarity™ (as the 'standard'), and additional Allplex™ HPV28 Detection results for distinguishing individual genotypes from pooled results, using STATA version 18.

3.6.6 Determining inter- and intra-run variation

In order to determine reproducibility of the assay, inter-run variation of positive HPV control specimens tested across eight different PCR runs was evaluated. Positive controls for HPV16 and 18 were from the AccuTrak™ HPV Genotype Qualification Panel QSH701 (SeraCare), while five confirmed HPV positive residual LBC specimens were used as positive controls for the other genotypes (HPV31, 35, 45, 52 and 58). To evaluate variation for the endogenous control gene, a negative control included in the AccuTrak™ HPV Genotype Qualification Panel QSH701, which consisted of cultured HPV negative human cells, was tested. Ct values from each control for all runs were used to calculate percentage coefficient of variation (%CV) and standard deviation (SD).

Repeatability was also evaluated for the duplex PCR by calculating intra-run variation. Seventeen residual clinical LBC specimens were tested in triplicate in one PCR run following initial testing on the duplex PCR. The specimens were selected to cover the full range Ct values reported on the BD Onclarity assay. Ct values <20 were defined as 'low', 20 – 30 as 'medium' and Ct >30 was defined as 'high' based on an internal laboratory protocol. The Ct values for each replicate were recorded and %CV and SD were calculated to show differences in Ct.

4. Results

4.1 DNA extraction method comparison

Magnetic bead-based DNA extraction, spin-column DNA extraction and heat lysis were performed on 46 residual LBC specimens, and the eluates were tested on real-time PCR targeting HPV16 and HPV18 in singleplex reactions using pre-designed commercial primers and probes. When compared against the BD Onclarity™ HPV Assay, PCR results from all extraction methods had high NPA, while PPA was more variable (Table 4.1). The average NPA across both PCR targets was 91.7%, 96.5% and 98.2% for magnetic bead-based DNA extraction, spin-column DNA extraction and heat lysis, respectively. Automated magnetic bead-based extraction had higher average PPA (92.9%) compared to spin column extraction (85.0%) and heat lysis (80.2%) across both HPV 16 and HPV18. Magnetic bead-based extraction was the most sensitive for HPV16 (PPA = 91.3%), while sensitivity for HPV18 was highest in spin column-based DNA extraction (PPA = 100%). Notably, extraction by heat lysis was more sensitive for the HPV16 target (PPA = 82.6%) compared to spin column-based extraction (PPA = 69.9%).

Overall agreement (Cohen's kappa (k)) in PCR results from spin-column extracted DNA was k=0.70 (good) for HPV16 and k=0.91 (very good) for HPV 18. For heat lysis, overall agreement was 0.83(very good) and 0.76 (good) for HPV16 and 18, respectively while automated magnetic bead-based extraction showed overall agreement of 0.78 (good) and 0.91 (very good) for HPV 16 and 18, respectively. Thus, the average agreement was also highest for automated magnetic bead-based extraction (k=0.85; very good) compared to spin-column DNA extraction (k=0.80; good) and heat lysis (k=0.80; good). Based on results from this analysis, automated magnetic bead-based extraction using the Tianlong Viral DNA/RNA Extraction Kit, T014H, was found to be the best performing extraction method and was used as the front-end processing method for HPV detection in the remaining study objectives.

Table 4.1: **Performance of extraction methods on HPV16 and HPV 18 real-time PCR assays (compared to BD Onclarity™ HPV assay)**

	Magnetic bead-based extraction			Spin column-based DNA extraction			Heat lysis		
	PPA, % (95%CI)	NPA, % (95%CI)	Overall Agreement% (95%CI)	PPA, % (95%CI)	NPA, % (95%CI)	Overall Agreement% (95%CI)	PPA, % (95%CI)	NPA, % (95%CI)	Overall Agreement% (95%CI)
HPV 16	91.3 (72.0 – 98.9)	87.0 (66.4 – 97.2)	89.1 (77.0 - 95.3)	69.9 (47.1 - 86.8)	100.0 (85.2 - 100.0)	84.8 (71.8 - 92.4)	82.6 (61.2 – 95.0)	100.0 (82.5 - 100.0)	91.3 (79.7 – 96.6)
HPV 18	94.4 (72.7 – 99.9)	96.4 (81.7 – 99.9)	95.7 (85.5 – 98.8)	100 (81.5 –100.0)	92.9 (76.5 – 99.1)	95.7 (85.5 – 98.8)	77.8 (52.4 – 93.6)	96.4 (81.7 – 99.9)	89.1 (77.0 – 95.3)
Average	92.9	91.7	92.4	85.0	96.5	90.3	80.2	98.2	90.2

4.2 Analytical performance diagnostic agreement: singleplex real-time PCR and BD Onclarity™ HPV assay

Using magnetic bead-based extraction, a second subset of specimens was extracted for extended singleplex real-time PCR testing targeting seven HR-HPV genotypes: HPV16, HPV18, HPV31, HPV35, HPV45, HPV52 and HPV58. For each target 10 HPV positive (confirmed by BD Onclarity™ HPV) and 10 negative specimens were tested. Overall, 51 HPV positive specimens were evaluated, including specimens with co-infections such that 15 specimens were tested on more than one singleplex assay, and 36 were only tested for one HR-HPV target. In addition, 10 specimens with expected HPV-negative results for all genotypes were tested across all the singleplex assays. The results were compared against BD Onclarity™ HPV assay.

When compared to the BD Onclarity™ HPV assay, the singleplex assays showed a range of 70.0 to 95.0% for overall agreement across targeted genotypes. Full results for agreement for singleplex and BD Onclarity™ HPV assay are shown on Table 4.2. Overall agreement was highest for HPV18, 31 and 52 (95%) and lowest for HPV35 (75%) and 58 (70%).

Table 4.2: **Agreement between singleplex real-time PCR and BD Onclarity™ HPV assay (n=61)**

*Singleplex PCR		*BD Onclarity™ HPV assay		Singleplex PCR v. BD Onclarity™ HPV assay	
		Positive	Negative	Overall Agreement, % (95%CI)	kappa (k)
HPV16	Positive	7	0	85.0 (64.0 – 94.8)	0.70 (0.40 to 1.00)
	Negative	3	10		
HPV18	Positive	9	0	95.0 (76.4 - 99.1)	0.90 (0.71 to 1.09)
	Negative	1	10		
HPV31	Positive	9	0	95.0 (76.4 - 99.1)	0.90 (0.71 to 1.09)
	Negative	1	10		
HPV35	Positive	5	0	75.0 (53.1 - 88.8)	0.50 (0.17 – 0.83)
	Negative	5	10		
HPV45	Positive	7	0	85.0 (64.0 - 94.8)	0.70 (0.40 – 1.00)
	Negative	3	10		
HPV52	Positive	9	0	95.0 (76.4 - 99.1)	0.90 (0.71 – 1.09)
	Negative	1	10		
HPV58	Positive	4	0	70.0 (48.1 - 85.5)	0.40 (0.08 -0.72)
	Negative	6	10		

*Individual genotype results shown as 2x2 tables for Singleplex PCR and BD Onclarity™ HPV assay

4.3 Analytical performance diagnostic agreement: singleplex real-time PCR and duplex real-time PCR

Once the utility of an extended singleplex PCR was determined, duplex combinations of HR-HPV targets were tested on the same set of specimens to establish a multiplex assay covering the same genotypes as the singleplex assay. The duplex assay was assessed against singleplex PCR to determine whether the results would be comparable. The multiplex PCR reactions' genotype combinations were performed as outlined in Table 3.2. Of the 10 negative BD Onclarity-confirmed specimens, one had a valid result (positive for internal control) on the singleplex assay but was negative for the human beta globin control (invalid) on the duplex assay, even after repeat testing, and excluded from singleplex versus duplex analysis. Furthermore, one HPV16 BD Onclarity-confirmed positive specimen was excluded from the evaluation of agreement for HPV16 detection due to an 'inconclusive' result on the duplex assay. This gave an error rate of 3.7% for this analysis. The duplex PCR had 100.0% (95%CI 83.2 - 100.0) agreement with the singleplex results for 4/7 HPV targets

- HPV31, 45, 52 and 58. The remaining genotypes had 'almost perfect' agreement (k = 0.89 - 0.90) with the exception of HPV35 which showed moderate agreement (k = 0.56) (Table 4.3). These results showed that detection outcomes similar to singleplex PCR could be achieved with an assay detecting more than one genotype in a single reaction.

Table 4.3: Agreement between singleplex and duplex real-time PCR (n=61)

	Singleplex real time PCR		Duplex real-time PCR		Singleplex PCR v. Duplex PCR	
			Positive	Negative	Overall Agreement, % (95%CI)	kappa (k)
Mix 1	**HPV16**	Positive	7	0	94.4 (74.2 - 99.0)	0.89 (0.67 to 1.10)
		Negative	1	10		
	HPV58	Positive	4	0	100.0 (83.2 to 100.0)	1.00 (1.00 -1.00)
		Negative	0	15		
Mix 2	**HPV18**	Positive	9	0	94.7 (75.4 - 99.1)	0.90 (0.70 to 1.09)
		Negative	1	9		
	HPV52	Positive	9	0	100.0 (83.2 - 100.0)	1.00 (1.00 - 1.00)
		Negative	0	10		
Mix 3	**HPV31**	Positive	9	0	100.0 (83.2 - 100.0)	1.00 (1.00 - 1.00)
		Negative	0	10		
	HPV45	Positive	7	0	100.0 (83.2 to 100.0)	1.00 (1.00 -1.00)
		Negative	0	12		
Mix 4 (paired with BGTL3 primers)	**HPV35**	Positive	3	2	84.2 (62.4 - 94.5)	0.56 (0.13 to 1.00)
		Negative	1	13		

4.4 Analytical performance diagnostic agreement: duplex real-time PCR and BD Onclarity™ HPV assay for n=61

Concordance between duplex PCR and BD Onclarity for this subset of specimens was similar to that reported in the singleplex assay. HPV16 and 18 had higher positive agreement with BD Onclarity (94.4% v. 85% and 95% v. 100%, respectively) compared to agreement with the singleplex assay. For HPV16, this increase in agreement may be attributed to exclusion of the 'inconclusive' specimen and subsequent decrease in sample

size for analysis. Notably, agreement was lower for HPV31, 35, 45, 52 and 58 (Table 4.4) on the duplex assay (in contrast to parallel comparison for singleplex v. BD Onclarity).

Table 4.4: **Agreement between duplex real-time PCR and BD Onclarity™ HPV assay (n=61)**

Duplex real time PCR		BD Onclarity™ HPV assay		Duplex PCR v. BD Onclarity™ HPV	
		Positive	Negative	Overall Agreement, % (95%CI)	kappa (k)
HPV16	Positive	8	0	94.4 (74.2 - 99.0)	0.89 (0.69 to 1.0)
	Negative	1	9		
HPV18	Positive	10	0	100.0 (83.2 - 100.0)	1.00 (1.00 – 1.00)
	Negative	0	9		
HPV31	Positive	9	0	94.7 (75.4 - 99.1)	0.90 (0.70 – 1.09)
	Negative	1	9		
HPV35	Positive	4	0	68.4 (46.0 - 84.6)	0.39 (0.07 – 0.71)
	Negative	6	9		
HPV45	Positive	7	0	84.2 (62.4 – 94.5)	0.69 (0.38 - 1.00)
	Negative	3	9		
HPV52	Positive	9	0	94.7 (75.4 - 99.1)	0.90 (0.70 – 1.09)
	Negative	1	9		
HPV58	Positive	4	0	68.4 (46.0 - 84.6)	0.39 (0.07 – 0.71)
	Negative	6	9		

4.5 Duplex real-time PCR analytical performance for final assay (n=175)

Ultimately, one hundred and seventy-five LBC specimens were tested on the duplex real-time PCR assay. The assay consisted of three reactions each targeting two HR-HPV genotypes: (1) = HPV16 and 58, (2) = HPV18 and 52, (3) = HPV31 and 45, and one reaction mix detecting HPV35 and BGLT3 (internal control gene) as shown in Table 3.2. Each specimen was tested on all four reactions (seven HPV targets and human beta globin internal control). Of the 175 specimens tested, four gave a negative result for the internal control gene and were re-tested. Following the repeat PCR, 3/4 were positive for human beta globin and considered valid, while one specimen remained invalid (negative for internal control) and was therefore excluded from the data

analysis. Across the valid specimen results, Ct values for BGLT3 amplification ranged from 23.874 to 34.131. Figure 4.1 shows amplification of BGLT3 in DNA extracted from 20 specimens within the sample set.

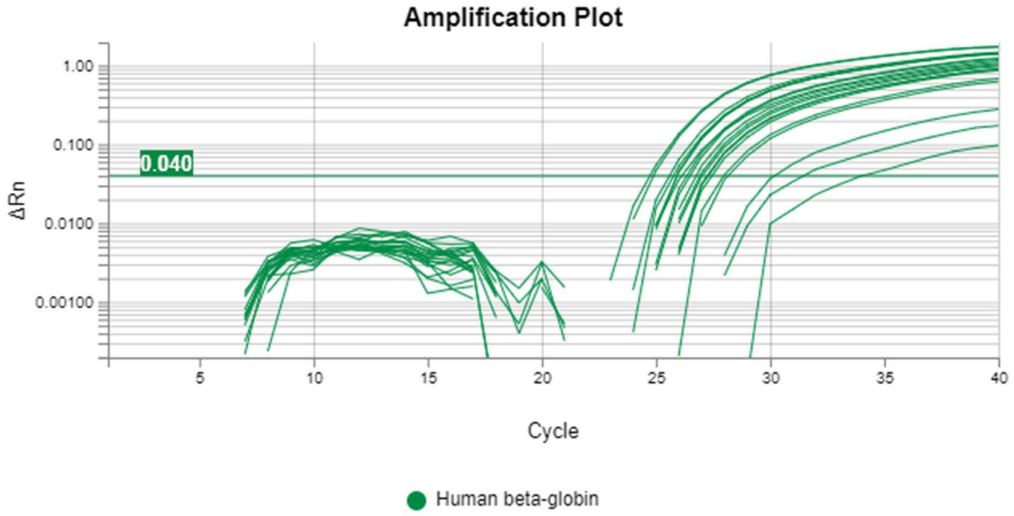

Figure 4.1: Amplification curves for BGLT3 in 20 specimens tested on the duplex PCR assay.

Human beta-globin = BGLT3, Delta Rn = change in fluorescence

4.6 Duplex PCR PPA, NPA and agreement for reporting of HPV

An evaluation of overall HPV results was determined by classifying specimens as 'HPV negative' (negative for all HPV genotype targets) or 'HPV positive' (amplification for ≥ 1 tested HPV genotype(s)). From the 174 valid PCR results, three specimens were negative for 6/7 and inconclusive for 1/7 of the HPV genotypes, were deemed inconclusive for HPV positivity and were not included in the analysis for overall reporting of HPV. Thus, combining the total number of invalid specimens (one) and inconclusive specimens (three) gave a total error of 2.3% (4/175) for overall reporting of HPV in the sample set, and a final data set of 171 specimens.

The duplex PCR reported 90/171 (52.6%) specimens as HPV positive and 81/171 (47.4%) negative for HPV. From the subset of specimens that tested positive for HPV, 40/90 (44.4%) had multiple HPV infections i.e. positive for more than one genotype (Figure 4.2). Infection with two HR-HPV genotypes was detected in 29/40 (72.5%) HPV positive specimens, and 10/40 (25%) specimens had DNA from three HPV genotypes. Only one specimen (2.5%) had co-infection with four HPV genotypes (Figure 4.3). In comparison, the BD Onclarity™ HPV assay detected HPV in 98/175 (56%) specimens and reported 77/175 (44%) as negative. In addition, multiple HPV infections were confirmed in 67/98 (68.4%).

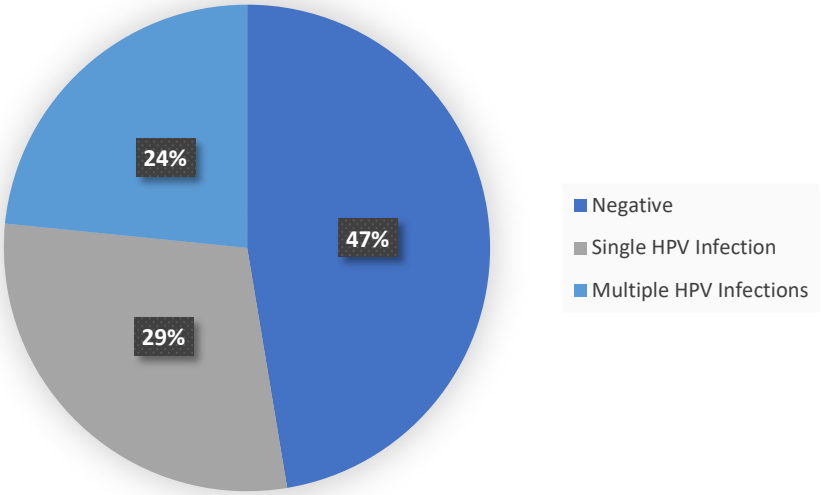

Figure 4.2: **Distribution of overall HPV results reported by duplex PCR (n=171)**

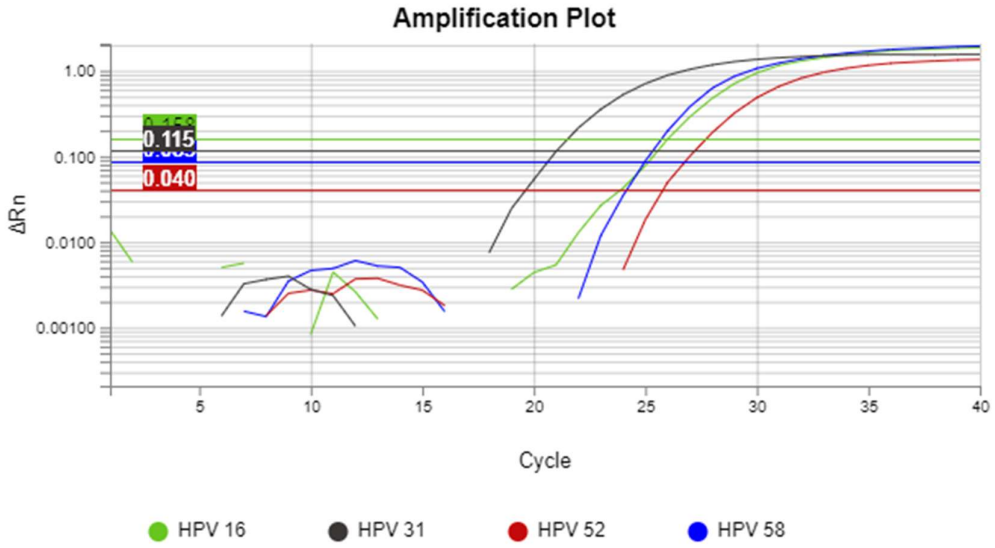

Figure 4.3: **Co-infection with HPV16, 31, 52 and 58 in a single LBC sample tested on the duplex real-time PCR assay**

The most common genotype in the sample set was HPV16 which was amplified in 42.2% (38/90) of the positive specimens, followed by HPV18 which was present in 30% (27/90) of the positive LBC specimens. HPV52 and HPV58 were detected in 24.2% (22/90) and 22.2% (20/90) of HPV positive results respectively. The least reported genotypes were HPV31, HPV45 and HPV35 at 16.7% (15/90), 12.2% (11/90) and 10% (9/90) respectively (Figure 4.4). When grouped by cytology, 38/90 (42.2%) HPV positives specimens were from HSIL, 33/90 (36.7%) were LSIL and 19/90 (21.1%) were categorized as NILM. HPV16 was also the

most frequently detected genotype across HSIL 20/38 (52.6%) and NILM 8/19 (42.1%), while HPV18 was the most frequent genotype detected in LSIL 12/33 (36.4%) (Figure 4.5).

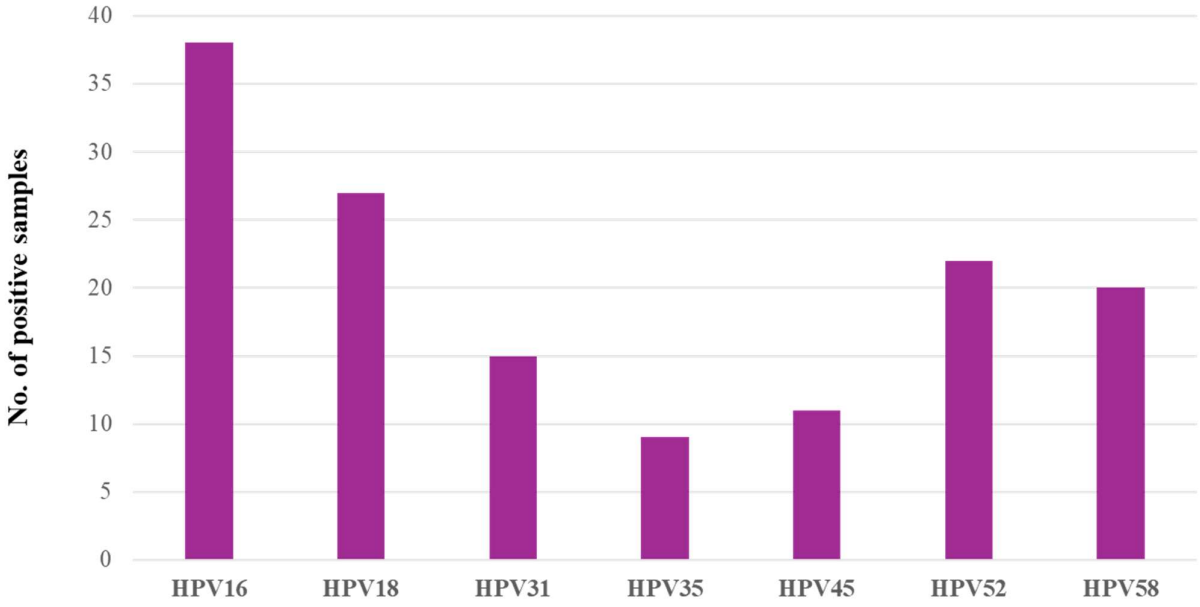

Figure 4.4: **HPV positivity stratified by individual genotype on duplex PCR**

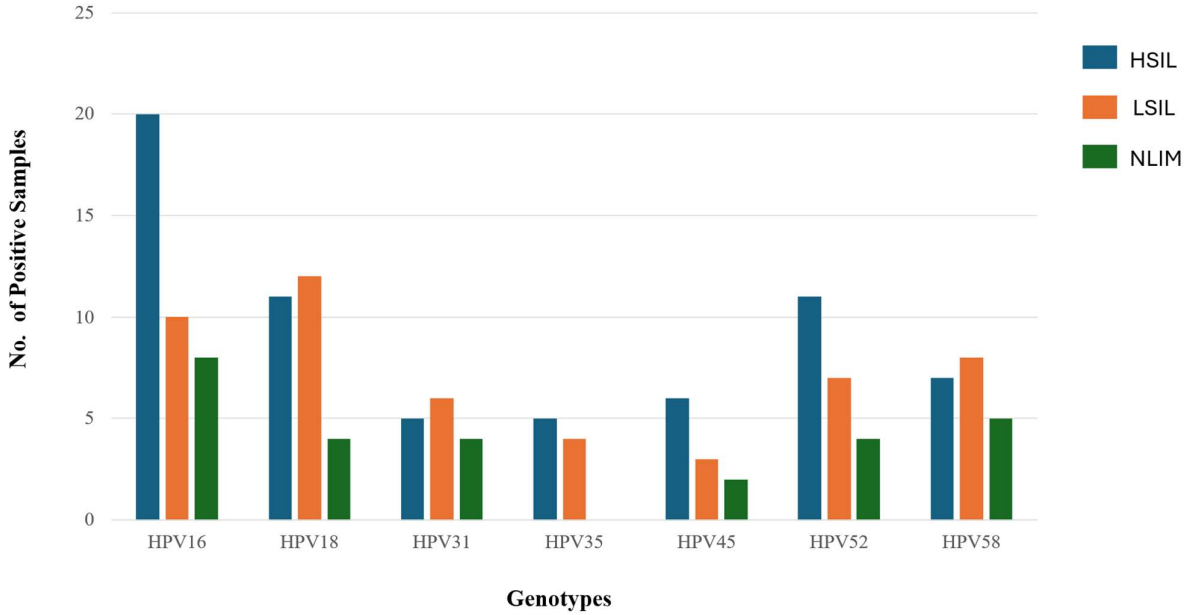

Figure 4.5: **Duplex PCR results stratified by SIL status**

Analytical performance of final duplex assay (n=174) in comparison to the BD Onclarity™ HPV assay is outlined in Table 4.5. There was good agreement between the duplex PCR and the BD Onclarity™ HPV for reporting of overall HPV (any HPV genotype) with 93.6% (95%CI 88.8 - 96.4) overall agreement and a kappa value of 0.87. The duplex assay also had strong analytical performance in terms of PPA (90.7% (95%CI: 83.1 - 95.7)) and NPA (97.3% (95%CI: 90.6 - 99.7)) for 'any HPV genotype'.

Table 4.5: **Agreement between duplex real-time PCR and BD Onclarity™ HPV (n=174)**

Duplex PCR		**BD Onclarity™ HPV assay**		**Duplex PCR v. BD Onclarity™ HPV**			
		Positive	**Negative**	**Overall Agreement, % (95%CI)**	**kappa, k (95%CI)**	**PPA, % (95%CI)**	**NPA, % (95%CI)**
Any HPV genotype	Positive	88	2	93.6 (88.8 - 96.4)	0.87 (0.80 - 0.94)	90.7 (83.1 - 95.7)	97.3 (90.6 - 99.7)
	Negative	9	72				
HPV16	Positive	36	2	93.0 (88.2 - 96.0)	0.81 (0.71 - 0.91)	78.3 (63.6 - 89.1)	98.4 (94.4 - 99.8)
	Negative	10	124				
HPV18	Positive	25	2	97.1 (93.4 - 98.8)	0.89 (0.80 - 0.99)	89.3 (71.8 - 97.7)	98.6 (95.1 - 99.8)
	Negative	3	142				
HPV31	Positive	15	0	99.4 (96.8 - 99.9)	0.96 (0.90 - 1.03)	93.8 (69.8 - 99.8)	100.0 (97.7 - 100.0)
	Negative	1	158				
***HPV35**	Positive	9	0	86.8 (80.9 - 91.0)	0.40 (0.21 - 0.57)	28.1% (13.7 - 46.7)	100.0 (97.4 - 100.0)
	Negative	23	142				
HPV45	Positive	11	0	97.1 (93.5 - 98.8)	0.80 (0.63 - 0.97)	68.8 (41.3 - 89.0)	100.0 (97.7 - 100.0)
	Negative	5	158				
HPV52	Positive	20	2	97.7 (94.2 - 99.1)	0.90 (0.80 - 1.0)	90.9 (70.8 - 98.9)	98.7 (95.3 - 99.8)
	Negative	2	150				
***HPV58**	Positive	18	2	89.5 (84.1 - 93.3)	0.61 (0.45 - 0.77)	52.9 (35.1 - 70.2)	98.6 (94.9 - 99.8)
	Negative	16	136				

*PPA for HPV35 and HPV58 were the initial values calculated prior to distinguishing individual genotypes from the pooled results of the BD Onclarity™ HPV assay.

Analysis of genotype-specific performance showed that PPA was highest for HPV31 (93.8%) and considerably lower for HPV35 (28.1%) and HPV58 (52.9%) as shown in Table 4.5. Due to the BD Onclarity™ HPV reporting HPV35 and 58 in pooled channels that also detect other genotypes, further confirmation using Allplex™ HPV28 Detection results, which report all its targets as individual genotypes, was done to determine true discordance. For HPV35, 23 specimens had discordant results where the specimen was positive on the BD Onclarity™ HPV P3 channel (HPV35/39/68) and negative for HPV35 on the duplex PCR. Only 9/23 had available Allplex™ HPV28 Detection results which showed 7/9 as actual positives for HPV35 and thus were reported as false negatives by the duplex PCR. When the discordant specimens with no available Allplex™ HPV28 Detection results (14/23) were excluded from PPA calculation, 160 directly comparative specimens could be analysed for HPV35 and the result increased from 28.1% to 56.2% (Table 4.6). For HPV58, 16 specimens were negative on the duplex assay and positive in the BD Onclarity™ HPV P1 channel (HPV33/58) and 6/16 had corresponding Allplex™ HPV28 Detection results. Five of the specimens were also negative for HPV58 on Allplex™ HPV28 Detection and positive for HPV33 and thus were not discordant with duplex results. However, one specimen was positive for both HPV58 and 33, and was thus a false negative for HPV58 on the duplex PCR. The false negative result may be attributed to a very low viral burden in the specimen as illustrated by the high Ct value of 39.93 reported by the Allplex™ HPV28 Detection for this genotype. By excluding specimens without confirmed individual results for the pooled channels of the BD Onclarity™ HPV assay, the duplex PCR had a PPA of 94.7% (Table 4.6)

Table 4.6: Adjusted PPA for HPV35 and HPV58 from pooled target result confirmation

Duplex PCR		**BD Onclarity™ HPV assay**		**Duplex PCR v. BD Onclarity™ HPV**			
		Positive	**Negative**	**Overall Agreement, % (95%CI)**	**kappa, k (95%CI)**	**PPA, % (95%CI)**	**NPA, % (95%CI)**
HPV35	Positive	9	0	95.6 (91.2 - 97.9)	0.70 (0.49 - 0.91)	56.2 (29.9 - 80.2)	100.0 (97.5 - 100.0)
	Negative	7	144				
HPV58	Positive	18	2	98.1 (94.7 - 99.4)	0.91 (0.81 - 1.01)	94.7 (74.0 - 99.9)	98.6 (95.0 - 99.8)
	Negative	1	141				

For negative agreement, HPV31, 35 and 45 had NPA of 100% and HPV16, 18, 52 and 58 each had >98% NPA. For overall reporting on any HPV genotypes, 11 specimens gave discordant results for HPV positivity. Two specimens were negative for HPV on the BD Onclarity™ HPV assay but positive on the duplex PCR (false positive), and 9 were HPV positive on BD Onclarity™ HPV but negative on the duplex platform (false negative).

Categorising the specimens into HSIL, LSIL and NILM showed similar overall agreement across all three cytological classifications (Table 4.7). For HSIL, overall agreement for reporting of any HPV genotype between the duplex assay and BD Onclarity™ HPV was 93.9%. For HPV detection in LSIL specimens, the two assays had 92.9% agreement. Finally, in the subset of specimens with no detected intraepithelial lesions – NILM – the duplex PCR had 93.9% overall agreement with the BD Onclarity™ HPV for detection of HPV in these specimens. Table 4.7 further outlines NPA and PPA values by each stratification which ranged between 95.7 – 100.0% and 89.2 – 92.7% respectively.

Table 4.7: Agreement between duplex real-time PCR and BD Onclarity™ HPV stratified by cytology

Target	PPA, % (95%CI)	NPA, % (95%CI)	Overall Agreement, % (95%CI)	kappa, k (95%CI)
Any HPV genotype in HSILs	92.7 (80.1 - 98.5)	100.0 (63.1 - 100.0)	93.9 (83.5 - 97.9)	0.81 (0.60 to 1.0)
Any HPV genotype in LSILs	89.2 (74.6 - 97.0)	100.0 (82.4 - 100.0)	92.9 (83.0 - 97.2)	0.85 (0.71 - 0.99)
Any HPV genotype in NILMs	89.5 (66.9 - 98.7)	95.7 (85.5 - 99.5)	93.9 (85.4 - 97.6)	0.85 (0.71 - 0.99)

4.7 Inter-run variation (reproducibility) in duplex PCR

During duplex PCR testing, positive controls were included in each run to validate PCR results and assay performance. A positive control was included for each of the seven HPV targets. In addition, a negative control – only positive for the endogenous gene – was included. Eight PCR runs were evaluated and data of Ct values from each control across all runs was used to calculate %CV for inter-run variation or reproducibility. In Table 4.8, mean Ct, SD and %CV for each control is shown. Notably, for most of the controls, with the exception of HPV45 and 52 positive controls, SD was < 1 for all other targets, and there was no result with greater than 3Ct (1 log) difference. The %CV was observed in the AccuTrak™ Negative control (BGLT3 positive) (1.85%).

Overall, %CV was less than 7% across all the tested controls, with a range between 2.08% and 6.01% for HPV targets specifically. Individual Ct values generated for control during the eight PCR runs are outlined in Table 4.9.

Table 4.8: Inter-run Ct variation for tested assay controls

Control	Mean Ct	SD	%CV
AccuTrak™ HPV16 (+)	32.9	0.80	2.42
AccuTrak™ HPV18 (+)	31.0	0.81	2.60
HPV31 (+)	23.3	0.61	2.63
HPV35 (+)	20.0	0.63	3.14
HPV45 (+)	19.3	1.16	6.01
HPV52 (+)	21.2	1.00	4.70
HPV58 (+)	23.3	0.48	2.08
AccuTrak™ Negative	28.2	0.52	1.85

(Ct= cycle threshold, SD = Standard deviation, %CV = percentage coefficient of variation)

Table 4.9: Ct values for PCR controls tested across eight different runs

Control	Result 1	Result 2	Result 3	Result 4	Result 5	Result 6	Result 7	Result 8
AccuTrak™ HPV16 (+)	32.2	32.9	31.9	33.5	34.021	33.869	32.9	32.2
AccuTrak™ HPV18 (+)	30.5	31.4	30.6	31.7	32.2	29.6	30.9	31.2
HPV31 (+)	24.0	23.3	23.7	23.0	24.0	22.9	22.2	23.3
HPV35 (+)	20.7	19.7	19.6	19.5	21.0	19.6	19.5	20.6
HPV45 (+)	20.1	17.5	20.4	19.0	19.1	19.6	17.8	20.7
HPV52 (+)	20.7	22.3	20.0	22.1	22.1	21.5	19.9	20.6
HPV58 (+)	23.9	22.9	23.2	22.9	23.2	22.9	23.0	24.1
AccuTrak™ Negative	28.3	28.0	27.6	27.8	29.0	28.7	27.5	28.4

4.8 Intra-run variation (repeatability) in duplex PCR

Following initial testing on the duplex assay, 17 specimens were re-tested in triplicate in a single PCR run to evaluate the assay's repeatability or intra-run variation. For each duplex reaction mix, at least one specimen was positive for one genotype (+/- or -/+) and one specimen positive for both targets (+/+) were tested. Variation in cycle threshold values across each replicate was determined using percentage coefficient of variation (%CV). The resulting mean, SD and %CV per specimen are summarised in Table 4.10. and details of Ct values for each replicate are given in Tables 4.11- 4.14. The %CV of the Ct values for HPV targets were acceptable, ranging from 0.08% and 2.84% across all replicates, while SD ranged from 0.02 and 0.99 (Table 4.10, overpage).

Table 4.10: Mean Ct, standard deviation and percentage coefficient of variation for specimen tested in triplicate for assessment of intra-run variation

Target	Specimen ID	Mean Ct	SD	%CV
HPV16	00016	31.5	0.13	0.42
	00062	33.71	0.34	1.02
	00442	23.84	0.17	0.73
HPV18	00180	20.43	0.30	1.47
	00415	32.26	0.02	0.08
	00104	29.03	0.06	0.21
HPV31	00309	30.34	0.12	0.39
	00315	21.46	0.13	0.58
HPV35	00175	18.62	0.12	0.62
	00450	26.20	0.15	0.58
	00081	25.56	0.14	0.56
HPV45	00309	34.83	0.99	2.84
	00078	27.17	0.16	0.60
HPV52	00180	22.27	0.22	0.98
	00415	36.20	0.06	0.17
	00188	30.41	0.42	1.38
HPV58	00062	32.92	0.20	0.61
	00442	26.07	0.19	0.75
	00085	28.32	0.17	0.60
BGLT3	00175	24.90	0.30	1.22
	00450	27.59	0.12	0.42
	00081	30.62	0.47	1.54
	00301	24.93	0.30	1.19
	00346	30.32	0.06	0.20
	00500	26.16	1.56	5.95

(Ct = cycle threshold, SD = standard deviation, %CV = percentage coefficient of variation)

Table 4.11: Intra-run variation in HPV16+HPV58 duplex reaction

Specimen ID	HPV16 (Ct)			HPV58 (Ct)		
	Result 1	Result 2	Result 3	Result 1	Result 2	Result 3
00016	31.4	31.7	31.5	-	-	-
00062	33.4	33.6	34.1	32.7	33.0	33.1
00442	24.0	23.7	23.8	26.3	25.9	26.0
00085	-	-	-	28.3	28.5	28.1

(Ct= cycle threshold)

Table 4.12: Intra-run variation in HPV18+HPV52 duplex reaction

Specimen ID	HPV18 (Ct)			HPV52 (Ct)		
	Result 1	Result 2	Result 3	Result 1	Result 2	Result 3
00180	20.6	20.1	20.6	22.4	22.0	22.4
00415	32.3	32.3	32.2	36.3	36.1	36.2
00104	29.0	29.0	29.0	-	-	-
00188	-	-	-	30.9	30.0	30.3

(Ct= cycle threshold)

Table 4.13: Intra-run variation in HPV31+HPV45 duplex reaction

Specimen ID	HPV31 (Ct)			HPV45 (Ct)		
	Result 1	Result 2	Result 3	Result 1	Result 2	Result 3
00309	30.3	30.3	30.5	34.3	34.2	36.0
00315	21.4	21.6	21.4	-	-	-
00078	-	-	-	27.1	27.1	27.4

(Ct= cycle threshold)

Table 4.14: Intra-run variation in HPV35+BGLT3 duplex reaction

Specimen ID	HPV35 (Ct)			BGLT3 (Ct)		
	Result 1	Result 2	Result 3	Result 1	Result 2	Result 3
00175	18.6	18.5	18.7	25.0	24.6	25.2
00450	26.3	26.3	26.0	27.7	27.6	27.5
00081	25.6	25.7	25.4	30.1	30.8	31.0
00301	-	-	-	25.0	24.6	25.2
00346	-	-	-	30.3	30.4	30.3
00500	-	-	-	27.1	27.0	24.4

(Ct= cycle threshold)

Figure 4.6 shows amplification curves for specimen 00415 which had the lowest difference (%CV) in Ct values (0.08%) on the HPV18 target. Analysis for individual genotypes showed that HPV31 had the lowest variation of Ct between its replicates with an average CV of 0.48%. This was followed by HPV18 and 35, each with an average CV of 0.59%. HPV16, 45, 52 and 58 had average CV of 0.72%, 1.72%, 0.84% and 0.65% respectively.

The repeatability for the internal control was also assessed on six specimens (3 HPV positive and 3 HPV negative) and had an average CV percentage of 1.75%. The highest %CV for a single specimen was 5.95% in specimen 00500 (Figure 4.7) and the range was 0.20 – 5.95% for the BGLT3 target.

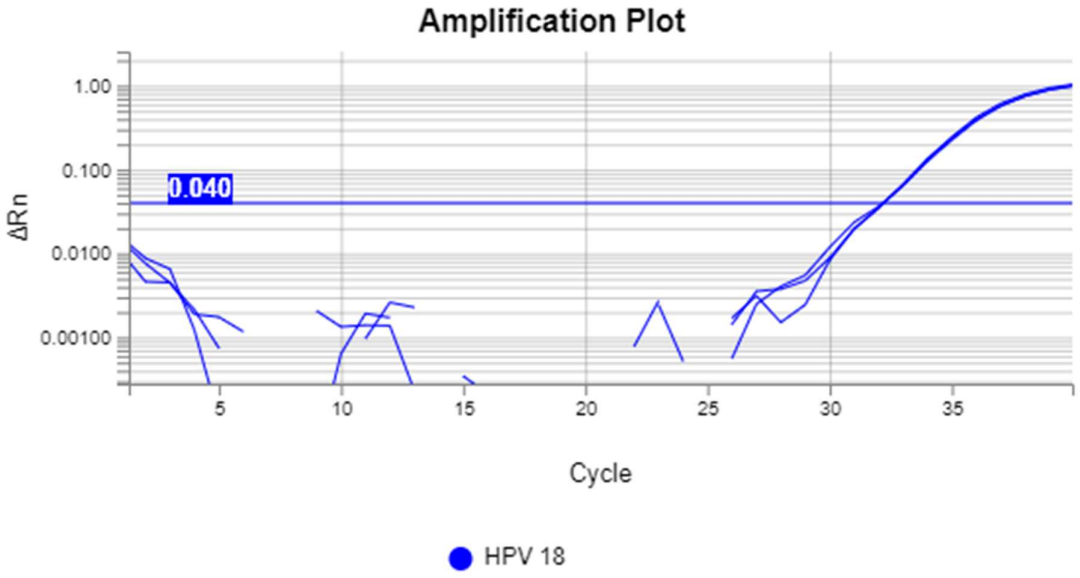

Figure 4.6: Amplification curves for specimen 00415 tested in triplicate. The resulting Ct values were of 32.281, 32.254 and 32.232.

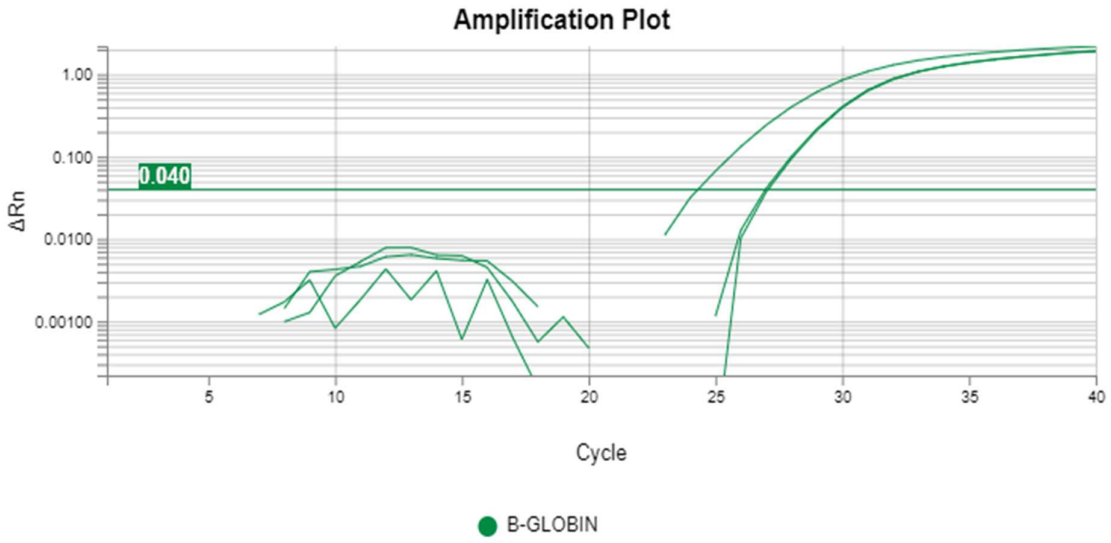

Figure 4.7: **Amplification curves for specimen 00500 tested in triplicate**. The reported Ct values for each replicate were of 27.111, 26.995 and 24,359. (B-GLOBIN = Human beta globin)

5. Discussion

5.1 Extraction methods

In this study, a comparison of automated magnetic bead-based (Tianlong Viral DNA/RNA Extraction Kit), spin column (QIAamp DNA mini kit) and heat lysis extraction was performed. An analysis of PPA, NPA and overall agreement was performed by evaluating parallel PCR results from DNA extracted using each method against correlated results of the BD Onclarity™ HPV. Although the QIAamp DNA mini kit and heat lysis in combination with Proteinase K digestion have been previously described (61,63) as front-end processing methods for HPV, the utility of the Tianlong Viral DNA/RNA Extraction Kit has not been previously reported for HPV DNA extraction. Automated magnetic bead-based extraction had higher overall performance for PPA (92.9%) compared to both manual extraction protocols (spin column extraction (85.0%) and heat lysis (80.2%)). This is in contrast with a similar comparison study which found manual extraction (AmpliLute) to have higher sensitivity (73.2%) than the automated magnetic bead-based (MagNa Pure system) protocol (67.32%) (60). The findings in our study also showed that although high, NPA for automated magnetic bead-based extraction was lower (91.7%) compared to spin-column DNA extraction (96.5%) and heat lysis (98.2%), which is consistent with other studies comparing manual and automated extraction (60). For screening purposes, a higher PPA (or sensitivity) for HPV detection takes precedence as it indicates a higher capacity to detect patients at high risk for cervical cancer which is critical in the context of regions with low screening coverage e.g. South Africa. However, future optimisation for higher specificity is still warranted to prevent over-treatment due to false-positive reporting (75,76).

Generally, literature on direct comparison of manual versus automated extraction was found to be limited and comparative analytical and clinical performance of automated and manual commercial extraction kits shows relative similarity between the techniques and performance differences observed often vary in significance (60,61). The findings from the extraction method comparison in this study thus adds to evidence on available automated magnetic bead-based systems that can used for HPV front-end processing for cost-effective but high-throughput processing. Although the automated method showed NPA results lower than the two manual methods, its value was still above 90%. In addition, unlike spin column extraction, magnetic bead-based extraction does not require multiple centrifugation steps and produces high purity DNA compared to crude heat lysis which carries over cellular debris and potential PCR inhibitors. Considerations to improve specificity of this front-end processing method could investigate minimising points of possible cross-contamination especially if the method is to be applied to high-throughput workflows.

Overall, magnetic bead-based extraction had fewer hands-on processing steps compared to the other two extraction protocols. Thus, consistent with literature (77), this is a more user-friendly front-end processing method for HPV, having potential for application in high-throughput workflows with more extensive genotypes which was shown with singleplex and multiplex (duplex) testing of HR-HPV targets in this study.

5.2 Singleplex and Duplex PCR for HPV detection

A singleplex PCR assay was evaluated on 61 LBC specimens and covered detection of seven HR-HPV genotypes. Agreement between this PCR and the BD Onclarity™ HPV ranged from 70% - 95% for all genotypes. When duplex testing was established with the same targets and tested on the same specimens, it had 100% correlation with singleplex PCR in 4/7 of the targets (HPV31,45,52 and 58) and some discordance in HPV16 (1 discordant specimen), HPV18 (1discordant specimen) and HPV35 (3 discordant specimens). These results showed that testing with duplex real-time PCR was feasible to provide a more efficient method of detection targeting multiple genotypes in a single reaction. While research studies usually have small to medium sample sets where singleplex can be feasible, large-scale testing required for diagnostic settings benefits from muti-target identification in a single well for saving on cost, reagents, volume of template (which is often limited) used and time taken for processing (78).

To our knowledge, this study is the first to evaluate pre-designed HPV-specific TaqMan® Gene Expression Assays for detection of HPV in residual LBC specimens and to compare the performance of the assays against the BD Onclarity™ HPV. Other publications have described custom TaqMan primer-probe sequences and established in-house PCR tests for different target pathogens. The limited studies that have reported use of TaqMan® Gene Expression Assays have applied it to the detection of circulating HPV DNA in plasma and DNA quantification in HPV DNA positive cancer cell lines (CaSki) (79,80). As described previously, the TaqMan® Gene Expression Assay consists of a pre-formulated primer-probe set detecting a single target, often requiring little to no optimization for singleplex testing. Duplex PCR using TaqMan® Gene Expression Assays is achieved by combining prime-probe sets containing different reporter dyes (e.g. FAM and VIC) to detect two targets in one reaction. Hein and Bodendorf (2007) found that duplex PCR using pre-designed TaqMan® Gene Expression Assays, targeting genes on human reference cDNA, and the QuantiTect Multiplex PCR Kit (Qiagen) had a correlation coefficient of 0.95 in comparison with singleplex PCR using the same targets, even without prior optimization. Furthermore, they observed that Ct values remained similar to those recorded in the singleplex PCR. However, it was noted that, in comparison, using Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) and Brilliant Multiplex Master Mix (Stratagene, San Diego, CA, USA) in duplex PCR produced considerably lower correlation with singleplex (0.42 and 0.47 respectively)

(78). This highlights the value in validating duplex PCR against singleplex results to confirm optimal performance even when using pre-formulated assays (71).

When the duplex real-time PCR was subsequently tested on a larger sample set (n=175), the most detected genotype was HPV16 which was present in close to half (42.2%) of the LBC specimens tested. However, it should be noted that the sample set in this study was selected from reference results from the BD Onclarity™ HPV Assay and thus the values for the genotypes are not directly correlated to prevalence in the general population. The duplex real-time PCR assay performed well against the BD Onclarity™ HPV, with both overall PPA and NPA above 90%. This puts the duplex PCR in line with validation criteria which require a test to achieve a sensitivity of 90% when compared against a comparator test. However, the results from duplex testing were not stratified by CIN category, therefore future evaluation with larger sample sets and including clinical data would be needed in order to fully confirm compliance with the guidelines as the criteria is applied to testing of specimens with ≥CIN2 grading (35).

This study did stratify specimens by SIL status, and the duplex PCR had a reported PPA of 92.7% in specimens with HSIL, giving a good indication of the assay's potential performance in specimens with a histological grading and screening for high-risk patients. In a similar study, the Seegene Anyplex II HPV28 (Seegene) which detects 28 HPV genotypes, was compared against the BD Onclarity™ HPV and its overall agreement was 90.1% with k = 0.8 (81) for genotypes correlating to the BD Onclarity™ HPV. In contrast to this, the duplex assay in this study had a higher agreement of 93.6% and a kappa value of 0.87 when tested in parallel with the BD Onclarity™ HPV. This was also higher than the reported concordance between the Roche cobas® and BD Onclarity™ HPV which was k=0.78 for overall HR-HPV (82). However, it should be noted that Seegene and Roche assays were evaluated for a larger set of HR-HPV targets (14 genotypes) and extension of the genotype coverage of the duplex assay, which covered seven genotypes, could provide more accurate head-to-head comparison.

Although the seven genotypes were a good representation of the critically prevalent HR-HPV genotypes in South Africa, evaluation of the duplex PCR's performance with the inclusion of additional HR-HPV genotypes can be performed to further extend the panel of genotypes detected. Systematic review data shows that genotyping can further improve the management of screened patients by correlating genotyping results to specific levels of risk of infection progression to cervical cancer based on the genotypes most associated with CIN (83). Furthermore, this stratification is reported to provide good guidance triaging patients for further testing or treatment (83). This has also been demonstrated by Botha *et.al.* where extended genotyping and grouping of HPV31, 33, 58 and 52 as moderate risk and HPV16, 18 and 45 as very high risk had high specificity (91.2%) for determining CIN2+ using the BD Onclarity™ HPV assay (84).

For individual genotypes, NPA was consistently high for all targets and ranged from 98.4 to 100% in duplex PCR. This indicates that the assay had high accuracy in correctly reporting HPV negative specimens. However, PPA (sensitivity) was much more variable with a range between 28.1% and 93.8%. The duplex assay PPA were lowest for HPV35 (28.1%) and HPV58 (52.9%). However, the values for HPV35 and 58 did not reflect the clinical performance of the assay for these targets as the BD Onclarity™ HPV assay results used for comparison report HPV35 and 58 pooled with other genotypes (HPV35/39/68 and HPV33/58). Therefore, specimens that are categorised as 'positive' for these genotypes on the BD Onclarity™ HPV in this study were potentially negative for HPV35 and 58 and positive for other genotypes targeted in the channel and thus not directly comparable with the results from the duplex PCR. Therefore, confirmatory results from Allplex™ HPV28 Detection providing individual results for these genotypes provided a more accurate indication of true positives or negatives for these targets specifically.

Not all discordant specimens had been previously tested on the Allplex™ HPV28 Detection and therefore not all discordant specimens could be confirmed for individual genotypes for HPV35 and 58. Using the available confirmatory results, 5/6 specimens were shown to be true negatives for HPV58 on the duplex PCR, increasing the PPA from 52.9% to 94.7% when specimens without available Allplex™ HPV28 Detection results were excluded. For HPV35 7/9 Allplex™ HPV28 Detection-tested specimens were false negatives on the duplex assay, which meant PPA remained relatively low (from 28.1% to 52.6). The poor performance of the HPV35 primer-probe set indicates the potential need for further optimisation or re-designing of this target. Although no direct comparison study using the same primers and probes for HPV35 was found, a study comparing performance of the PapilloCheck® against HC2-confirmed specimens also found that the lowest sensitivity in the assay was for the HPV35 genotype (72.2%) (85).

A possible explanation for these false negative results for HPV35 may also be that the target was in a duplex combination with the BGLT3 target for human beta globin. It can be assumed that the internal control gene is more abundant or highly expressed, and non-target specific reagents during PCR, i.e. components within the TaqPath™ 1-Step RT qPCR Master Mix, were more rapidly used up BGLT3 amplification during the reaction, leaving less available for HPV35 to produce detectable amplicons. Primer limiting, which involves using lower concentration of target-specific primers for genes that are more highly expressed compared to those of relatively less abundant genes, is known to reduce competition for reagents in multiplex reaction (86). In our study's duplex PCR, BGLT3-specific TaqMan® Gene Expression Assay used was not primer-limited and thus could have restricted amplification of HPV35. Therefore, any future optimisation should also adjust the concentration of the internal control gene primers within the multiplex assay. Improving the sensitivity of

HPV35 is particularly important to ensure cases of HPV35 are not missed given its increasing prevalence in South Africa (13).

The duplex assay was also evaluated for repeatability (intra-run) and reproducibility (inter-run) which both demonstrated high precision. Inter-run CVs were between 2.08% and 6.01% across all HPV targets and intra-run variation ranged from 0.08% and 2.84% showing the assay produced high reproducibility and repeatability. In comparison, a study on clinical specimen performance characteristics of the BD Onclarity™ HPV assay reported that the test had %CV values between from 2.6% -5.4% for 'within run' (intra-run) assessments and variation ranging from 0 – 1.0% between tests (inter-run) for Ct scores on pooled clinical specimens (87). This shows that the duplex PCR could potentially produce better outcomes for repeatability on individual genotypes than the BD Onclarity™ HPV assay, however, the variation between tests is more pronounced in comparison. The duplex PCR reproducibility was similar to that of the cobas® HPV test which had reported %CV ranging from 1.1 – 6.5% (88). In the case of non-commercial HPV PCR assays, a digital droplet PCR (ddPCR) was described for the detection of HPV16, 18, 33 and 45 on LBCs and formalin fixed paraffin embedded (FFPE) tissue specimens and had recorded average inter-assay variation between 3.4 - 7.0% and 2.6 – 8.2% intra-assay variation (89).

In summary, with a maximum coefficient of variation of 6.01%, the duplex real-time PCR proved to be an accurate assay with good reproducibility, with performance similar to that of currently available commercial HPV PCR tests and relatively better performance than some in-house methods reported in literature. Future work on this duplex HPV assay can include testing a larger sample set to evaluate inter- and intra-laboratory agreement against Meijer's criteria which requires "a percentage of agreement with a lower confidence bound not less than 87%" when testing ≥500 specimens with a 30% positivity rate confirmed by a validated reference assay (35).

5.3 Limitations and further considerations

The sampling of LBCs for this study was done through pre-selection of specimens, based on BD Onclarity™ HPV results, to meet a set rate of positivity and genotype distribution with all HPV types represented in the selection. Future testing to further validate the duplex PCR for implementation would require the inclusion of a clinical performance evaluation of residual specimens from clinical cohorts and, subsequently, testing of clinical feasibility to determine if similar assay performance is observed.

When comparing the extraction methods, selection of the best performing method was determined by the PCR result compared to the BD Onclarity™ HPV and specimen validity through amplification of the housekeeping

gene. DNA eluted was not quantified by concentration to give a scope of the difference in DNA yield obtained from each method. Optimising in future studies can evaluate exact DNA yield from this platform and tackle possible strategies to maximise efficiency.

There were a number of 'discordant' results from pooled reporter channels which could not be resolved at individual genotype level as results from a comparator test were not available. This affected calculations for those genotypes specifically, however reporting of overall HPV positivity (any HPV genotype) was not affected as these specimens had infection with other genotypes and thus still reported as positive.

As previously mentioned, the scope of HR-HPV genotypes included in this study was limited to seven HR-HPV genotypes compared to 14 HR-HPV genotypes reported by the BD Onclarity™ HPV and would benefit from expansion of the targeted genotypes. Moreover, with the proposed expansion of genotypes, further development of the assay can include optimising for detection of more targets within a single well i.e expanding from duplex to triplex or quadruplex PCR, to accommodate these additional genotypes more efficiently. Additionally, the optimised TaqMan® Gene Expression Assays can be set up on custom array plates which would consist of the optimised primer-probe sets pre-loaded and dried onto the plate (90) to further scale up the testing throughput, decrease hands-on time the workflow and decrease time taken to produce results for patients. This mechanism could even be evaluated to establish the assay as a near point-of-care method that would require minimal handling or pipetting.

In addition, evaluation of the duplex PCR can be expanded to include other specimen types, including both clinician- and self-collected swabs. Demonstrating utility of this assay in self-collected swabs would add value to current work aiming to couple easier specimen collection methods that increase screening uptake with efficient low-cost molecular tests.

6. Conclusion

In this study, automated magnetic bead-based extraction was shown to perform better than manual extraction protocols, and method comparison demonstrated that it was more feasible for application to high-throughput processing of LBC specimens for detection of HPV DNA.

The study also successfully applied the TaqMan Gene Expression Assays for HPV16,18, 45, 35 31, 52 and 58 and BGLT3 in a duplex PCR format. The established duplex PCR was able to accurately detect high-risk HPV genotypes in LBC specimens and had near perfect overall percentage agreement when compared to the BD Onclarity™ HPV. It was also highly accurate in terms of reproducibility and repeatability. There was high NPA for all targets, however, marked variability in PPA for the specific genotypes still warrants further investigation.

Overall, this research highlighted a sensitive and cost-effective workflow for scaled testing for the presence of HPV. It shows potential for application to high-throughput testing that will offer a platform with high sensitivity and efficiency from its initial processing for DNA extraction to detection of HR-HPV genotypes. Its format can be easily adapted to include additional genotypes and even development towards its use as a near-point of care test.

7. References

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8. Appendices

Appendix A: Ethical clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO: Profs W Stevens & LE Scott; Dr L Hans; Ms L Noble
Wits Diagnostic Innovation Hub
Faculty of Health Sciences
Medical School
University

E-mail: Lara.Noble@wits.ac.za

CC: Supervisor: Not applicable
<>
and <HREC-Medical Research Office@wits.ac.za>

FROM: Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2023/10/25

REF: R14/49

PROTOCOL NO: **M230649** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)

PROJECT TITLE: *The evaluation of cervical Human Papillomavirus (HPV) molecular tests and specimen collection devices in South (HPV-MDev protocol)*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

WITWATERSRAND
JOHANNESBURG

R49 Profs W Stevens & LE Scott; Dr L Hans; Ms L Noble

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M230649

NAME: Profs W Stevens & LE Scott; Dr L Hans; Ms L Noble **DEGREE:** N/A
(Principal and Co-Investigators)

DEPARTMENT: Wits Diagnostic Innovation Hub
Faculty of Health Sciences
Medical School
University

PROJECT TITLE: *The evaluation of cervical Human Papillomavirus (HPV) molecular tests and specimen collection devices in South (HPV-MDev protocol)*

DATE CONSIDERED: 2023/06/30

DECISION: Approved conditionally

CONDITIONS: (1) This clearance applies to female participants only, until further notice; (2) This clearance applies only to the study sites listed in Annex 1 attached - further sites may be added on receipt of evidence of management approval

NOTE: If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>

SUPERVISOR: Not applicable

APPROVED BY: _____
Professor P Ruff, Chairperson, HREC (Medical)

DATE OF APPROVAL: 2023/10/25 **EXPIRY DATE:** 2028/10/24

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

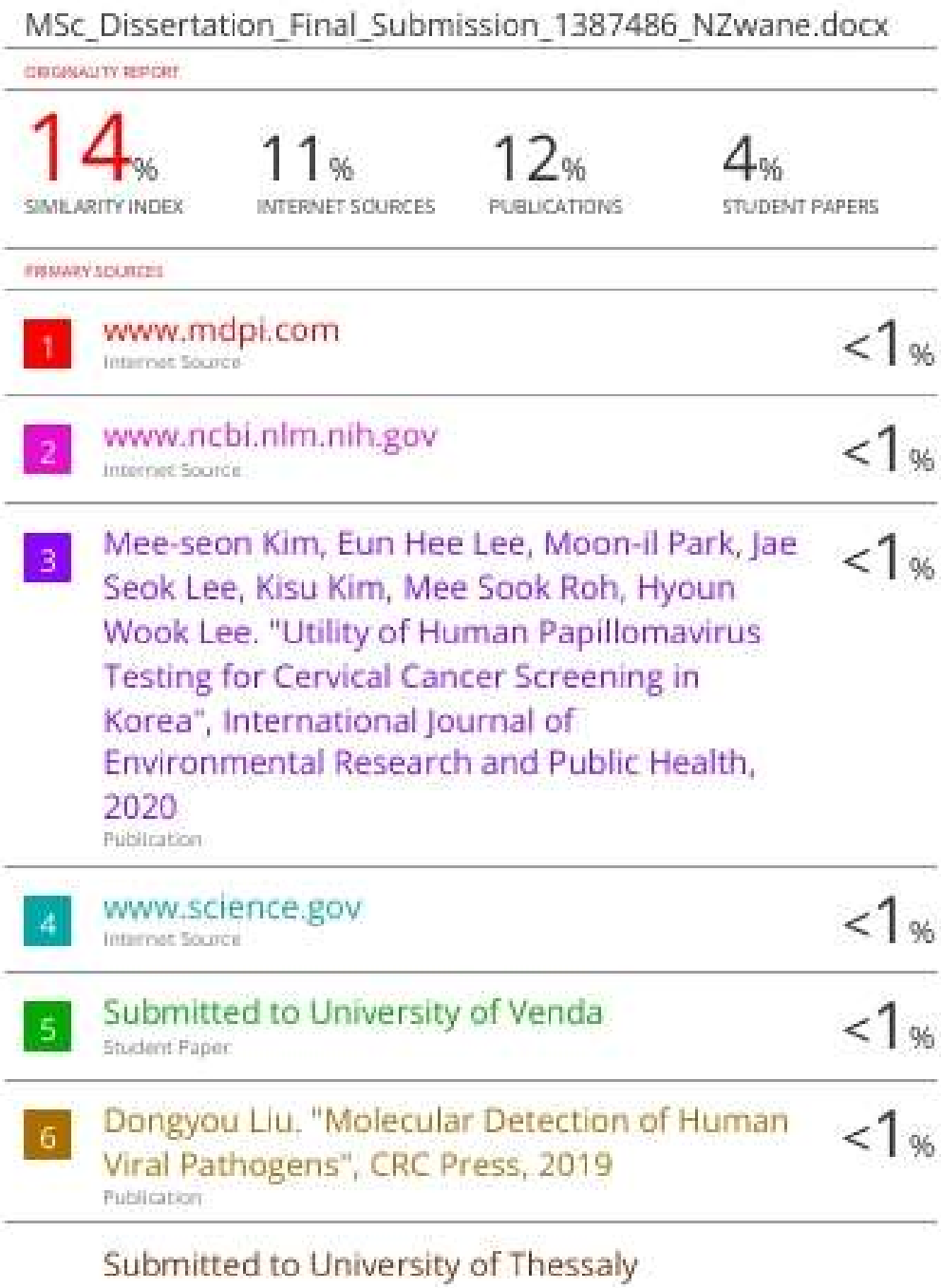
DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **June** and therefore reports and re-certification will be due in the month of **June** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

_____ _____
Signature of Principal Investigator Date

Appendix B: Turnitin report



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4	www.science.gov Internet Source	<1%
5	Submitted to University of Venda Student Paper	<1%
6	Dongyou Liu. "Molecular Detection of Human Viral Pathogens", CRC Press, 2019 Publication	<1%

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