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Validation of the Xpert breast cancer STRAT 4 essay on GeneXpert instruments to assess hormone receptor status, KI-67 and HER-2 mutational status in breast cancer tissue samples

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VALIDATION OF THE XPERT® BREAST CANCER STRAT4 ASSAY ON THE GENEXPERT INSTRUMENT TO ASSESS HORMONE RECEPTOR STATUS, KI-67 AND HER-2 MUTATION STATUS IN BREAST CANCER TISSUE SAMPLES.

Lina Sewanywa

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of requirements for the degree of Master of Medicine (Haematology)

DECLARATION

I, Lina Sewanywa, declare that this Research Report is my own, unaided work. It is being submitted for the degree of Master of Medicine (Haematology) at the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree of examination at any other university.



_____ (Signature)

09 day of June 2022 in Johannesburg, South Africa.

To my parents, Andrew and Joy Sewanywa

PUBLICATIONS FROM THIS STUDY

This article will be submitted to Applied Immunohistochemistry and Molecular Morphology journal for peer review and publication.

ABSTRACT

Breast cancer is the commonest cause of cancer-related mortality in African females where patients often present later and with advanced disease. Causes for delayed diagnosis include restricted diagnostic access and international controversy on interpretation of ancillary tests like immunohistochemistry (IHC). Fine needle aspirates (FNAC) are an attractive alternative although may have reduced sensitivity. The STRAT-4 assay (Cepheid, Sunnyvale) is a semi-quantitative reverse-transcription RNA assay which detects mRNA expression in breast samples for Oestrogen Receptor (*ESR1*), Progesterone receptor (*PGR*), human epidermal growth factor receptor (HER2/*ERBB2*) and the proliferation marker, Ki67. We assessed the performance of this assay on both formalin-fixed paraffin-embedded (FFPE, n=31) and matched FNAC (n=20) samples from patients presenting with breast cancer to the Johannesburg academic hospitals, as a pilot study. IHC and Fluorescent *in-situ* hybridisation analysis (performed on HER2-indeterminate samples) was compared to the expression of the target genes for FFPE and mRNA expression on FNAC samples were compared to the FFPE results for both mRNA expression and IHC. Concordance was highest for hormone receptor (HR) expression with an overall percent agreement (OPA) for *ERBB2* of 96.7% and of 81.3%, for *ESR1* expression of 93.3% and 93.3% and for *PGR* expression at 86.7% and 94.4% and lowest for Ki67 expression at 83.9% and 66.7% for FFPE and FNAC samples respectively. This suggests that the STRAT-4 assay may be a useful ancillary test in determining HR and Ki67 status in FFPE samples and that use on FNAC samples is feasible. Future studies should expand the sample numbers and establish locally relevant cut-offs.

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LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ASCO	American Society of Clinical Oncology
CAP	College of American Pathologists
CHBAH	Chris Hani Baragwanath Academic Hospital
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CNB	Core needle biopsy
CT	Cycle threshold
dCT	Delta cycle threshold
ER	Oestrogen receptor protein
ERBB2	*Erb-B2* receptor tyrosine kinase 2 gene
ESR1	Oestrogen receptor 1 gene
FFPE	Formalin fixed paraffin embedded
FNA	Fine needle aspiration
FNAC	Fine needle aspiration cytology
HER-2	Human epidermal growth factor receptor – 2 protein
IAC	International Academy for Cytology
IHC	Immunohistochemistry
FISH	Fluorescent in situ hybridisation
HR	Hormone receptor
IQR	Interquartile range
M	Maui
MKi67	Marker of proliferation Ki67 gene
mRNA	Messenger ribonucleic acid
NHLS	National Health Laboratory Service
NPA	Negative percent agreement
OPA	Overall percent agreement
PCR	Polymerase chain reaction

PPA	Positive percent agreement
PR	Progesterone receptor protein
PGR	Progesterone receptor gene
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
UICC	Union for International Cancer Control
Q	Quick
WHO	World Health Organisation

1. INTRODUCTION/BACKGROUND

Cancer accounts for 7.2% of deaths in Africa [1]. Breast cancer is the commonest cause of cancer-associated death in women worldwide. In South African women, breast cancer accounts for 22% of all cancers, but presents at younger ages and at a more advanced stage than in high-income countries [2] with a higher mortality[3]. Factors contributing include poor early detection strategies, socio-economic barriers to receiving treatment and advanced disease at presentation[2, 4].

Breast cancer diagnosis can be made by histological or cytological assessment of tissue samples with additional investigations to detect hormone receptor (HR) status: specifically, the oestrogen receptor(ER), progesterone receptor (PR) and the human epidermal growth factor receptor (HER2 or ERBB2) expression by breast cancer cells. Ki67, a measure of tumour cell proliferation, is also measured routinely [5]. Detection of HR status allows for classification of breast cancer into subtypes with prognostic and therapeutic significance (table 1)[4, 6-8]. Subtyping guides treatment decisions including extent of surgery, use of neoadjuvant therapy (chemotherapy and/or radiation therapy) and use of biological and endocrine agents[4].

Immunohistochemistry (IHC) is the gold-standard for determining HR and Ki67 status in breast cancer although the technique has limitations [13]. Several published guidelines attempt to standardise interpretation of HR and Ki67 testing and to establish clinically relevant cut-offs although there is still not consensus on positive ranges. The cut-off value for Ki67 positivity varies in the literature between 10% and 30% tumour cells positive [5, 13]. IHC shows significant variability between different operators and different reagent clones and Ki67% particularly has repeatedly shown poor reproducibility [14]. The American Society of Clinical Oncology/College of American Pathologists have published joint guidelines on HER2 testing, with recommendations for secondary testing using fluorescent *in-situ*-hybridisation (FISH) in cases with IHC scores of 2+[7].

Core needle biopsy (CNB) is the recognised standard diagnostic procedure for breast tissue biopsies. Fine needle aspiration cytology (FNAC) represents an attractive cost-effective alternative in low-resource settings, provides a faster turnaround time and has acceptable accuracy [15, 16]. FNAC is included in the South African breast cancer screening programme[17] and utilises the International Academy of Cytology (IAC) Yokohama System to provide a standardised assessment [18, 19].

Reverse Transcription Polymerase Chain Reaction (RT-PCR) assays analyse messenger RNA (mRNA) levels of different gene products thus assessing gene expression. There are commercially available RT-PCR assays for breast cancer[20] . The GeneXpert® platform (Cepheid, Sunnyvale, CA, USA), is an automated system that performs semi-quantitative RT- PCR (RT-qPCR) on samples[21]. The GeneXpert® MTB/RIF assay is endorsed by the WHO as an initial diagnostic tool for the assessment of HIV-associated TB, particularly in high-risk countries. In South Africa, the GeneXpert® MTB/RIF has a footprint of 314 devices spread across 207 laboratories[21]. The widescale accessibility, robustness and ease-of-use makes the GeneXpert® attractive for implementation in other diagnostic algorithms including for cancer.

The Xpert® Breast Cancer STRAT4 Assay is an RT-qPCR, cartridge-based assay, that runs on the GeneXpert® machine and measures mRNA levels of ER, PR, HER2 and Ki67 in formalin-fixed paraffin embedded (FFPE) breast tissue samples. The assay offers fast and accurate detection of these biomarkers with a high concordance with standard methods (IHC and FISH) when performed under standardised conditions in a College of American Pathologists certified facility[22] . The performance of the assay has not been widely assessed in resource-constrained settings where IHC may be less standardised. Additionally, use of the STRAT4 in fine-needle aspirate samples has not been widely assessed.

This study aimed to evaluate the performance of the STRAT4 assay on samples from patients attending a South African tertiary breast cancer clinic including both FFPE and FNA samples.

2. MATERIALS AND METHODS

2.1 ETHICS CLEARANCE

Ethical clearance for this study was given by the University of Witwatersrand Human Research and Ethics Committee (M180731) and the National Health Laboratory Service (NHLS) Academic Affairs and Research Management System. All samples were anonymised.

2.2 PATIENT SAMPLES

Samples were obtained from the Chris Hani Baragwanath Academic Hospital (CHBAH) breast unit, the NHLS cytology unit and the NHLS histopathology units at CHBAH and Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) (Figure 1). Fifteen retrospective FFPE samples from patients diagnosed with malignant breast cancer at CMJAH/CHBAH after 2017 were retrieved from an established database. A nurse collected 26 FNAC samples prospectively following informed consent from patients presenting to the CHBAH breast unit with a breast mass. Six samples that had no traceable corresponding histology report or those with benign or inadequate cytological evaluation were excluded. Sixteen residual FFPE samples matched by unique patient identifiers to prospectively collected FNA samples were processed. A further four FNAC were included. These had associated FFPE samples with IHC/FISH results available, although residual FFPE blocks were not available for analysis via RT-qPCR.

2.3 PATHOLOGICAL AND MOLECULAR CLASSIFICATION OF SAMPLES

Tissue samples were reported by the NHLS Anatomical Pathology Department, an accredited tertiary laboratory servicing a large metropolitan area in South Africa. IHC and FISH results were retrieved from the laboratory information system, TrakCare®, InterSystems (Massachusetts, USA).

Different antibody clones were used for IHC depending on the reporting site, including clone SP1 (Ventana, Tucson, USA) for ER and clone PgR 636 (Dako Corp, CA, USA) or 1E2 (Ventana) for PR. Samples were classified positive if ≥1% of cells were positive regardless of staining intensity based on ASCO/CAP guidelines with 1-9% considered as weakly positive[8]. HER2 status was assessed immunohistochemically using IHC with a 4B5 (Ventana) or c-erbB-2 Oncoprotein (Dako Corp) clone. Tumours were classified as HER2 positive if intense (3+) staining of at least 10% of tumour cells was present. No (0) or weak (1+) staining was diagnosed as HER2 negative and moderate (2+) staining as indeterminate [23]. All indeterminate results were tested by FISH using a Vysis Pathvysion HER2 DNA probe kit (Vysis-Abott, IL, Chicago, USA). Ki67 immunostaining was performed using antibody clone MIB-1 (Dako Corp) or 30-9 (Ventana) and reported positive if ≥20% cells were positive[24].

Pathological grading of breast tumours was based on a modified Bloom and Richardson algorithm from grade I (well differentiated) to grade III (poorly differentiated). Pathological staging was based on the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) 2017 guidelines [25], in patients in whom a mastectomy was performed. Breast tumours were sub classified into subtypes Luminal A, Luminal B, HER2 enriched and triple negative based on HR status, HER2 expression and Ki67expression as described in the St Gallen guidelines [26].

2.4 SAMPLE CONTROLS

FFPE samples, previously assessed by the manufacturer during assay optimisation were processed to ensure assay validity. The reference gene (*CYFIP1*) and assay-specific internal controls were used to confirm sample quality and adequate performance of reagents.

2.4.1 FFPE SAMPLES

Each sample comprised two slides with 4um-thick unstained FFPE tissue. Single unstained FFPE slides were scraped using a blade into an Eppendorf tube and treated with 1.2mL of FFPE lysis reagent and 20uL of proteinase K. The sample was vortexed at a high speed for 15 seconds and pulse-spun before being heated at 80°C for 30 minutes. The entire lysate was added to 1.2mL of > 95% ethanol. This was vortexed at a high speed for 15 seconds and pulse-spun. An aliquot of 520uL was transferred to the STRAT4 cartridge and analysed in the GeneXpert®. Nucleic acid purification, amplification, and real-time PCR analysis were automated by the instrument, with results available within 75 minutes.

For samples that were smaller than $10mm^2$ or produced an “indeterminate” or “invalid” result, a retest procedure using a concentrated lysis preparation was performed on the second unstained FFPE slide using the same methodology.

2.4.2 FNA SAMPLES

Each FNAC sample comprised four air-dried unstained glass slides. Two different methods of lysis were performed on each FNA sample with approximately half of each FNA smear assigned to each method. In the quick lysis method, 600uL of FFPE lysis buffer was placed in a 1.5mL Eppendorf tube, the FNA scraping was added, and the sample was vortexed at a high speed for at least 10 seconds then pulse-spun. Subsequently, 600uL of ethanol was added, the sample was vortexed for at least 10 seconds and pulse-spun. In the Maui lysis method, 1.2mL of Maui lysis reagent was added to the FNA scraping and the sample was vortexed at a high speed for at least 10 seconds. An aliquot of 520uL was transferred to the STRAT4 cartridge for processing in the GeneXpert® for both methods.

2.5 GENE XPERT DATA ANALYSIS

The mRNA levels of *ESR1*, *PGR*, *ERBB2* and *MKi67* were derived from the cycle threshold (Ct) of detection of each target gene, normalised against the mRNA value of an internal control reference gene (*CYF1P1*) and expressed as a delta CT value (dCT) based on the formula (dCT = [Ct $_{CYF1P1}$] – [Ct $_{target\ gene}$]).

All target gene results were automatically analysed and reported using a preset algorithm defined on previous FFPE studies which identified the most appropriate dCT values to define a positive result as "-1" for *ESR1* and *ERBB2*, as "-3,5" for *PGR* and at "-4" for *MKi67* [27].

2.6 STUDY DATA ANALYSIS

A qualitative result was reported for each target gene based on the dCT cut-off value and a semi-quantitative dCT value was analysed for each case. Descriptive statistics were derived for all dCT values and results were compared using a student T-test or a Mann-Whitney analysis using GraphPad Prism (Graphpad Prism, San Diego, USA).

Qualitative IHC/FISH results for HR and Ki67 were compared to qualitative results obtained for *ESR1*, *PGR*, *ERBB2* and *MKi67* mRNA on the GeneXpert®. Concordance / overall percent agreement (OPA), positive percent agreement (PPA) and negative percent agreement (NPA) were calculated for each target gene. A Cohen's Kappa statistic (κ) was used to determine the degree of agreement, based on standard criteria for interpretation [28]. A p value of <0.05 was considered statistically significant.

IHC/FISH results for HR and Ki67 were analyzed as categorical clinically significant ranges and compared to semi-quantitative results derived for *ESR1*, *PGR*, *ERBB2* and *MKi67* mRNA levels based on dCT values.

3. RESULTS

3.1 OVERVIEW OF THE SAMPLE COHORT

A total of 35 patients were enrolled including 15 patients with retrospective FFPE samples, 16 patients with prospectively matched FFPE and FNAC samples, and 4 patients with prospectively collected FNAC samples (Figure 1). Of these patients, 77%, 69% and 24% had tumours that were ER, PR and HER2 positive respectively. 77% of tumours expressed a Ki67 of ≥20%. The most commonly diagnosed subtype was Luminal B (HER2 negative), followed by Luminal A, Luminal B (HER2 positive), Triple negative and HER2 overexpressing (Table 2).

3.2 COMPARISON OF IHC AND mRNA RESULTS – FFPE

Valid test results were obtained for all 31 FFPE samples, with the exception of one indeterminate *PGR* result despite repeat testing using a more concentrated lysate which was excluded from analysis.

There was high concordance between ER protein and *ESR1* mRNA expression, with an OPA of 90,3%, PPA of 88% and a NPA of 100%. This improved to an OPA of 93,3%, a PPA of 91,1% and a NPA of 100% when weakly positive ER cases were excluded (Table 3). ER protein expression on IHC appeared discrete with cases either negative or showing bright ER expression in >66% of the cells. The dCT values for *ESR1* reflected a similar pattern with a significant difference in dCT for cases with no expression and cases positive for ER expression in ≥10% cells ($p<0,0001$). Discrepant cases were shown to cluster around the dCT cut-off level of "-1" and to show intermediate ER protein expression (1-33% cells positive) (Figure 2A).

There was an OPA of 86,7%, a PPA of 100% and a NPA of 55,6% (Table 3) between PR and *PGR* mRNA . PR protein staining was less discrete and more variable than ER expression, with a significant proportion (29%) of cases showing weak positive PR expression (Figure 2B). A significant difference in dCT was noted for cases with no or weak expression of PR in <10% of cells when compared to cases positive for PR expression in ≥10% cells (p<0,001). No significant difference between dCT values was present when comparing cases with absent and weak PR expression.

There was high concordance between HER2 positive cases (based on IHC and FISH results in indeterminate cases) and *ERBB2* mRNA expression, with an OPA of 96,7%, PPA of 87,5% and a NPA of 100% (Table 3). A single discordant case was IHC 3+ for HER2 expression, but negative for *ERBB2* mRNA expression. HER2 FISH analysis performed on the discordant case was negative, with an average HER2 copy number of 3 signals/cell and a HER2/CEP17 ratio of 1,1. A significant difference in dCT was noted for HER2 negative compared to HER2 positive cases (p<0,001), whilst there were no significant differences in dCT values in cases with HER2 IHC reported 0, 1+ or 2+ (FISH negative) and in cases HER2 IHC reported 2+ (FISH positive) or 3+ (Figure 2C).

The agreement between Ki67 and *MKi67* was weaker with a continuum of results (Figure 2D) and an OPA of 83,9%, PPA of 91,7% and a NPA of 57,1% (Table 3). A significant difference was noted in dCT value when considering cases with <10% and >20% Ki67 expression (p<0,004), although no significant differences were noted for intermediate cases with between 10-20% expression.

3.3 CORRELATION OF RESULTS FOR FNAC SAMPLES

A total of 20 FNAC samples were included: 16 samples had a matched FFPE which had IHC/FISH results available and RT-qPCR performed on the FFPE for comparison and a further 4 with corresponding FFPE results available for IHC/FISH for comparison only. Not all cases had sufficient material available for

analysis using both the Maui (M) and Quick (Q) lysis methods, with valid results obtained for 19 (M) and 16 (Q) samples respectively. The result for *PGR* and *MKi67* was indeterminate for one sample after using a more concentrated lysate and these were excluded from analysis.

OPA was good for *ESR1*, *PGR* and *ERBB2* mRNA when comparing both FFPE IHC/FISH and mRNA results to matched FNA Xpert mRNA results (75%-94,4%), although the correlation for *MKi67* was poorer (62-68%) (Table 4).

4. DISCUSSION

Breast cancer is a common cancer in African women and a leading cause of cancer-related mortality worldwide [(1)]. We here provide a small pilot project, investigating the performance characteristics of the Xpert® Breast Cancer STRAT4 Assay on FFPE and FNA samples from South African women with breast cancer.

Although tissue biopsy is considered the gold standard for diagnosis of cancer, this is not always readily available in low- and middle-income countries resulting in delays in diagnosis and linkage-to-care[(11, 29)]. Patients present later with advanced disease and increased morbidity and mortality. In addition, pre-analytical delays can influence the performance of adjunctive testing modalities like immunohistochemistry and FISH. FNAC is a cost-effective and minimally invasive technique which can be performed in the clinic setting [(16, 30)]. Ancillary testing which could improve the diagnostic accuracy of FNAC is critical to a public health screening programme.

Concordance between immunohistochemistry and STRAT4 determination on FFPE blocks was generally acceptable, particularly when considering variables such as ER and HER2. In these target genes, OPA

between IHC and RT-qPCR was >90% and Kappa (κ) suggests substantial to near-perfect agreement. There was a single discordant HER2 case, although in this case the IHC (3+) and HER2 FISH (negative) results were also discordant, which may reflect either heterogeneous HER2 expression in the tumour, increased expression of HER2 in an intraductal tumour component or activating mutations in the *ERBB2* gene[31, 32]. The concordance between PR and Ki67 was more moderate, with OPA 84-87% and κ suggesting moderate (Ki67) to substantial (PR) agreement. This may reflect the expression patterns for both and a number of cases where expression was weakly positive for PR. Previous studies have shown that approximately 50% of weakly positive PR cases were correctly classified by the STRAT4 assay [27]. Furthermore, up to 20% of breast cancer cases may be incorrectly classified based on IHC for ER and PR receptors[7, 8, 33, 34] and the PR IHC positive cut-off is controversial with a cut-off of >20% cells positive considered more clinically relevant[35]. Ki67 IHC is also poorly reproducible in both international and local studies and cut-offs for clinical relevance are not standardized[13, 14]. For these target genes, the analyser cut-off settings should be optimized by individual laboratories based on clinically relevant levels of expression.

Notably, previous evaluations of the STRAT4 test have employed standardised IHC panels where inter-operator variability was minimised [22, 27, 36]. In this study, to evaluate the robustness of the assay, no attempt was made to influence the processing or histological evaluations of the samples. In addition, variation in interpretation of the IHC and the cut-offs used for positivity, use of different antibody clones and potential pre-analytical confounders (including warm and cold ischaemic time, delayed or prolonged fixation) [31, 32] may all have negatively impacted IHC interpretation.

FNA samples were also a feasible sample alternative for the STRAT4 assay and produced adequate quality results in the majority of cases. There was good concordance with FFPE for IHC and when results were compared to the FFPE results on the STRAT4 assay. Despite being a small study, the results suggest the potential of the Xpert® Breast Cancer STRAT4 Assay as an ancillary test in FNAC evaluation. Importantly, IHC is not performed directly on the FNA cytospin at our facility. Although all FNAs were evaluated by an expert cytopathologist to ensure that tumour cells were present, the possibility of sampling bias in a heterogeneous tumour population could not be excluded. To overcome this, it may be useful to obtain multiple samples from the same tumour. In addition, the manufacturer derived settings and pre-set algorithms were validated by the manufacturer on FFPE samples and optimal settings for FNAs would require validation on a much larger cohort of FNAC samples.

This study had some limitations. Sample sizes were small and for the retrospective evaluation, full clinical information was not always available. FNAC are not a registered sample type for the assay and more extensive formal validation is required prior to implementation. This pilot project did, however, confirm that performance of the assay in a middle-income country is feasible and may provide valuable diagnostic information to improve and streamline patient management.

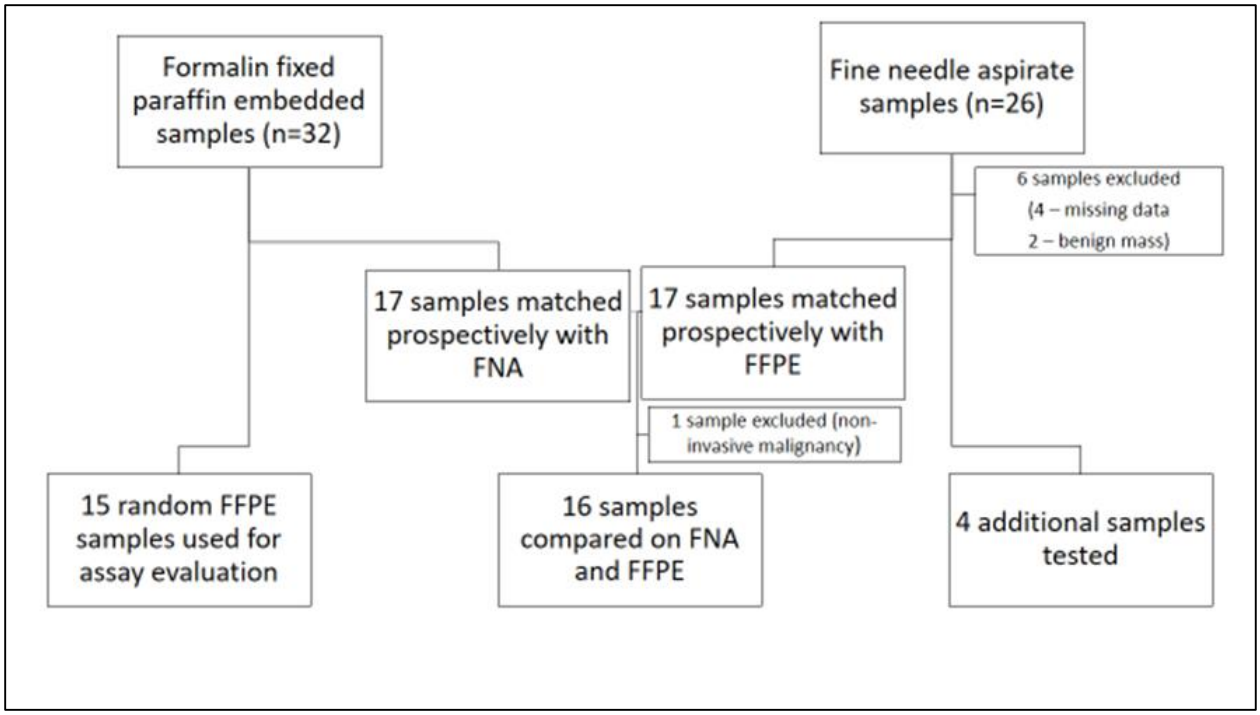


Figure 1: Summary of the procedure followed for collection of FFPE and FNAC samples. FFPE, formalin-fixed paraffin-embedded; FNAC, fine needle aspirate cytology

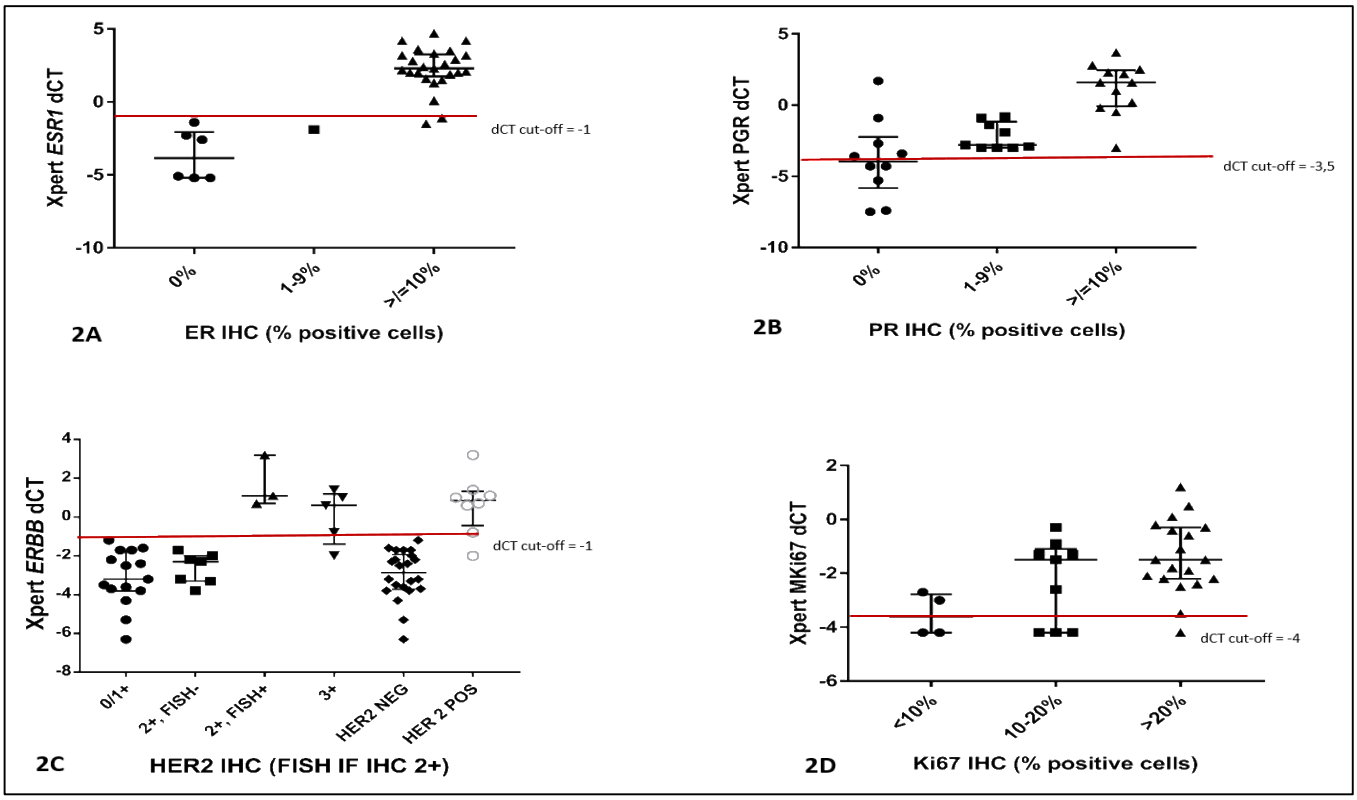


Figure 2: Comparison between IHC and/or FISH results for ER, PR, HER2 and Ki67 and mRNA expression of *ESR1* (Fig 2A), *PGR* (Fig 2B), *ERBB2* (Fig 2C) and *MKi67* (Fig 2D) derived from the Xpert® Breast Cancer STRAT4 Assay in FFPE samples.

dCT, delta CT value; ER, Oestrogen receptor; *ERBB2,* Erb-B2 receptor tyrosine kinase 2 gene; *ESR1,* Oestrogen receptor gene; FFPE, formalin-fixed paraffin-embedded; FISH, *fluorescence in-situ hybridization*; HER2, Human epidermal growth factor receptor 2; IHC, immunohistochemistry; *MKi67*, Marker of proliferation Ki67 gene; *PGR,* Progesterone receptor gene; PR, Progesterone receptor

Table 1: Biomarkers for breast cancer classification [7-12]

	HER2	ER	PR	Ki67 %	SOUTH AFRICAN PREVALENCE N=499 *	INTERNATIONAL PREVALENCE	TREATMENT	PROGNOSIS AND CLINICAL OUTCOME
Definition of positive	>10% tumour cells 3+ staining	>1% tumour cells stain positive	>1% tumour cells stain positive	>20-30% ***				
Definition of indeterminate or weak positivity	>10% tumour cells 2+ staining**	<10% tumour cells positive	<10% tumour cells positive	10-30%				
Definition of negative	0/1+ staining	<1% cells positive	<1% cells positive	<10-15%				
SUBTYPE								
Luminal A	Negative	Positive	Positive	Low <15-20%	10.9%	40-76%	Endocrine	Good
Luminal B					70.6%	11-30%	Endocrine	Intermediate
HER2-	Negative	Positive	Negative or dimly positive	High >15-20%			Chemotherapy Targeted therapy	
HER2+	Positive	Positive	Any	Any				

HER2 enriched	Positive	Negative	Negative	Any	6.5%	5-20%	Targeted therapy Chemotherapy	Poor
Triple negative/ **Normal breast-like**	Negative	Negative	Negative	Any	11.9%	11-20%	Chemotherapy PARP inhibitors	Poor

*Based on a study performed at CHBAH

** Indeterminate IHC for HER2 should be confirmed by FISH for HER2

*** Definition of Ki67% positivity varies in different studies

ER, Oestrogen receptor; FISH, fluorescence *in-situ* hybridisation; HER2, Human epidermal growth factor receptor 2; PR, Progesterone receptor

Table 2: Patient and tumour characteristics

PATIENT CHARACTERISTICS		
Median age at diagnosis (years) [range] n = 35	48 [33-94]	
Age range n (%) n = 35	<40years	7 (20,0%)
	40-49 years	13 (37,1%)
	50-59 years	6 (17,1%)
	60-69 years	4 (11,4%)
	70-79 years	4 (11,4%)
	>80 years	1 (2,9%)
SAMPLE CHARACTERISTICS		
Sample type n (%) n = 35	Mastectomy	6 (17,1%)
	Core needle biopsy	29 (82,9%)
TUMOUR CHARACTERISTICS		
Pathological tumour stage n (%) n=19 *	IA	4 (22,2%)
	IIA	6 (33,3%)
	IIB	3 (16,7%)
	IIIA	2 (11,1%)
	IIIB	1 (5,6%)
	IIIC	2 (11,1%)
Pathological tumour grade n (%) n = 35	I = well-differentiated	4 (11,4%)
	II = moderately differentiated	18 (51,4%)
	III = poorly differentiated	13 (37,1%)
Hormone receptor status n (%) n = 35	ER positive	27 (77,1%)
	PR positive	24 (68,6%)

HER2 IHC and/or FISH result n (%) n=34 **	HER2 overexpression	8 (23,5%)
Ki67 expression n=35	Ki67 expression ≥20%	27 (77,1%)
Subtype n (%) n=34 #	Luminal A: ER+, PR+, HER2-, Ki67<20%	8 (23,5%)
	Luminal B (HER2-): ER+, PR+/- or Ki67≥20%, HER2-	13 (38,2%)
	Luminal B (HER2+): ER+, HER2+ any PR and Ki67	6 (17,6%)
	HER2 enriched: ER-, PR-, HER2+	2 (5,9%)
	Triple negative: ER-, PR-, HER2-	5 (14,7%)

* Tumour staging performed after mastectomy and lymph node dissection, ** One sample was indeterminate after HER2 IHC and FISH testing, # One sample unclassifiable due to indeterminate HER2 results

ER, Oestrogen receptor; FISH, *fluorescence in-situ hybridization*; HER2, Human epidermal growth factor receptor 2; IHC, immunohistochemistry; IQR, interquartile range; PR, Progesterone receptor

Table 3: FFPE tissue samples comparison of mRNA (RT-qPCR) and protein expression (IHC)

PARAMETER	FFPE SAMPLES (n)	OVERALL PERCENT AGREEMENT (OPA) %	COHEN'S KAPPA STATISTIC (95% CI)	POSITIVE PERCENT AGREEMENT (PPA) %	NEGATIVE PERCENT AGREEMENT (NPA) %
ESR1 mRNA / ER IHC *	31	90,3%	0,739 (0,469 to 1)	88%	100%
ESR1 mRNA / ER IHC **	30	93,3%	0,811 (0,571 to 1)	91,7%	100%
PGR mRNA / PR IHC *	30 [#]	86,7%	0,646 (0,327 to 0,945)	100%	55,6%
ERBB2 mRNA / HER2 IHC or FISH [×]	30 [¥]	96,7%	0,9111 (0,741-1)	87,5%	100%
MKi67 mRNA/ Ki67% IHC [±]	31	83,9%	0,514 (0,144 to 0,884)	91,7%	57,1%
MKi67 mRNA/ Ki67% IHC [€]	22	86,4%	0,492 (0-0,984)	94,4%	50%

* IHC cut-off 1% cells ER or PR positive (as per ASCO-CAP guidelines)

** Excludes case with IHC cut-off 1-9% cells ER positive

[×] Positive if HER2 IHC 3+ or FISH positive for HER2 amplification (as per ASCO-CAP guidelines)

[±] IHC cut-off ≥ 20% cells Ki-67 positive; € IHC cut-off <10% negative and >20% Ki-67 positive. 10-20% excluded as indeterminate

[#] *PGR* result indeterminate for one sample; [¥] HER 2 IHC / FISH results indeterminate for one sample

CI, confidence interval; ER, oestrogen receptor; *ESR1,* oestrogen receptor gene; *ERBB2,* Erb-B2 receptor tyrosine kinase 2 gene; FFPE, formalin-fixed paraffin-embedded; FISH, *fluorescence in-situ hybridization*; HER2, Human epidermal growth factor receptor 2; IHC, immunohistochemistry; *MKi67*, Marker of proliferation Ki67; PR, Progesterone receptor; *PGR*, progesterone receptor gene

Table 4: FNAC samples comparison of mRNA (RT-qPCR) and protein expression (IHC)

PARAMETER	LYSIS METHOD BUFFER (n)	OVERALL PERCENT AGREEMENT (OPA) %	POSITIVE PERCENT AGREEMENT (PPA) %	NEGATIVE PERCENT AGREEMENT (NPA) %
mRNA FNA compared to FFPE IHC / FISH				
ESR1 mRNA / ER IHC *	M (n=19)	84,2%	85,7%	80%
	Q (n=16)	81,3%	83,3%	75%
ESR1 mRNA / ER IHC **	M (n=18)	88,9%	92,3%	80%
	Q (n=15)	86,7%	90,9%	75%
PGR mRNA / PR IHC *	M (n=18) #	94,4%	91,7%	100%
	Q (n=16)	81,3%	72,7%	100%
ERBB2 mRNA / HER2 IHC or FISH ×	M (n=19)	78,9%	50%	82,4%
	Q (n=16)	81,3%	50%	100%
M Ki67 mRNA/ Ki67% IHC ±	M (n=18) #	66,7%	66,7%	66,7%
	Q (n=16)	62,5%	64,3%	50%
mRNA FNA compared to mRNA FFPE				
ESR1 mRNA / ER IHC *	M (n=15)	93,3%	100%	80%
	Q (n=12)	91,7%	100%	75%
PGR mRNA / PR IHC *	M (n=14) #	85,7%	81,8%	100%
	Q (n=12)	75%	70%	100%
ERBB2 mRNA / HER2 IHC or FISH ×	M (n=15)	80%	100%	78,6%
	Q (n=12)	76,9%	100%	81,8%
M Ki67 mRNA/ Ki67% IHC ±	M (n=14) #	64,3%	63,6%	66,7%
	Q (n=12)	66,7%	70%	50%

* IHC cut-off 1% cells ER or PR positive (as per ASCO-CAP guidelines); ** Excludes case with IHC cut-off 1-9% cells ER positive.

[×] Positive if HER2 IHC 3+ or FISH positive for HER2 amplification (as per ASCO-CAP guidelines); [±] IHC cut-off ≥ 20% cells Ki-67 positive; € IHC cut-off <10% negative and >20% Ki-67 positive. 10-20% excluded as indeterminate; [#] *PGR* and M Ki67 result indeterminate for one sample.

ER, oestrogen receptor; *ERBB2,* Erb-B2 receptor tyrosine kinase 2 gene; FFPE, formalin-fixed paraffin-embedded; FISH, fluorescence *in-situ* hybridization; FNAC, fine needle aspirate cytology; HER2, Human epidermal growth factor receptor 2; IHC, immunohistochemistry; M, Maui lysis method; PR, Progesterone receptor; Q, Quick lysis method

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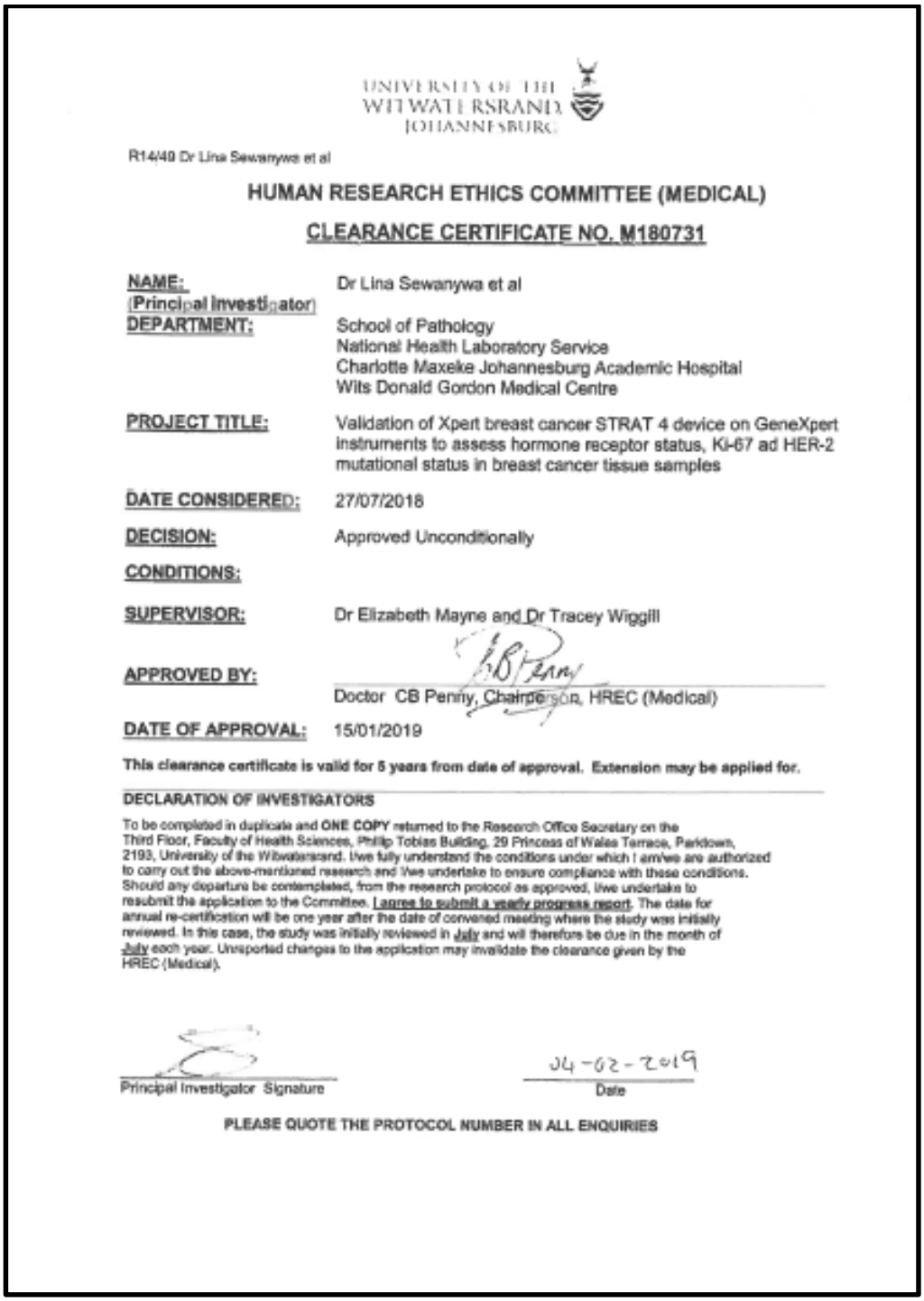
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6. APPENDICES

6.1 APPENDIX A: ETHICS CLEARANCE CERTIFICATE



UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/40 Dr Lina Sewanywa et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M180731

NAME: (Principal Investigator)	Dr Lina Sewanywa et al
DEPARTMENT:	School of Pathology National Health Laboratory Service Charlotte Maxeke Johannesburg Academic Hospital Wits Donald Gordon Medical Centre
PROJECT TITLE:	Validation of Xpert breast cancer STRAT 4 device on GeneXpert instruments to assess hormone receptor status, Ki-67 ad HER-2 mutational status in breast cancer tissue samples
DATE CONSIDERED:	27/07/2018
DECISION:	Approved Unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Elizabeth Mayne and Dr Tracey Wiggill
APPROVED BY:	Doctor CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	15/01/2019

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

DECLARATION OF INVESTIGATORS

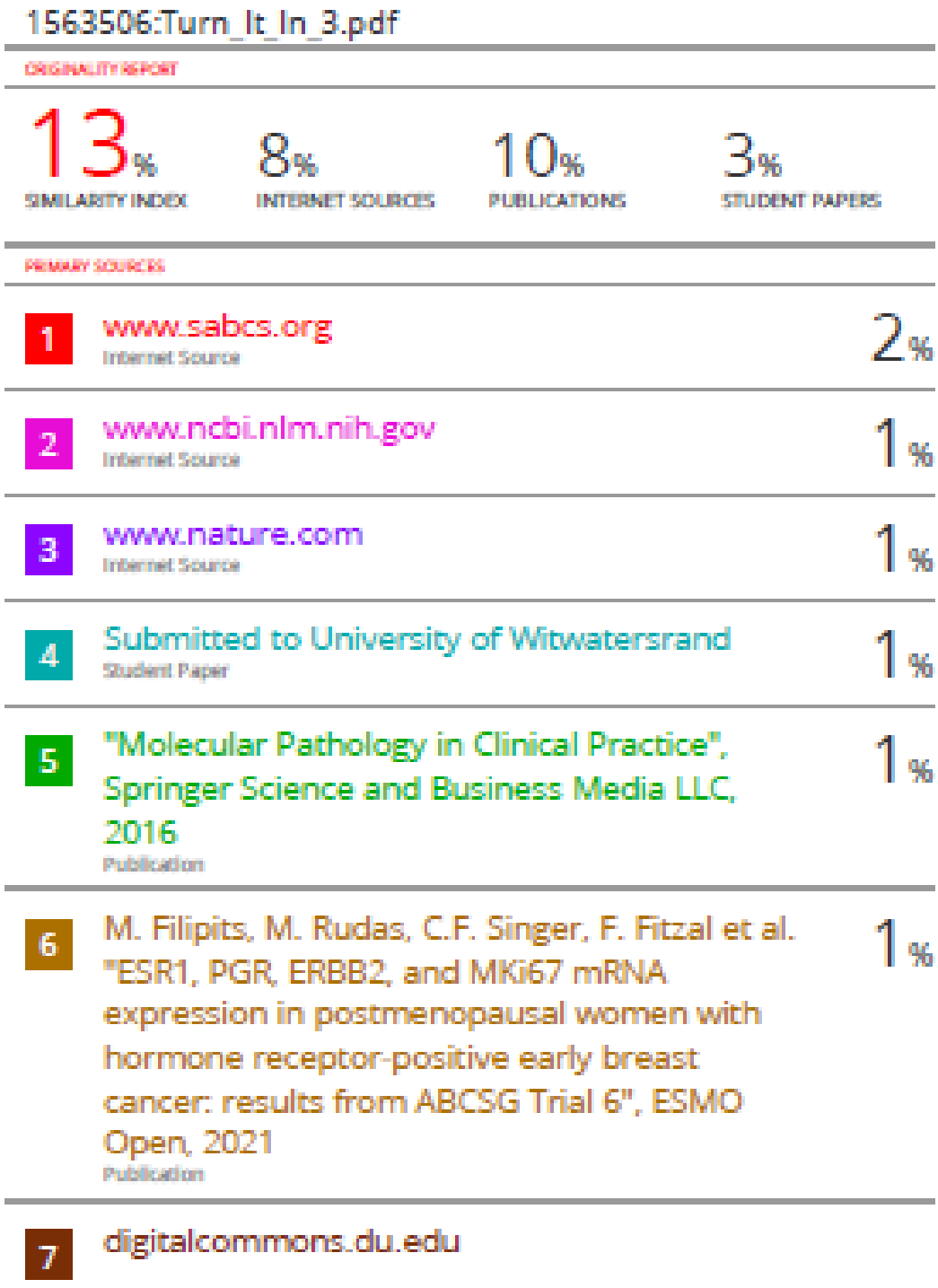
To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are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

Principal Investigator Signature

04-02-2019
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

6.2 APPENDIX B: Turn-it-in Report



1563506:Turn_It_In_3.pdf

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

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4	Submitted to University of Witwatersrand Student Paper	1%
5	"Molecular Pathology in Clinical Practice", Springer Science and Business Media LLC, 2016 Publication	1%
6	M. Filipits, M. Rudas, C.F. Singer, F. Fitzal et al. "ESR1, PGR, ERBB2, and MKi67 mRNA expression in postmenopausal women with hormone receptor-positive early breast cancer: results from ABCSG Trial 6", ESMO Open, 2021 Publication	1%
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13	Susan Mahler Zneimer. "Breast cancer – HER2 FISH analysis", Wiley, 2014 Publication	< 1 %
14	"Breast", Laboratory Investigation, 2013. Publication	< 1 %

15	Lulu Sun, Molly C. Schroeder, Ian S. Hagemann, John D. Pfeifer et al. "Expression of Potential Biomarker Targets by Immunohistochemistry in Cervical Carcinomas", International Journal of Gynecological Pathology, 2022 Publication	<1 %
16	Junxian Li, Chenyang Li, Ziwei Feng, Luyang Liu et al. "Effect of Estradiol as a Continuous Variable on Breast Cancer Survival by Menopausal Status: a Cohort Study in China", Research Square Platform LLC, 2021 Publication	<1 %
17	bjp.sagepub.unboundmedicine.com Internet Source	<1 %
18	Ming Ni, Xiaoming Zhou, Jingwei Liu, Haiyang Yu, Yuanxiang Gao, Xuexi Zhang, Zhiming Li. "Prediction of the clinicopathological subtypes of breast cancer using a Fisher discriminant analysis model based on radiomic features of diffusion-weighted MRI", Research Square, 2019 Publication	<1 %
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24	Witness Mapanga, Shane A Norris, Ashleigh Craig, Oluwatosin A. Ayeni et al. "Drivers of Disparities in Stage at Diagnosis Among Women With Breast Cancer: South African Breast Cancers and HIV Outcomes Cohort", Research Square Platform LLC, 2021 Publication	<1 %
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26	L Kiesel. "Expression of Endothelin-A-Receptor predicts unfavourable response to neoadjuvant chemotherapy in locally advanced breast cancer", British Journal of Cancer, 08/02/2004 Publication	<1 %
27	Soo Jung Lee, Jeeyeon Lee, Wan Wook Kim, Jin Hyang Jung, Ho Yong Park, Ji-Young Park,	<1 %

Yee Soo Chae. "Del-1 Expression as a Potential Biomarker in Triple-Negative Early Breast Cancer", Oncology, 2018
Publication

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Exclude bibliography	On		

6.3 APPENDIX C: APPROVED RESEARCH PROTCOL

Validation of the Xpert® Breast Cancer STRAT 4 assay on the GeneXpert instrument to assess hormone receptor status, Ki-67 and HER-2 mutation status in breast cancer tissue samples

Lina Sewanywa

STUDENT NUMBER: 1563506

MMED: Haematology

Research Protocol

Dr Elizabeth Mayne MBBCh (Wits); MMed Haematology (Wits)

Dr Tracey Wiggill MBBCh (Wits); MMed Haematology (Wits)

Prof Martin Hale MBChB, LRCP (Edin), LRCS (Edin) LRCP&S (Glas), FCPath

1. INTRODUCTION / BACKGROUND

Cancer is an acquired genetic disease characterised by uncontrolled proliferation. This may occur in solid tissue (for example carcinomas in epithelial cells and sarcomas) or in blood (leukaemias and lymphomas). Cancer arising in solid organs can expand locally by tissue invasion or systemically by metastasis. Tumour cells may arise de novo or be preceded by a premalignant state (for example, carcinomas *in situ*), as with ductal carcinoma in situ (DCIS) or *de novo* (1). Cancer is the second leading cause of death worldwide with 8.2 million people dying from this disease in 2012, as reported by the World Health Organisation (2).

Breast cancer, arising most commonly from the epithelial cells lining the ducts in breast, is the most common type of cancer in women and causes the highest number of cancer-related deaths in women in spite of efforts to improve detection at earlier stages where treatment strategies are less aggressive and outcomes are better (3). In the United Kingdom, the National Health System breast cancer screening programme was associated with a decrease in breast cancer mortality by 21% from 1991 - 2005 (4). The beneficial effects of screening are illustrated in other neoplasms as well. For example, a modelling study by J. Lew et al, which looked at the effects of the nation-wide population screening for colorectal cancer, showed the National Bowel Screening programme has the potential to prevent 92 200 cancer cases and 59000 deaths over a period 2015 – 2040 (5) Currently, breast cancer screening is reliant on patient self-examination and mammography although access to mammography in many settings may be restricted (6,7).

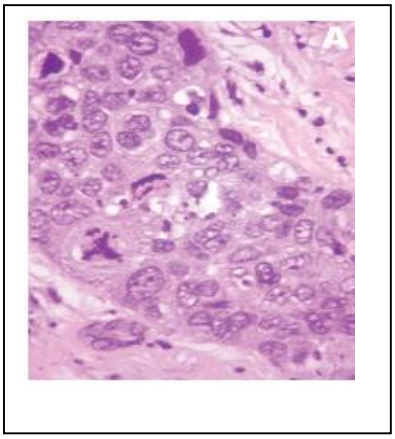


Figure 1.1 Breast cancer histology

Image showing stained breast tissue biopsy of a high grade invasive ductal carcinoma. Image adapted from http://www.pathologyoutlines.com/topic/breastmalignantsuperpagebreastcancer.html

The diagnosis of breast cancer is histological, see figure 1.1. Auxiliary investigations detect hormone receptor (HR) status: specifically, the oestrogen receptor, progesterone receptor (PR) and the human epidermal growth factor receptor (HER-2 or ERBB2) expression by breast cancer cells (3,6). Detection can be molecular or by immunohistochemistry. HR expression impacts prognosis and is used for subtyping (8). ER positive breast cancers responds to hormone

blocking therapies like tamoxifen which competitively inhibits oestrogen receptors (9). Some evidence suggests that progesterone positive tumours may also respond favourably to hormone blockade (10). HER-2 expression is associated with more aggressive tumours and a poorer prognosis (3,8). These tumours may respond to biological agents which block this receptor (e.g. Herceptin) (6). Table 1.1 further illustrates breast cancer subtyping.

Table 1.1 Breast cancer subtyping

Subtype	Ki -67 (Indication of histological grade)	ER status	PR status	Her-2/ Neu status	Prognosis
Luminal A	Low	+	+	-	Good/ Best
Luminal B	High	+	+	-/ +	Better for Her-2+
HER-2+/ erbB2+	Any	-	-	+	Poor
Triple negative/ Basal tumours	Any	-	-	-	Poor

(+): Positive, (-): Negative, (-/+): Positive or negative. Triple negative breast cancers are associated with breast cancer susceptibility gene 1 (BRCA1) mutations which increase the cancer risk from that of the normal 10% to as high as 70% (3,12).

Ki-67 is a proliferation marker that identifies actively dividing cells in histology tissue samples (11). Tumours with a greater percentage of cells that are actively dividing are more aggressive and will have a higher ki-67 (given as a percentage of the total tumour cell population). In breast cancer, ki-67 is useful in early identification of more aggressive tumours (11).

1.1 BREAST CANCER IN THE SOUTH AFRICAN CONTEXT

Breast cancer incidence is reported to be higher in developed compared to less developed countries, but the impact of under-reporting from less developed countries is not clear (13). The World Health Organization-International Agency for Research on Cancer (WHO/IARC) reports increasing trends world-wide (14). Breast cancer mortality is higher in developing countries with up to 58% of breast cancer related deaths occurring in developing countries (15). The South African (SA) National Cancer Registry (NCR) is a pathology-based registry, established in 1986, which provides SA national cancer statistics. The most recent report, released in 2013, indicated that breast cancer accounts for 11% of all cancers and 22% in women with an age-standardised incidence of 32.6 per 100 000 per year (16). Breast cancer in South African females is more common at younger ages, presents at a more advanced stage and has a higher mortality than in more developed countries (17–19). Breast cancer prognosis is more favourable in higher income countries, with an overall five-year survival rate of 80%, compared to lower and middle income countries with a five–year survival rates of approximately 50% (3,17). Factors contributing include poor early detection strategies, barriers to receiving treatment as well as advanced disease at presentation (15,17). The newly released South African Breast Cancer policy guidelines aim to address these challenges by improving breast cancer detection and access to health care (6).

A recent systematic review of breast cancer receptor subtypes in populations in Africa, found that despite heterogeneity in the ER and PR status, overall, the majority of breast cancer in Africa are ER positive (18). Recent studies in South Africa have found similar hormone receptor profiles – suggesting that most women present with Luminal A tumours, however a greater proportion of black women had HER-2 positive and triple negative breast cancer (20). Table 1.2 summarises the findings of HR and HER-2 status in this study.

1.2 DIAGNOSIS OF BREAST CANCER

Screening strategies are essential for early detection of breast cancer, which results in better patient outcomes. Improving education and awareness, genetic testing in high risk patients, breast examination and mammography form part of the screening strategies used. In South Africa, diagnosis of breast cancer is based on a triple assessment of clinical examination, imaging and histological confirmation with molecular testing where available (6).

Table 1.2 Hormone receptor status at a single centre in Johannesburg South Africa in patients diagnosed between 2006 – 2012.

	Incident cases breast cancer from 2006 – 2012	ER Positive	PR Positive	HER-2 Positive	Luminal A	Luminal B	Erb2+	Triple Negative
Number of cases	1247 (%)	696 (64.9%)	567 (53.1%)	267 (26%)	551 (53.7%)	150 (14.6%)	117 (11.04%)	209 (20.4%)

Table adapted from McCormack VA, Joffe M, van den Berg E, Broeze N, dos Santos Silva I, Romieu I, et al. Breast cancer receptor status and stage at diagnosis in over 1,200 consecutive public hospital patients in Soweto, South Africa: a case series. Breast Cancer Res [Internet]. 2013 Oct 17;15(5):R84. Available from: http://breast-cancer-research.biomedcentral.com/articles/10.1186/bcr3478

With regards to histological assessment, core needle biopsy (CNB) is considered the gold standard (3,6). Fine needle aspiration cytology (FNAC) is also in widespread use particularly in screening of breast lumps and as a cheaper diagnostic alternative (6,21). Limitations of FNAC include inability to definitively differentiate "grey-zone" lesions (which contain atypical cells that may represent malignant or benign tumours) (22,23). Advantages of FNAC include lower cost, faster procedure time, faster turnaround time acceptable sensitivity and specificity. These make FNAC an attractive diagnostic modality, particularly in lower and middle-income countries and FNAC has been included in South Africa's breast cancer prevention and control policy. The fact that molecular testing in the form of HR and HER-2 can be performed on FNAC is of particular interest in our setting (22).

Immunohistochemistry is the historic gold-standard for determining HR and Ki-67 status in breast cancer tissues. However, this technique is known to have significant degree of variability (24). Processing requirements and staff constraints can result in reporting times 5-7 days on average. Equivocal HER-2 results (HER-2 value of 2+) necessitates repeat evaluation by *Fluorescent in situ hybridisation* (FISH). Large published series suggest that approximately 50% of cases will give equivocal results for HER-2 status and require FISH analysis (25,26). Immunohistochemical testing identifies excess amount of the protein product whilst, FISH identifies amplification in the genes responsible for HER-2 formation by visualisation of fluorescently labelled probes that bind to targeted DNA sequences.

1.3 MOLECULAR TESTING

Adjunctive molecular testing has been increasingly used in cancer diagnosis and prognostication (27). Molecular testing offers more accurate testing processes with earlier identification of disease and prognostic information with more effective tailoring of therapeutic options (10,28).

1.3.1 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) - BASED ASSAYS

Reverse transcription Polymerase Chain Reaction assays analyse messenger RNA (mRNA) levels of different gene products thus assessing gene expression. Examples of commercially available RT-PCR assays in breast cancer include Mammaprint ™ and Oncotype DX ® (28). The HERmark ™ assay measures HER2 protein (H2T) and functional HER2 homodimer (H2D) levels on the cell surface of FFPE breast cancer tissue using fluorescent labelled tags which are quantified using capillary electrophoresis (10).

The GeneXpert® platform (Cepheid), is an automated system that performs semi-quantitative RT- PCR on samples. Samples are inserted into a cartridge which contains the reagents and PCR components needed to run the test (29). The GeneXpert® MTB/RIF assay has been widely implemented for detection of Mycobacterium Tuberculosis and to identify strains with resistance to Rifampicin. This assay is currently endorsed by WHO as an initial diagnostic tool for the assessment of HIV-associated TB, particularly in high risk countries. In South Africa, the GeneXpert® MTB/RIF has a footprint of 314 devices spread across 207 microscopy centres in the country and has played a role in improving management of the disease since its national roll-out in 2011 (30). With such a wide footprint and advantage of improved sensitivity in detecting molecular markers, this platform has offerings including providing assays for detection of molecular markers in cancer (31).

Xpert® Breast Cancer STRAT 4 Assay

The Xpert® Breast Cancer STRAT4 Assay is a (RT-qPCR), cartridge-based assay, that runs on the GeneXpert® machine. It measures mRNA levels of ER, PR, HER-2 and Ki-67 in formalin-fixed paraffin embedded (FFPE) breast tissue samples. The assay offers a fast and accurate detection of these biomarkers with a high concordance with standardised methods (IHC and FISH) routinely used to detect these markers (29,32). This assay still requires evaluation in a South African setting and there have been limited studies in other sample types including FNA samples.

2. STUDY AIM AND OBJECTIVES

The aim of this study is to validate the Xpert® Breast Cancer Strat 4 for determining HR and Ki-67 status as well as assessing for HER-2 mutations in FFPE breast cancer tissue samples. In addition, this study aims to assess the ability of the Xper® t Breast Cancer STRAT 4 to determine HR, Ki-67 and HER-2 status from breast cancer FNAC samples.

The objectives of this study are to be carried out in a retrospective and prospective arm as follows:

2.1 RETROSPECTIVE ARM

1. To assess HR and Ki-67 status as well as HER-2 mutations in breast cancer FFPE tissue samples, which were previously tested using standardised techniques (immunohistochemistry for HR and Ki-67 status and immunohistochemistry and / or FISH for HER-2 mutation), using the Xpert Breast Cancer STRAT 4 assay.

2. To compare results obtained from the Xpert® Breast Cancer STRAT 4 assay in testing these samples to those obtained using routine standard techniques.

2.2 PROSPECTIVE ARM

1. To optimise the methodology (including extraction techniques) for FNAC breast cancer specimens on the Xpert Breast Cancer Strat 4 assay in consultation with Cepheid Oncology Research Development team.

2. To assess HR and Ki-67 status as well as HER-2 mutations in breast cancer FNA specimens, using the Xpert Breast Cancer Strat 4 assay.

3. To assess the concordance of results of the IHC marker status on FNAC samples obtained from the Xpert® Breast Cancer STRAT 4 assay, to the IHC results obtained from the initial/diagnostic breast cancer tissue sample through standardised techniques.

3. METHODS

3.1 SITE OF STUDY

The study will involve three tertiary hospitals in Johannesburg, namely Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), Chris Hani Baragwanath Academic Hospital (CHBAH) and The Wits Donald Gordon Medical Centre (DGMC), a private teaching hospital. Human research ethics approval will be obtained before any sample collection occurs.

Processing and analysis of the samples will take place centrally at the NHLS laboratory of Charlotte Maxeke Academic Hospital.

3.2 SAMPLE SIZE

75 samples will be analysed:

1. 25 residual breast cancer FFPE tissue slides
2. 25 residual breast cancer FFPE tissue slides (matched to the cytology slides)
3. 25 residual FNAC samples with tumour cells

A sample size of 25 allows for a margin of error of 14% for this study with a confidence interval of 95%. This was established to be acceptable on personal communication with the developers of the assay.

3.3 SAMPLE MATERIAL

This study will comprise residual FFPE samples collected from the department of Anatomical Pathology. The diagnostic results of these tissues using standardised techniques (IHC and/or FISH), will be retrieved from the laboratory Information System (LIS). Briefly, the samples will comprise 5 -10 um unstained tissue sections prepared on glass slides and processed on the STRAT4 assay as per the manufacturer's protocol. The results obtained from the STRAT4 assay will be compared to those obtained using IHC and / or FISH. Comparison of results will be performed once samples have been analysed on the STRAT4 assay.

Residual FNAC samples with breast tissue cells will be collected from the department of cytology.

3.4 INCLUSION/ EXCLUSION CRITERIA

3.4.1 INCLUSION CRITERIA

1. Residual FFPE tissue blocks with sufficient material to make two 5-10um slides with areas representative of the tumour cell.
2. Use of the FFPE tissue block in this study will not exhaust all the residual material.
3. The slides prepared from breast cancer FFPE tissues have approximately 30% of the tissue represented by tumour cells or will have this amount post macro-dissection.
4. FNAC samples contain breast tissue cells

3.4.2 EXCLUSION CRITERIA

Applicable to retrospective arm:

1. Male patients with breast cancer
2. FFPE breast tissue samples with no IHC and / or FISH assessments or results for HR, KI-67 and HER-2 status using standardised techniques.

3.5 TEST OPTIMISATION

The primary investigator will receive focussed training on sample processing and optimisation of the assay using different lysis buffers in Sunnyvale California, under the guidance of Cepheid scientists. Samples included will be 20 FNA and 5 FFPE samples and all material will be covered with a material transfer agreement.

3.6 SAMPLE PREPARATION

3.6.1 FFPE samples:

1. Two 5 – 10µm sections will be cut using a microtome from breast cancer FFPE tissues
2. Cut slides will be overlaid onto respective diagnostic slides with IHC staining to ensure they are representative of the tumour area
3. Tumour tissue will be scraped off and placed in a 1.5mL tube and 1.2mL of lysis buffer will be added followed by 20 µLProteinase K (PK).
4. Sample will be vortexed and incubated at 80° and micro-centrifuged to remove liquid from the lid.
5. Contents will be added to 95% ethanol to 5mL vial and vortexed to produce the FFPE lysate.
6. The sample will be added into the Xpert® STRAT4 cartridge which will be put into the GeneXpert® chamber for analysis (approximately 75 minutes).
7. Results will be captured before comparison to results obtained using IHC and or FISH.

3.6.2 FNAC samples

1. FNA samples will be collected and spread onto slides (10 per patient) and allowed to air dry.
2. One of the FNAC samples will be stained to confirm the presence of tumour cells.
3. Breast tissue FNA sample will be scraped off the slide and placed in a 1.5 mL tube.
4. Samples will be processed as for FFPE although lysis buffer and PK volumes will be reduced.
5. Additional slides will be used for troubleshooting and optimisation.

3.7 CONTROLS

The Xpert® Breast Cancer STRAT 4 assay has a built-in sample adequacy or quality control ensuring the amount of RNA in the sample is sufficient to obtain adequate results.

3.8 BIAS ELIMINATION

To minimize bias in specimen analysis by the Xpert® Breast Cancer STRAT4 Assay, the following practices shall be employed:

Prospective samples will be analysed using the STRAT4 assay prior to retrieving and comparison of diagnostic results.

4. PATIENT DATA COLLECTION

For each sample, the following data will be collected. Please see Appendix A.

1. Date Xpert® Breast Cancer STRAT4 performed
2. Ethnicity
3. Age at diagnosis
4. Family history of breast cancer
5. Year diagnosed with breast cancer
6. Tumour histology Tumour stage at diagnosis
7. Tumour size
8. Tumour grade
9. Nodal status: Positive or negative (if positive, number of positive nodes)
10. IHC results for: ER, PR, HER2 and Ki67
11. HER2 FISH analysis
12. Results obtained for ER, PR, HER2 and Ki67 using STRAT4 assay
13. Any additional genetic testing (e.g BRCA1 mutation)

5. CONSENT

Patient consent will be obtained for the use of FNAC samples for analysis in this study and by Cepheid developers abroad. Consent will not be required for use of the breast cancer FFPE tissue samples as these are residual samples.

Patient identity will be kept confidential by use of a unique sample number for all specimens. Where FNA and FFPE tissue samples belong to the same patient, a similar identification number will be used to aid data analysis.

6. DATA ANALYSIS

The Cepheid sponsors will be available to assist with data analysis. Results obtained from the Xpert® Breast Cancer Strat 4 assay will be compared to those obtained through standardised techniques using a standard 2 x 2 table.

Calculation of the positive predictive value, negative predictive value and overall concordance of the Xpert® Breast Cancer STRAT4 in breast cancer FFPE tissues as well as its utility for FNAC samples will be recorded. Receiver operator curves (ROC) will be used to plot each marker tested and will include the area under the curve, the standard error, 95% confidence interval and p values. A Kappa statistic will be calculated to rate the overall agreement between the different methods.

7. ETHICS

An application has been made to the Human Research Ethics Committee (HREC) and is pending.

Age without birth dates will be submitted and all data will always be kept confidential.

8. TIMING

Table 8.1 Study period

TASK	MONTHS (2019 – 2020)											
	03	04	05	06	07	08	09	10	11	12	01	02
Training on specimen preparation												
Data collection and processing												
Data Analysis												
Write up & manuscript												

1. FUNDING

An application will be made to the University of Witwatersrand for an Individual Research grant.

- The Xpert Breast Cancer STRAT 4 cartridges will be sponsored and supplied by Cepheid ™ (Sunnyvale, CA).
- GeneXpert instrumentation already present at the hospital centre will be used for running samples.

Table 9.1 Estimated costs

Item required	Rands
STRAT4 assay cartiridges	~ Funded by Cepheid
FFPE lysis reagent	~ Funded by Cepheid
Proteinase K	~ Funded by Cepheid
1.5mL tubes	~ R1100/ x 500 units (x1)
5mL vials	~ R1400/x 500 units (x1)
20mL syringes	~ R60/ box of 100 (x 1)
Needles	~ R400/box of 50 (x 2)
Pipette tips without aerosol filters	~ R100/bag of 1000 (x 2)
Pipette tips with aerosol filters	~ R1400/bag of 50 (x2)
Glass slides	~ R200/box 50 (x10)
Disposable gloves	~ R80/box 100 (x3)
Sterile gloves	~ R100/box 50 (x3)
Boards/containters to transporting slides in	~ R200/tray (x10)
Fuel for travelling to respective hospitals	~ R1600
Travel to Sunnyvale California	~ Self funded
Total	~ R 12240

2. LIMITATIONS

1. Testing FNAC samples with the Breast Cancer Xpert® STRAT4 assay has not previously been documented and this will be a pilot study to assess its utility for this, thus optimisation of this technique may require trouble shooting with possible adjustment to the methodology.
2. Obtaining results from FNAC samples may be hampered by sample integrity/stability.

Limited availability of FNAC samples, which is determined by the number of patients that undergo breast surgery, may be overcome by collection of samples from three tertiary level hospitals.

3. Training for use of histopathology equipment may require longer period.

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