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Developing Paper-Based Enzyme-Linked Immunoassays for Highly Sensitive Diagnosis at the Point-of-Care

By

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ABSTRACT

Developing Paper-Based Enzyme-Linked Immunoassays for Highly Sensitive Diagnosis at the Point-of-Care

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Cervical cancer is a leading cause of cancer death among women in low-resource settings, largely due to disparities in the availability and affordability of cancer screening and early detection programs. Access to screening and diagnostic tests are often limited by high per-test costs, infrastructure requirements, and the need for highly trained personnel. Because of this, over 85% of cervical cancer deaths occur in resource-limited areas.

This thesis describes the development of a highly sensitive paper-based enzyme-linked immunoassay (ELISA) platform that is low-cost and easy-to-use. The platform is then applied towards developing two point-of-care assays for cervical cancer: a human papillomavirus (HPV) DNA assay and an HPV E7 oncoprotein assay for cervical cancer screening and diagnosis respectively, as HPV is the etiologic agent for most cervical cancers.

First, this work presents the development of an HPV DNA paper assay. The assay is low-cost, does not require expensive equipment or infrastructure, and runs within an hour. Moreover, the assay is easy-to-use, with seven user steps and acceptable System Usability

Scale (SUS) scores from usability assessments in El Salvador and Mozambique. Furthermore, the assay has equivalent sensitivity to the gold standard *digene* Hybrid Capture 2 assay. In a set of 16 clinical samples, the point-of-care assay was able to determine HPV status with 93.75% accuracy.

Next, this work describes an HPV E7 paper oncoprotein assay for higher specificity in diagnosis of precancerous lesions. Similar to the HPV DNA paper assay, the HPV E7 paper assay has a low per-test cost, no infrastructure requirements, and can be completed in five simple user steps. In a set of 10 clinical samples, the sample-to-answer assay was able to detect CIN2+ samples with 90% accuracy.

In combination, the paper-based HPV DNA and oncoprotein tests demonstrate comparable performance to gold standard technologies in a point-of-care format appropriate for use in resource-limited settings. After additional clinical validation, these assays have the ability to improve access to cervical cancer screening and diagnosis in resource-limited settings.

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"And whatever you do, in word or deed, do everything in the name of the Lord Jesus, giving thanks to God the Father through him."

- Colossians 3:17

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Nomenclature

ACP	Achromopeptidase
AIS	Adenocarcinoma in-situ
ASCUS	Atypical squamous cells of undetermined significance
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CIN	Cervical intraepithelial neoplasia
DAB	3,3'- Diaminobenzidine
DNA	Deoxyribonucleic acid
DPI	Dots-per-inch
ELISA	Enzyme-linked immunoassay
FBS	Fetal bovine serum
HC2	Hybrid Capture 2
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSIL	High-grade squamous intraepithelial neoplasia
IRB	Institutional Review Board
LEEP	Loop electrosurgical excision procedure
LFA	Lateral flow assay
LMICs	Low- and middle-income countries
LSIL	Low-grade squamous intraepithelial neoplasia
LOD	Limit of detection
mRNA	Messenger ribonucleic acid

NILM	Negative for intraepithelial lesion or malignancy
NPV	Negative predictive value
NS	Not significant
NLC	No lysis control
PBST	Phosphate Buffered Saline with 0.05% Tween 20
Pf HRP2	*Plasmodium falciparum* histidine-rich protein 2
RDT	Rapid diagnostic test
RNA	Ribonucleic acid
ROI	Region-of-interest
SBR	Signal-to-background ratio
SUS	System Usability Scale
TMB	3,3',5,5'-Tetramethylbenzidine
2DPN	Two-dimensional paper network
VIA	Visual inspection with acetic acid
VILI	Visual inspection by Lugol's iodine
WHO	World Health Organization

Chapter 1

Introduction

1.1. Objectives and Specific Aims

This dissertation aims to develop a paper-based enzyme-linked immunoassay (ELISA) platform with gold standard sensitivity and to translate that platform towards sample-to-answer and point-of-care technologies for screening and diagnosis of cervical cancer and precancer in resource-limited settings. The specific aims are as follows:

Specific Aim 1: Design a low-cost, paper-based assay that is equivalent in sensitivity to a traditional 96-well ELISA

Specific Aim 2: Develop a sample-to-answer HPV DNA paper assay that detects high-risk HPV for cervical cancer screening in resource-limited settings

Specific Aim 3: Develop a point-of-care HPV E7 paper oncoprotein assay for more specific diagnosis of cervical neoplasia in resource-limited settings

1.2. Overview

This dissertation is organized as follows:

Chapter 2 provides a summary of cervical cancer screening and diagnostic protocols used in low-resource settings and highlights the need for point-of-care and low-cost technologies for HPV DNA and HPV oncoprotein detection in resource-limited settings. Sections of chapter 2 were previously published in *Expert Review of Molecular Diagnostics* with additions and revisions included in this chapter.

Chapter 3 describes the development of a paper ELISA platform with equivalent sensitivity to a traditional 96-well ELISA gold standard assay. The platform is low-cost, runs within 90 minutes, and does not require infrastructure or expensive machinery to achieve highly sensitive detection. The contents of this chapter were previously published in *Analytical Chemistry* and have been rewritten for the purpose of this thesis.

Chapter 4 describes a point-of-care, sample-to-answer HPV DNA paper assay which is able to detect high-risk HPV DNA with equivalent sensitivity to commercially available hybrid capture HPV DNA tests. The assay is evaluated with synthetic DNA, cellular materials, and with clinical samples collected from a screening population in San Salvador, El Salvador in a small pilot study.

Chapter 5 describes a sample-to-answer, low-cost HPV E7 paper oncoprotein assay that can be performed in five simple user steps without the need for instrumentation. The assay was evaluated with cellular materials as well as with clinical samples collected from a referral population in Houston, TX in a small pilot study.

Chapter 6 provides a summary of the research described in this thesis with suggested future research directions.

Chapter 2

Cervical Cancer Screening and Diagnosis with Human Papillomavirus in High-Resource and Low-Resource Settings

Sections of this chapter were previously published in the journal article: KA Kundrod, CA Smith, B Hunt, RA Schwarz, K Schmeler, R Richards-Kortum. Advances in technologies for cervical cancer detection in low-resource settings. Expert Rev Mol Diagn. 2019;19:695-714. PMID: 31368827. Additions and revisions have been included in this chapter.

2.1. Abstract

Cervical cancer is a preventable disease with a disproportionate burden in low- and-middle- income (LMIC) countries, mainly due to disparities in the availability and affordability of HPV vaccines and cancer screening and early detection programs. While screen-and-treat programs can be implemented in resource-limited areas, these screening technologies are often limited either by performance variability, in the case of visual inspection with acetic acid (VIA), or by the cost and infrastructure requirements of human papillomavirus (HPV) testing. Additionally, a lack of confirmatory diagnosis after screening can lead to overtreatment due to poor specificity of screening tests. In this review, I describe screening and diagnostic technologies currently used in resource-limited settings, as well as novel technologies being developed. Finally, I discuss the need for truly point-of-care and low-cost screening and diagnostic tests to enable rapid and effective screening in LMICs and improve access to cervical cancer detection programs.

2.2. Introduction

2.2.1. Cervical Cancer Incidence and Mortality

Cervical cancer is a disease that can be detected and treated early, greatly improving outcomes for women. However, cervical cancer remains a leading cause of cancer death among women in the developing world [1–3]. Each year, 570,000 new cases are detected, and approximately 311,000 women die (**Figure 2-1)** [3]**.** Over 85% of these deaths occur in low-resource areas, mainly due to disparities in the availability and affordability of cancer screening and early detection programs [4,5], and this percentage is predicted to reach 95%

by 2030 [6]. In contrast, the implementation of screening tests in the United States have decreased the number of deaths due to cervical cancer by over 60% since the 1940s [7].

Cervical cancer can be prevented through vaccination against high-risk human papillomavirus (HPV), the etiologic agent for cervical cancer, and through screening, diagnosis, and treatment of precancerous lesions. Although an effective vaccine has been developed, many women do not have access to the vaccine, and global vaccination rates remain low [8,9]. The expensive cost ($420 to $720) is especially prohibitive to those living in non-GAVI eligible countries where subsidies for the vaccine are absent, or those living in low-resource areas of higher income countries like the United States [10]. Recent reports show that less than half of eligible children in the US undergo HPV vaccination [11,12]. In addition, HPV vaccines do not treat pre-existing infections, and millions of women who did not receive the vaccine at an early age are at risk for developing cervical cancer and in need of low-cost diagnostics [13,14]. Therefore, screening, diagnosis, and treatment methods for cervical cancer and precancer will be needed for the foreseeable future.

In this review, I first describe the standard of care for screening and diagnosis in high- and low-resource settings; I review biomarkers associated with cervical precancer and cancer; and I discuss needs for new tests. I then discuss promising new technologies that could increase access to cervical cancer screening and diagnosis in LMICs. Finally, I discuss the need for continued innovation to reduce rates of cervical cancer incidence and mortality globally.

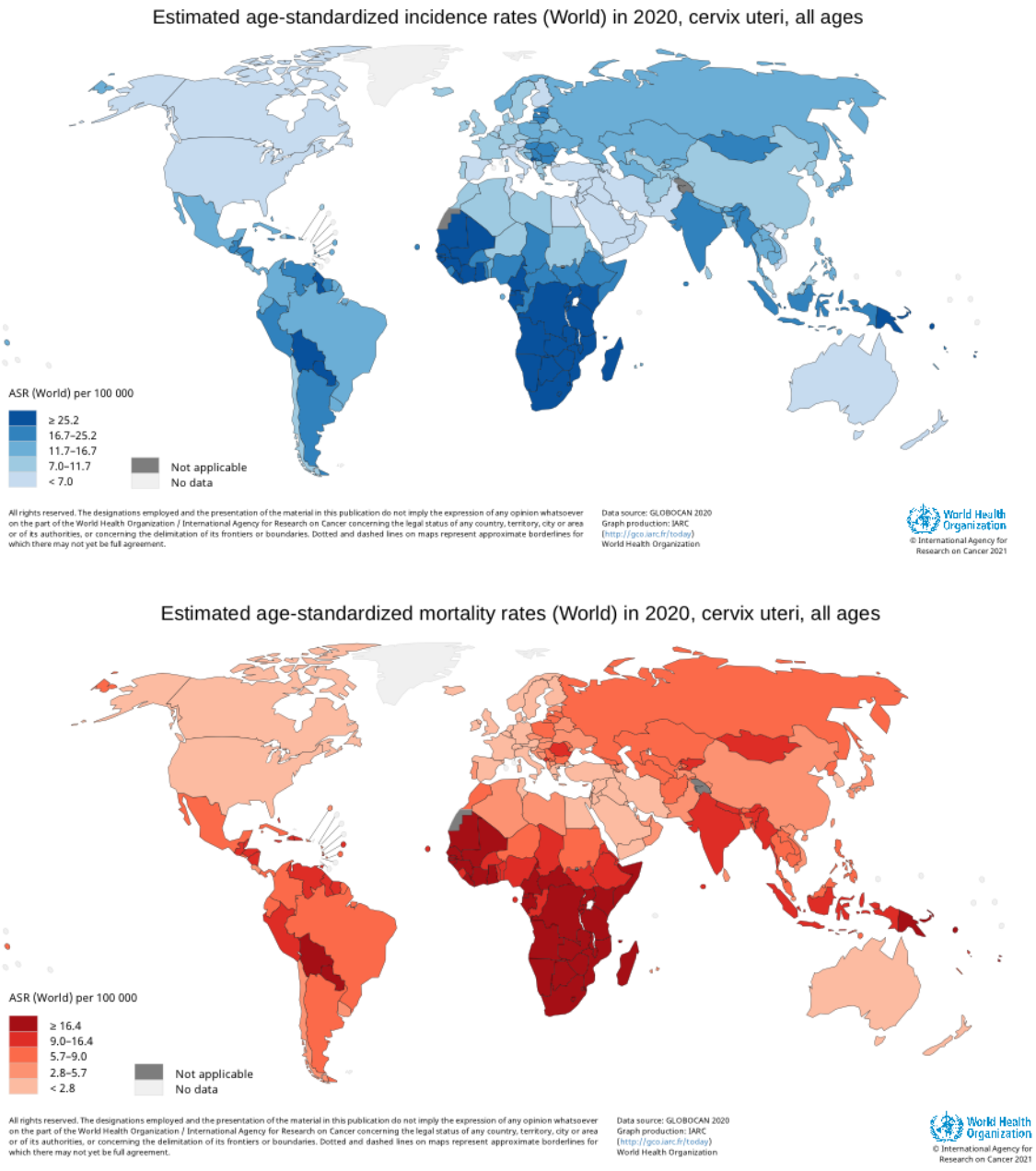

Figure 2-1: Cervical cancer incidence and mortality worldwide. Incidence (top) and mortality (bottom) rates per 100,000 people are depicted, with highest values shown in darker colors. Reproduced from [2].

2.2.2. Current Methods for Cervical Cancer Screening and Diagnosis

Several technologies and methods are used for cervical cancer screening and diagnosis. In this section, I first describe the gold standard diagnostic test against which the clinical performances of new technologies are measured. I then describe the currently recommended practices for cervical screening and diagnosis in high- and low-resource settings.

2.2.3. Gold Standard for Cervical Cancer Diagnosis

The gold standard for the diagnosis of both cervical dysplasia and invasive cancer is histopathologic examination of biopsied specimens to identify premalignant and malignant conditions of the cervix. In this process, a pathologist examines the biopsied epithelium of the cervix and classifies it according to the fraction of the epithelial layer that displays abnormal cellular morphology. For squamous epithelium, cervical intraepithelial neoplasia (CIN) 1 or low-grade squamous intraepithelial neoplasia (LSIL) is diagnosed when a third or less of the epithelium has undergone cellular changes; CIN2 and 3 (high-grade squamous intraepithelial neoplasia (HSIL) is diagnosed when greater than one-third of the squamous epithelium displays abnormal cellular morphology. Adenocarcinoma-in-situ (AIS) is diagnosed when the columnar epithelium shows abnormal morphology and may be associated with CIN. Cancer is diagnosed when neoplastic cells have invaded the stroma beneath the basement membrane in squamous epithelium (squamous cell carcinoma) or glandular epithelium (adenocarcinoma). If left untreated, CIN2 or more severe diagnoses (referred to as CIN2+ diagnoses) can progress to invasive cancer and therefore are commonly treated by ablation or excision to prevent disease progression [15]. More detailed

definitions of tumors and their precursors are outlined in the World Health Organization (WHO) Blue Book [16].

2.2.4. Standard of Care Screening and Diagnosis in High-Resource Settings

Screening and diagnostic tests are generally evaluated in terms of clinical sensitivity and specificity relative to the gold-standard of biopsy-proven CIN2+; the sensitivities and specificities reported throughout this article follow this convention. In high-resource settings, the standard of care for cervical cancer screening includes cervical cytology and/or high-risk HPV DNA or RNA testing, as the vast majority of cervical cancers are caused by infection with HPV. Cytology, commonly referred to as Pap testing, involves examining the morphology of exfoliated cervical cells under a microscope and generally has a low sensitivity (53-55.4%) and high specificity (84.2-94.5%) [17–20]. Cytology performance varies greatly, even within the United States, due to interpretative variability [21]. In low-resource settings, the challenge of achieving high-quality cytology is greater because of a lack of medical capacity and even logistical capacity to get high-quality reagents into the country. Therefore, sensitivity may be even lower in low-resource regions than in high-resource settings, where it is at best moderate, because validated Pap staining and/or liquid-based cytology is not available. As such, quality assurance of cytology is important to achieve similar preventive impact on cervical cancers compared with validated cytological methods. To compensate for low sensitivity in the United States, cytology testing efficacy comes from repeated, regular screening [22].

HPV DNA testing, in comparison, has relatively higher sensitivity (90.2-96.1%) and lower specificity (84.2-94.5%) in screening populations [17–19,23]. U.S. guidelines suggest that women below age 30 receive cytology testing in three year intervals, whereas women

over the age of 30 can receive cytology testing in three year intervals, HPV screening in five year intervals, or co-testing with cytology and HPV screening in five year intervals [24].

Screening by cytology requires infrastructure to obtain, store, and transport a cytology specimen, as well as a skilled technician or automated reader to process the sample. Currently FDA-approved HPV tests similarly require significant laboratory and transportation infrastructure and/or skilled technicians. Innovations in digital cytology [25] and HPV testing [26,27] could increase access to standard-of-care practices in low-resource settings, although the technical complexity, infrastructure requirements, and cost are significant barriers.

A positive screening test result triggers standard diagnostic procedures, including colposcopy and biopsy. In colposcopy, a trained provider examines the cervix, using a colposcope, which is a low magnification optical microscope. Visually abnormal areas are biopsied, excising small samples of cervical tissue for histopathological examination. Given the reliance on highly trained providers, it is challenging to scale diagnostic procedures in low-resource settings [28–34].

An example flowchart for HPV screening and diagnosis in high-resource settings is shown in **Figure 2-2.** In this flowchart, primary screening for cervical cancer occurs via HPV testing in 5-year intervals, with positive results referred to cytology testing. A high-grade cytology result, or known HPV16 or HPV18 status when genotyping is available, leads to follow-up diagnosis through colposcopy. A positive HPV test with negative cytology receives follow-up testing in a year. This is one example of cervical cancer screening and management in high-resource settings, but other protocols may be used. In general, positive

screening results will lead to confirmatory diagnostic colposcopy and biopsy, followed by treatment if the lesion is precancerous.

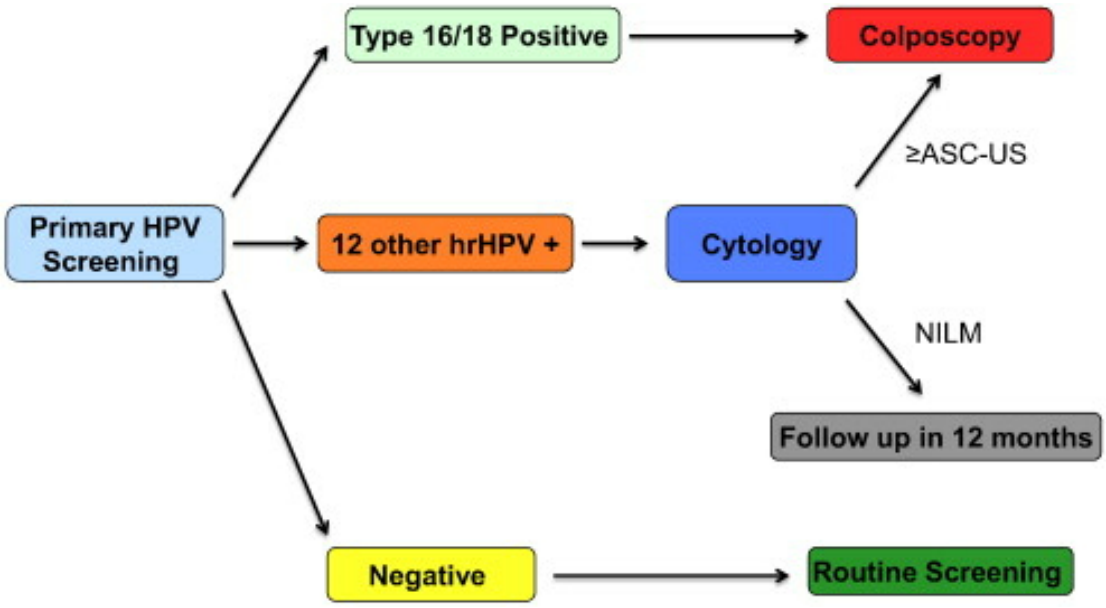

Figure 2-2: Example of a cervical cancer screening and management algorithm when using primary HPV screening. Reproduced from [35] with permission from Elsevier. *hrHPV: high-risk HPV; ASC-US: atypical squamous cells of undetermined significance; NILM: negative for intraepithelial lesion or malignancy.*

2.2.5. Standard of Care Screening and Diagnosis in Low-Resource Settings

Many barriers to implementing cervical cancer screening programs exist in low-resource settings, including but not limited to: lack of trained providers, lack of laboratory supplies, lack of laboratory infrastructure, socio-religious and cultural barriers to pelvic examination, unsustainable rates of overtreatment, and limited physical access to patient populations [36]. Decisions regarding appropriate screening and diagnostic technologies are made primarily based on available resources. For example, the 2014 World Health

Organization guidelines for implementing cervical cancer screening in a low-resource setting are shown in **Figure 2-3**.

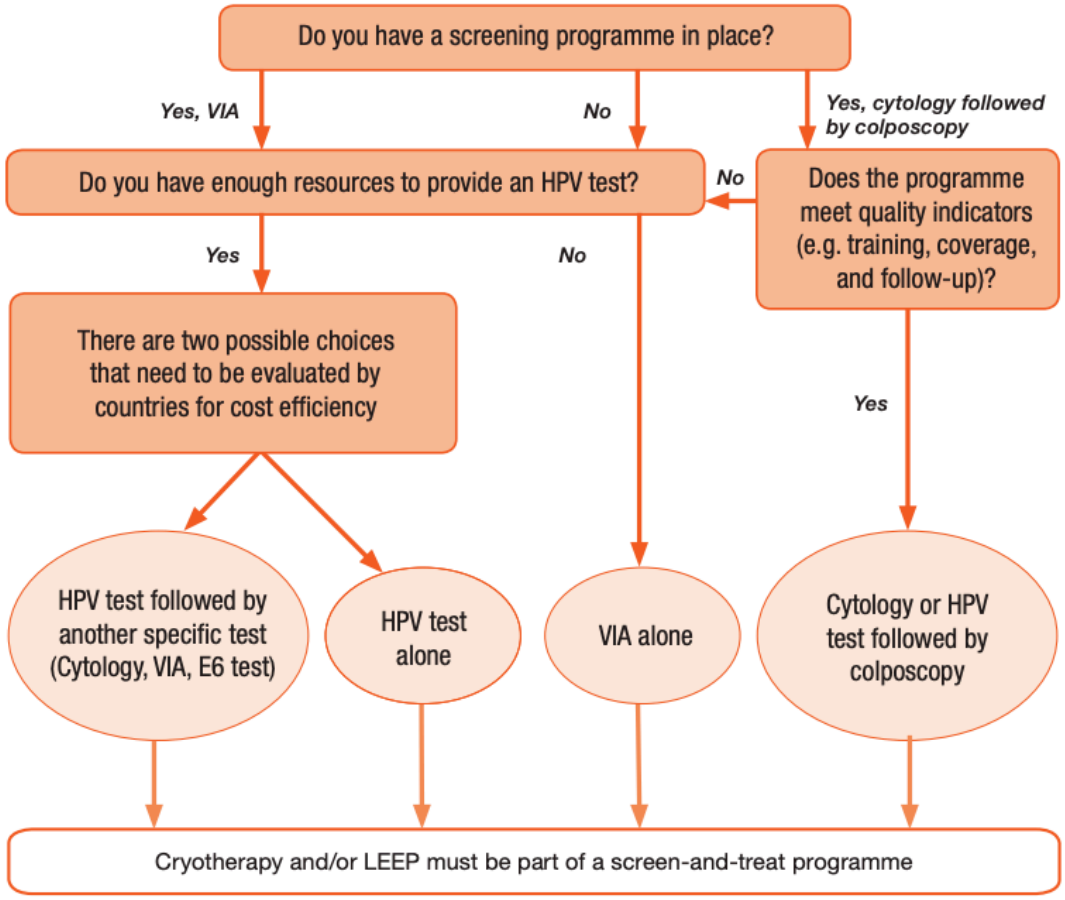

Figure 2-3: Decision-making flowchart for implementing screen-and-treat programs in low-resource settings. Decisions to implement HPV testing, VIA, cytology, and colposcopy for screening are made primarily on the basis of available resources. Reproduced from [37]. *HPV: human papillomavirus; VIA: visual inspection by acetic acid; LEEP: loop electrosurgical excision procedure.*

In addition to the screening test options available in high-resource settings, visual inspection with acetic acid (VIA) and by Lugol's Iodine (VILI) have been recommended for use in LMICs due to their low cost and limited infrastructure requirements. VIA and VILI involve applying acetic acid or Lugol's Iodine, respectively, to the cervix and observing color

changes, which indicate precancerous or cancerous lesions. In a large study of a screening population in rural India, sensitivity and specificity of VIA were reported as 41.4% and 94.5%, respectively. These methods are highly dependent upon user training and environmental considerations, such as lighting conditions. Therefore, VIA and VILI have highly variable clinical performance. In one report, for example, the range of VIA sensitivity was 55-96% and specificity was 49-98%, and the range of VILI sensitivity was 44-98% and specificity was 75-91% [38]. Additional study with pathologic endpoints is needed to determine the true sensitivity and specificity of VIA and VILI, but have been challenging to perform in low-resource settings where these tests are in use because of the lack of medical capacity and infrastructure to do colposcopy and pathology. While visual inspection tests are inexpensive and have limited supply chain requirements, Silkensen *et al* argue significant scale-up challenges, problematic accuracy, and insufficient reproducibility will limit their use moving forward [39].

Where feasible, objective tests with improved performance, like HPV testing, are recommended for use in LMICs; however, technology to support HPV DNA testing remains inaccessible in much of the world. Self-sampling for HPV DNA tests could help reduce barriers to screening program implementation in LMICs. Socio-religious and cultural barriers or unpleasant subjective experiences including discomfort with conventional physician-collected swabs can reduce participation in cervical cancer screening programs. However, recent studies have shown good agreement between self-collected cervicovaginal swabs and physician-collected cervical swabs for HPV DNA testing[40]. In addition, self-collection is strongly accepted and preferred according to a meta-analysis with nearly 20,000 women from 24 countries [41]. Technologies for self-collection of cervical samples have previously been reviewed [42]. Self-sampling could help remove barriers to HPV screening

in LMICs without compromising test performance and could reduce the total time required during a screen-and-treat visit [43].

Similarly to the challenges faced in implementing screening programs in LMICs, availability of highly trained personnel and infrastructure often limit the accessibility and performance of diagnostic follow-up to positive screening tests. When available, colposcopy and biopsy are used for diagnosis in LMICs. When diagnosis is not available, screen-and-treat programs are implemented. Screen-and-treat programs include a screening test, such as VIA, and immediate treatment by cryotherapy or loop electrosurgical excision procedure (LEEP) of any positive-appearing cervical tissue. With currently available technologies, these programs may lead to overtreatment due to poor specificity [44].

2.3. Relevant Biomarkers for Cervical Cancer Screening

Molecular cervical cancer screening tests can target a number of clinically relevant biomarkers, primarily relating to HPV infections. Virtually all cases of cervical cancers are caused by HPV, a virus that integrates within the genome of host cells to disrupt normal cellular function. While there are over 200 types of HPV, only 14 are considered carcinogenic, or high-risk [45]. Several biomarkers related to high-risk HPV correlate to infection and, in some cases, progression toward cancer.

2.3.1. HPV DNA

Tests that detect high-risk HPV DNA have high negative predictive values (NPVs) of over 98% for cervical precancer [46–48]. HPV DNA tests have high sensitivities (90.2-96.1%) and lower specificities (84.2-94.5%) in screening populations [17–19,23]. With low

rates of false negatives, HPV DNA testing is often used as a first line screening test for cervical precancer and cancer. However, the U.S. Centers for Disease Control and Prevention (CDC) estimates that 90% of HPV infections are cleared within two years [45]. Therefore, confirmatory diagnosis for cervical precancer or cancer is necessary after an HPV DNA screen to avoid overtreatment. The risk of overtreatment associated with HPV DNA testing is lower in older patients, as rates of transient infections tend to decrease, and rates of type-specific persistence, which is required for cancer progression, tend to increase with age [49].

2.3.2. HPV mRNA

While the presence of HPV DNA indicates an infection, progression to cancer occurs when the infection persists, the viral genome integrates, mRNA overexpression of oncogenes begins, and oncoproteins are produced. mRNA overexpression of the E6 and E7 genes is the precursor for the production of E6 and E7 oncoproteins, which interfere with tumor suppressors p53 and pRB, respectively [50]. Therefore, evaluation of HPV E6 and E7 mRNA overexpression provides a more accurate assessment of progression to cancer compared with HPV DNA. HPV mRNA testing has been shown to have comparable sensitivity and improved specificity for biopsy-proven CIN2+ compared with DNA testing [51]. Despite its advantages, current high-risk HPV mRNA tests remains too costly and complex for implementation in low-resource settings.

2.3.3. HPV Oncoprotein

Like HPV mRNA, E6 and E7 oncoprotein detection has been shown to have high specificity for pre-cancer and cancer [52,53]. Both E6 and E7 are involved in the progression

of HPV infection into precancer and cancer. Up-regulation of these proteins is needed for malignant conversion of HPV-infected cells, and over-expression implies high risk of progressive disease [50]. While oncoprotein detection improves specificity, sensitivity is generally lower than DNA or mRNA detection [52].

2.4. Needs for New Tests

Molecular testing provides opportunities to increase access to cervical cancer screening in LMICs through enabling accurate see-and-treat strategies and self-sampling. Health systems in LMICs generally can support limited numbers of patient encounters, so high-sensitivity screening tests, such as an HPV DNA test, allow providers to identify at-risk patients at the time of their first visit. Studies have shown that a single HPV DNA test, coupled with appropriate treatment, can reduce cervical cancer mortality by 50% [54]. Current limitations in HPV DNA tests include high per-test cost, instrumentation cost, infrastructure requirements, and complexity of use. Further, molecular testing makes the possibility of self-testing more realistic, as sample adequacy requirements are much more stringent for cytology than for DNA testing [55]. An inexpensive, point-of-care HPV DNA test or oncoprotein test that uses self-sampling techniques could greatly increase access to cervical cancer screening and diagnosis in resource-limited settings.

2.5. Recent Advances in Molecular Tests

Molecular testing is clinically useful as a first line screening method for cervical cancer. As previously described, HPV testing is often used in conjunction with cytology or as a standalone test for primary cervical cancer screening. In LMICs, HPV testing is a

recommended screening practice when sufficient resources are available. Here, I describe commercialized and in-development advances in molecular tests for cervical cancer screening in LMICs.

2.5.1. Commercialized Tests

Several assays for HPV testing in LMICs are commercialized and in routine use. Some of the tests are packaged as assays that require standard laboratory equipment, and others are fully integrated and are sold with all required instrumentation. The tests target different biomarkers, including DNA, RNA, and protein. In addition, the partial genotyping capability of each test varies. This review focuses on tests that are currently in routine use in low-resource settings, subsidized for certain LMICs, or use a detection method that could be translatable to the point-of-care, such as isothermal amplification. A summary of the tests discussed in this section can be found in **Table 1**. The selected tests do not include all FDA-approved HPV screening tests. For example, the Roche cobas test is in fairly widespread use in the United States; however, because of the large instrument footprint, reliance on advanced infrastructure, and the presence of an alternative DNA test that I see as more appropriate for use in LMICs, the GeneXpert test, I have not included Roche cobas in this review. Summaries of high-resource commercialized HPV tests [56,57] and their enabling methods [58] have previously been described. I acknowledge the challenges of comparing test parameters, e.g. citing comparable manufacturing cost estimates or performance data across different sites; in this section, I present representative values as cited in the literature.

Table 1: Summary of selected commercially available HPV tests for cervical cancer screening

Test	Bio-marker	Detect. method	Partial geno-typing?	LOD	Se (%)[c]	Sp (%)[c]	Pop.	Per-test cost (USD)	Inst. cost (USD)	Sample prep. Int.?	Bat ch-ing
***digene* HC2 (Qiagen)**	DNA	Hybr., Chem.	No	100,000 copies/mL [59]	85.7–97.5 [48,60]	81.8–85.4 [48,60]	Scr. [61], Ref. [48]	$71 [62]	-- [f]	No	Yes
careHPV (Qiagen)	DNA	Hybr., Chem.	No	100,000 copies/mL [63]	85.7–88.1 [48,52]	83.1–83.7 [48,52]	Ref. [48], Scr. + Ref. [52]	$5–42 [26,64,65]	$20,000 [65]	No	Yes
GeneXpert HPV (Cepheid)	DNA	qPCR, fluor.	16, 18/45	2903 to 50,493 copies/mL[d] [66]	94% [67]	83% [67]	Scr. + Ref. [67]	$20 (est.) [68]	$11,530–71,500 [69]	Yes	No
Aptima HPV (Hologic)	RNA	TMA, fluor.	16, 18/45	60 to 1220 copies/mL[e] [59]	97.5 [61]	90.2 [61]	Scr, [61]	$12[a] [70] ($30 [69])	$0[a] [70] ($150,000 [69])	Yes	No
NucliSENS EasyQ (bio Mérieux)	RNA	NASBA, fluor.	16, 18, 31, 33, 45[b]	230 to 30,000 copies/mL [48]	69–79.3 [71–73]	36–72.6 [71–73]	Ref. [71–73]	$23 (est.) [69]	$45,000–65,000 [69]	No	Yes
Proofer (PreTect)	RNA	NASBA, fluor.	16, 18, 31, 33, 45[b]	4000 to 5000 copies/ mL [74,75]	78.1 [76]	75.5[76]	Scr. + ref. [76]	-- [f]	-- [f]	No	Yes
OncoE6 (ArborVita)	Protein	Sandwich assay, LFA	16, 18[b]	20,000 cells/mL [77]	31.3–53.5 [52]	98.9–99.4 [52]	Scr. + ref. [52]	-- [f]	$2,000 [57]	No	No

Detect.: detection; LOD= limit of detection; Se: sensitivity; Sp: specificity pop.: population; inst.: instrument; prep.: preparation; int.: integrated; hybr.: hybridization; chem.: chemiluminescence; qPCR: quantitative polymerase chain reaction; fluor.: fluorescence; TMA: transcription-mediated amplification; NASBA: nucleic acid sequence-based amplification; LFA: lateral flow assay; scr.: screening; ref.: referral

[a] *subsidized cost in eligible countries under the Hologic Global Access Initiative with unsubsidized costs in parentheses; (est.): estimated test costs based on different assay using same platform*

[b] *only types detected for these tests; the remaining tests produce a pooled high-risk result plus partial genotyping.*

[c] *all reported sensitivities and specificities are compared against a gold standard of biopsy-proven CIN2+*

[d] *values converted from international units/milliliter (IU/mL) to copies/mL using the WHO International Standard, NIBSC code 06/202* [78]

[e] *values calculated from copies/reaction.*

[f] *to our knowledge, values are not found in the literature*

2.5.1.1. HPV DNA Tests

Hybrid capture HPV tests rely upon hybridization of target DNA to synthetic RNA. The DNA/RNA hybrids are then detected in enzyme-linked immunosorbent assay (ELISA) format. Hybrid capture approaches generally are less sensitive than amplification methods but detect DNA in the clinically relevant range.

The *digene* HC2 DNA Test (Qiagen) is a hybrid capture assay that relies on standard laboratory equipment and protocols and for which all required reagents for high-risk HPV detection are packaged and sold. The result is a qualitative indicator of the presence of any high-risk HPV types without genotyping. The test is complex and requires significant hands-on time. It is also expensive, at an estimated cost of US$71 per test [62]. The test has high sensitivity and relatively high specificity, though there is some cross-reactivity with low-risk types [79]. One of the biggest challenges with implementing HC2 is the required laboratory infrastructure and instrumentation [48], including a plate reader, shaker, calibrated set of pipettes, and refrigerator.

In an attempt to bring HPV DNA testing closer to the point-of-care, Qiagen developed careHPV, a test that utilizes the same hybrid capture testing principles as HC2 in a more point-of-care-friendly format. Similarly to HC2, careHPV produces a pooled high-risk result without genotyping. Along with the required reagents, careHPV packages the necessary instruments, which still include a plate reader and orbital shaker. While the single source of all testing equipment is helpful, the total cost of instrumentation is still estimated to exceed US$20,000, and a stable power supply is necessary [65]. In addition, test complexity remains a major challenge with careHPV. The 96-well plate format requires

training for users to competently and confidently run the test. The per-test cost can be as low as US$5, but only if a batch of 90 samples are run at a time [26,64]. Additionally, the low per-test cost assumes scale-up to 20,000 tests and that no additional capital investments will be required. Per-test cost estimates, considering both equipment and supplies, were reported as US$42 in a pilot careHPV implementation program in Myanmar [65]. Batching requirements can lengthen turnaround time in low-throughput clinical settings. The four-hour testing time and batching-related delays mean patients almost always have to come back for a second visit to receive results, which increases the likelihood of losing patients to follow-up [26]. Despite the challenges faced by careHPV, many groups have implemented careHPV and evaluated clinical performance in large-scale studies. For example, in a multi-country study with over 16,000 patients, sensitivity and specificity of careHPV on physician-collected cervical samples were 81.5% and 91.6% and on self-collected vaginal samples were 69.6% and 90.9%, respectively. In comparison, sensitivity and specificity of VIA were 59.8% and 84.2% and cytology were 58.4% and 87.7%, respectively [80]. Other studies have validated the use of careHPV in screening programs in low-resource settings [63,81–83] and with self-collected samples [40,84].

Several amplification-based HPV DNA assays including GeneXpert have been developed, as shown in Table 1. These tests are more sensitive than hybrid capture assays; however, most amplification-based HPV DNA tests are not appropriate for low-resource settings due to their high cost, need for trained personnel, and expensive equipment [85]. Removing infrastructure and limiting cost creates technical challenges difficult for translating these highly sensitive diagnostics to low-resource settings. A truly point-of-care and infrastructure-free test would greatly increase access to screening in many areas with the highest prevalence (**Figure 2-1**).

2.5.1.2. Oncoprotein Tests

In comparison with HPV DNA tests, oncoprotein tests generally have lower sensitivity and higher specificity. Arbor Vita (Fremont, CA) has commercialized a lateral-flow based E6 oncoprotein test, OncoE6, for HPV types 16, 18, and 45 [52]. The lateral flow readout is point-of-care-friendly and has separate detection lines for each HPV type, allowing for partial genotyping [53]. Reported clinical sensitivities and specificities of the OncoE6 test range from 31.3% to 53.5% and 98.9% to 99.4%, respectively [52]. When restricting analysis to patients who were positive for the three genotypes covered by the test, the sensitivity increased to 64.5%; therefore, sensitivity limitations are not solely attributable to missed genotypes [85]. Equipment for the OncoE6 test is fairly affordable at an estimated US$2,000. However, the test requires over 45 minutes of sample preparation with several pipetting and centrifugation steps, and therefore is not yet an optimal solution for low-resource settings [53,57,85]. Automating sample preparation and limiting hands-on testing time, as well as increasing the number of genotypes detected, could improve the performance and usability of the OncoE6 test.

2.5.2. HPV Tests in Development

While careHPV and GeneXpert have increased access to HPV testing in some low-resource environments, their cost and infrastructure requirements limit their potential for large-scale screening. To increase accessibility, several promising technologies are being developed to reduce the cost and infrastructure necessary for HPV molecular testing (**Table 2, Figure 2-4**).

Table 2: Summary of in-development HPV tests for cervical cancer screening

Test	Bio-marker	Detect. method	Partial geno-typing?	LOD	Sample prep. Int.?	Phase of Development
Q-POC (QuantuM Dx)	DNA	PCR, fluor.	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68a, 68b	10 to 50 copies/ reaction [86]	yes	Pilot study under ideal conditions (n=70; concordance analysis in progress) [86]; pursuing multisite clinical evaluation [87]
Paper-fluidic chip (Klapperich lab)	DNA	LAMP, LFA	16	100,000 copies/mL [88]	No	Addressing limitations of current prototype after initial pilot test (n=10 in ideal conditions) [88]
Onco E6/E7 Eight HPV Type Test (Arbor Vita)	Protein	Sandwich assay, LFA	16, 18, 31, 33, 35, 45, 52, 58	20,000 to 100,000 cells/mL [77]	No	Pilot study completed under ideal conditions with laboratory technicians (n=259, 31 CIN2+; Se: 67.7%, Sp: 89.5%) [89]; currently pursuing larger-scale validation study

Detect.: detection; LOD= limit of detection; prep.: preparation; int.: integrated; PCR: polymerase chain reaction; fluor.: fluorescence; LAMP: loop mediated isothermal amplification; LFA: lateral flow assay

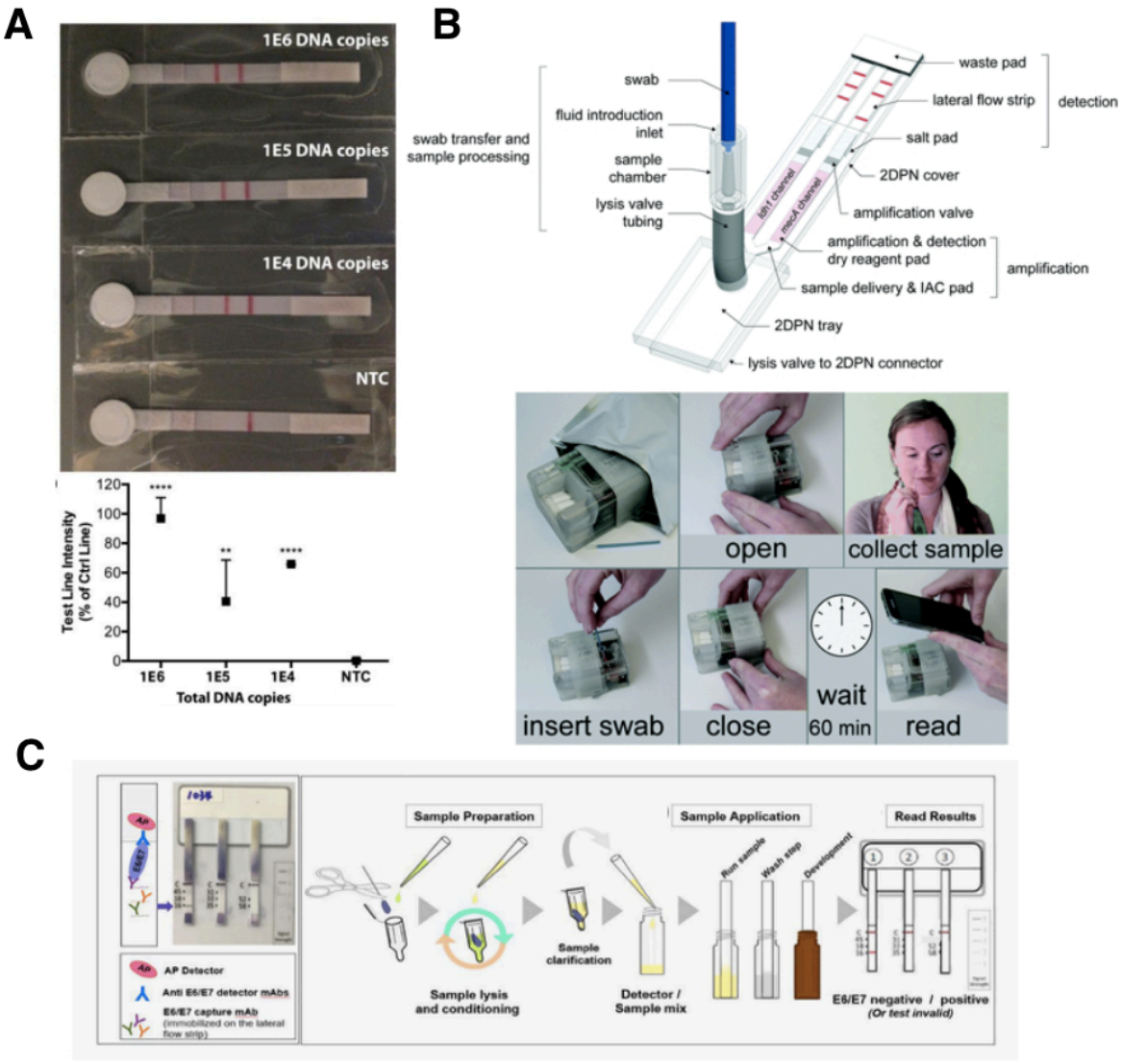

Figure 2-4: In-development paperfluidic HPV tests. (A) Paperfluidic test developed by Rodriguez et al. Example results with input DNA copies ranging from 1E4 to 1E6 and a no-target control (NTC) are shown. Test line intensities indicate positive signals were formed at the test line in the presence of DNA over 1E4 total input copies of HPV 16 DNA; no test line signal formed with the NTC condition, indicating analytical specificity of the test. (B) MAD NAAT test developed by LaFleur et al. Internal components, including sample inlet port, lysis chamber, sample elution mechanisms, amplification reagents, and lateral flow detection are shown (top). The method of use is also shown (bottom), with 5 user steps spanning roughly an hour. (C) OncoE6 8-type test developed by Zhao et al. Left: example lateral flow strips and capture chemistries are shown. Capture antibodies are embedded in lateral flow strips and capture E6/E7 proteins. Detection antibodies form a sandwich assay with the immobilized proteins, and alkaline phosphatase (AP) binds and produces a purple colorimetric signal. Three total test strips are used to detect 8 high-risk types. Right: method of use of

OncoE6 8-type test is shown. First, a cervical swab is placed in buffer, and the sample is lysed and conditioned. Next the sample is added to detection buffer, and lateral flow strips are placed into the mixture. The lateral flow strips are then moved into different buffers for washing and signal development. Finally, test strips are read by ensuring control lines are positive on all strips and identifying colorimetric signals at any of the test lines. In this figure, the sample is positive for type 16. (A) and (B) reproduced from [86] and [88], respectively, with permission of the Royal Society of Chemistry from; permission conveyed through Copyright Clearance Center, Inc. (C) reproduced from [85] with permission of John Wiley and Sons.

QuantuMDx Q-POC and the paperfluidic chip by the Klapperich lab are two DNA amplification assays that are lower cost and more suitable to low-resource settings (**Figure 2-4a,b**). Other groups have developed paper platforms for individual components of nucleic acid testing including sample preparation, amplification, and detection, which are discussed in a recent review article [90]. Many of these devices use isothermal amplification of DNA with a single temperature heater, or body heat in the case of recombinase polymerase amplification (RPA), to reduce equipment and infrastructure needs [91]. Despite these advances, no truly low-cost and low-infrastructure platform for DNA or RNA amplification has been validated with large-scale clinical studies in low-resource settings. Although GeneXpert has proven accurate for large-scale clinical validation in resource-limited settings[69-71], an assay with lower per-test and equipment cost could further increase access to critical HPV screening in areas with the highest prevalence.

Arbor Vita, in collaboration with PATH, has recently developed a new prototype of their OncoE6 test (**Figure 2-4c**). The prototype, the Onco E6/E7 Eight HPV Type Test, expands detection to both E6 and E7 oncoproteins and includes two additional lateral flow strips for oncoprotein detection of HPV types 16, 18, 31, 33, 35, 45, 52, and 58 [77,89]. The test works in a similar method to the OncoE6, with individual test lines for genotyping and with relatively complex and user-intensive sample preparation. In a small clinical study

(n=259), the new prototype had a sensitivity and specificity of 67.7% and 89.5%, respectively [89]. To evaluate true sensitivity and specificity, larger studies on broader populations will need to be conducted. The increased test sensitivity relative to the three-type test is likely due to the increased number of HPV types tested. However, the sample preparation and sensitivity limitations of the OncoE6 test remain the same with this prototype. This test will need to be further evaluated clinically to understand its potential role in screening and triage.

2.6. Conclusion

Despite the increasing burden of cervical cancer incidence and mortality in LMICs, screening for cervical cancer in low-resource settings remains limited by cost, equipment, and complexity. Commercially available HPV DNA tests such as careHPV and GeneXpert are being used to screen women in LMICs; however, the per-test cost and infrastructure requirements limit their sustainability and scalability for country-wide screening. Recent advances toward point-of-care molecular testing, including paper-based approaches and emerging technologies like the QuantuMDx Q-POC test, hold promise; however, these technologies still need to be evaluated for clinical use. These innovations help bring molecular testing closer to the point of care so that screen-and-treat options can be effectively implemented in LMICs. Additionally, without accessible diagnostic tests, screen-and-treat programs can lead to high rates of overtreatment [44]. With self-collected HPV DNA swabs, a truly point-of-care screening test, and a lower cost diagnostic test, a single visit including accurate screening, diagnosis, and treatment can become the new standard of care in LMICs and reduce the burden of cervical cancer globally.

Chapter 3

Highly Sensitive Two-Dimensional Paper Network Incorporating Biotin–Streptavidin for the Detection of Malaria

This chapter describes the development of paper-based platform with equivalent sensitivity to a traditional 96-well enzyme-linked immunoassay. The contents of this chapter were previously published in the journal article: BD Grant, CA Smith, K Karvonen, R Richards-Kortum. Highly Sensitive Two-Dimensional Paper Network Incorporating Biotin–Streptavidin for the Detection of Malaria. Anal Chem. 2016;88(5):2553-7. PMID: 26824718 [92]. *The chapter has been rewritten for the purpose of this thesis.*

3.1. Abstract

Recent development of two-dimensional paper networks (2DPN) have increased the ability to detect highly sensitive antigens at the point-of-care. However, to date, these 2DPNs are 30X less sensitive than their gold standard counterparts, 96-well enzyme-linked immunoassays (ELISAs), limiting clinical utility. By adapting the layout of previous 2DPNs and incorporating more complex signal amplification, we achieved equivalent sensitivity to a traditional ELISA. The assay produces a visual readout within 90 minutes, costs less than $1, and runs without the need for complex infrastructure or skilled laboratory personnel. We demonstrated the performance of the improved paper-based ELISA format using an assay that detects malaria protein *Pf*HRP2. However, this platform can be easily adapted towards other assays for highly sensitive detection in resource-limited settings.

3.2. Introduction

Paper-based microfluidic devices are infrastructure-free rapid diagnostic tests (RDTs) which use capillary action and wicking properties of absorbent materials to manipulate fluid flow. The most simple form of paper-based microfluidic devices, lateral flow assays (LFAs), are widely used immunoassays that detect clinically relevant antigens either directly or in a sandwich format [93–96]. LFAs are low-cost, rapid, and easy-to-use because of their simplicity; however, they can detect only higher concentrations of clinically relevant antigen, limiting their usefulness for assays requiring higher sensitivity [95–97].

Only recently has the biomedical community focused on translating more sensitive diagnostics to the point-of-care. Most notably, researchers at the University of Washington have developed inexpensive paper-based enzyme-linked immunoassays (ELISAs) using two-

dimensional paper networks (2DPN) [93,98–103]. These assays use a series of reagents stored on glass fiber pads to deliver traditional ELISA components down a nitrocellulose membrane. Reagents flow sequentially to a detection region using capillary action with no additional user input after initiation of flow. Furthermore, the reagents are stable for months at 37°C when dried into the pads, and results can be visually interpreted, making 2DPNs ideal for use in low-resource settings [101,103].

One application for highly sensitive RDTs include the development of a point-of-care test for malaria elimination efforts. The World Health Organization has programs focused on eliminating and eradicating malaria, similar to the polio elimination efforts of the past [104–109]. These programs aim to detect subclinical infection of malaria in the asymptomatic population because people with low-level parasite loads serve as vectors of transmission. Additionally, submicroscopic blood infections are common among pregnant women with malaria since parasites sequester in the placenta, and these submicroscopic infections may affect health outcomes for both mother and child [110].

Malaria LFAs tests are ubiquitous in LMICs. Approximately 2.1 billion malaria RDTs were sold to sub-Saharan African countries from 2010-2019 [111]. These tests typically detect *Plasmodium falciparum (Pf)* parasite using the antigen *Pf* histidine-rich protein 2 (*Pf* HRP2). *Plasmodium falciparum* releases *Pf*HRP2 into blood plasma throughout its life cycle, so *Pf*HRP2 concentrations indicate total body parasite biomass including the sequestered load [112]. LFAs typically measure high, symptomatic concentrations of parasite, with limits of detection near 100 parasites/μL [95,96,104,106]. Similarly, blood microscopy smears enable observation of parasites at 10 to 100 parasites/μL [104,106,107,113]. More appropriate for malaria elimination programs, qRT-PCR-based tests can detect parasite DNA to values as

low as 0.02 parasites/μL, and traditional ELISAs reliably measure 0.11 ng/mL, corresponding to about 0.7 parasites/μL [97,104,114,115]. However, with half of the world's population at risk for malaria, both PCR-based methods and traditional ELISAs are not appropriate for malaria elimination goals, as both are high-cost, infrastructure-dependent, and time-intensive [104,106,109,115]. While much more sensitive than lateral flow tests, recent 2DPNs report a limit of detection of 2.9 ng/mL, over 25 times greater than standard ELISAs[102]. Sensitivity on par with the gold standard tests of less than a parasite/μL (approximately 0.38 ng/mL) is desired for full efficacy in malaria elimination programs [101,102].

One way to enhance sensitivity of 2DPNs is through signal amplification using streptavidin-biotin detection. Biotin can be conjugated to antibodies without denaturation due to its small size (244 Da), and biotin has both high affinity and high specificity for streptavidin (Kd ~ 4×10^{-14}) [116,117]. Biotin-streptavidin inclusion would amplify the signal for samples with low *Pf*HRP concentrations. Multiple biotin molecules can be conjugated to one antibody, so biotinylating the detection antibody and adding streptavidin inherently increases the signal [116]. Furthermore, Fitzgerald has conjugated streptavidin to polymers of 400 horseradish peroxidase molecules (poly-HRP80), greatly enhancing the number of HRP enzyme molecules per captured *Pf*HRP protein and therefore amplifying signal from a small amount of protein.

Here, we demonstrate successful development of a paper-based ELISA platform which is low-cost, easy to use, and equivalent in sensitivity to a traditional 96-well ELISA test. We validate our design with a range of *Pf* HRP2 concentrations spiked into FBS and

compare paper platform results to a gold standard kit, Malaria Ag CELISA. This platform can be used to develop additional assays with high sensitivity for resource-limited settings.

3.3. Methods

3.3.1. Malaria Ag CELISA Kit

In order to confirm the gold standard limit of detection, the commercially available Malaria Ag CELISA assay (Cellabs, Sydney, Australia) was performed. CELISA is considered the gold standard HRP2 ELISA[104]. Linear dilution was used to create a range of recombinant *Pf*HRP2 (CTK Biotech, San Diego, CA) spiked into fetal bovine serum (FBS) (Bio-Techne, Minneapolis, MN): 0 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.25 ng/mL, 0.5 ng/mL, and 1 ng/mL. Each concentration was run in triplicate according to the CELISA protocol. Briefly, 100 µL of sample was added to each well and incubated in a humid chamber for 1 hour at room temperature. Following the sample incubation, the plate was washed with 300 µL of Phosphate Buffered Saline (PBS) with 0.05% Tween 20 (PBST) for five times. Next, 100 µL of the kit detection reagent was added to each well and incubated in a humid chamber for 1 hour at room temperature. The wash protocol was then repeated, with 300 µL of PBST for five times. Finally, 100 µL of the substrate from the CELISA kit was added to each well and incubated in the dark for 15 minutes at room temperature, before adding the stopping solution. The absorbance of the wells at 450 nm was read using a plate reader (Tecan, Zürich, Switzerland). Two-sided t-tests with p-values less than 0.05 were used to determine values significantly different than zero.

3.3.2. Traditional 96-well ELISA

As an additional gold standard control, a traditional in-house 96-well ELISA was performed using our reagents. A linear range of *Pf*HRP2 spiked into FBS was created as described above with a larger range: 0 ng/mL, 0.001 ng/mL, 0.01 ng/mL, 0.1 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 25 ng/mL, 100 ng/mL, and 1000 ng/mL. Samples were run in triplicate, and positivity threshold was determined as the average negative signal plus three standard deviations. For the ELISA protocol, first 100 µL of the capture antibody was plated. An IgM antibody to *Pf*HRP2 (Immunology Consultants Laboratory, Portland, OR) was diluted to 5 µg/mL in 1X ELISA coating buffer (Biolegend, San Diego, CA), and incubated overnight at 4°C. The next day, the plate was washed with 300 µL PBST three times, and 300 µL of blocking solution which consisted of 3% bovine serum albumin (BSA) (Sigma-Aldrich Inc, St. Louis, MO) in PBS was added for 2 hours at room temperature on an orbital shaker. After sample incubation, the plate was washed with 300 µL PBST three times, and 100 µL of each sample dilution was added in triplicate to the plate and incubated for 1 hour at room temperature on an orbital shaker. The plate was washed again with 300 µL PBST three times. Then, 100 µL of biotinylated detection antibody at 2 µg/mL was added to each well and incubated at room temperature on an orbital shaker for 1 hour. The unconjugated IgG detection antibody (Immunology Consultants Laboratory, Portland, OR) was biotinylated with 20mM biotin using the EZ-Link™ Sulfo-NHS-Biotin biotinylation kit (Thermo Fisher Scientific, Waltham, MA). After conjugation, unbound biotin was removed by running the antibody solution three times through desalting Zeba columns (Thermo Fisher Scientific, Waltham, MA), and the concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA). After detection antibody incubation, the plate was washed with 300 µL PBST four times. Next,

100 µL of the secondary antibody, 0.1 µg/mL (1:10,000) of streptavidin poly-HRP80 (Fitzgerald, Acton, MA), was incubated at room temperature on an orbital shaker for 1 hour. Before the final colorimetric detection, all wells were washed with 300 µL PBST six times. Then, 50 µL of room temperature 1-Step Ultra TMB (3,3',5,5'-tetramethylbenzidine) ELISA Substrate (Thermo Fisher Scientific, Waltham, MA) was added to the plates, incubated for 15 minutes at room temperature, and stopped with 50 µL H_2SO_4 (2N). Any air bubbles were removed from the plate, and the absorbance of the wells at 450 nm was read using a plate reader (Tecan, Zürich, Switzerland) Two-tailed t-test were performed between each concentration to determine significance.

3.3.3. Two-Dimensional Paper Network Device

A two-dimensional paper network layout was adapted from Fu et al [102]. The device, shown in **Figure 3-1**, consists of a 4 mm backed nitrocellulose strip (HF135, Millipore, Billerica, MA), six glass fiber pads (grade 8951, Ahlstrom, Helsinki, Finland), and a cellulose wicking pad (C083, Millipore, Billerica, MA) on a plastic backing made of 10 mm Dura-Lar (Blick Art Supplies, Galesburg, IL) and 5 mm adhesive-backed Dura-Lar (Blick Art Supplies, Galesburg, IL). All components were cut using an in-house CO_2 laser cutter (Universal Laser Systems, Scottsdale, AZ).

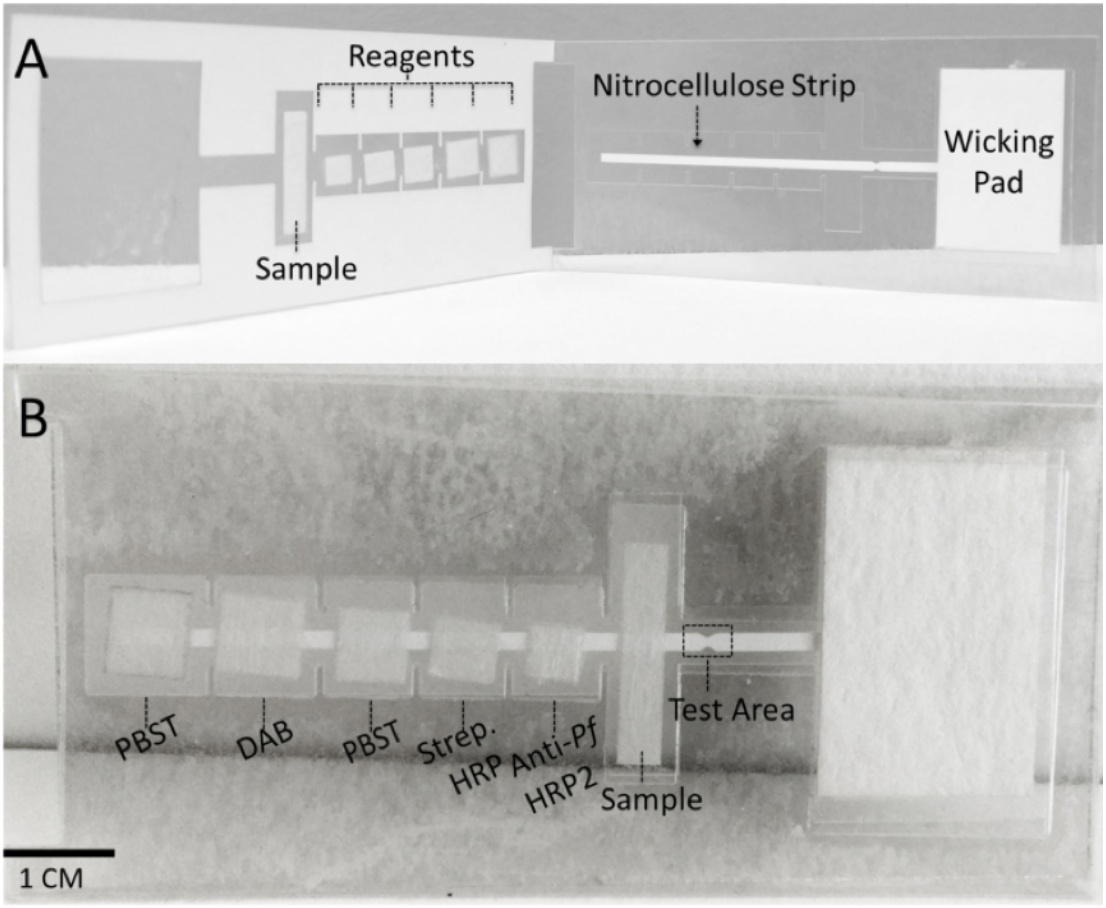

Figure 3-1: Paper-based ELISA format. (A) Linear device design highlighting all major paper and plastic components. (B) Folded device with reagent pads labeled and the test area with capture antibody represented as a dotted box. Image from [92].

3.3.4. Fluid Flow Characterization

Delivery and timing of reagents were recorded using food dye (McCormick, Sparks, MD) diluted in PBST for two different assay layouts: a layout with inlets perpendicular to the nitrocellulose membrane ("leg") which had been used in previous 2DPNs and a novel linear layout ("linear"), as shown in **Figure 3-3.** Different colors were applied to each reagent pad as follows: 50 μL PBST in the sample pad, followed by 15 μL of blue dye, 20 μL of green dye, 25 μL of yellow dye, 30 μL of orange dye, and 35 μL of red dye. Devices were folded in

half to start fluid flow, and images were obtained at the following time points using a flatbed color scanner at 600 dots-per-inch (DPI): 15, 25, 40, 50, 60, and 80 minutes. Using these data, the timing of the assay was estimated, and the differences between perpendicular and linear layouts were observed.

3.3.5. *Pf*HRP2 2DPN Assay Preparation

First, capture antibody was spotted onto the nitrocellulose membrane at the test area. 0.4 μL of 1 mg/mL IgM murine capture antibody diluted in PBS was pipetted onto the test area twice with 10 minutes between each application. After spotting, strips were dried in the incubator at 37°C for 1 hour. Next, strips were blocked for 30 minutes at room temperature on an orbital shaker to prevent nonspecific binding of ELISA reagents and reduce any background signal. The blocking solution, modified from Fu et al, included 2% BSA, 0.25% polyvinylpyrrolidone (PVP) (40KD mW, Sigma-Aldrich, St. Louis, MO), and 5% sucrose in PBST[11]. Following blocking, the nitrocellulose was dried in the incubator at 37°C for 1.5 hours to ensure the membrane was completely dry.

To assemble the paper-based ELISA, mirrored cutouts of Dura-Lar and adhesive-backed Dura-Lar were placed on top of an adhesive Dura-Lar backing **(Figure 3-1**). Glass fiber pads were placed in assigned cut-out locations on one side, where as the nitrocellulose membrane with capture antibody was placed on the opposite side. The wicking pad was placed overlapping the outer end of the nitrocellulose membrane, so as to ensure differential fluidic pressure and fluidic flow by capillary action.

3.3.6. Running the Paper-Based ELISA

To run the paper-based ELISA after assay preparation, all reagents are added to the glass fiber pads as follows: 50 µL of sample is added to the sample glass fiber pad, followed by 15 µL of 40 µg/mL of biotinylated IgG detection antibody diluted into PBST, 20 µL of 8 µg/mL streptavidin polyHRP-80 diluted into PBST, 25 µL PBST, 30 µL of the colorimetric solution, and a final 35 µL PBST wash step (**Figure 3-1)**. The colorimetric solution consisted of a 0.5 mg/mL solution of 3,3'- diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) in DI water with 0.5 % sodium percarbonate (Sigma-Aldrich, St. Louis, Missouri) as the source of hydrogen peroxide for the reaction. To do this, 7.5 mg of sodium percarbonate was added to 1.5 mL of DAB immediately before running the assay.

Once all reagents are added to glass fiber pads, remove the paper membrane from the adhesive Dura-Lar backing, and fold the assay in half. The reagents will begin to flow sequentially down the nitrocellulose membrane towards the test area. If sample is present in the assay, it will bind to the capture antibody in the test area, followed by the detection antibody and subsequently the streptavidin polyHRP-80. Then the DAB, sodium percarbonate, and HRP bound at the test area will react to create a brown precipitate signal which you can visually detect. In the absence of sample, no streptavidin polyHRP-80 should be bound to the test area, and no visible signal should be detected. Paper-based assays were imaged at 60 and 90 minutes using a flatbed color scanner at 600 dots-per-inch (DPI).

3.3.7. 2DPN Assay Signal-to-Background Analysis

Signal-to-background analysis using MATLAB compared the signal intensity from a region-of-interest (ROI) at the detection zone to the signal intensity from a background ROI

of the same size (**Figure 3-4, G**). In the custom MATLAB code, a fixed-size ROI was placed over the test zone and a background ROI was automatically placed equidistant from the signal ROI and the wicking pad. The code first computed the complement of the ROI regions, so that an increase in pixel intensity correlated with an increase in signal. Then, a line scan was performed over the ROI, the maximum pixel value in each row was determined, and these values were averaged to create an overall signal intensity. The signal ROI value was divided by the signal from the background ROI to create the signal-to-background ratios. Two-sided t-tests of unequal variance were used to determine the limits of detection for both perpendicular and linear device layouts, with p-values less than 0.05 considered significant.

3.3.8. Validation in Spiked Samples

A linear dilution was used to create a range of recombinant *Pf*HRP2 samples spiked into fetal bovine serum (FBS): 0 ng/mL, 0.1 ng/mL, 0.25 ng/mL, 0.5 ng/mL, and 1 ng/mL. This linear range was run in triplicate for both perpendicular, or "leg", and linear device formats. Signal-to-background analysis using the MATLAB algorithm described above was performed, and two-sided t-tests of unequal variance were used to determine the limits of detection for both perpendicular and linear device layouts, with p-values less than 0.05 considered significant.

3.3.9. Detection in Whole Blood Samples

As a proof-of-concept experiment, the assay was amended to incorporate a plasma separation membrane (GE Healthcare, Chicago, IL) for detection in whole blood samples. Because the plasma separation membrane filters plasma from whole blood laterally and not

vertically, an inlet had to be added to the nitrocellulose membrane at the sample pad. The remaining aspects of the assay did not change. Concentrations of 0, 1, and 10 ng/mL recombinant *Pf*HRP spiked into whole blood were run on the device, imaged, and analyzed using the MATLAB algorithm described above.

3.3.10. Statistical Analysis

Two-sided t-tests of unequal variance were used to determine the limits of detection for all analyses, with p-values less than 0.05 considered significant.

3.4. Results

We successfully developed a highly sensitive 2DPN that is acceptable for use at the point-of-care. While previous 2DPNs achieved a limit of detection of 2.9 ng/mL, highly sensitive tests that equal the CELISA sensitivity of 0.11 ng/mL were desired[102]. Reducing the sensitivity 30X without infrastructure or equipment required engineering both the assay components as well as assay layout. In order to accomplish this goal, we enhanced both signal amplification methods and assay layout to develop a more complex and highly sensitive 2DPN.

First, biotin-streptavidin signal amplification was incorporated into the assay. To incorporate biotin-polystreptavidin signal amplification and ensure maximum sensitivity, the 2D paper networks was extended to include six inlets (**Figure 3-1)**. Sample was added to the first pad, followed by 1) biotinylated detection antibody, 2) poly-HRP80 as the enzyme complex, 3) a PBST wash step, 4) DAB with sodium percarbonate as the source of hydrogen peroxide, and 5) a final PBST wash step. The first wash step was incorporated to reduce

background signal and prevent DAB from oxidizing horseradish peroxidase and precipitating while travelling down the nitrocellulose strip. Additionally, the size of the sample pad was increased compared to previous 2DPNs to enable detection of lower concentrations of protein. At small scale production, the assay costs $0.86 (**Table 3)**. We predict that large-scale production and additional optimization of the paper and plastic components could further reduce cost.

Item	**Cost**
Paper-based materials	$0.065
Plastics	$0.207
Antibodies	$0.403
Signal Amplification Reagents	$0.108
Buffers	$0.080
Total	**$0.862**

Table 3. Cost of highly sensitive paper-based ELISA.

Furthermore, the assay layout was rearranged into a linear design. Previous 2DPNs added reagents via perpendicular inlets to the nitrocellulose membrane ("leg"), which decreased sensitivity and increased cost. We hypothesized that the non-sequential flow caused by the perpendicular addition of reagents would decrease sensitivity, as the full quantity of reagents would not be delivered uniformly over the detection spot. To test this hypothesis, fluid flow was characterized, and limit of detection tests were run on both perpendicular and linear layouts.

Here, we describe results that characterize: 1) linear range and limit-of-detection of the CELISA gold standard kit with spiked samples; 2) fluid flow characterization for both perpendicular and linear assay layouts; 3) linear range and limit-of-detection of the 2DPNs with spiked samples; and 4) proof of concept testing using whole blood. Together, these

results demonstrate a point-of-care platform for highly sensitive antigen detection in resource-limited settings.

3.4.1. Limit-of-Detection with Malaria Ag CELISA Kit

As reported in literature, the Malaria Ag CELISA assay produced a limit of detection of 0.1 ng/mL **(Figure 3-2, A**). All p-values were significant, except between 0.05 ng/mL and 0 ng/mL. Likewise, when a traditional 96-well ELISA was run using the same reagents from our paper-based assay, 0.1 ng/mL was observed as the limit of detection (**Figure 3-2, B**). The p-values for all concentrations were significant ($p<0.05$), except between the samples 0.001 ng/mL, 0.01 ng/mL, and 0 ng/mL, and between 5 ng/mL and 25 ng/mL. Therefore, the gold standard for protein-based malaria *Pf*HRP detection was determined to be 0.1 ng/mL, or 10 pg of protein total since both assays require 100µL of sample.

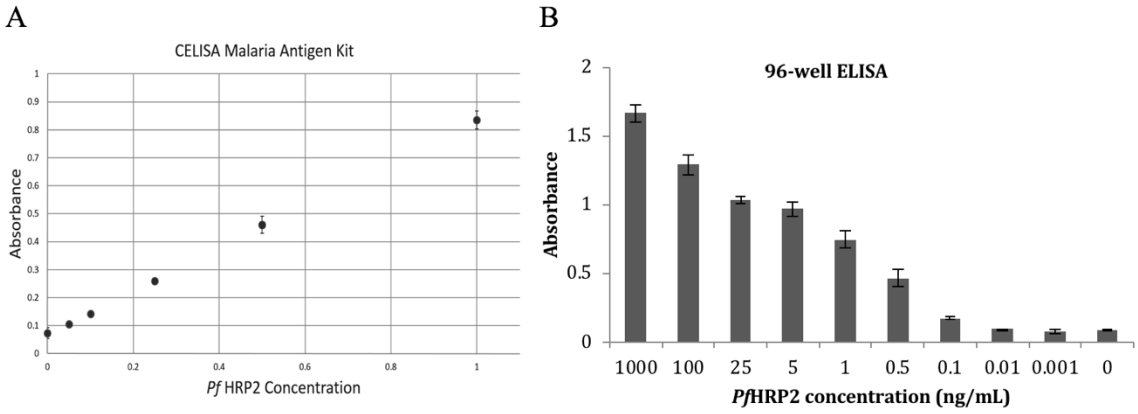

Figure 3-2: Results of Malaria Ag CELISA and traditional 96-Well ELISA gold standard assay. (A) After running a range of spiked *Pf*HRP2 concentrations from 0ng/mL to 1ng/mL on the Malaria Ag CELISA, the limit-of-detection was determined to be 0.1 ng/mL (n=3). A two-tailed t-test was performed between each concentration to determine significance. All p-values were significant ($p<0.05$), except between 0.05 ng/mL and 0 ng/mL. Image from [92]. (B) The limit-of-detection of 0.1 ng/mL also was observed for an in-house traditional 96-well ELISA using the same reagents used in the paper-based device (n=3). A two-tailed t-test was performed between each concentration to determine significance. There was no significant differences between the samples 0.001 ng/mL, 0.01

ng/mL, and 0 ng/mL, and between 5 ng/mL and 25 ng/mL. As the p-value between 0.1 ng/mL and 0 ng/mL was less than 0.05, this was determined to be the limit-of-detection.

3.4.2. Sequential Flow Comparison

Flow data showed that the linear layouts delivered reagents consecutively, while the perpendicular (or "leg") layout resulted in non-sequential flow. As shown in **Figure 3-3**, parallel flow of food dyes is noticeable at almost every time point during the perpendicular assay. In contrast, each reagent of the linear layout is fully delivered to the detection zone before the next reagent is delivered. Furthermore, the linear design is completed within 80 minutes, as the last reagent (red) has fully reached the detection region. The perpendicular design is still delivering the second to last reagent (orange) across part of the detection zone at 80 minutes. While the difference in time is not substantial, the linear design did perform faster.

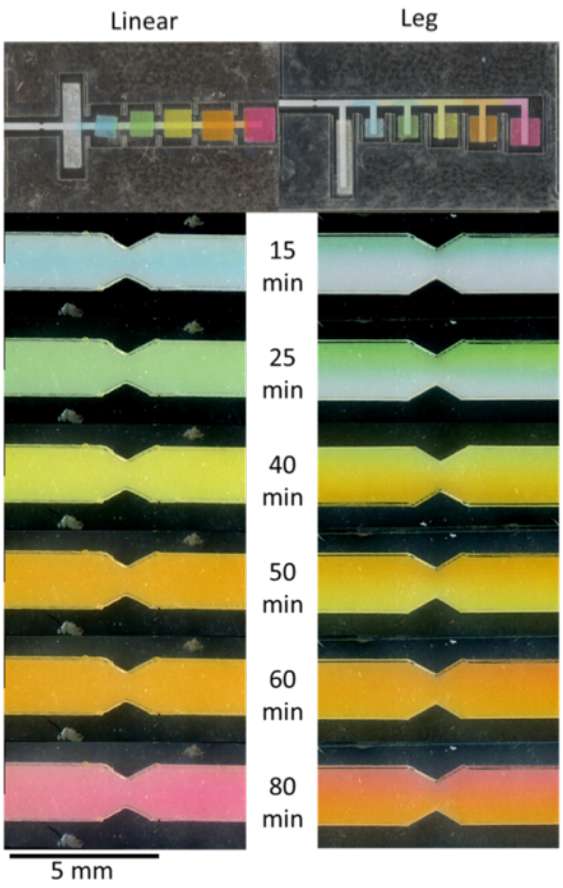

Figure 3-3: Flow profile comparing the linear and perpendicular devices. Food dye spiked into PBST shows flow over the course of 80 minutes. Only the linear device produced sequential flow of reagents; the perpendicular device created laminar flow of multiple reagents at once. Image from [92].

3.4.3. Limit-of-Detection with 2DPN Assay

Our highly sensitive paper-based ELISA with biotin-streptavidin signal amplification was tested in triplicate over a range of 0 to 100 ng/mL, and the signals were analyzed at both 60 and 90 minutes (**Figure 3-4**). Signal-to-background analysis using MATLAB compared the signal intensity from the ROI at the detection zone to the signal intensity from a background ROI of the same size.

Using this MATLAB code, the device produced a limit of detection of 0.1 ng/mL after 90 minutes, or 5 pg of protein total since the assay requires 50μL of sample (**Figure 3-4, J**). This value equals the sensitivity and limit of detection reached by both the commercially available CELISA and the traditional 96-well ELISA with the same reagents. A colorimetric signal could be visually observed at the limit of detection after 90 minutes as well, confirming that the test can be analyzed without expensive equipment **(Figure 3-4, A-C**). Signal-to-background of 0.1 ng/mL was not significantly different from 0 ng/mL at 60 minutes however, indicating that for full sensitivity, the test takes 80 or more minutes to run. This observation agrees with data from the flow characterization experiment.

Additionally, the corresponding perpendicular layout was tested in triplicate over a range of 0 to 100 ng/mL, and the signals also were analyzed at both 60 and 90 minutes (**Figure 3-4, H**). In contrast to the linear design, no statistical differences in signal were noted at 60 or 90 minutes. While signal was observed at 0.5 ng/mL and 1 ng/mL, high standard deviation prevented significant conclusions about limit of detection, likely due to the variability in parallel flow as noted by the flow characterization experiments (**Figure 3-4, D-F**). Also, while the signal in the linear design spanned across the entire nitrocellulose width, the perpendicular format showed signal only in the center of the strip. We hypothesize that the strip center was the only area to receive an adequate amount of each reagent.

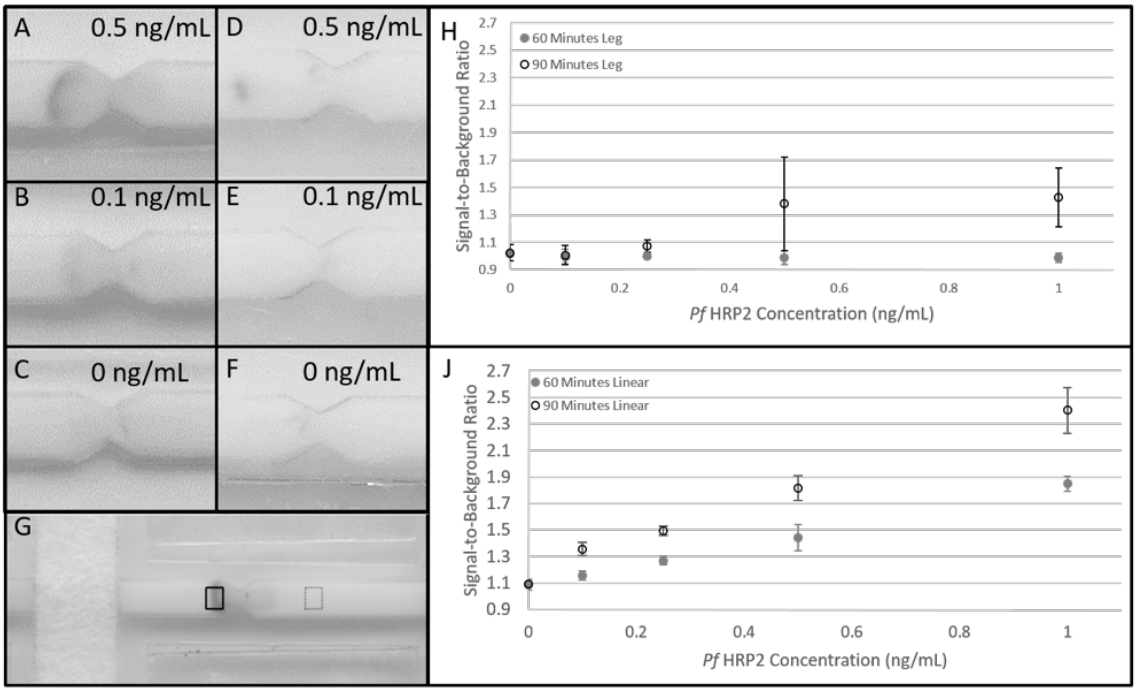

Figure 3-4: Highly sensitive assay validation. Images are shown for the linear assay detection region at (A) 0.5 ng/mL, (B) 0.1 ng/mL, and (C) 0 ng/mL *Pf*HRP2. The signal is visible by eyesight as low as 0.1ng/mL, so high-cost equipment is not necessary for qualitative results. Images are also shown for the perpendicular assay at D) 0.5 ng/mL, (E) 0.1 ng/mL, and (F) 0 ng/mL *Pf*HRP2. Signal in the perpendicular assay is present only in the center of the nitrocellulose, whereas the linear assay displays signal across the membrane width. (G) Example of MATLAB signal-to-background regions are shown, with the solid black box as the signal ROI and the dotted box as the background ROI. (H) The perpendicular assay showed no significant differences in signal-to-background at 60 or 90 minutes due to the high inter-strip variability. (J) For the linear assay, a limit of detection of 0.1 ng/mL *Pf*HRP2 was observed after 90 minutes, equivalent to the limits reported for the CELISA and the traditional 96-well ELISA in lab. At 60 minutes, 0.1 ng/mL data were not significantly different from 0 ng/mL, but after 90 minutes, all data were statistically significant. Image from [92].

3.4.4. Proof of Concept with Whole Blood

Finally, as a proof-of-concept, the assay was altered and tested for use with whole blood samples. A nitrocellulose inlet was added at the sample pad, and GE Healthcare plasma separation membranes were utilized to separate plasma from whole blood. **Figure 3-5** shows a proof-of-concept, with 10 ng/mL and 1 ng/mL of *Pf*HRP2 spiked into whole

blood reported into a modified version of the assay. Future validation studies should test clinical samples from patients with subclinical infection of malaria.

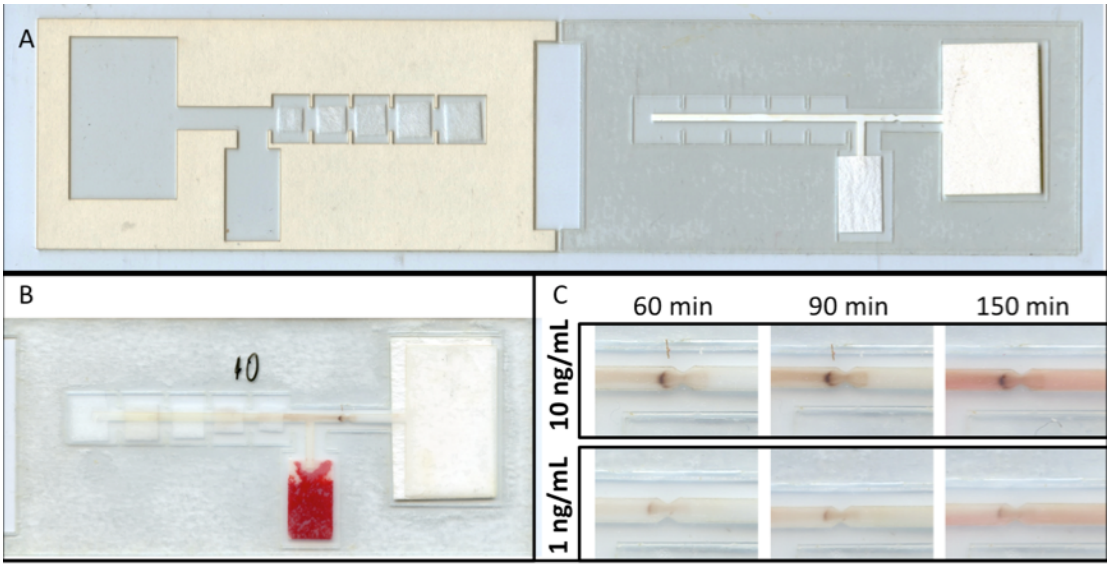

Figure 3-5: Whole blood proof-of-concept. (A-B) Device design was altered to accommodate a plasma separation pad for sample inlet, and is shown before and after folding. (C) Images of the signal at the detection zone for 10 ng/mL and 1 ng/mL over a few hours. Signal is visible even after lysis of red blood cells.

3.5. Discussion

We have demonstrated a paper-based ELISA platform which achieved equivalent sensitivity to the gold standard Cellabs CELISA Malaria Antigen Kit and a traditional 96-well ELISA. A comparison of the platform with a traditional 96-well ELISA is shown in **Table 4**. In the future, the assay could be useful for the WHO malaria elimination goals, if desired as

a low-cost method to screen asymptomatic patients; however, the assay would first need to be validated with clinical testing.

	96-Well ELISA	Paper-Based ELISA
Cost	$0.75-$3.50 per test; Equipment: ~$500 orbital shaker; ~$20,000 plate reader	$0.86 per test; no equipment costs
Sensitivity	0.1 ng/mL	0.1 ng/mL
Ease-of-Use	Highly trained laboratory technicians	Minimally trained healthcare workers
Time	4+ hours	60-90 minutes
Infrastructure Requirements	Electricity	None

Table 4. Comparison of traditional 96-well ELISAs with the paper-based ELISA for *Pf*HRP2.

While relativity point-of-care friendly, improvements to the assay design could enhance the utility in resource limited settings. In order to minimize user input, ELISA reagents could be lyophilized into the glass fiber pads, so that a user would only need to reconstitute the pads with buffer. This would reduce user steps and remove the need for refrigeration of antibodies and enzymes; however, studies addressing the stability of lyophilized reagents in heat and humidity would need to be conducted to ensure the 2DPN was appropriate for resource-limited settings. Additionally, we acknowledge that the time to result for our 2DPN is higher than a typical LFA. However, for highly sensitive applications, 90 minutes is an improvement upon traditional ELISAs (4+ hours), and the 2DPN provides an infrastructure-free, low-cost solution. The platform is low-cost and can be easily adapted to develop additional assays with high sensitivity for resource-limited settings.

Chapter 4

An HPV DNA Paper Assay for Cervical Cancer Screening in Low-Resource Settings

This chapter describes the development of a point-of-care HPV DNA paper assay which uses hybrid capture technology on a paper ELISA platform. The assay was developed and evaluated with synthetic DNA, cellular materials, and clinical samples. Parts of Chapter 4 were presented at the 2017 SelectBio Conference on Point-of-Care Diagnostics, Global Health, and Emerging Viral Disease *in Coronado Island, California*; *the 2018* 12th Stop Cervical, Breast, & Prostate Cancers in Africa Conference (SCCA) *in Maseru, Lesotho*; and the *2018* Rice University Fifth Annual Innovation Symposium *in Houston, Texas. A publication involving the contents of Chapter 4 is currently in preparation for submission.*

4.1. Abstract

High-risk human papillomavirus (HPV) DNA testing is the most sensitive screening technology for cervical cancer; however, per-test cost and infrastructure requirements limit HPV DNA testing in resource-limited settings that could benefit most from screening programs. To overcome this challenge, I have developed a paper-based hybrid-capture assay that detects high-risk HPV DNA within an hour. The assay is equivalent in sensitivity to the *digene* Hybrid Capture 2 (HC2) assay without the need for expensive read-out equipment. The test has an estimated per-test cost of less than $3 without the need for batching. I demonstrate performance with DNA standards, cellular ranges, and a small pilot study (n=16), showing 93.75% accuracy compared to reported careHPV results. Furthermore, usability studies in El Salvador and Mozambique report the assay as acceptable to perform. After additional clinical validation, the HPV DNA paper assay could serve as a low-cost, point-of-care screening test for resource-limited settings.

4.2. Introduction

Cervical cancer is preventable, yet 570,000 new cases and 311,000 new deaths are reported annually [1–3]. The burden for cervical cancer lies largely in resource-limited settings, where access to screening and diagnostic programs may be limited [4,5]. Countries with accessible screening programs, such as the United States, have largely seen reduction in deaths since their implementation [7]. On the other hand, low-and-middle-income countries (LMICs) have an increasing share of the global burden, with over 85% of mortality cases in resource-limited areas [6]. This is disparity is largely driven by lack of availability of screening programs and technologies.

The high-risk human papillomavirus (HPV) DNA test is widely accepted as the most sensitive screening method, as almost all cervical cancers are caused by HPV, a virus that integrates with host genome to produce oncogenes involved in the malignant conversion of cells [45,50]. Tests that detect high-risk HPV strands (HPV 16/18/31/33/35/39/45/51/52/56/58/59/68) have high negative predictive values (NPVs) over 98% [48]. With very few false negatives, HPV DNA testing is a great first line screen for cervical cancer. Recent studies have shown a single screen using HPV DNA testing is effective at reducing up to 50% of advanced cervical cancers and cervical cancer deaths, more than a single screen using VIA or cytological testing [54]. Additionally, studies have shown that self-collected cervical swabs produce equivalent results with physician-collected swabs [118,119]. Self-collection improves access for many women who either do not have access to healthcare providers or do not feel comfortable with pelvic exams due to cultural or religious reasons.

As mentioned in Chapter 2, amplification-based HPV DNA tests are often inappropriate for low-resource settings due to per-test cost, necessary instrumentation, and need for highly trained personnel [61,120]. Other commercially available, hybrid capture HPV DNA tests are expensive (*digene* HC2 HPV DNA Test $71/test) and require expensive infrastructure to read the assay [48,62]. The careHPV test is a HPV DNA detection method developed specifically for use in low-resource settings. However, despite a low per-test cost of $5/test, samples must be run in batches of 90 to be cost-effective [26,64]. Because patient samples are batched and not run individually, women are not treated in the same visit as their screen, and may be lost to follow-up. Moreover, both of these hybrid capture assays must be performed in a lab with an orbital shaker and plate reader to read luminescence, costing at least $20,000 and requiring stable power sources [65]. A low-cost, equipment-free HPV DNA test is required to truly be effective at the point-of-care.

To overcome these limitations, I have developed a low-cost, point-of-care high-risk HPV DNA paper assay, which uses hybrid capture technology on a highly sensitive paper platform to produce a low-cost and accurate screening test. This work builds upon the paper ELISA platform from chapter 3, and is therefore both low cost and highly sensitive [92]. The assay workflow requires seven user steps with only a heater needed for instrumentation. First, I characterize and assess the point-of-care sample preparation protocol on the HPV DNA paper platform using DNA standards. Next, I demonstrate performance with cellular samples using fully lyophilized reagents. Finally, I perform a small pilot study (n=16) and usability assessment (n=44) to determine assay accuracy compared to gold standard HPV DNA testing and ease-of-use, respectively.

4.3. Methods

4.3.1. HPV DNA Paper Assay Components

The HPV DNA paper assay was assembled as described in Chapter 3 [92], with some modifications. Briefly, an in-house laser cutter (Universal Laser Systems, Scottsdale, AZ) was used to create device components, including adhesive plastic (5 mm Dura-Lar, Blick Art Supplies, Galesburg, IL), membrane (backed CN95, Sartorius, Goettingen, Germany), glass fiber pads (grade 8951, Ahlstrom, Helsinki, Finland), and a wicking pad (C083, Millipore, Billerica, MA). The device components are shown in

Figure 4-1**.**

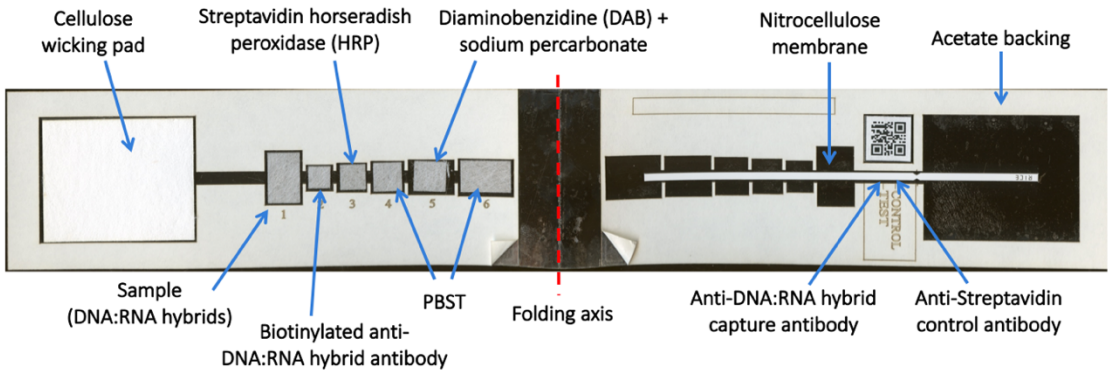

Figure 4-1: HPV DNA paper assay components. The paper assay includes a nitrocellulose membrane with capture antibodies spotted using a sciFLEXARRAYER S3 system, a wicking pad, glass fiber pads with lyophilized HPV DNA ELISA reagents, and an adhesive acetate backing. A QR code adjacent to the test and control lines is used by low-cost readers to locate the area for signal analysis (Brady Hunt thesis) and can also monitor batches of reagents for quality control.

A sciflexarrayer S3 machine printed control and test antibodies onto the nitrocellulose membrane: 80 nL of 250 μg/mL streptavidin monoclonal antibody (S10D4, Thermo Fisher Scientific, Waltham, MA) and 400 nL of 1 mg/mL anti-DNA-RNA hybrid antibody (MABE1095, Millipore, Billerica, MA) respectively. Once antibodies were printed, the nitrocellulose membranes were dried at 37°C for 60 minutes, blocked for 30 minutes with 0.5% BSA, 4% trehalose, and 1% sucrose in PBST, and dried for an additional 90 minutes at 37°C before storage at 4°C in a foil pouch with desiccant.

Enzyme-linked immunoassay (ELISA) reagents included 16 μg/mL biotinylated anti-DNA-RNA-hybrid detection antibody (ENH001, Boston, MA), 15 μg/mL streptavidin poly-HRP80, 1 mg/mL diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO), and sodium percarbonate (Sigma-Aldrich, St. Louis, Missouri). The sodium percarbonate was added to diaminobenzidine directly before running the assay with fresh reagents at 0.5% w/v, or lyophilized onto a glass fiber pad and stacked with lyophilized DAB pad for all lyophilization experiments. A solution of 1% BSA, 1% trehalose, and 1% sucrose in PBST was used as the wash buffer to separate the poly-horseradish polymerase (HRP) enzyme and colorimetric reagents while flowing down the nitrocellulose membrane. Volumes for reagents are as follows: 1) 50 μL sample, 2) 15 μL detection antibody, 3) 20 μL streptavidin poly-HRP80, 4) 25 μL wash buffer, 5) 30 μL colorimetric reagents, and 6) 50 μL final wash buffer.

4.3.2. Point-of-Care Sample Preparation Protocol

Achromopeptidase (ACP) was used as a lysis agent because the proteolytic enzyme has previously been shown to effectively lyse samples in a point-of-care friendly format [121–123]. ACP (MilliPore Sigma A3547, Burlington, MA) was reconstituted into 10mM Tris (pH 8.0) with 5% trehalose at 20U/μL. For the point-of-care sample preparation protocol, 0.5 μL of HPV RNA (*digene* Hybrid Capture 2, Qiagen, Germantown, MD), 18.25 μL of nuclease free water with 5% trehalose, and 1.25 μL 20U/uL ACP were mixed together, incubated for 5 minutes at room temperature, and heated at 95 °C for 10 minutes to fragment the RNA. After removal from the heater, 5 μL of 10X STE (Thermo Fisher Scientific, Waltham, MA) was added as a source of EDTA. At this point, the combined RNA and lysis solution was lyophilized, and when ready to run the assay, 25 μL of sample was added directly to the lyophilized pellet. Alternatively, 25 μL of sample was added directly to the RNA mixture without lyophilization to run the assay with fresh reagents. After sample addition, the solution was incubated for 5 minutes at room temperature, followed by a heating step at 95 °C for 5 minutes to denature and fragment the DNA. Upon cooling, the DNA hybridized to RNA in the solution, and the resultant DNA-RNA hybrids were added directly to the sample pad on the paper assay.

4.3.3. Lyophilization

Before lyophilization, detection antibody and streptavidin poly-HRP 80 were reconstituted in 1% BSA, 5% trehalose, and 5% sucrose. DAB and sodium percarbonate were reconstituted in nuclease free water with 5% trehalose. Wash pads were prepared using 1% BSA, 1% trehalose, and 1% sucrose. Reagent pads and RNA with ACP were flash frozen for 20 seconds using liquid nitrogen before lyophilization for at least 24 hours. Reagents were stored at -20°C with desiccant until use.

4.3.4. Point-of-Care Workflow

To run the assay, first the sample was lysed and DNA-RNA hybrids were formed using the protocol described above. Sample was added to pad 1, and other ELISA reagents were added to pads 2-6 for fresh reactions or rehydrated using PBST for lyophilized reagents. Once the paper backing on the adhesive was removed, the assays were folded in half to initiate fluid flow. After 45 minutes, tests were analyzed. If a colorimetric signal appears at the test and control lines, the assay is positive for high-risk HPV DNA; if signal only appears at the control line, the assay is negative for high-risk HPV DNA. Lack of any signal indicates an invalid result. All assays were imaged 600 dots-per-inch (DPI) with a flatbed color scanner. The full workflow is shown in **Figure 4-2**.

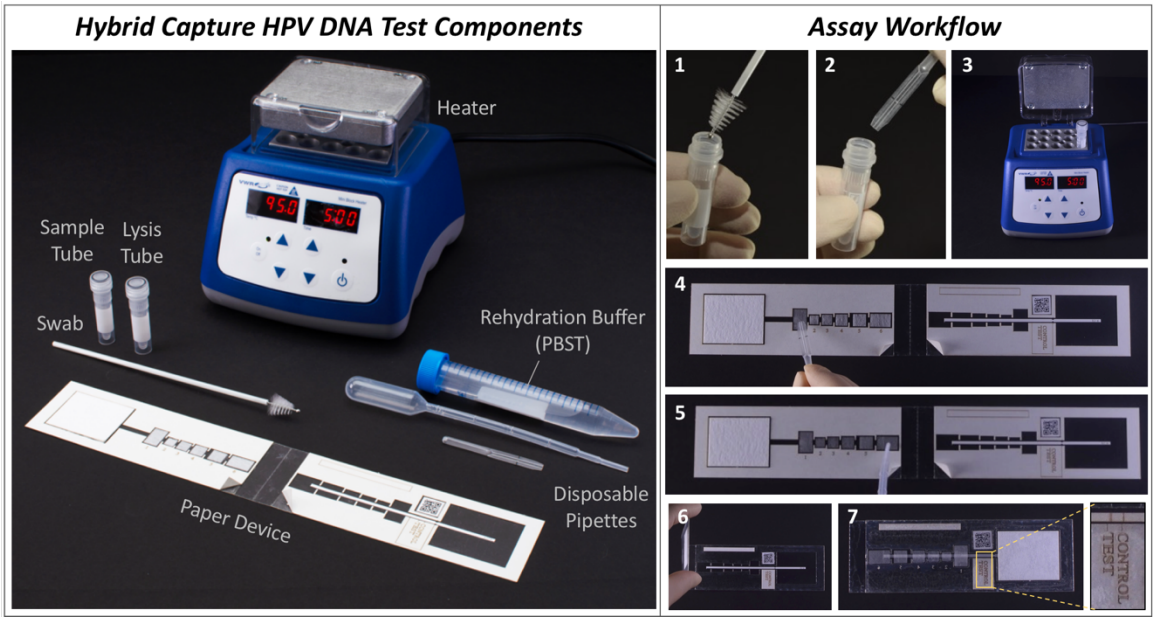

Figure 4-2: HPV DNA paper assay workflow. A point-of-care HPV DNA test that detects all high-risk HPV types using hybrid-capture technology. (Left) All necessary components for the assay are shown, including the swab, sample tube, lysis tube, disposable pipettes, HPV DNA paper device, rehydration buffer, and heater. (Right) The workflow involves seven user steps: 1) Swab the cervix with a brush and place into the sample tube. 2) Using an exact volume disposable pipette, add sample into a vial with lyophilized hrHPV RNA and Achromopeptidase (ACP) for lysis. Mix and incubate for 5 minutes at room temperature. 3) Heat at 95°C for 5 minutes. 4) Add sample to the first pad on the paper device. 5) Rehydrate lyophilized pads 2-6 with PBST (phosphate-buffered saline with 0.05% Tween20) rehydration buffer. 6) Peel paper backing to reveal sticky acetate and fold assay in half to initiate fluid flow, and 7) after 45 minutes observe signal visually or with a low-cost automated reader. For visual interpretation, two visible lines indicate a positive result. For automated interpretation, a portable reader [124] or mobile phone-based app can be used [Brady Hunt thesis].

4.3.5. Fragmentation Experiment

Theorizing that secondary structure from full length DNA and RNA could cause false positive results on the HPV DNA paper assay, I completed a fragmentation experiment, in which I created HPV DNA and HPV RNA fragments with differing sizes by heating with ACP at 95 °C over various time points.

4.3.5.1. Gel Electrophoresis for DNA

SiHa DNA was extracted using the DNeasy®Blood & Tissue Handbook (Qiagen, Germantown, MD), and then added to a solution containing 1X STE and ACP to a final concentration of 0.5 U/μL. Samples were incubated at room temperature for 5 minutes and then heated for 0, 0.5, 1, 2, 5, 10, or 30 minutes at 95 °C. Products were run on a 2% agarose gel at 140V for 1.5 hours.

4.3.5.2. Gel Electrophoresis for RNA

SiHa RNA was extracted using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA), including performing Genomic DNA Removal and RNA Cleanup. RNA was added to a solution containing 1) ACP to a final concentration of 0.5 U/μL without EDTA, or 2) a solution of 1X STE with ACP to a final concentration of 0.5 U/μL. Samples were incubated at room temperature for 5 minutes and then heated for 0, 0.5, 1, 2, 5, 10, or 30 minutes at 95 °C without EDTA; with EDTA, samples were run for 0, 5, 10, 15, or 30 minutes. Products were run on a 1% agarose gel at 70V for 2 hours.

4.3.5.3. Fragmentation Assessment on HPV DNA Paper Assay

I then tested both a high-risk HPV DNA control (5.0E+5 copies/mL HPV16, *digene* HC2 high-risk quality control standard, Qiagen) and a low-risk HPV DNA control (5.0E+5 copies/mL HPV6, *digene* HC2 low-risk quality control standard, Qiagen) in duplicate using the following heat profiles. With EDTA, DNA and RNA were heated for 0.5, 5, or 10 minutes at 95 °C after the addition of ACP and 5 minute room temperature incubation. Without EDTA, RNA was first heated for 0.5, 5, or 10 minutes with ACP as described above. After heating, EDTA in the form of 1X STE was added to the vial along with the DNA. Samples were mixed, incubated for 5 minutes at room temperature, and then heated a second time for 0.5, 5, or 10 minutes at 95 °C. Resultant hybrids were tested on the HPV DNA paper assays using the workflow described above. Results are shown in **Figure A1**.

4.3.6. *Digene* Hybrid Capture 2

The *digene* Hybrid Capture 2 test was performed on samples according to kit instructions. Briefly, DNA was denatured using a sodium hydroxide-based denaturant for 45 minutes at 65 °C, followed by addition of probe RNA and annealing at 65 °C for 60 minutes. 100 µL of the hybrid solution was added to the *digene* HC2 capture plate and incubated at room temperature on a shaker at 1100 rpm for 60 minutes. Next, 75 µL of Detection Agent 1 was added and incubated for 45 minutes at room temperature. Wells were washed 6X with the Wash Buffer before 75 µL of Detection Agent 2 was added and incubated for 15 minutes at room temperature. Finally, the chemiluminescence was measured using a plate reader (Tecan, Zürich, Switzerland).

4.3.7. Short HPV Synthetic Probe Targets

Short synthetic HPV16 and HPV6 DNA targets were hybridized to HPV16 RNA probes and tested with both *digene* HC2 and the HPV DNA paper assay. Briefly, a linear dilution of HPV16 short synthetic targets were created from 5E+13 copies/mL to 5E+9 copies/mL. These standards, along with a buffer control and 5E+13 copies/mL of low-risk HPV6 DNA, were combined with 10 µM complementary HPV 16 RNA in 1X STE solution and heated for 0.5 minutes at 95 °C to denature DNA and create DNA-RNA hybrids. The resultant hybrids were tested in both *digene* Hybrid Capture 2 and on the HPV DNA paper assays as described in the workflow above. HPV16 and HPV6 sequences are shown below.

HPV16 Double Stranded DNA	5'- CCC GAA AAG CAA AGT CAT ATA CCT CAC GTC GCA GTA -3'
HPV16 RNA	5'- rUrArC rUrGrC rGrArC rGrUrG rArGrG rUrArU rArUrG rArCrU rUrUrGrCrUrU rUrUrC rGrGrG -3'
HPV6 Double Stranded DNA	5'- ATC AAA GTG TCT ATA TTG GTT AAT TTT TCC ATG AAA -3'

4.3.8. HPV DNA Paper Assay Ranges with Qiagen Standards

A range of Qiagen standards from the *digene* HC2 kit, including 5.0E+5, 2.5E+5, and 1.0E+5 copies/mL of high-risk HPV16 DNA, 5.0E+5 copies/mL of low-risk HPV6 DNA, and a negative calibrator were run on the HPV DNA paper assays with various sample preparation protocols. The samples were also run per manufacturer's instruments in the *digene* HC2 kit.

4.3.8.1. *Digene* HC2 Sample Preparation

DNA-RNA hybrids were prepared using the standard *digene* HC2 sample preparation method, which includes a 2-hour process involving sodium hydroxide denaturant, as described above. After sample preparation, the assays were run on an orbital shaker to ensure fluidic flow.

4.3.8.2. Point-of-Care Sample Preparation with Achromopeptidase

The range of standards were also run using ACP lysis as described above. Two heating profiles were tested: 1) heating RNA for 0.5 minutes at 95 °C, followed by EDTA addition and heating DNA for an additional 0.5 minutes at 95 °C; and 2) the standard point-of-care protocol which includes heating RNA for 10 minutes at 95 °C, followed by EDTA addition and heating DNA for an additional 5 minutes at 95 °C. Samples were run in triplicate on HPV DNA paper assays.

4.3.9. HPV DNA Paper Assay Ranges with Cellular Samples

Cellular ranges were created by diluting SiHa (HPV16) and HeLa (HPV18) cells into C33A (HPV negative) cells, keeping the cell count consistent at 1 million cells/mL. These cellular samples were tested using the point-of-care ACP protocol described above with lyophilized pellets of RNA and ACP and lyophilized glass fiber pads. Samples were run in triplicate on the HPV DNA paper assays and imaged after an hour.

4.3.10. Collection Buffer Assessment

HeLa and C33A cells were reconstituted in buffers common to HPV DNA collection, namely SurePath preservation buffer (Becton Dickinson, Franklin Lakes, NJ) and PreservCyt (Hologic, Marlborough, MA). For SurePath samples, 1mL of sample was centrifuged at 4000 RPM for 10 minutes, buffer was converted to a 10mM Tris, and samples were heated at 120 °C for 20 minutes to reverse formalin-induced crosslinking. PreservCyt buffers were converted to 10mM Tris using the Sample Conversion Kit (Qiagen, Germantown, MD) per kit instructions. After conversion, positive (HeLa) and negative (C33A) controls were tested on the HPV DNA paper assay to determine buffer compatibility (**Figure A2)**.

4.3.11. Clinical Testing and Validation

4.3.11.1. Clinical Samples from Houston, TX

Samples were collected as part of a cross-sectional study performed at The University of Texas MD Anderson Cancer Center and Lyndon Baines Johnson General Hospital (LBJ) in Houston, TX. Women were eligible to participate if they 1) were over the age of 18; 2) were able to provide consent; and 3) had either histologically confirmed cervical, vaginal or vulvar high-grade dysplasia, invasive squamous cell carcinoma, invasive adenocarcinoma, or adenocarcinoma-in-situ (AIS) or high-grade intraepithelial lesion (HSIL) from a routine Pap test. Participants provided written informed consent, and the protocol was reviewed and approved by the Institutional Review Board (IRB) at both The University of Texas MD Anderson Cancer Center and Rice University. Two cervicovaginal swabs were collected from each participant. One swab was tested per clinical standard of care for HPV DNA using the Roche cobas HPV test. The second swab was collected into SurePath buffer for testing the Rice HPV DNA Paper assay. All samples were deidentified prior to testing at Rice University. Before clinical testing, SurePath buffer was converted to a 10mM Tris buffer as described above. Sensitivity, specificity, and accuracy were determined using Roche cobas HPV results.

4.3.11.2. Clinical Samples from El Salvador

Clinical validation was also performed using samples collected from a screening study at Basic Health International in El Salvador. Women were eligible to participate if they 1) were over the age of 30; 2) received a negative pregnancy test; 3) had an intact cervix; 4) had no history of invasive cervical cancer; and 5) were able and willing to provide consent. Participants provided written informed consent, and the protocol was reviewed and approved by the Rice Institutional Review Board (IRB), the University of Texas MD Anderson Cancer Center IRB, and the Comité Nacional de Ética de El Salvador. A standard of care cervicovaginal swab was collected and tested using careHPV to determine the participant's HPV status. A second cervicovaginal swab was collected and placed into PreservCyt buffer for testing at Rice University. For women with colposcopic lesions, a cervical biopsy was also obtained according to standard of care clinical protocols, and histopathologic diagnosis was performed using standard criteria.

Before clinical assessment with the point-of-care HPV DNA paper assay, clinical samples were converted from PreservCyt buffer to 10mM Tris using the Qiagen Sample Conversion Kit as described above. Sensitivity, specificity, and accuracy were determined using careHPV results.

4.3.12. Signal-to-Background Analysis

Signal-to-background analysis of the HPV DNA paper assay was calculated as described in Chapter 3 [92]. Briefly, a custom MATLAB code measured pixel intensities across the region-of-interest (ROI) associated with the test line and from a corresponding ROI of the nitrocellulose background. Signal-to-background was calculated by dividing the values from these two ROIs.

4.3.13. Statistical Analysis

Two-sided t-tests with values <0.05 were used to determine significance. Probit analyses were used to determine reported limits-of-detection. First, a positivity threshold was determined as the average negative signal plus three standard deviations. Then, test results were binarized as positive or negative compared to that positivity threshold, and probit analysis calculated the limit of detection with a probability value of 0.95 (XLSTAT, Addinsoft, Paris, France).

4.4. Results

I developed a highly sensitive point-of-care HPV DNA paper assay which is easy to use and appropriate for low-resource settings. First, I characterize the assay with short synthetic DNA and RNA sequences, followed by full genome HPV DNA synthetic standards. Next, I assess sensitivity using fully lyophilized reagents with a range of HPV positive cells. I validate the HPV DNA paper assay in a small pilot study using deidentified cervical samples obtained from a study with Basic Health International. Finally, I demonstrate ease-of-use of the HPV DNA paper assay through results from two usability studies- one in El Salvador and one in Mozambique.

4.4.1. Short HPV Synthetic DNA Targets

Probit analysis of short HPV synthetic DNA targets shows a limit of detection of 3.4E+10 copies/mL using *digene* HC2 and a limit of detection of 3.4E+11 copies/mL with the HPV DNA paper assay **(Figure 4-3).** In both assays, the positivity threshold was determined to be the average of the negative signal plus three standard deviations, and the low-risk HPV DNA created from HPV6 DNA produced a negative result, as expected. These data show that the HPV DNA paper assay performs well with high sensitivity and specificity using short targets.

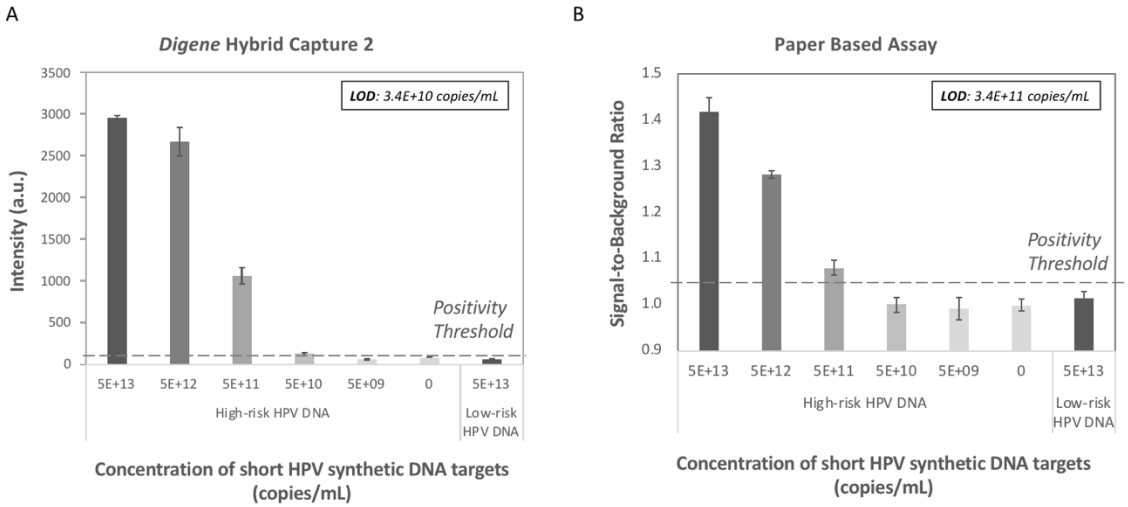

Figure 4-3: *Digene* Hybrid Capture 2 and HPV DNA paper assay with short HPV synthetic DNA targets. Signal intensity vs. target concentration for short HPV synthetic DNA-RNA hybrids tested in (A) the *digene* Hybrid Capture 2 assay and (B) the HPV DNA paper assay. Probit analysis showed the LOD for *digene* Hybrid Capture 2 is 3.4 E+10 copies/mL while the LOD for the HPV DNA paper assay is 3.4E+11 copies/mL. *High-risk HPV DNA = HPV16 target; Low-risk HPV DNA= HPV6 target; Dashed Line= positivity threshold determined as average negative signal ± three standard deviations.*

4.4.2. Qiagen Standards

When Qiagen DNA standards, including full genome HPV16 and HPV6 DNA sequences, were tested on the HPV DNA paper assay using *digene* HC2 sample preparation methods, I received a false positive result with the low risk HPV DNA (**Figure 4-4, A**). Likewise, when the standards were treated with an ACP lysis solution, and heated for 0.5 minutes at 95°C, the low risk HPV DNA sequences also produced a false positive result (**Figure 4-4, B**). I theorized this false positive signal was due to secondary structure of unfragmented DNA and RNA sequences sterically binding to the anti-DNA-RNA capture antibody in paper. A fragmentation experiment supported this theory (**Figure A1**), and from the results, I determined that the optimal sample preparation protocol included: mixing RNA with ACP, incubating for 5 minutes at room temperature, heating for 10 minutes at 95°C, adding EDTA and DNA, incubating for 5 minutes at room temperature, and finally heating the final solution for 5 minutes at 95°C. For end use, the RNA and ACP are lyophilized after heating and then EDTA addition, so the user simply needs to add DNA and heat for 5 minutes at 95°C. With this point-of-care sample preparation protocol, no false positive signal resulted with the low risk HPV DNA when tested on the HPV DNA paper assay (**Figure 4-4, C**). Standard *digene* HC2 was performed using the *digene* HC2 sample preparation methods as a control and to show that the false positive issue did not occur in ELISA format, only on paper (**Figure 4-4, D**).

Probit analysis was performed on the HPV DNA paper assay using the final point-of-care sample preparation and on the standard *digene* HC2 assay. In both cases, the limit of detection was determined to be 6.6E+4 copies/mL, showing that the HPV DNA paper assay has an equivalent limit of detection. Probit analyses were not conducted on the HPV DNA paper ranges using alternate sample preparation protocols due to the false positives. In

all cases, the positivity threshold was determined from the average signal of the negative calibrator plus three standard deviations.

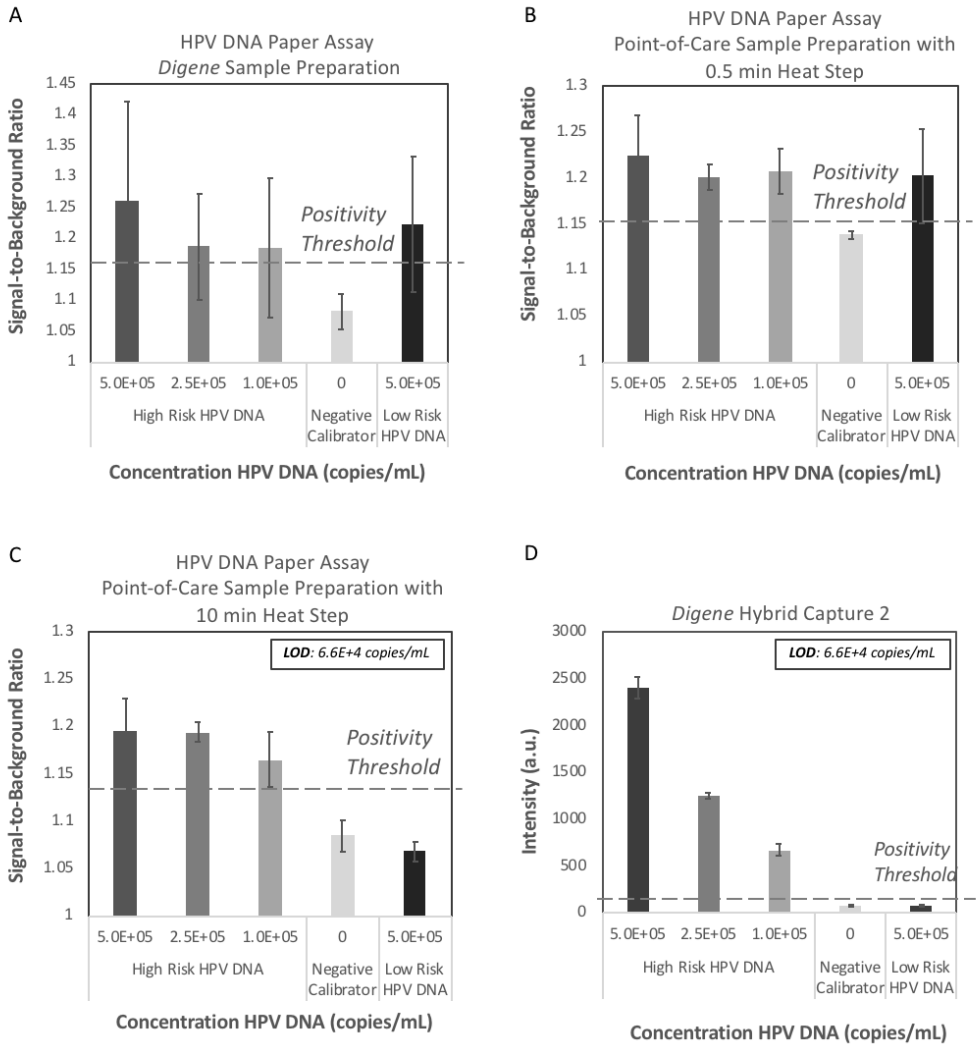

Figure 4-4: *Digene* Hybrid Capture 2 and HPV DNA paper assay with Qiagen standards. Signal intensity vs. target concentration for Qiagen standards (n=3 for each condition). The HPV DNA Paper assay was tested with three sample preparation methods: *digene* sample preparation (A); and a point-of-care sample preparation method that includes lysis with Achromopeptidase and heating at 95°C for 0.5 minutes (B) or 10 minutes (C). As shown in (A), the *digene* sample preparation method produced a false positive result for the low risk HPV DNA control on the paper device. As shown in (B), the point-of-care method with a 0.5 minute heat step also produces false positive results for low risk HPV DNA control. As shown in (C), the point-of-care sample preparation method with a 10 min heat step results in no signal for the negative calibrator and low-risk HPV DNA controls. The limit of detection for high risk HPV DNA is 6.6E+4 copies/mL, which is equivalent to that of the *digene* Hybrid Capture 2 assay shown in (D). *High-risk HPV DNA = HPV16 target; Low-risk HPV DNA= HPV6 target; Dashed Line= positivity threshold determined as average negative signal ± three standard deviations.*

4.4.3. Linear Range of Cellular Samples using Lyophilized Reagents

Linear ranges of high-risk HPV+ cellular samples were run in triplicate on the HPV DNA paper assay using fully lyophilized reagents **(Figure 4-5**). SiHa (HPV16+) and HeLa (HPV18+) were spiked into C33A (HPV negative) cells to ensure a consistent cell count. The cellular samples were added to lyophilized RNA and ACP, and subsequently treated using the point-of-care sample preparation protocol described in Figure 4-4C to lyse cells and create DNA-RNA hybrids. Samples were run on the HPV DNA paper assay using fully lyophilized reagent pads.

Probit analysis shows a limit of detection for SiHa of 1.43E+5 cells/mL and a limit of detection for HeLa of 6.87E+4 cells/mL. SiHa cells have approximately 1-2 copies of HPV16 per cell, whereas HeLa cells have 10-50 copies of HPV18 per cell [125], a lower limit of detection was expected for HeLa. Both cellular ranges showed strong signal with high copy numbers of HPV+ cells and minimal signal for C33A HPV negative cells.

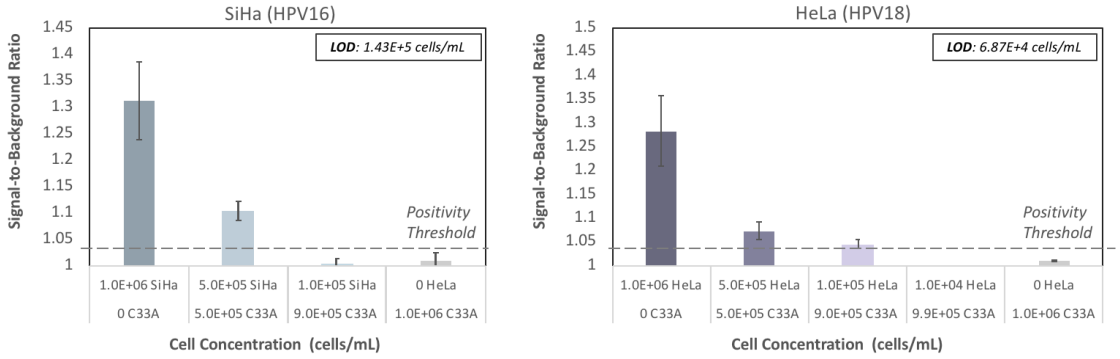

Figure 4-5: Linear range of cellular samples using lyophilized reagents. To assess assay performance and determine limit of detection with lyophilized reagents, linear dilutions of HPV+ cell lines were tested using the full workflow described in Figure 4-2. The HPV+ cells (SiHa, HPV16; HeLa, HPV18) were combined with HPV- cells (C33A) before lysis to ensure a consistent cell count. Using probit analysis, the limit of detection for SiHa cells was 1.43E+5 cells/mL, and the limit of detection for HeLa cells was *6.87E+4* cells/mL. *Dashed Line= positivity threshold determined as average negative signal ± three standard deviations.*

4.4.4. Clinical Assessment

I tested 16 samples from a screening population in conjunction with Basic Health International, El Salvador. All samples were collected into PreservCyt buffer. Using careHPV as the gold standard comparison test, 8 of the samples tested positive for high-risk HPV, and 8 tested negative for high-risk HPV. All 16 samples were tested on the HPV DNA paper assay with the point-of-care sample preparation protocol. The HPV DNA paper assay was determined to be positive if the test had a signal-to-background ratio above the positivity threshold determined as the average C33A signal in PreservCyt plus three standard deviations (**Figure A2**). Notably, results were the same if using any of the following positivity thresholds: the average C33A signal in PreservCyt plus three standard deviations (**Figure A2,** positivity threshold = 1.144), the average C33A signal in Tris plus three standard deviations (**Figure A2,** positivity threshold = 1.138), or the average negative calibrator signal plus three standard deviations (**Figure 4-4, C**; positivity threshold = 1.132).

The results of clinical testing are shown in **Figure 4-6** and

Table 5**.** The HPV DNA paper had an 87.5% sensitivity, 100% specificity, and 93.75% accuracy when compared to careHPV results. One sample characterized as high-risk HPV positive by careHPV tested negative in the HPV DNA paper assay. The rest of the HPV DNA paper results matched the corresponding careHPV results; seven samples characterized as hrHPV positive by careHPV tested positive in the HPV DNA paper assay, and eight samples characterized as HPV negative by careHPV tested as negative in the HPV DNA paper assay.

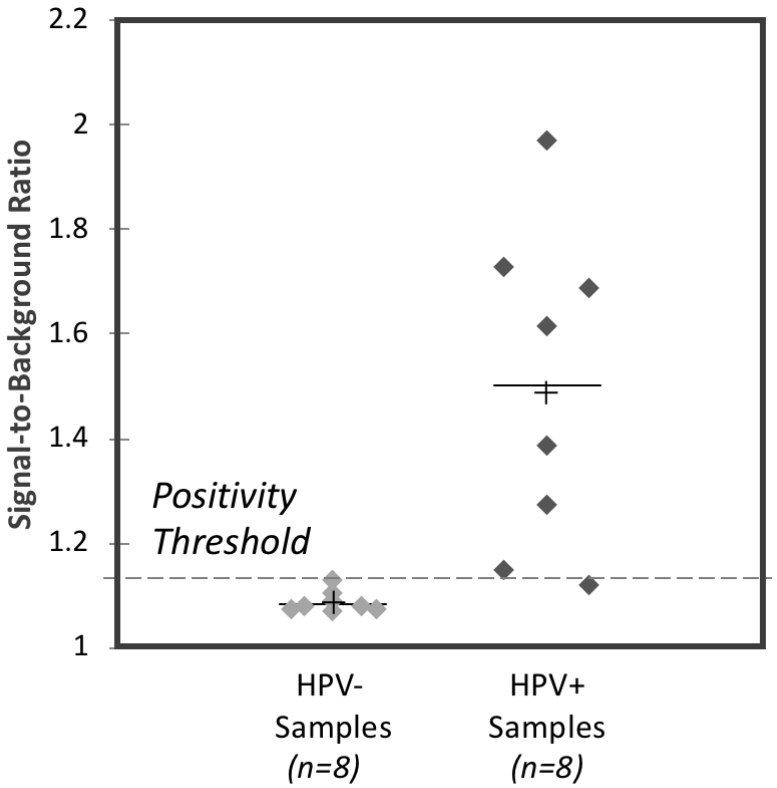

Figure 4-6: Clinical assessment of HPV DNA paper assay. Signal to background ratio for HPV DNA paper assay when performed with clinical samples collected into PreservCyt buffer and stratified by the gold standard, careHPV. The positivity threshold was determined using the negative C33A signal plus three standard deviations from **Figure A2**. There was a statistically significant difference in the

mean signal-to-background ratio of HPV- and HPV+ samples (p= 0.007). *Dashed Line= positivity threshold determined as average negative signal ± three standard deviations. + = mean; line = median.*

		Gold Standard: careHPV	
		HPV+	HPV-
HPV DNA Paper Assay	HPV+	7	0
	HPV-	1	8

Sensitivity (Se): 87.5%
Specificity (Sp): 100%
Accuracy: 93.75%

Table 5: Clinical results. Sensitivity, specificity, and accuracy of the HPV DNA paper assay for clinical samples compared to the gold standard HPV DNA test, careHPV (n=16).

4.4.5. Usability Assessment

Participants in both El Salvador and Mozambique filled out a System Usability Scale (SUS) survey after running the HPV DNA paper assay with mock samples (**Table 4-2**). The average results are shown in **Figure 4-7**. In El Salvador, the average SUS score was 82.07, while in Mozambique the average SUS score was 76.25. Scores over 70 indicate a test that is acceptable to use [126], and therefore the HPV DNA paper assay was determined as usable at both locations.

Site	Urban or Rural	Occupation
El Salvador (n= 30)	Urban (n=10)	Physicians (n=8)
		Nurses (n=1)
		Lab Technician (n=1)
	Rural (n= 20)	Physicians (n=20)
Mozambique (n=14)	Urban (n=14)	Physician or Nurse (n=13)
		Lab Technician (n=1)

Table 6: Usability participants. Number of participants from each site, stratified by location in an urban or rural setting and by occupation.

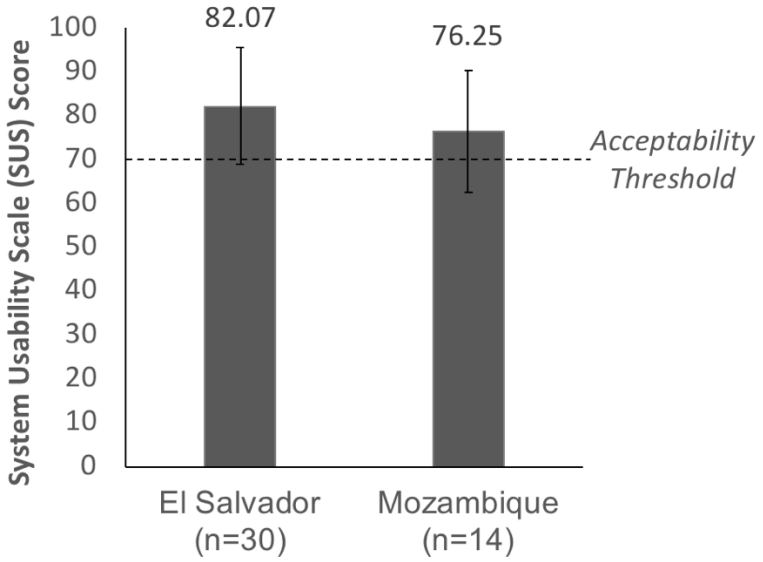

Figure 4-7: Usability testing. System usability scale (SUS) scores for the HPV DNA paper assay for users in two locations. Usability of the HPV DNA assay was assessed in El Salvador (n=30) with physicians practicing in rural (n=20) and urban locations (n=8), a nurse (n=1), and a lab technician (n=1); and in Mozambique (n=14) with physicians and nurses (n=13) and a lab technician (n=1). Participants performed two mock HPV DNA paper assays with the assistance of a job aid and subsequently filled out a usability survey. All groups rated the HPV DNA assay as acceptable to use (SUS score ≥ 70, indicated with dashed line).

4.5. Discussion

The HPV DNA paper assay is highly sensitive, with a comparable sensitivity to the gold standard *digene* HC2 assay. While probit analysis determined that the limit of detection with short HPV synthetic probes in the HPV DNA paper assay was an order of magnitude greater than the limit of detection with *digene* HC2, the limits of detection were determined to be equivalent for full length HPV standards, which are more clinically relevant. Both the HPV DNA paper assay with the point-of-care sample preparation and the *digene* HC2 had limits of detection of 6.6E+4 copies/mL when tested with Qiagen standards. Additionally,

both testing formats did not produce false positive results when tested with high levels of low-risk HPV6 DNA. I believe the HPV DNA paper assay is specific only with the developed point-of-care sample preparation protocol based upon the data from **Figure 4-4** and **Figure A1**. Steric binding of full-length nucleic acids to the capture antibody on a paper membrane produces false positive results; alternatively, fragmenting the DNA and RNA sequences with ACP plus heat at 95°C reduces secondary structure of nucleic acids and therefore nonspecific binding to the capture antibody.

The ability of the HPV DNA paper assay to work well with cellular samples and lyophilized reagents ensures that 1) achromopeptidase is effectively lysing cells; and 2) that the assay can be performed in a point-of-care manner as shown in **Figure 4-2**. With this workflow, users in El Salvador and Mozambique with no previous training were able to accurately perform the HPV DNA paper workflow, and rated the test as acceptable to use. Feedback from the usability study rated the timing of the DNA heating step and use of disposable pipettes as the most difficult aspects of the HPV DNA paper assay. A self-timed heater could remove the need for precise timing of sample preparation (5 minutes at 95°C), which could be difficult in a busy clinical setting. Additional testing is required to determine if boiling water is a suitable alternative to 95°C, for a more infrastructure-free workflow.

HPV DNA paper clinical results are promising when tested with samples in PreservCyt buffer and when compared to a gold standard HPV status determined by careHPV, resulting in an accuracy of 93.75% (**Figure 4-6, Table 5**). Additional clinical validation is necessary for a more accurate assessment of clinical sensitivity and specificity. Of note, the HPV DNA paper assay is based upon hybrid-capture technology, and using an amplification-based assay, such as GeneXpert, for gold standard HPV status will likely

produce a lower clinical sensitivity. Additional work is also necessary to process clinical samples in other collection buffers, such as SurePath preservative buffer (**Figure A3**). When samples collected into SurePath were tested, false negative and false positive results were reported. These issues could be due to incomplete reversal of nucleic acid and protein crosslinks induced by the formalin in SurePath buffer. Additional heating and washing of SurePath samples are necessary to determine if false positive signal can be reduced with SurePath samples in the HPV DNA paper assay.

Whereas the *digene* HC2 test is expensive ($71/test), requires expensive infrastructure to read the assay, and takes over four hours to produce a result [48,62], the HPV DNA paper assay is inexpensive (<$3/test) without the need for batching, requires only a heater for instrumentation, and produces a result within an hour. CareHPV is low cost at $5 per test, but requires batching in groups of 90 samples at a time to achieve this low cost and uses expensive readout equipment. As a result of batching, women have to come back to receive their HPV results and are often are lost to follow-up. The HPV DNA paper assay eliminates the need for batching and runs within an hour, making it potentially useful as a screening test in a see-and-treat clinic. A comparison of the HPV DNA paper test to other commercially available HPV DNA screening tests is shown in **Table 7.**

	Digene Hybrid Capture 2 (HC2)	careHPV	GeneXpert	HPV DNA Paper Test
Commercially Available?	Yes	Yes	Yes	No
Batching Required?	No	Yes	No	No
Limit of Detection (per literature)	100,000 copies/mL	100,000 copies/mL	2903 to 50,493 copies/mL	100,000 copies/mL
Limit of Detection (as evaluated in this work)	66,000 copies/mL			66,000 copies/mL
Time to Result	4.5+ hours	3+ hours	1 hour	1 hour
Cold Storage Requirements	Refrigerator	Refrigerator	None	None
Level of Lab Expertise Required	High	Medium	Low	Low

Table 7: Characteristics of commercially available HPV DNA tests and HPV DNA paper assay. The newly developed HPV DNA paper assay is compared to *digene* Hybrid Capture 2, careHPV, and

GeneXpert in the following: commercial availability, batching requirement, limit-of-detection, time to result, cold storage requirements, and level of lab expertise required [120].

4.6. Conclusion

I developed a sample-to-answer screening test for high-risk HPV DNA that is sensitive, low-cost, and easy-to-use. The assay is equivalent in sensitivity to commercially available hybrid-capture HPV DNA tests, with a 93.75% accuracy compared to reported careHPV results in a small pilot study. Furthermore, the test is complete within an hour, is low-cost without batching, and requires only seven user steps to perform. A heater is required for instrumentation, but no further complex or expensive machinery, reducing the level of infrastructure necessary to run the assay. Together, these characteristics could prove useful in a screen-and-treat setting for resource-limited areas with the highest cervical cancer prevalence. Once validated, the HPV DNA paper assay could serve as a rapid, point-of-care screening test for cervical cancer and precancer, helping to enable sustainable and inclusive access to cervical cancer screening and prevention for women in low-resource areas.

Chapter 5

A Paper-Based Assay for HPV E7 Oncoprotein Detection and Cervical Neoplasia Diagnosis at the Point-of-Care

This chapter describes the development of a point-of-care HPV E7 oncoprotein assay which uses the paper ELISA platform. The assay was developed and evaluated with recombinant HPV18 E7 protein, cellular materials, and clinical samples. Parts of Chapter 5 were presented at the National Cancer Institute in Bethesda, Maryland in 2017 and 2018. A publication involving the contents of Chapter 5 is currently in preparation for submission.

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5.1. Abstract

Although preventable if detected and treated early, cervical cancer remains a global issue with a high burden in resource-limited settings. Lack of affordable and easy-to-use screening and diagnostic tests contribute to the disparity, as several commercially available tests are not appropriate for use in low-and-middle income countries (LMICs). Specifically, HPV mRNA and oncoprotein tests that diagnose cervical cancer and precancer with high specificity require complex sample preparation protocols and expensive instrumentation. To address these limitations, I developed an HPV E7 oncoprotein assay for HPV16, HPV18, and HPV45 that is appropriate for use at the point of care. The assay is paper-based, easy-to-use, and does not require instrumentation to run. I demonstrate a clinically-relevant limit of detection with cellular samples using five simple user steps. Additionally, I assess clinical performance with a small pilot study (n=10), in which the HPV E7 paper assay is shown to have 90% accuracy in comparison to the gold standard of histopathology. With further clinical validation, this assay could provide a point-of-care diagnostic assay that is infrastructure-free and appropriate for use in resource-limited settings.

5.2. Introduction

Although preventable if detected and treated early, cervical cancer remains a leading cause of death among women in resource-limited settings, with 570,000 new cases and 311,000 deaths annually [1–3]. Over 85% of deaths due to cervical cancer occur in resource-limited settings, mainly due to lack of accessibility of early screening and diagnosis programs [4,5]. Expensive per-test costs, significant infrastructure requirements, and a lack of trained personnel prevent women in resource-limited settings from receiving potentially life-saving early detection measures.

In its 2014 guidelines for implementing screening programs in resource-limited settings, the World Health Organization recommends performing human papillomavirus (HPV) DNA testing, followed by a second more specific test, such as an oncoprotein test or cytology (**Figure 2-3**) if available [37]. High-risk HPV DNA tests are highly sensitive, with negative predictive values over 98% for cervical cancer and its precursors [46–48]. However, these HPV DNA tests are not specific for cervical neoplasia, as most HPV infections are cleared from the body within a few years; because of this, screen-and-treat programs based on HPV DNA testing alone can lead to high levels of overtreatment and wasted resources [45].

HPV mRNA and oncoprotein tests provide more diagnostic specificity for cervical precancer [52,53]. After HPV integrates into its cellular host, HPV mRNA begins to overexpress oncogenes, which in turn produce oncoproteins like E6 and E7 [50]. These oncoproteins can inhibit tumor suppressors such as p53 and pRB, leading to malignant transformation of infected cells. Therefore, overexpression of HPV E6 or E7 mRNA and/or oncoprotein production are key biomarkers for identifying high risk of cervical precancer and progression to cancer [52].

Several commercially available HPV mRNA tests exist, as shown in Chapter 2, with high diagnostic specificity; however, high per-test costs and the need for complex sample preparation and instrumentation limit utility of mRNA tests in resource-limited settings [120]. Arbor Vita has commercialized OncoE6, a diagnostic test that detects HPV16, HPV18, and HPV45 E6 oncoprotein [57]. The assay uses 100 uL of sample and detects as low as 2,000 cells per test. The clinical results from a screening and referral population showed very high specificity of the test (98.9-99.4%) with lower sensitivity (31.3-53.5%) when compared to histological CIN2+ pathology [52]. A second novel test in development, the Arbor Vita Onco E6/E7 Eight HPV Type Test, detects oncoprotein associated with additional HPV types (16, 18, 31, 33, 35, 45, 52, 58) at 2,000 to 10,000 total cells per assay [77]. With a pilot study (n=259, 31 CIN2+), the sensitivity for the assay was 67.7%, and specificity was 89.3% when compared to CIN2+ pathology; notably the sensitivity increased to 100% when compared with CIN3+ pathology (n=259, 10 CIN3+) [89]. While the Arbor Vita assay does require a $2000 instrument to read results, the main challenge for use in resource-limited settings is a complex 45-minute sample preparation process, which requires extensive user interaction and centrifugation when processing the sample [57]. The requirements for instrumentation and trained personnel limit use in settings where diagnostic testing is most desired.

To address these gaps, I developed a sample-to-answer, paper-based HPV E7 oncoprotein assay, which is low-cost and easy-to-use. Expanding upon the work of Chapter 3, the assay is a paper-based enzyme-linked immunoassay (ELISA) with high sensitivity due to signal amplification [92]. The assay can be performed in five simple steps, including sample preparation and lysis. Furthermore, no instrumentation or infrastructure is needed to run the assay, making it appropriate for use in resource-limited settings. Here, I first describe the workflow and characterize the point-of-care sample preparation and lysis protocols. Next, I assess the performance of the assay with HPV16, HPV18, and HPV45 cell lines in both the traditional 96-well ELISA and paper-based ELISA format. Finally, I validate the assay with clinical cytology samples from patients with histologic diagnoses of CIN2+ and <CIN2 in a pilot clinical study.

5.3. Methods

5.3.1. Cell Lines

Five cell lines were used to evaluate the oncoprotein assay: HeLa (HPV18, HTB-35), SiHa (HPV16, CCL-2), CaSki (HPV16, CRL-1550), MS751 (HPV45, HTB-34), and C33A (HPV negative, HTB-31). All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured using DMEM (Corning, Tewksbury, MA) with 10% fetal bovine serum (FBS, Bio-Techne, Minneapolis, MN) and Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, MA), and passaged no more than ten times. After passaging, cells were counted and pelleted, media was removed, and the dry pellets were stored at -80ºC until use.

5.3.2. Lysis Evaluation

Four buffers were tested for point-of-care lysis: 1) Tissue Protein Extraction Reagent (T-PER, Thermo Fisher Scientific, Waltham, MA); 2) Mammalian Protein Extraction Reagent (M-PER, Thermo Fisher Scientific, Waltham, MA); 3) NP-40 (Thermo Fisher Scientific, Waltham, MA); and 4) xTractor Buffer (Takara Bio, Mountain View, CA). Each buffer was compared to a no lysis control (NLC) and to a freeze-thaw positive lysis control. Five different cell types were tested, including HeLa (HPV18), SiHa (HPV16), CaSki (HPV16), MS751 (HPV45), and C33A (HPV negative).

For each point-of-care lysis method, buffer was added to a cell pellet at 10M cells/mL, briefly mixed, and incubated for 10 minutes at room temperature. No lysis controls were reconstituted in Phosphate Buffered Saline (PBS); the freeze-thaw samples were reconstituted into ice cold PBS with 0.05% Tween 20 (PBST) with 1 mg/mL EDTA-free protease inhibitor (Roche, Basel, Switzerland). For freeze-thaw, samples were frozen with liquid nitrogen and thawed in a 37°C water bath four successive times to achieve lysis. After sample preparation, all samples were centrifuged at 13,000 rcf for 10 minutes, and the resultant supernatant was diluted 1:2 in PBS before assessment using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA). Total protein concentration in the supernatant was used to characterize the lysis ability of each buffer. The fold change in lysis compared to freeze thaw was also calculated for each buffer by taking the ratio of protein concentration to the freeze thaw concentration of the corresponding cell type.

5.3.3. Lysis Buffer Comparison

Assay performance was compared for cells lysed in all four point-of-care lysis buffers using a traditional 96-well ELISA. The 96-well ELISA was performed using the protocol described in Chapter 3, with HPV E7 antibodies in the place of malaria antibodies, namely anti-HPV18 E7 monoclonal capture antibody (MBS310529, MyBioSource, San Diego, CA) and biotinylated anti-HPV E7 detection antibody (Ab100953, Abcam, Cambridge, MA). A small range of HeLa cells were spiked into C33A cells, so that the total cell number remained constant at 50,000 cells. Cellular samples were lysed using the point-of-care buffers with a 10 minute incubation step at room temperature, and added directly to ELISA plate for sample incubation. As a control, the same cellular range was prepared using standard freeze-thaw lysis as describe above.

5.3.4. HPV E7 Paper Assay Components and Workflow

The HPV E7 paper ELISA assays were prepared in a manner similar to those described in Chapter 3 [92]. Briefly, the devices consist of a nitrocellulose membrane (backed CN140, Sartorius, Goettingen, Germany), glass fiber pads (grade 8951, Ahlstrom, Helsinki, Finland), adhesive-backed plastic backing (5 mm Dura-Lar, Blick Art Supplies, Galesburg, IL), and a cellulose wicking pad (C083, Millipore, Billerica, MA), all cut using an in-house CO2 laser cutter (Universal Laser Systems, Scottsdale, AZ). An example of the paper device is shown in Figure 5-1.

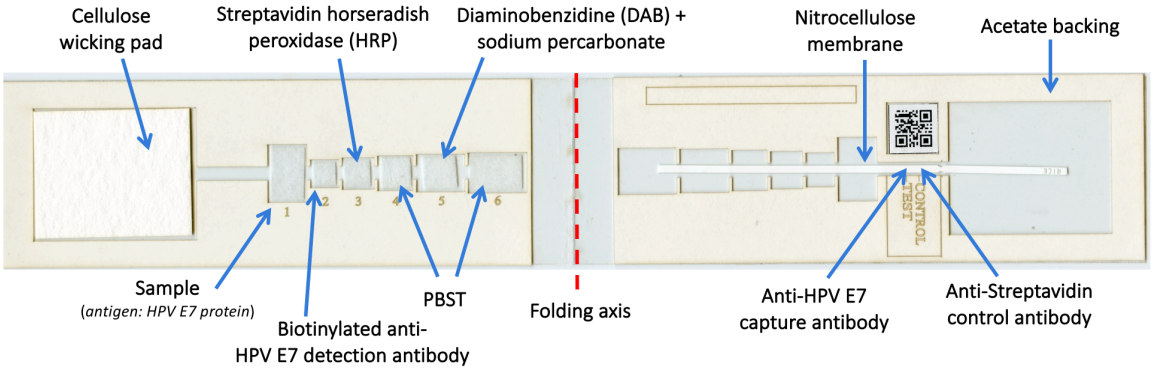

Figure 5-1: HPV E7 paper assay components. The HPV E7 paper assay includes a cellulose wicking pad, six glass fiber pads with lyophilized ELISA reagents, a nitrocellulose membrane printed with test antibody (anti-HPV E7) and a control antibody (anti-streptavidin) all on top of an acetate backing.

Capture lines were printed onto the nitrocellulose membrane using the sciFLEXARRAYER S3 (scienion, Berlin, Germany) printer. The control line consisted of 80 nL of 250 μg/mL streptavidin monoclonal antibody (S10D4, Thermo Fisher Scientific, Waltham, MA), while the test line consisted of 400 nL of 1 mg/mL anti-HPV18 E7 monoclonal antibody (MBS310529, MyBioSource, San Diego, CA). After printing, strips were dried for 1 hour in a 37° C incubator. Next, the nitrocellulose strips were blocked on an orbital shaker for 30 minutes with 0.5% BSA, 4% trehalose, and 1% sucrose in PBST. Finally, strips were dried for 1.5 hours in a 37°C incubator before being stored in a foil pouch with desiccant at 4° C until use.

When ready to run the assay, the nitrocellulose strips and glass fiber pads were added onto the adhesive-backed Dura-Lar backing. The following reagents were then added to the glass fiber pads as follows: 15 μL of 10 μg/mL biotinylated detection antibody (Ab100953, Abcam, Cambridge, MA), 20 μL of 20 μg/mL streptavidin poly-HRP80, 25 μL of wash buffer (1% BSA, 1% trehalose, 1% sucrose in PBST), 30 μL of the colorimetric solution, and 35 μL wash buffer (1% BSA, 1% trehalose, 1% sucrose in PBST). The colorimetric solution consisted of 2 mg/mL solution of diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) with 0.5% sodium percarbonate (Sigma-Aldrich, St. Louis, Missouri) added immediately before running the assay. Alternatively, lyophilized antibody, enzyme, colorimetric reagent, and wash pads were placed upon the acetate backing and rehydrated with PBST before folding the assay in half.

After adding 50 µL of sample to the first glass fiber pad and rehydrating the remaining lyophilized pads, the paper covering for the adhesive Dura-Lar was removed, and the assay was folded in half. Each component of the ELISA then flowed sequentially down the nitrocellulose to the test zone, where a reaction occurred if any oncoprotein was captured on the test line. The colorimetric reagents react with the streptavidin HRP captured at the control or test lines to form a brown precipitate; the results can be read visually. If E7 oncoprotein is present in the sample, two lines should appear: a control and test line. If the sample does not contain oncoprotein, only one line should appear: the control line. Absence of any lines indicates issues with the stored reagents, and results should be considered invalid. Paper-based ELISAs were imaged using a flatbed color scanner at 600 dots-per-inch (DPI). A complete workflow is shown in **Figure 5-2**.

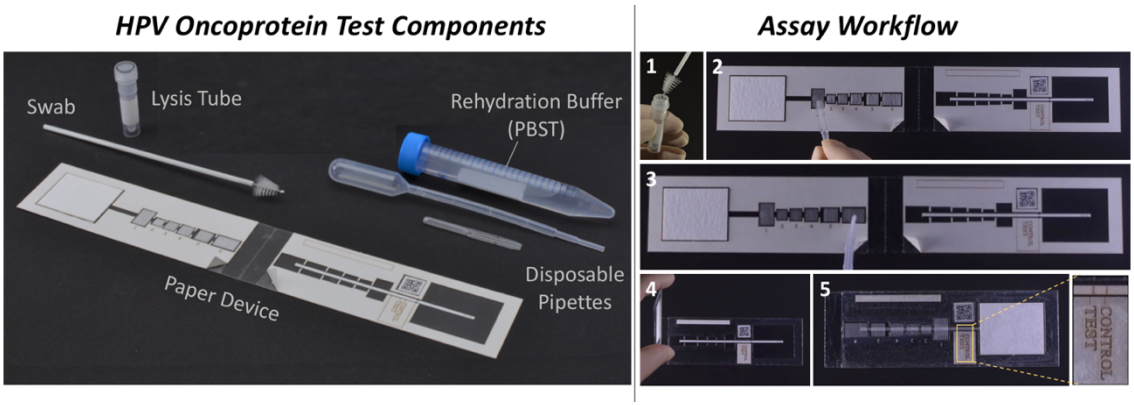

Figure 5-2: HPV E7 paper assay workflow**.** (Left) The complete set of reagents needed to run the HPV E7 assay includes a cervical swab, a tube containing lysis buffer (xTractor), a paper device, disposable pipettes, and rehydration buffer (PBS with Tween20 (PBST)). (Right) To perform the assay, only five user steps are required: 1) Place the cervical swab into the xTractor lysis buffer, mix, and incubate for 10 minutes at room temperature. 2) Add sample to the first pad using a disposable pipette. 3) Rehydrate the other reagent pads (2-6) with PBST using a second disposable pipette. 4) Peel off the paper backing and fold the assay in half. 5) After an hour, observe the test area. Colorimetric signal appears at the test line if HPV E7 oncoprotein is present in the sample.

5.3.5. Lyophilization

Biotinylated detection antibody, streptavidin poly-HRP80, DAB, sodium percarbonate, and wash pads were lyophilized as following. Detection antibody and streptavidin poly-HRP80 were diluted into a lyophilization solution (1% BSA, 5% trehalose, and 5% sucrose) at 10 μg/mL and 40 μg/mL, respectively. DAB and sodium percarbonate were prepared in water with 5% trehalose at 2 mg/mL and 2.5 mg/mL (0.25%), respectively. Wash pads consisted of 1% BSA in PBST. Reagents were added to glass fiber pads with the following volumes: 15 μL for biotinylated detection antibody, 20 μL for streptavidin poly-HRP80, 30 μL for DAB, 15 μL for sodium percarbonate, and 25 μL and 35 μL for the wash pads. DAB and sodium percarbonate were lyophilized onto separate glass fiber pads to prevent interaction before the pads are rehydrated. Reagents were flash frozen in liquid nitrogen for at least 20 seconds, and lyophilized for a minimum of 24 hours (LabConco FreeZone 12, Kansas City, MO). Reagents were stored in a foil pouch with desiccant at -20°C until use. During assembly, the lyophilized sodium percarbonate pad was placed onto the adhesive backing first and covered with the lyophilized DAB pad. When rehydrated, the two reagents mix before travelling down the nitrocellulose to the capture zone.

Lyophilized reagents were compared to freshly prepared reagents on a paper ELISA platform using positive (HeLa) and negative (C33A) samples. For each sample type, cell pellets were reconstituted at 1M cells/mL using xTractor buffer, incubated for 10 minutes at room temperature, and added directly to the sample pad. Lyophilized reagents were reconstituted with PBST.

5.3.6. BSA Assessment

To reduce any false positive results on the paper ELISA, various concentrations (1-3% w/v) of the blocking agent BSA were added to the reagent and wash pads and tested with 50,000 total HeLa and C33A cells in duplicate. HeLa and C33A cells were lysed with xTractor buffer as described previously. The optimal condition was defined as one that eliminates false positives (signal-to-background of C33A samples < 1), while retaining positive HeLa signal.

5.3.7. Reagent Optimization

The concentrations of paper ELISA components were optimized to maximize signal-to-background ratio of HPV+ cell lines while retaining a negative signal for C33A samples. HeLa and C33A samples were lysed with xTractor buffer and run in duplicate on the paper ELISA platform with the following conditions: baseline, 2X detection antibody concentration, 2X streptavidin poly-HRP80 concentration, 2X DAB concentration, and 0.1X sodium percarbonate concentration. As described previously, the baseline condition included 10ug/mL detection antibody, 20ug/mL streptavidin HRP, 1mg/mL DAB, and 0.5% sodium percarbonate. The optimal condition was defined as one that eliminates false positives (signal-to-background of C33A samples < 1), while retaining positive HeLa signal.

5.3.8. Assay Performance with a Range of Cellular and Recombinant Protein Concentrations

Samples with a range of HPV+ cell concentrations were created by diluting HeLa (HPV18), SiHa (HPV16), CaSki (HPV16), or MS751 (HPV45) cells into C33A (HPV negative) cells, so that the total cell number remained constant at 50,000 total cells. Each HPV positive cell type was tested over the following range: 50,000 cells, 25,000 cells, 10,000 cells, 5000 cells, 2500 cells, 1000 cells, 500 cells, and 0 cells diluted into HPV negative cells, plus a no cell control. Cells were lysed using xTractor buffer for 10 minutes at room temperature, then added directly to the 96-well ELISA plate or to the sample pad of the HPV E7 paper assay. Additionally, a range of HPV18 E7 recombinant protein (Biomatik, Wilmington, DE) was created by linear dilution into xTractor buffer. Each HeLa cell has approximately 1 fg of HPV18 E7 protein [127], so the following amounts of total recombinant protein were tested to correspond to the cellular HeLa range: 50 pg, 25 pg, 10 pg, 5 pg, 2.5 pg, 1 pg, 0.5 pg, and 0 pg. Cellular and recombinant protein ranges were tested in both traditional 96-well ELISA and HPV E7 paper assay, using the respective protocols described above.

5.3.9. Clinical Testing and Validation

Clinical validation was performed using cervicovaginal cytology swabs collected into SurePath preservation buffer (Becton Dickinson, Franklin Lakes, NJ) at the colposcopy clinic of The University of Texas MD Anderson Cancer Center or Lyndon Baines Johnson General Hospital (LBJ) in Houston, TX. Women were eligible to participate if they were 1) 18 years of age or older; 2) able to provide written consent; and 3) had either histologically confirmed cervical, vaginal or vulvar high-grade dysplasia, invasive squamous cell carcinoma, invasive adenocarcinoma, or adenocarcinoma-in-situ (AIS) or high-grade intraepithelial lesion (HSIL) from a routine Pap test. Participants provided written informed consent and the protocol was reviewed and approved by the Institutional Review Boards (IRB) at both The University of Texas MD Anderson Cancer Center and Rice University. Two cervicovaginal cytology swabs were collected from each participant. One was evaluated for clinical purposes using the Roche cobas HPV test. A second swab was evaluated using the HPV E7 paper assay; all samples were deidentified prior to testing at Rice University. For women with colposcopic lesions, a cervical biopsy was obtained according to standard of care clinical protocols and histopathologic diagnosis was performed using standard criteria.

For clinical testing, 1 mL of each sample was centrifuged at 4000 RPM for 10 minutes to pellet the cells. The SurePath buffer was removed and replaced with 100 μL of xTractor buffer. I note that future samples could be collected directly into xTractor buffer to eliminate the need for this centrifugation step. Samples were mixed and incubated for 10 minutes at room temperature before addition to the sample pad of the paper ELISA platform. Sensitivity and specificity were determined using pathology as the gold standard.

5.3.10. Signal-to-Background Analysis

Signal-to-background analysis of the HPV E7 paper strips were determined as previously described in Chapter 3 [92]. Briefly, a custom MATLAB code was used to assess the pixel intensities from a region-of-interest (ROI) at the test line and from a corresponding background ROI. A ratio of the two ROIs then determined the signal-to-background value.

5.3.11. Statistical Analysis

To assess whether differences in means were significant between conditions, a two-sided t-test was performed; p-values <0.05 were determined to be significant. For limit-of-detection analyses, a positivity threshold was first created using the average negative signal plus three standard deviations. Using that threshold, values were binarized, and probit analysis was performed to determine limit of detection using a probability value of 0.95 (XLSTAT, Addinsoft, Paris, France).

5.4. Results

5.4.1. Point-of-Care Sample Preparation

Of the four buffers I tested for point-of-care sample preparation, all four achieved lysis equivalent to or greater than the freeze-thaw positive control (**Figure 5-3,** n=2). xTractor buffer performed the best across all five cell types, with a 1.35-1.45 fold change in lysis compared to freeze-thaw. These results indicate that the 10 minute point-of-care protocol at room temperature is able to effectively lyse cellular samples.

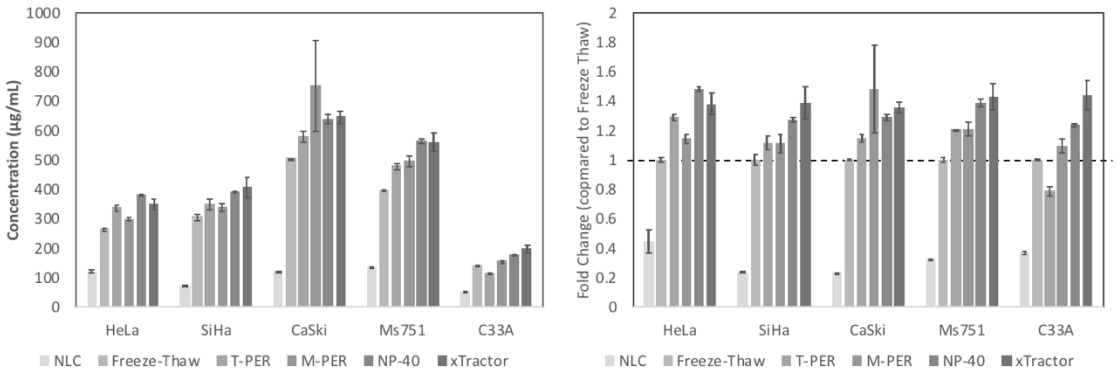

Figure 5-3: Point-of-care sample preparation: cell lysis. Four buffers were tested for point-of-care lysis: T-PER, M-PER, NP-40, and xTractor. (*Left*) The protein concentration in the resulting supernatant was compared to a standard negative lysis control (NLC) and a positive lysis control (Freeze-Thaw) using a BCA assay. *(Right)* Lysis efficiencies for the different buffers were assessed by comparison to the freeze-thaw condition for each cell type. All four buffers resulted in equivalent or greater lysis than the freeze-thaw positive control, with xTractor performing best across all five cell types. *NLC= no lysis control; HeLa = HPV18+; SiHa= HPV16+; CaSki= HPV16+; MS751= HPV45+; C33A = HPV negative.*

To assess the effect of the point-of-care lysis buffers on assay sensitivity, I performed a traditional 96-well ELISA over a range of cellular samples using all four lysis buffers as well as freeze-thaw lysis (**Figure 5-4**, n=2). All lysis methods produced an appropriate response in absorbance to HPV E7 oncoprotein levels in the HeLa samples. However, freeze-thaw and xTractor were the only lysis methods that had a significant difference ($p<0.05$) in absorbance between 1,000 HeLa cells and 50,000 C33A cells. Additionally, xTractor had a strong positive signal at higher HeLa concentrations of 50,000 HeLa cells and 10,000 HeLa cells compared to other lysis options. Therefore, I selected xTractor as the lysis buffer for future experiments.

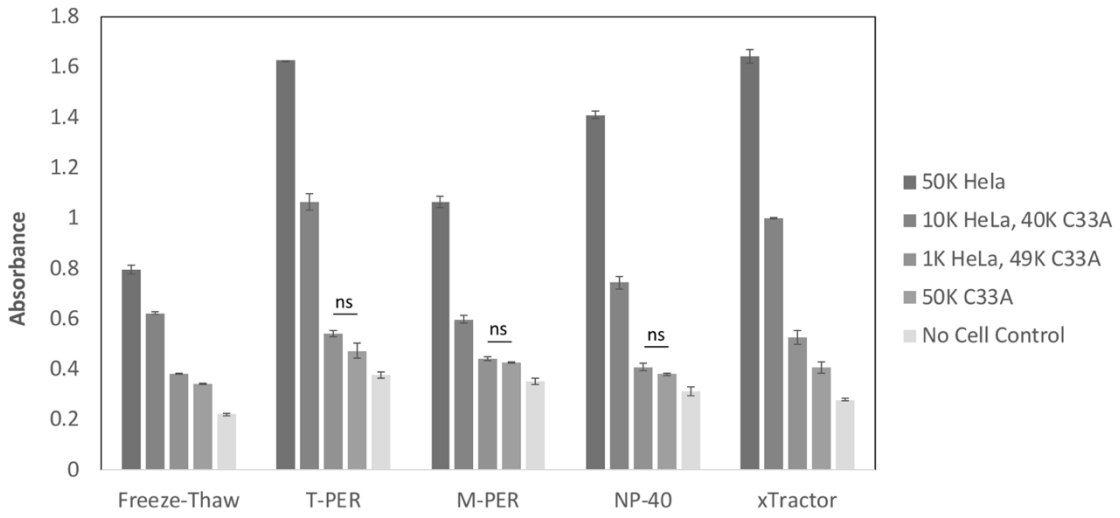

Figure 5-4: Traditional 96-well ELISA with different lysis buffers. A range of HeLa cells spiked into C33A cells were tested in a traditional 96-well E7 ELISA format using five lysis options: the positive control (Freeze-Thaw), T-PER, M-PER, NP-40, and xTractor buffers. Although all buffers performed well, Freeze-Thaw and xTractor sample preparation methods were the only two that resulted in a statistically significant difference in absorbance between 1,000 HeLa cells and 50,000 C33A cells. Additionally, xTractor had a strong positive signal at higher HeLa concentrations. Therefore, xTractor was selected as the lysis buffer for future experiments. *HeLa = HPV18+; C33A = HPV- negative; ns= no significant difference.*

5.4.2. Limit of Detection with 96-Well ELISA

Next, I tested a range of HeLa (HPV18), SiHa (HPV16), CaSki (HPV16), andMS751 (HPV45) cells spiked into C33A (HPV negative) in the traditional 96-well ELISA format; results are shown in **Figure 5-5** (A-D, respectively, n=2). I also tested a range of HPV18 E7 recombinant protein that corresponds to the HeLa cellular range (**Figure 5-5 E,** n=2). The positivity threshold was determined to be the average C33A signal plus three standard deviations, and probit analysis was performed using this threshold for positivity. The limits of detection for HPV+ cells were determined as: 135 total HeLa cells, 2,533 total SiHa cells,

6,210 total CaSki cells, and 1,823 total MS751 cells. The limit of detection for HPV18 E7 recombinant protein (135 fg) correlated well that of HeLa cells (135 total cells).

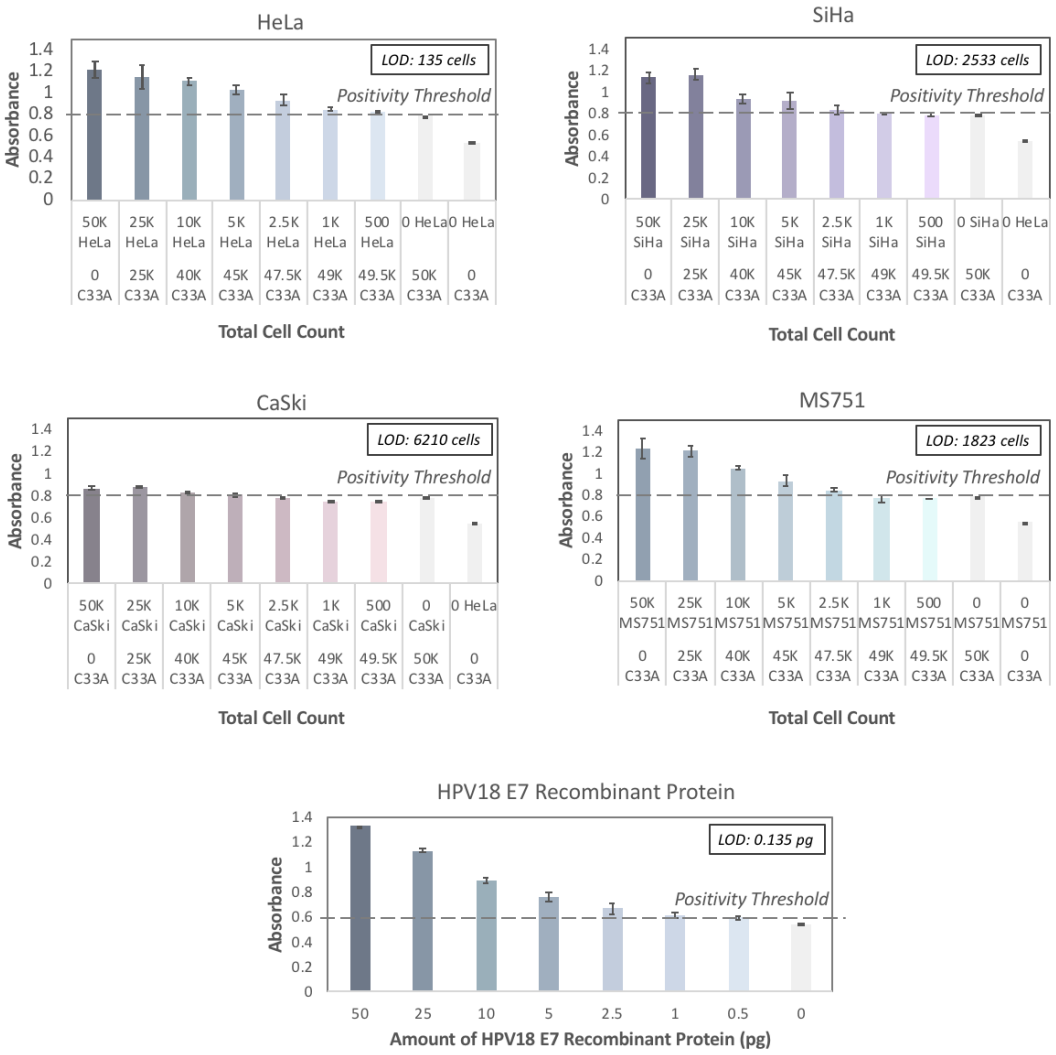

Figure 5-5: Range in a traditional 96-well E7 ELISA with xTractor sample preparation. *(A-D)* A range of HeLa (HPV18), SiHa (HPV16), CaSki (HPV16), and MS751 (HPV45) cells were spiked into C33A (HPV negative) cells so that total cell number remained constant. After point-of-care lysis using xTractor buffer, the samples were immediately tested in a traditional 96-well E7 ELISA (*n=2*). Using probit analysis, the limits-of-detection for HPV+ cells were determined as: 135 total HeLa cells, 2,533 total SiHa cells, 6,210 total CaSki cells, and 1,823 total MS751 cells. The positivity threshold represents the average negative signal ± three standard deviations. *(E)* A range of HPV18 E7 recombinant protein spiked into xTractor buffer (*n=2*). The limit-of-detection for HPV18 E7 recombinant protein is 135 fg which correlates to 135 total HeLa cells. *HeLa = HPV18+; SiHa=*

HPV16+; CaSki= HPV16+; MS751= HPV45+; C33A = HPV negative; Dashed Line= positivity threshold determined as average negative signal ± three standard deviations.

5.4.3. Limit of Detection with HPV E7 Paper Assay

All samples from the 96-well ELISA in **Figure 5-5** were also tested in the HPV E7 paper assay **(Figure 5-6**, n=3). I first determined the optimal amount of BSA in the glass fiber pads and showed that 1% w/v BSA in both wash and reagent pads can reduce false positive signal (**Figure B1**). I also optimized all paper components to achieve maximum signal-to-background for HPV positive cells while remaining clean for HPV negative cellular samples (**Figure B2**). Once again, I set the positivity threshold as the average C33A signal plus three standard deviations and performed probit analysis on results using that cut-off value. The limits of detection for HPV+ cells were determined as: 328 total HeLa cells, 15,968 total SiHa cells, 12,287 total CaSki cells, and 3,513 total MS751 cells. These values were likely limited by the inherent variability of the paper assay, as a larger coefficient of variation between the negative cellular signal resulted in a higher positivity threshold. Nevertheless, the limits of detection of all four cell types are within 16,000 total cells which is encouraging for a point-of-care assay that does not require sample manipulation. The assay is particularly promising for HPV18 E7 oncoprotein, with a limit of detection below 500 total HeLa cells. Additionally, the limit of detection for HPV18 E7 recombinant protein is 0.331 pg which correlates to 331 total HeLa cells. This value matches well with the HeLa limit of detection.

Finally, I ensured that paper ELISA performance was comparable for paper devices prepared with fresh reagents and fully lyophilized reagents (**Figure B3**). With point-of-care

lysis and lyophilized reagents, the HPV E7 paper test requires minimal user input while retaining good performance.

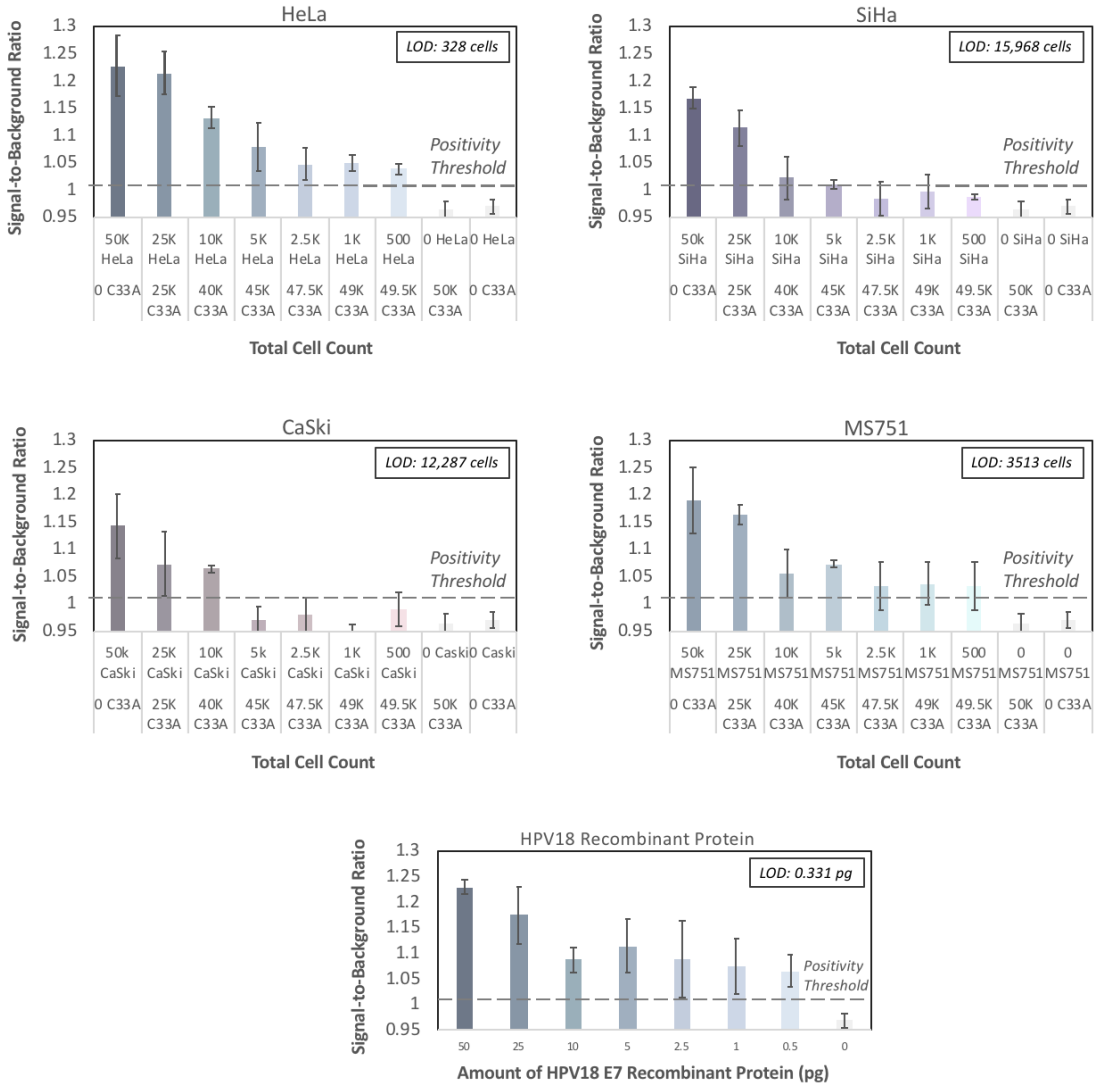

Figure 5-6: Range in HPV E7 paper assay with xTractor sample preparation. *(A-D)* A range of HeLa (HPV18), SiHa (HPV16), CaSki (HPV16), and MS751 (HPV45) cells were spiked into C33A (HPV negative) cells to maintain a constant number of cells, lysed using xTractor buffer, and tested on the HPV E7 paper assay (*n=3*). Using probit analysis, the limits-of-detection for the HPV E7 paper assay were: 328 total HeLa cells, 15,968 total SiHa cells, 12,287 total CaSki cells, and 3,513 total MS751 cells. The limit-of-detection was determined to be the average negative signal ± three standard deviations. *(E)* The corresponding linear range of HPV18 E7 recombinant protein spiked into xTractor buffer correlated well to the HeLa range when tested in the HPV E7 paper assay *(n=3)*. The limit-of-detection for HPV18 E7 recombinant protein is 0.331 pg which correlates to 331 total HeLa

cells. *HeLa = HPV18+; SiHa= HPV16+; CaSki= HPV16+; MS751= HPV45+; C33A = HPV negative; Dashed Line= positivity threshold determined as average negative signal ± three standard deviations.*

5.4.4. Clinical Assessment

A small pilot study was conducted using 14 samples from a referral population in conjunction with the University of Texas MD Anderson Cancer Center and Lyndon Baines Johnson General Hospital (LBJ) in Houston, TX. All samples were collected into PreservCyt buffer and tested positive for high-risk HPV DNA by the Roche cobas HPV test. A summary of samples tested is shown in **Table 8**. Ideally, I would test an equivalent number of HPV positive samples with benign pathology to ensure that the HPV E7 paper assay was specific and only produced a positive result when oncoprotein was overproduced. However, due to obtaining samples from a referral population at MD Anderson Cancer Center, only 5 HPV16+ samples and no HPV18+ samples received a benign pathology report. In addition, only HPV16 and HPV18 clinical samples had corresponding pathology reports; therefore, additional HPV types such as HPV45 were not tested.

	Roche cobas HPV Result	**Pathology**
Samples (n=14)	HPV16 (n=12)	<CIN2 (n=5)
		CIN3 (n=7)
	HPV18 (n=2)	CIN2 (n=1)
		CIN3 (n=1)

Table 8: Summary of clinical samples. Samples were characterized with HPV DNA according to the Roche cobas HPV test and with pathology according to standard histopathologic diagnosis of a biopsy.

The results of clinical testing are shown in **Figure 5-7** and **Table 9.** The HPV E7 paper assay was determined to be positive if the test had a signal-to-background ratio above the positivity threshold determined by the cellular ranges in **Figure 5-6**; this positivity

threshold was set at the average signal for 50,000 C33A cells plus three standard deviations. Of the 14 samples tested, only 10 had a valid result; the other four samples did not flow down the strip, likely due to the high viscosity of the samples. Of the valid samples, 9 out of 10 produced an accurate result that corresponded with known histopathologic biopsy results, with 5 of 6 samples with known CIN2+ pathology testing positive in HPV E7 paper assay and 4 of 4 samples with benign pathology testing negative in the HPV E7 paper assay. The pilot study reported one false negative test on a sample with known CIN3 pathology; this sample tested positive for HPV16 with the Roche cobas HPV DNA test.

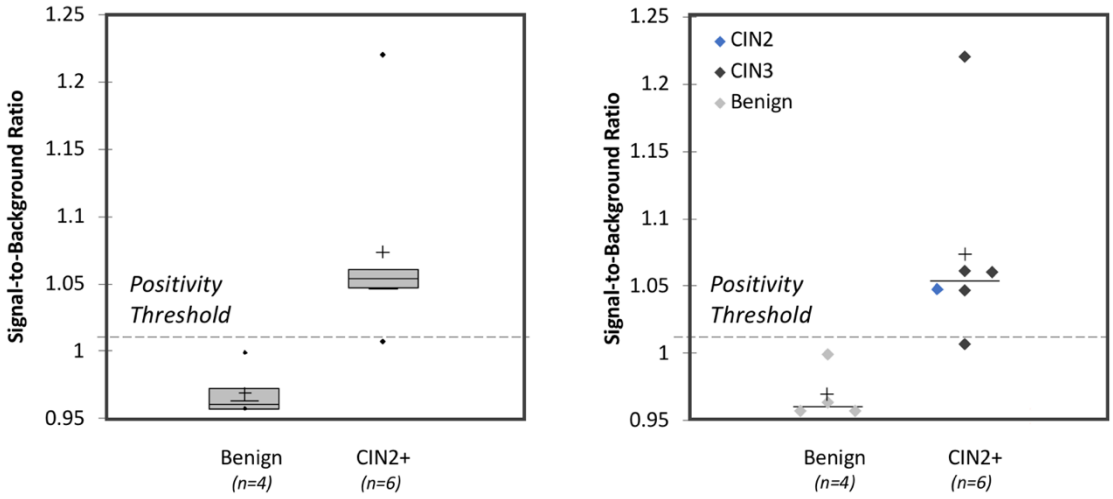

Figure 5-7: Clinical assessment. Signal to background ratio for HPV E7 paper assay performed with clinical samples collected into SurePath buffer. All clinical samples tested positive for high-risk HPV using the Roche cobas test. Samples are stratified by the histopathologic diagnosis of a confirmatory biopsy. There is a significant difference in the mean signal-to-background ratio for samples with CIN2+ pathology compared to samples with benign pathology (p= 0.017). *Dashed Line= positivity threshold determined as average negative signal ± three standard deviations; + = mean; line = median.*

		Histopathologic Diagnosis	
		CIN2+	Benign
HPV E7 Paper Test	Positive	5	0
	Negative	1	4

Sensitivity (Se): 83.3%
Specificity (Sp): 100%
Accuracy: 90%

Table 9: Clinical results. Sensitivity, specificity, and accuracy of clinical samples compared to pathology (n=10). Four additional samples had an invalid result.

5.5. Discussion

I developed a point-of-care assay for HPV E7 oncoprotein which is able to detect precancerous lesions with minimal user input and without the need for instrumentation or infrastructure. Sample preparation with xTractor buffer effectively lyses cellular samples without the need for centrifugation, a key component in creating a test appropriate for use at the point-of-care in resource-limited settings. The xTractor sample preparation protocol in addition to successful lyophilization of reagent pads ensures that the workflow is easy-to-perform with five simple user steps that can be accomplished within 15 minutes. In addition, the HPV E7 paper assay costs less than $1 per test for small-scale manufacturing, or $1.46 including costs for cervical collection brush, lysis tube, and disposable pipettes (**Table B1**). The lack of instrumentation, ease of use, and low cost make the HPV E7 paper assay appropriate for use in resource-limited settings.

The desired sensitivity was determined using the Arbor Vita oncoprotein test as a benchmark. As discussed in Chapter 2, Arbor Vita OncoE6 has a limit of detection of 2,000 total cells for HPV types 16, 18, and 45 [77]. However, the test requires an extensive 45-

minute sample preparation process with several centrifugation steps as well as a $2000 instrument [57]. My goal was to create an assay with less than 2,000 total cells per assay for HPV16, 18, and 45 to match the performance of the Arbor Vita test without the need for complex sample preparation or instrumentation. After probit analysis, the limits-of-detection of the HPV E7 paper oncoprotein assay were determined to be: 328 HeLa cells, 15,968 SiHa cells, 12,287 CaSki cells, 3,513 MS751 cells. I achieved my desired limit-of-detection for HPV18 E7 and close to the limit of detection with HPV45 E7. Siha and CaSki limits of detection were slightly higher, although still reasonable for a truly point-of-care assay at less than 16000 total cells detected. With these data, I determined the HPV E7 paper assay was able to sufficiently quantify HPV18, HPV45, and HPV16 E7 oncoprotein, although HPV16 detection can be improved in the future with the addition of a secondary HPV16 E7 detection antibody if necessary after clinical evaluation.

Future work will focus on decreasing the invalid rate of the paper ELISA oncoprotein devices. Mucus or other large and viscous cellular components are likely the inhibitors of fluid flow; next steps to reduce invalid rate include mechanical agitation on an orbital shaker, sample dilution, mucolytic chemical reagents, and/or paper-based membrane filters may improve validity rate. In addition, total assay performance would likely improve if the paper assays were produced under strict manufacturing conditions, and additional HPV16 antibodies might further reduce SiHa and CaSki limits of detection.

Further clinical validation will be necessary to ensure assay sensitivity and specificity. Increasing sample volume or adding in additional HPV16 E7 oncoprotein antibodies might improve accuracy for HPV16+ samples, as the one false negative HPV E7 paper result was for sample with CIN3 pathology and positive for HPV16 by Roche cobas testing.

Furthermore, the signal-to-background ratio for the positive samples could be increased by reconstituting the SurePath sample into a smaller volume of xTractor during buffer conversion or by collecting samples directly into xTractor buffer. With SurePath, a swab is collected into 10mL of solution, whereas with xTractor, a swab could be added directly to 300μL of lysis buffer. Reconstituting a cervical swab into a smaller buffer volume could increase oncoprotein levels in the sample vial and improve test performance.

Nevertheless, despite the need for larger-scale validation, the HPV E7 paper assay performs well with cellular and clinical samples, and I was able to detect both HPV16+ and HPV18+ samples with pathology greater than CIN2 with an accuracy of 90%. As such, the assay could serve as a follow-up diagnostic for women who test positive for high risk HPV DNA in a screen-and-treat program in resource-limited settings. Having a paper-based, low-cost test to diagnose women likely to have CIN2+ lesions could prevent overtreatment, while allowing a patient to be screened, diagnosed, and treated within the same visit to reduce loss to follow-up.

5.6. Conclusion

I demonstrated successful creation of a sample-to-answer HPV oncoprotein assay, with five simple user steps, no infrastructure requirements, and a low-cost platform. I validated the assay with HPV16, HPV18, and HPV45 cellular samples as well as with a pilot clinical study, producing an overall accuracy of 90%. Further clinical validation is necessary; nevertheless, with promising performance and a truly point-of-care format, the HPV E7 paper oncoprotein assay could prove a helpful tool for diagnosing precancerous and cancerous lesions in resource-limited settings.

Chapter 6

Conclusions

6.1. Summary of Results

This thesis describes the development of two novel sample-to-answer diagnostics for the detection of HPV DNA and HPV E7 oncoprotein in resource-limited settings. Both assays use a highly sensitive paper platform that is low-cost, easy-to-use, and requires minimal infrastructure to perform.

6.1.1. Paper ELISA Platform

In Chapter 3, a highly sensitive paper-based platform was presented. The flow and timing of the paper ELISA were characterized with food coloring, and the assay was optimized using malaria protein *Pf*HRP2. The paper ELISA platform had equivalent sensitivity to a traditional 96-well ELISA gold standard assay at 0.1 ng/mL *Pf*HRP2, cost less than $1 per test, and produced results within 90 minutes.

6.1.2. HPV DNA Paper Assay

In Chapter 4, a point-of-care, sample-to-answer HPV DNA paper assay was presented. The assay had a limit of detection equivalent to *digene* Hybrid Capture 2 without detecting low risk HPV DNA. A point-of-care sample preparation method was demonstrated with a workflow that required seven user steps and only a heater for instrumentation. Usability studies in El Salvador and Mozambique determined the HPV DNA paper assay was acceptable to use. The assay was assessed using HPV positive and negative cells and validated in a small pilot study using clinical samples, achieving 93.75% accuracy.

6.1.3. HPV E7 Paper Oncoprotein Assay

In Chapter 5, a point-of-care HPV E7 paper assay for more specific oncoprotein detection was presented. A sample-to-answer workflow was demonstrated with only five user steps and no instrumentation or infrastructure requirements. As part of this workflow, a point-of-care sample preparation protocol was validated, with xTractor buffer shown to effectively lyse multiple cell lines with 1.35-1.45 fold greater lysis compared to a standard freeze-thaw protocol. The HPV E7 paper assay was validated using HPV positive cells and clinical samples collected from a referral population in Houston, TX, demonstrating an accuracy of 90%.

6.2. Conclusion

In conclusion, this thesis accomplished three specific aims:

Specific Aim 1: Designed a low-cost, paper-based assay that is equivalent in sensitivity to a traditional 96-well ELISA

Specific Aim 2: Developed a sample-to-answer HPV DNA paper assay that detects high-risk HPV for cervical cancer screening in resource-limited settings

Specific Aim 3: Developed a point-of-care HPV E7 paper oncoprotein assay for more specific diagnosis of cervical neoplasia in resource-limited settings

As discussed in Chapter 2, access to cervical cancer screening and diagnostic measures are often limited in low-resource settings due to high per-test costs, infrastructure requirements, or need for highly trained personnel. To address these limitations, I first developed a paper platform with equivalent sensitivity to a gold standard ELISA in Specific Aim 1. Leveraging this low-cost platform, I designed a point-of-care, sample-to-answer HPV DNA paper assay for cervical cancer screening in Specific Aim 2. With a limit of detection matching commercial hybrid capture tests, an estimated per-test cost less than $3, and only a heater required for instrumentation, this assay could provide more accessible screening in resource-limited settings. Furthermore, the test requires minimal training to perform, with new users in El Salvador and Mozambique rating the test as acceptable to use. While the HPV DNA paper test provides a low-cost alternative for cervical cancer screening, overtreatment can occur without additional follow-up diagnostic testing. To provide more specific diagnosis of precancerous lesions, I developed a sample-to-answer HPV E7 paper assay in Specific Aim 3. This assay costs less than $1.50 without the need for infrastructure and requires five simple user steps to perform. In combination, the two diagnostic tests

could serve as low-cost options for screen-and-treat programs in resource-limited settings to increase access to care.

6.3. Future Research Directions

Future research directions for this work include larger-scale clinical evaluations for both the HPV DNA paper test and the HPV E7 paper test. Both assays were assessed in small pilot studies, and larger validation is necessary to accurately evaluate clinical performance. Furthermore, clinical evaluations should be performed in resource-limited settings to validate assay robustness and performance in the intended setting. Larger scale evaluation of the HPV DNA paper assay is planned using additional samples from San Salvador, El Salvador and Maputo, Mozambique, and further evaluation of the HPV E7 paper assay will be completed using additional samples from Houston, Texas and samples from San Salvador, El Salvador.

For the HPV DNA paper test, future directions include creating in-house high-risk HPV RNA probes for a low per-test cost and adding an internal cellular control to the assay. Although the current anti-streptavidin control line determines viability of reagents, an internal cellular control would be more valuable with self-collected swabs, to ensure adequacy of sample collection. Finally, further optimization is necessary for testing cervicovaginal swabs collected into SurePath buffer.

For the HPV E7 paper test, additional future work focuses on improving the invalid rate and reducing the limit of detection for HPV16 cell lines if necessary after additional clinical validation. Next steps to improve flow rate include mechanical agitation, sample dilution, addition of mucolytic reagents, and/or physical membrane filters. Sensitivity for

HPV16 E7 may be improved with additional antibodies if needed. Like with the HPV DNA paper assay, the HPV E7 paper assay could also be enhanced by adding in an internal cellular control line.

The variability with both paper assays could be reduced with stricter manufacturing conditions, and the coefficient of variation over several antibody batches, days, and ambient conditions should be assessed. Notably, stability studies of the assays in heat and humid environments are necessary for end use in resource-limited settings. Finally, the highly sensitive paper platform and research presented in this thesis can be applied to other targets for use in resource-limited settings.

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Appendix A

This appendix contains supplemental information for Chapter 4, including effect of DNA/RNA fragment size, buffer assessment, and a clinical pilot study of cervicovaginal samples collected into SurePath buffer.

DNA/RNA Fragment Size

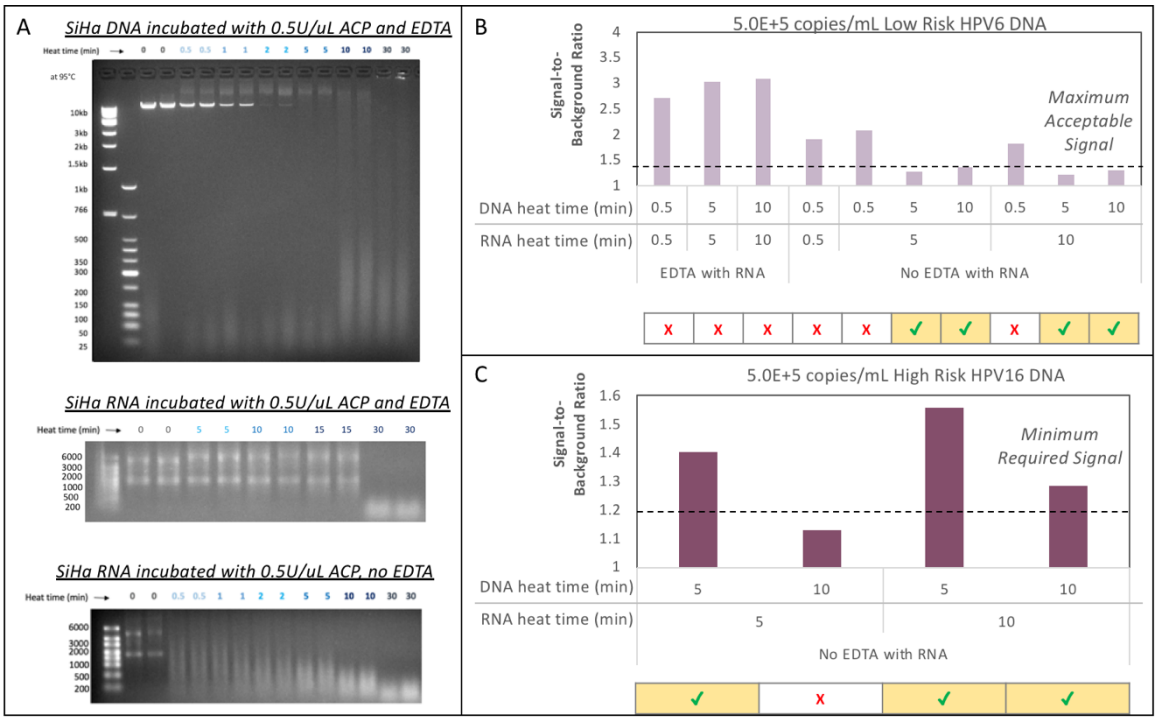

Figure A1: Effect of DNA/RNA fragment size. Effect of heating time on DNA and RNA fragment size (A). SiHa DNA in 1 mM EDTA (*top*) and RNA (*middle in 1 mM EDTA; bottom with no EDTA*) were incubated with 0.5U/uL ACP for 5 minutes at 23°C, heated at 95°C for various times, and run on agarose gels. (B) Result of HPV DNA paper assay for a negative control (5.0E+5 copies/mL of low-risk HPV6 DNA) incubated with 0.5U/uL ACP for 5 minutes at 23°C, heated at 95°C for various times (n=2). False positive results are shown with red Xs, and acceptable conditions are shown with green check marks. (C) Result of HPV DNA paper assay for a positive control (5.0E+5 copies/mL of high-risk HPV16 DNA) performed with the acceptable conditions identified in (B), (n=2). Acceptable conditions are denoted with green check marks. Signal was strongest for high-risk HPV16 DNA with a 5 minute DNA and 10 minute RNA heat time. In practice, the RNA would be heated for 10 minutes prior to lyophilization. Therefore, the user workflow for the optimized sample preparation protocol would include adding DNA to pre-fragmented and pre-lyophilized RNA,

incubating with 0.5U/uL ACP for 5 minutes at 23°C, and finally heating for 5 minutes at 95°C. This heating profile describes the point-of-care sample preparation.

Clinical Assessment in SurePath Buffer

In addition to the samples from Basic Health International that were collected into PreservCyt buffer, I tested samples from a referral population in conjunction with the University of Texas MD Anderson Cancer Center and Lyndon Baines Johnson General Hospital (LBJ) in Houston, TX. These samples were collected into SurePath preservative buffer, which contains formalin, an agent that crosslinks proteins with nucleic acids. Because SurePath preservation buffer is known to be difficult to work with when testing for nucleic acids, I first tested HPV positive (HeLa) and HPV negative (C33A) cells collected into SurePath buffer with the HPV DNA paper assay. I used a sample preparation protocol described above that reverses formalin-induced crosslinking using heat at 120 °C for 20 minutes followed by buffer conversion to 10mM Tris. These results, shown in **Figure A2**, show that cellular samples collected into SurePath buffer performed comparably to cellular samples collected directly into Tris-based solution or into PreservCyt buffer, with no significant differences between buffers for comparable samples.

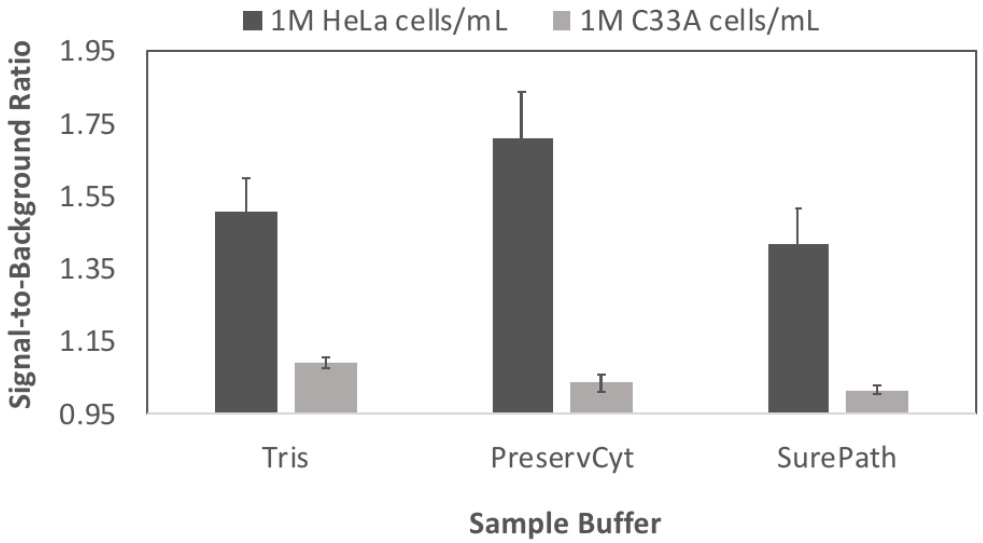

Figure A2: Buffer assessment with spiked cellular samples**.** Signal-to-background ratio for the HPV DNA paper assay evaluated with Hela and C33A cells constituted in a Tris-based buffer and sample buffers commonly used with clinical HPV DNA tests. For the latter, cellular samples were stored for over 48 hours in either PreservCyt or SurePath buffer before sample conversion to a Tris-based solution and testing on the HPV DNA paper assay. The HPV DNA paper assay performed comparably with all buffer types, with no significant differences between buffers for comparable samples.

Based upon these data, I tested 14 samples from the referral population, collected into SurePath preservative buffer. According to Roche cobas HPV gold standard results, seven of these samples were positive for high-risk HPV, and seven were negative for HPV. All 14 samples were tested on the HPV DNA paper assay with the point-of-care sample preparation protocol, after buffer conversion to a Tris-based solution. The HPV DNA paper assay was determined to be positive if the test had a signal-to-background ratio above the positivity threshold determined as the average C33A signal in SurePath plus three standard deviations (**Figure A2,** positivity threshold = 1.06). With this cutoff, six of the seven HPV

positive samples tested positive with the HPV DNA paper assay, producing one false negative result. Likewise, four of the seven HPV negative samples tested negative by the HPV DNA paper assay, producing three false positive tests. There was no statistically significant difference in the mean signal for samples with a gold standard HPV- or HPV+ result ($p= 0.184$) (**Figure A3**). I also analyzed the data using different positivity threshold: the average C33A signal in Tris plus three standard deviations (**Figure A2,** positivity threshold = 1.138) and the average negative calibrator signal plus three standard deviations (**Figure 4-4, C**; positivity threshold = 1.132). With either alternative threshold, four of the seven HPV positive samples tested positive with the HPV DNA paper assay, and five of the seven HPV negative samples tested negative.

With SurePath and Roche cobas HPV tests as the gold standard, I would expect false negative results. Roche is an amplification-based HPV DNA test and inherently more sensitive than hybrid-capture HPV DNA tests such as the HPV DNA paper assay. Likewise, the buffer conversion process necessary to process SurePath samples could lead to loss of high-risk DNA during centrifugation steps. The strong false positive results are unexpected, and I theorize they could be caused by incomplete crosslink reversal during the buffer conversion process. If nucleic acids were still bound to protein, the resultant complex could be sterically bound by the DNA-RNA capture antibody and result in false positive signal. Additional heating and washing of SurePath samples are necessary to determine if false positive signal can be reduced with SurePath samples in the HPV DNA paper assay.

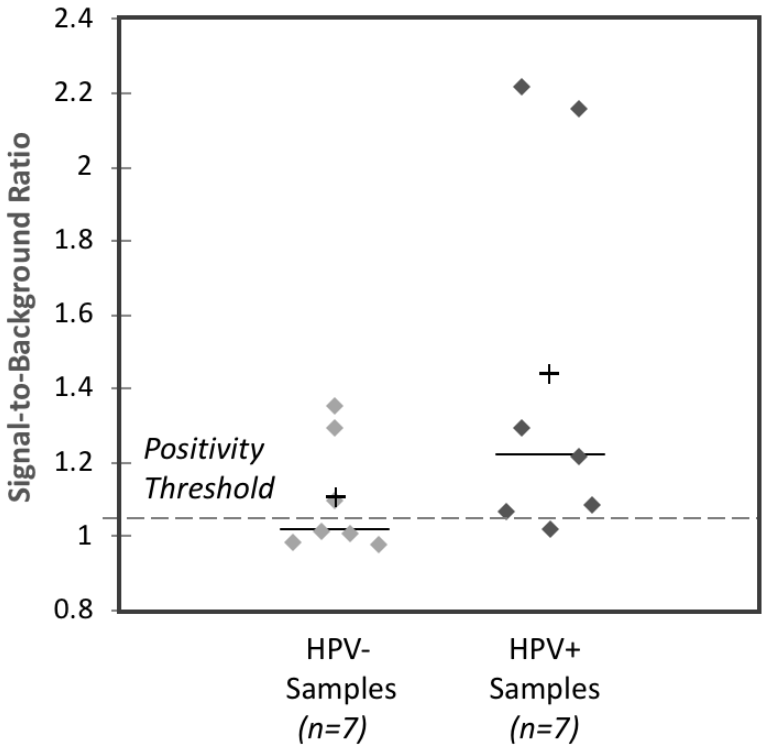

Figure A3: Clinical sample assessment with SurePath preservative buffer**.** Signal to background ratio for HPV DNA paper assay performed with clinical samples collected into SurePath buffer. The positivity threshold was determined using the negative C33A signal in SurePath plus three standard deviations from **Figure A2**. Roche cobas HPV was used as the gold standard HPV result. There was no statistically significant difference in the mean signal for samples with a gold standard HPV- or HPV+ result (p= 0.184). *Dashed Line= positivity threshold determined as average negative signal ± three standard deviations. + = mean; line = median*

Appendix B

This appendix contains supplemental information for Chapter 5, including BSA optimization, paper ELISA reagent optimization, and lyophilization.

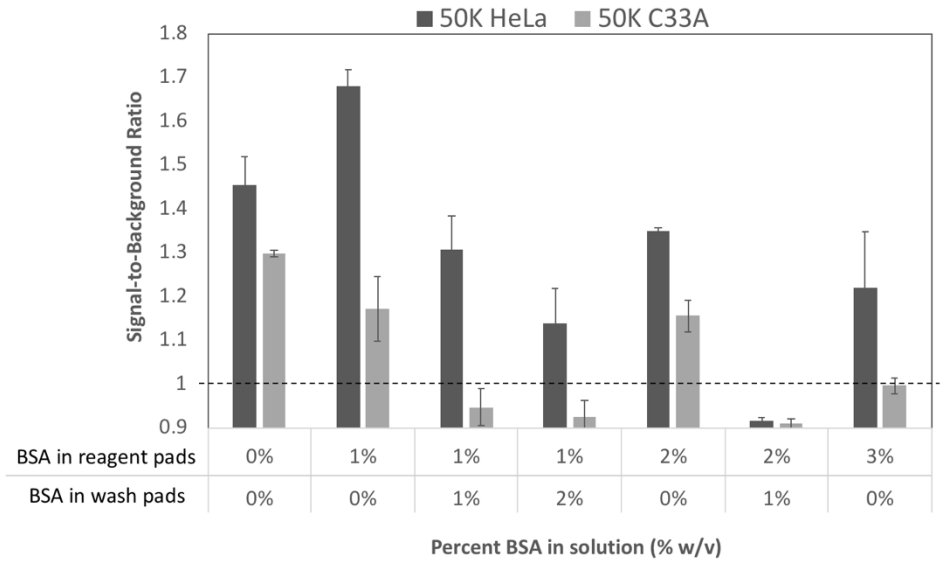

Figure B1: Reduction of false positives in HPV E7 paper assay with BSA**.** To reduce non-specific binding in the HPV E7 paper assay, varying concentrations of BSA were added into the reagent and wash pads. HeLa (HPV18) and C33A (HPV negative) cells were lysed with xTractor buffer and tested in duplicate for each condition. Adding 1% BSA in both reagent and wash pads reduced non-specific binding, so that the C33A signal-to-background ratio was below 1. Increasing BSA concentration in the reagent or wash pads beyond 1% decreased the positive HeLa signal.

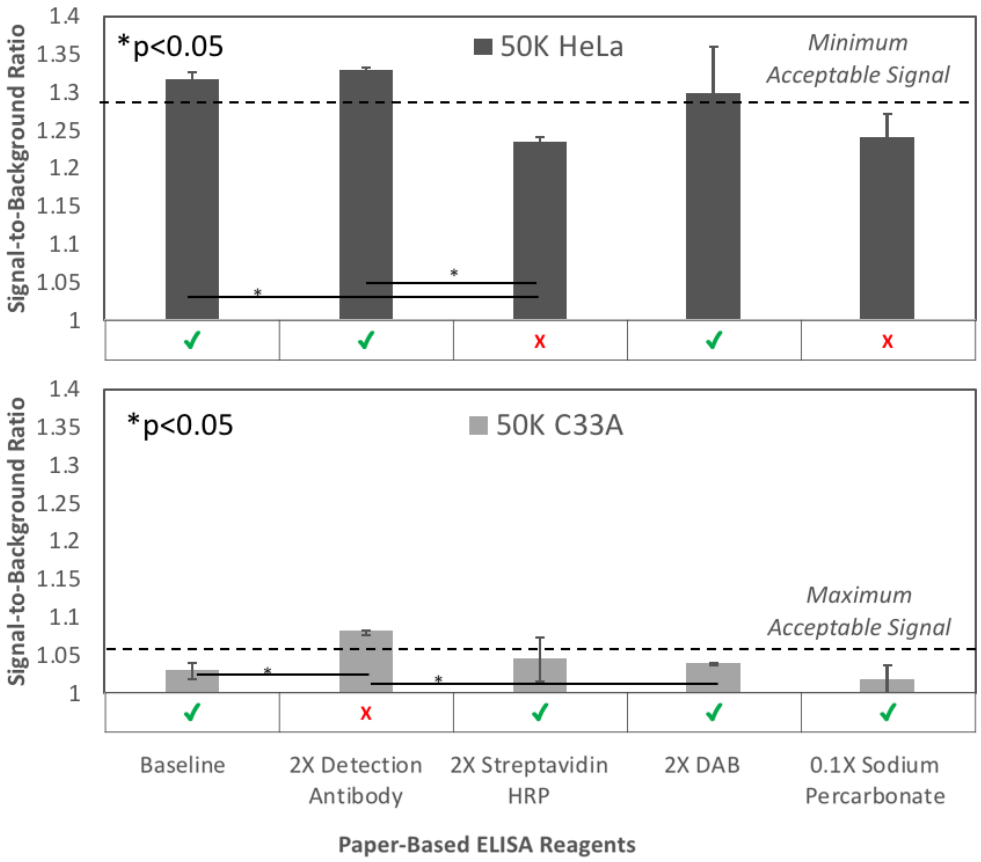

Figure B2: Reagent optimization in paper. Reagents for the HPV E7 paper assay were optimized by assessing the signal-to-background ratios of positive (HeLa; HPV18, *top*) and negative (C33A; HPV negative, *bottom*) cellular samples with increasing concentrations of detection antibody, streptavidin HRP, diaminobenzidine (DAB), and sodium percarbonate. Acceptable conditions are denoted with green check marks. Increasing the concentration of streptavidin HRP reduced the positive (HeLa) signal, compared to the baseline and increased detection antibody conditions ($p<0.05$). However, increasing the concentration of detection antibody increased the negative (C33A) signal compared to the baseline and increased DAB conditions. *Minimum acceptable signal= average baseline HeLa signal ± three standard deviations; Maximum acceptable signal= average baseline C33A signal ± three standard deviations.*

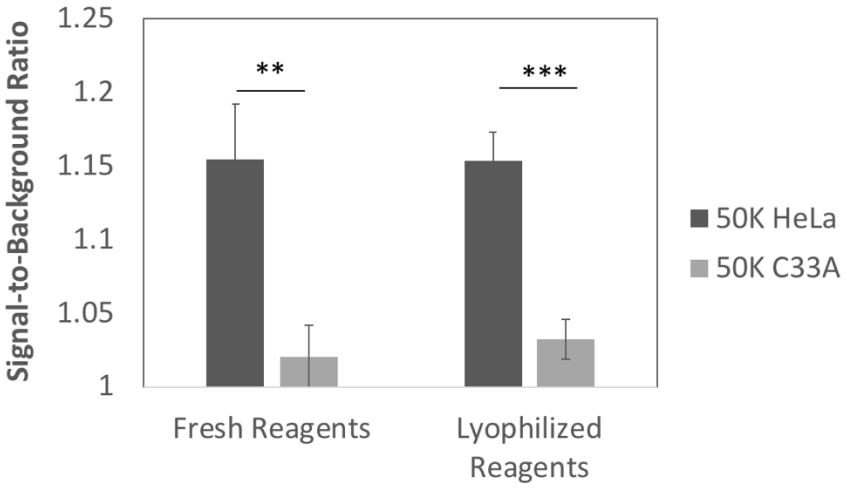

Figure B3: Lyophilization**.** Positive (HeLa) and negative (C33A) samples were tested with freshly prepared and lyophilized reagents (n=3). There was a significant difference between the positive and negative controls for both fresh ($p<0.01$) and lyophilized reagents ($p<0.005$). There were no significant differences in either positive (HeLa) signal or negative (C33A) signal between freshly prepared reagents and lyophilized reagents. ** $p<0.01$; *** $p<0.005$

Sample Collection and Preparation	xTractor Buffer	$ 0.21
	Cervical Brush	$ 0.19
	Tubes and Pipettes	$ 0.14
HPV E7 Paper Assay	Antibodies	$ 0.26
	Paper and Plastic	$ 0.34
	Other Reagents	$ 0.33
All Sample Collection and Preparation		$ 0.54
All HPV E7 Paper Assay		$ 0.92
Total Cost		**$ 1.46**

Table B1. HPV E7 paper assay cost. Small-scale cost estimations of sample collection and preparation materials as well as the HPV E7 paper assay are shown.