RICE UNIVERSITY

Development of a Diagnostic Assay for the Multiplexed Detection of Ovarian Cancer Biomarkers at the Point-of-Care

By

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

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HOUSTON, TEXAS
DECEMBER 2014
ABSTRACT

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Point-of-care (POC) diagnostic platforms have the potential to enable low-cost, large-scale screening. As no single biomarker is shed by all ovarian cancers, multiplexed biomarker panels promise improved sensitivity and specificity to address the unmet need for early detection of ovarian cancer. We have configured the programmable bio-nano-chip (p-BNC) - a multiplexable, microfluidic, modular platform - to quantify a novel multimarker panel comprised of CA125, HE4, MMP-7 and CA72-4. The p-BNC is a bead-based immunoanalyzer system with a credit-card-sized footprint that integrates automated sample metering, bubble and debris removal, reagent storage and waste disposal, permitting POC analysis. Multiplexed p-BNC immunoassays demonstrated high specificity, low cross-reactivity, low limits of detection suitable for early detection, and a short analysis time of 43 minutes. Day-to-day variability, a critical factor for longitudinally monitoring biomarkers, ranged between 5.4-10.5% – well below the biological variation for all four markers. Biomarker concentrations for 31 late-stage sera correlated well ($R^2 = 0.71$ to 0.93 for various biomarkers) with values obtained on the Luminex® platform. In a 31-patient cohort encompassing early- and late-stage ovarian cancers along with benign and healthy controls, the multiplexed p-BNC panel was able to distinguish cases from controls with 68.7%
sensitivity at 80% specificity. Utility for longitudinal biomarker monitoring was demonstrated with pre-diagnostic sera from 2 cases and 4 controls.

An updated card capable of being manufactured on a large-scale was developed that enabled the transition to true point-of-care measurement. A custom backpack-sized analyzer capable of precise actuation of blister packs (attached to each card) was used to control fluid rates of the various stages and contains all hardware necessary for imaging and analysis. Without sacrificing analytical performance, the multiplexed assay was reduced to 17 minutes in order to return results during an average patient visit. In a 22-patient study, blood was shown to correlate well to plasma and serum in the p-BNC, demonstrating promise for whole blood fingerstick measurements. Taken together, the p-BNC shows strong promise as a diagnostic tool for large-scale screening and takes advantage of faster results and lower costs while leveraging possible improvement in sensitivity and specificity from biomarker panels.
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Chapter 1: Early Detection of Ovarian Cancer and Towards a Point-of-Care Screening Solution

1.1 Thesis Overview

This dissertation details the development of the programmable Bio-Nano-Chip (p-BNC) immunoanalyzer as an ovarian cancer diagnostic and screening tool. By leveraging multiplexability in a point-of-care (POC) format, the p-BNC ovarian cancer diagnostic test aims to overcome challenges associated with proposed general population screening tools, including diagnostic performance, cost and patient comfort. The measurement of circulating protein biomarkers has been demonstrated as a useful tool for ovarian cancer detection, treatment response and remission, but the ovarian cancer research community has not accepted any specific combination of biomarkers and additionally no method of multiplexed analysis of ovarian cancer biomarkers is readily available. Multiplexed analysis of biomarkers with short assay times, high sensitivity and specificity, good precision, and low cross-reactivity can serve to validate biomarkers for ovarian cancer detection and has high potential for use as a diagnostic platform for screening at the POC.

This introduction details the motivation of developing a screening tool for ovarian cancer and describes the requirements and challenges associated with the diagnosis of ovarian cancer. The terminology used throughout the thesis is also described, as well as background on the development and treatment of ovarian cancer in order to frame current limitations and illuminate the best way to improve survival. This chapter also addresses the methods currently used to diagnose ovarian cancer and in addition to summarizing previous attempts of screening for ovarian cancer, which serve to clarify the reasoning for the approach adopted in this thesis. Furthermore, the advantages and requirements of a POC solution are described and the ability of the p-BNC to meet those needs are discussed.
Finally, the remaining chapters of this dissertation are outlined to frame the necessary work and to summarize the objectives achieved.

1.2 Motivation

In 2013, an estimated 22,000 women in the United States were diagnosed with ovarian cancer and 14,000 women died from the disease.¹ Ovarian cancer occurs more frequently in postmenopausal women² and has one of the highest case-to-fatality rates, likely due to a combination of lack of distinctive symptoms in the early stages of disease and the nonexistence of a screening method for the general population.³ Improved surgical management combined with advanced chemotherapy have improved treatment, but there is still less than a 30% cure rate overall.⁴ Significantly, when the disease is detected in early stage (stage I), survival rates up to 90% can be achieved,⁵ but unfortunately only 20-25% of cases are diagnosed at an early stage.⁶ The development of a screening test could greatly improve survival rates but given the low prevalence of ovarian cancer in post-menopausal women (1 in 2500), any screening test for ovarian cancer must result in fewer than 10 surgical operations per case of ovarian cancer detected.⁵

Due to the inaccessibility of the ovaries and discomfort created from invasive procedures, there is significant interest in non-invasive biomarker-based strategies for the first line of screening. While the biomarker CA125 is commonly used in clinics for disease progression, its sensitivity and specificity are not high enough to meet the requirements created by clinicians for a general population screening method.⁵ A screening combination of CA125 measurements and subsequent referral to ultrasound is currently under investigation and showing promise in a large clinical trial,⁷ but there is a significant amount of turnaround
time between awaiting lab results of the CA125 test and scheduling an ultrasound appointment. Utilizing a microfluidic immunoanalyzer system to enable POC analysis could significantly shorten wait times to the point where a high CA125 value could immediately refer patients to ultrasound within the same visit. Further, the inherent advantages of microfluidic immunoanalyzer platforms could also be leveraged to measure multiple biomarkers simultaneously, a possible solution to the limited sensitivity and specificity of CA125 alone.⁸

1.3 Ovarian Cancer Background and Biology

1.3.1 Ovarian Cancer Classification

Ovarian cancer refers to an extremely heterogeneous group of tumors, which are classified by invasiveness into the three groups:⁹

- **Benign** – lack excessive proliferation and invasive behavior
- **Borderline** – excessively proliferate but do not have invasive behavior
- **Malignant** – excessively proliferate and have invasive behavior

Most ovarian cancers form from three types of cells: epithelial cells, sex cord stromal cells and germ cells, each of which has a significant number of subtypes. Over 90% of malignant ovarian cancer tumors form from epithelial cells, which is further divided into serous, mucinous, endometrioid, clear cell and transitional cells.¹⁰ Microscopic investigation of tumor cells is required for effective classification of ovarian cancer types and subtypes; this classification is used for predicting tumor behavior and determining treatment options.⁹
As the vast majority of malignant ovarian tumors refer to neoplasms formed from epithelial cells, the term ‘ovarian cancer’ will be used in this thesis to refer to epithelial ovarian tumor subtypes unless specified otherwise.

1.3.2 Ovarian Cancer Staging and Metastasis

Table 1-1: FIGO staging of ovarian cancer.\textsuperscript{11,12}

<table>
<thead>
<tr>
<th>FIGO Stage</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>Tumor confined to ovaries</td>
</tr>
<tr>
<td>IA</td>
<td>Tumor on 1 ovary</td>
</tr>
<tr>
<td>IB</td>
<td>Tumor on both ovaries</td>
</tr>
<tr>
<td>IC</td>
<td>Tumor limited to 1 or both ovaries</td>
</tr>
<tr>
<td>IC1</td>
<td>Surgical spill</td>
</tr>
<tr>
<td>IC2</td>
<td>Capsule rupture before surgery or tumor on ovarian surface</td>
</tr>
<tr>
<td>IC3</td>
<td>Malignant cells in ascites or peritoneal washings</td>
</tr>
<tr>
<td>II</td>
<td>Tumor on 1 or both ovaries with pelvic extension or primary peritoneal cancer</td>
</tr>
<tr>
<td>IIA</td>
<td>Extension on uterus and/or Fallopian tubes</td>
</tr>
<tr>
<td>IIB</td>
<td>Extension to other pelvic intraperitoneal tissues</td>
</tr>
<tr>
<td>III</td>
<td>Metastasis to the peritoneum outside the pelvis and/or to the retroperitoneal lymph nodes</td>
</tr>
<tr>
<td>IIIA1</td>
<td>Positive retroperitoneal lymph nodes only</td>
</tr>
<tr>
<td>IIIA2</td>
<td>Microscopic extrapelvic peritoneal involvement</td>
</tr>
<tr>
<td>IIIB</td>
<td>Macroscopic ≤2cm extrapelvic peritoneal metastasis</td>
</tr>
<tr>
<td>IIIC</td>
<td>Macroscopic &gt;2cm extrapelvic peritoneal metastasis</td>
</tr>
<tr>
<td>IV</td>
<td>Distant metastasis</td>
</tr>
<tr>
<td>IVA</td>
<td>Pleural effusion</td>
</tr>
<tr>
<td>IVB</td>
<td>Hepatic and/or splenic metastasis and/or metastasis to extra-abdominal organs</td>
</tr>
</tbody>
</table>

Tumor stage classification is necessary for standardized communication between researchers and clinicians and facilitates the prognosis and recommended course of treatment. For ovarian cancer, two main staging classifications are used: (1) Tumor, Node and Metastasis (TNM) and (2) International Federation of Gynaecology and Obstetrics
(FIGO), both of which describe the degree of metastasis. For this thesis, FIGO staging, listed in detail in Table 1-1 and graphically displayed in Fig. 1-1, will be used when describing stages of ovarian tumors.

Figure 1-1: Graphical representation of the metastasis sites of ovarian cancer tumors in relation to FIGO staging. Reprinted with permission from Cancer Research UK (“Stages of Ovarian Cancer” from www.cancerhelp.org.uk copyright 2014).

Ovarian tumors are staged surgically with laparotomy and biopsy and require histological/cytological confirmation. Generally (Fig. 1-2), ovarian cancer starts on one or
both ovaries and begins to spread once the tumor capsule is ruptured. Metastasis to adjacent tissues then occurs, which includes the uterus, fallopian tubes, peritoneum (mesothelial lining of the pelvic cavity) and omentum (adipose pad covering the bowel and abdominal cavity). Tumor cells also spread via the lymphatics to the pelvic and paraaortic lymph nodes. Unlike most cancers, ovarian cancer metastasis to distant organs via the blood stream is clinically rare.\textsuperscript{15,16}

\textbf{Figure 1-2}: Common sites of ovarian cancer tumor cell progression. Reprinted by permission from Macmillian Publishers Ltd: Nature Reviews Cancer,\textsuperscript{15} copyright 2006.
1.3.3 Treatment and Prognosis

An extensive review of treatment options of ovarian cancer is beyond the scope of this dissertation and has been published elsewhere. Briefly, following an exploratory laparotomy for histological confirmation and tumor debulking, cytoreductive surgery is performed, often including a hysterectomy. Removing nodules larger than 1.5cm in dimensions is considered optimal and using a 5mm cut-off has shown even better outcomes. Importantly, referral to specially trained gynecologic oncologists for surgery improves overall survival. Following surgery, platinum- and taxane-based chemotherapy is usually administered and has been found effective in over 70% of ovarian cancers.

Although 50% of advanced stage ovarian cancer patients who have undergone chemotherapy following surgery have complete remission, often-uncurable disease recurrence continues to be a major problem. Comfort and symptom management are the primary goal of most recurrent disease treatment, but second-line chemotherapy and experimental agents with 10-20% cure success rates are often used.

1.3.4 Ovarian Cancer Disease Statistics

In 2013, an estimated 22,000 women in the United States were diagnosed with ovarian cancer and 14,000 women died from the disease. While ovarian cancer is not one of the most common forms of cancer, it has one of the highest case-to-fatality rates, possibly due to the lack of distinctive symptoms in the early stages of disease. Superior surgical management combined with carboplatin and paclitaxel chemotherapy has improved treatment, but there is still less than a 30% cure rate overall.
Table 1-2: Impact of the level of ovarian cancer metastasis on 5-year survival rates.¹

<table>
<thead>
<tr>
<th>Disease status</th>
<th>Stage at diagnosis</th>
<th>5-year survival rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localized</td>
<td>15%</td>
<td>92%</td>
</tr>
<tr>
<td>Regional</td>
<td>17%</td>
<td>72%</td>
</tr>
<tr>
<td>Distant</td>
<td>61%</td>
<td>27%</td>
</tr>
</tbody>
</table>

Significantly, when ovarian cancer is detected in early stage (stage I), survival rates up to 90% can be achieved⁵ (Table 1-2). However, only 15-20% of cases are diagnosed at stage I,¹ which is largely due to the fact that there is no screening test currently recommended for the general population at average risk and because symptoms are not apparent in early stages.³ Late stage prognoses are significantly worse, with 5-year survival rates falling to under 20% for stage IV diagnoses¹⁴ (Fig. 1-3), demonstrating the importance of early diagnosis for ovarian cancer.

Figure 1-3. 5-year survival rates of ovarian cancers detected at different stages. Reprinted from International Journal of Gynecology & Obstetrics with permission from Elsevier.¹⁴
1.4 Currently Used Ovarian Cancer Detection Methods

Despite 89% of women with stage I/II ovarian cancers reporting symptoms prior to diagnosis,\textsuperscript{22} the nonspecific nature of the symptoms, in addition to lack of a non-invasive screening method, still prevents any more than a quarter of cases being diagnosed in early stages.\textsuperscript{6} Common symptoms include pelvic and abdominal pain, increased abdominal bloating, feeling full and more frequent urination, all of which are found in other gastrointestinal, genitourinary and gynecological conditions.\textsuperscript{22} The inability to rely on these symptoms, especially in early stages, creates an important need for an ovarian cancer detection method. No general population screening option has met the requirements set forth by ovarian cancer researchers and clinicians, but several studies are ongoing to evaluate promising detection methods as screening tools.

1.4.1 Transvaginal Sonography and Other Imaging Techniques

Transvaginal sonography (TVS) is commonly used in hospitals and clinics for investigating suspected pelvic masses and for precise imaging of the ovary. First line screening methods using TVS have been conducted and have shown some success approaching the 10% PPV required for an ovarian cancer screening test.\textsuperscript{23,24} However, other studies focusing on the early detection of ovarian cancer have shown poor specificity distinguishing benign masses from malignant, prompting 30 operations for each case of ovarian cancer diagnosed.\textsuperscript{25,26} Additionally, 56% of postmenopausal women without gynecological disorders have small benign masses that could trigger a positive TVS result.\textsuperscript{26} Though TVS could be useful as a second or third line screen, high cost and discomfort caused by invasiveness prohibits TVS from being used as a first line screen.\textsuperscript{5}
Due to the increase in blood flow to the ovary when a malignant lesion is present, color-flow Doppler imaging has been suggested as a means of ovarian cancer detection.\textsuperscript{27} While some studies have had success with Doppler imaging,\textsuperscript{28} it is generally accepted that Doppler imaging as a standalone method lacks specificity and even when combined with TVS minimal benefit is achieved with significant additional cost.\textsuperscript{29-31} Other non-invasive techniques such as computed tomography (CT), magnetic resonance imaging (MRI) and radioimmunoscintigraphy are too expensive to be used in a screening method for ovarian cancer.\textsuperscript{32}

### 1.4.2 Ovarian Cancer Biomarkers

Cancer biomarkers refer to any biological molecule or process capable of indicating the presence of cancer in the body.\textsuperscript{33} These biomarkers can be used for screening, diagnosis, staging, recommending and predicting response to treatment, evaluating treatment efficacy\textsuperscript{34} and in some cases are targets for therapeutic intervention.\textsuperscript{35} Using mass spectroscopy, protein chip data and bioinformatics, biomarker discovery has been employed to distinguish serum proteomic signatures of healthy and ovarian cancer patients, in addition to early and late stage cancers.\textsuperscript{36,37} Over 200 biomarkers have been linked to ovarian cancer.\textsuperscript{38}

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) are used to evaluate the performance of biomarkers (Table 1-3). Sensitivity refers to the proportion of diseased patients that receive a positive test result, while specificity refers to the number of non-diseased patients that receive a negative result. PPV denotes the percentage of patients that received a positive result and actually have the disease, while
NPV refers to the percentage of patients that received a negative result and do not actually have the disease.\textsuperscript{39}

**Table 1-3:** Diagnostic performance calculations for sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

<table>
<thead>
<tr>
<th></th>
<th>Diseased</th>
<th>Non-Diseased</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test Positive</strong></td>
<td>True Positive (TP)</td>
<td>False Positive (FP)</td>
</tr>
<tr>
<td><strong>Test Negative</strong></td>
<td>False Negative (FN)</td>
<td>True Negative (TN)</td>
</tr>
</tbody>
</table>

\[
\text{Sensitivity} = \frac{TP}{TP + FN} \quad \text{Specificity} = \frac{TN}{TN + FP} \]

\[
\text{PPV} = \frac{TP}{TP + FP} \quad \text{NPV} = \frac{TN}{TN + FN}
\]

For ovarian cancer, diagnostic test performance is critical because a positive test refers a patient to surgery for biopsy, where significant risk of complications by this invasive surgery exists.\textsuperscript{7} As a result, clinicians and researchers have agreed upon an arbitrary 10% PPV requirement for an ovarian cancer screening test, meaning that 1 case of ovarian cancer is confirmed from every 10 referrals to surgery.\textsuperscript{40} At 99.6% specificity, a sensitivity >75% is required to achieve a 10% PPV,\textsuperscript{41} which no single screening test has been able to realize.\textsuperscript{42}

**1.4.2.1 CA125 Measurement**

The blood biomarker Cancer Antigen 125 (CA125) is elevated in 90% of advanced stage ovarian cancers\textsuperscript{43} and 80% of all ovarian cancers.\textsuperscript{44} As a result, CA125 is an FDA-approved biomarker and has been used in clinics for more than three decades to monitor disease response, evaluate pelvic masses, estimate prognosis, and improve clinical trial
design. For detection, 35U/mL has classically been defined as the cut-off for a patient to be at risk of ovarian cancer. However, clinical utility of CA125 measurement as a screening tool for early detection has yet to be established. A total of 40-50% of early stage ovarian cancers and 20% of all ovarian cancers do not have elevated CA125 levels, likely due to the heterogeneity of ovarian cancer. Additionally, CA125 is elevated in some benign gynecological issues, other forms of cancer and up to 1% of the normal population, limiting its specificity for diagnosing ovarian cancer. The combined limitations of the sensitivity and specificity of CA125 make it difficult to achieve a 10% PPV with CA125 alone, making it necessary for a general population screening method to include additional information in order to effectively diagnose early staged ovarian cancers while limiting the number of surgeries required.

Further information on statistics, use and biology of CA125 is described in Section 2.3 of this dissertation.

1.5 Early Detection and Screening Methods Under Evaluation

Including all stages of diagnoses, ovarian cancer only has a 43% 5-year survival rate, one of the lowest of all types of cancers. Diagnosing ovarian cancer while the tumor is still localized to the reproductive system (stage I/II) significantly increases 5-year survival rates from 30% (advanced stage) to 90%, making it a potential candidate for a general population screening method. Despite the relatively low prevalence, ovarian cancer meets many of the criteria set forth by the World Health Organization for general population screening, including being a major source of death, being well-characterized, and having treatment that
improves outcomes. However, it is still unclear whether a screening test can detect ovarian cancer while achieving the necessary 10% PPV.

1.5.1 Multimodal Screening – TVS and CA125

Multimodal screening refers to a screening effort that utilizes multiple diagnostic methods. In two of the two largest ovarian cancer screening trials, a combination of CA125 measurement and TVS evaluation has been employed. The Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial enrolled 74,000 women aged 55 to 74 years in the United States. In the intervention arm, women were given a yearly TVS evaluation for 3 years and a yearly CA125 blood test for 5 years while the control arm continued with normal care. The entire population of the study was monitored for 13 years following the end of the trial to monitor health status and cause of death. In the intervention group, 212 ovarian cancer cases were diagnosed compared to 176 cases for the control group, but unfortunately no statistically significant reduction in mortality was found in the intervention group. The lack of mortality reduction was likely due to the disease cut-offs used for TVS (10cm³) and CA125 (35U/mL) being too high for levels found in the early stages of the disease. By not using levels associated with the early stages of the disease, symptoms were apparent in the control arm at a similar timing to the confirmation with CA125 and TVS of the intervention arm.

In the United Kingdom, the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) enrolled over 200,000 women aged 50-74. The intervention arm was split into two screening groups – one that underwent yearly TVS examination and the other yearly CA125 measurement. Patients who underwent TVS and had abnormal results were referred
to more frequent TVS scans or a higher level of examination using TVS. Patients in the CA125 group were initially classified into (1) normal, (2) intermediate or (3) elevated risk of ovarian cancer based on CA125 level and age. Normal risk patients (90.9% of intervention group) underwent annual CA125 measurements, intermediate risk patients (8.6%) returned in 12 weeks for an additional CA125 measurement, and high risk patients (0.5%) were referred to a TVS scan. After the first measurement, risk of ovarian cancer was calculated from absolute CA125 value and the longitudinal monitoring of CA125 values, where the rate of change affected risk even if the CA125 value was still considered normal. Ultimately, 87 women were diagnosed with ovarian cancer, 42 in the CA125 group and 45 in the TVS group. There was, however no difference in stage distribution between CA125 and TVS groups, although 48% of cancers diagnosed in both groups were discovered in stage I/II, compared to only 20-25% reported in other studies. Importantly, only 97 women were referred to surgery in the CA125 group compared to 845 women in the TVS group. A total of 24 of the 942 surgeries had major complications, emphasizing the importance of limiting referral to surgeries from screening methods.

While showing promise, multimodal screening methods are still under evaluation as no definitive clinical utility has been determined for any combination of TVS and CA125.

1.5.2 Longitudinal Monitoring of CA125

As mentioned in the previous section, the UKCTOCS findings from 2009 utilized longitudinal measurements of CA125 rather than using a strict cut-off value as seen in the PLCO trial. Early iterations of the UKCTOCS also used a strict cut-off for CA125 at 30U/mL, but it was found that most healthy patients had a flat profile of CA125 values that
fluctuated around the patient’s baseline value, while ovarian cancer patients’ values increased prior to diagnosis (Fig. 1-4). By monitoring serial CA125 values rather than looking at a binary cut-off, sensitivity increased from 62% to 86% while maintaining 98% specificity. This significant increase in sensitivity led future UKCTOCS studies to utilize serial information for CA125.7,53

**Figure 1-4:** Graphical representation of flat baselines for healthy patients (Women 1 and 2), even if they are above the classically used disease threshold, and a rising biomarker value for a cancer patient (Woman 3). Reprinted with permission from Springer Science and Business Media and Advances in Experimental Medical Biology.55

A Risk of Ovarian Cancer Algorithm (ROCA) was created based on the data collected from multiple longitudinal screening trials. The ROCA is used to identify the change-point from fluctuations around the patient’s baseline to an actual increase in CA125 values. The algorithm returns a risk percentage – for example, a 2% value represents a one in 50 chance of developing ovarian cancer over their lifetime.56 In ongoing longitudinal
monitoring, every new CA125 measurement results in a recalculation of the algorithm, where the result recommends screening action (continued yearly CA125 measurement, more frequent CA125 measurement, or ultrasound) depending on risk percentage. Using the ROCA on two retrospective trials resulted in PPV values of 16% and 19%. When combined in a multimodal screening with TVS in the UKCTOCS study, a PPV of 35.1% was achieved. In another study using the same strategy in a different population, a PPV of 40% was demonstrated. While these results combining ROCA with TVS in a multimodal screen are very promising, no other clinical trial has shown the efficacy of screening the general population. Further demonstration of clinical utility with larger numbers of ovarian cancer patients is needed before widespread acceptance. Additionally, more data on the sensitivity of the screening method and its impact on mortality needs to be demonstrated.

1.5.3 Multimarker Screening

Using multiple biomarkers has been proposed as a solution to the aforementioned limited sensitivity and specificity of CA125. Multiplexing 2-4 biomarkers together in the same screening test aims to overcome the heterogeneity of ovarian cancer and the lack of elevated CA125 in many early stage ovarian cancers.

In a study from 2010, 96 serum biomarkers (Table 1-4) were analyzed and all possible combinations of two, three and four biomarker panels were evaluated to determine the best sensitivity at 95% specificity. Several panels in this study were found to improve upon the sensitivity of CA125 alone (61%), the best of which consisted of CA125, HE4, CEA and VCAM-1 (86%). Several other studies have found increased sensitivity and specificity by using other biomarkers in addition to CA125, but no combination of
biomarkers without a multimodal strategy has been clinically validated with the sensitivity
and specificity required for a 10% PPV ovarian cancer screening test.

**Table 1-4:** List of 96 possible biomarkers for ovarian cancer.\(^8\)

<table>
<thead>
<tr>
<th>Biological Group</th>
<th>Biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines/chemokines</td>
<td>Eotaxin-1, TNFR1, TNFR2, IL-1Rα, IL-2R, IL-6R, IL-1b, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-13, G-CSF, GM-CSF, IFNγ, TNF-α, IP-10, MCP-1, MIP-1α, MIP-1β, MIF, CD40L, fractalkine</td>
</tr>
<tr>
<td>Growth/angiogenic factors</td>
<td>EGF, VEGF, bFGF, HGF, NGF, EGFR, Her2/neu, IGFBP-1</td>
</tr>
<tr>
<td>Antiangiogenic factors</td>
<td>Angiostatin, endostatin, thrombospondin</td>
</tr>
<tr>
<td>Cancer antigens</td>
<td>CA125, CA15-3, CEA, CA19-9, CA72-4, AFP, SCC</td>
</tr>
<tr>
<td>Apoptotic proteins</td>
<td>Cyfra 21-1, DR5, Fas, FasL</td>
</tr>
<tr>
<td>Proteases(binding proteins)</td>
<td>Kallikreins 8 and 10, MMP-1 to MMP-13, TIMP-1 to TIMP-4</td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td>ICAM, VCAM, E-selectin</td>
</tr>
<tr>
<td>Hormones</td>
<td>Prolactin, TSH, LH, ACTH, GH, βHCG</td>
</tr>
<tr>
<td>Adipokines</td>
<td>Adiponectin, leptin, resistin</td>
</tr>
<tr>
<td>Other markers</td>
<td>Mesothelin, HE4, tPAI-1, active PAI-1, MPO, Apolipoproteins (Apo) A1, A1, AII, B, CII, CIII, E, TTR, insulin, osteopontin, osteoprotegerin, osteocalcin</td>
</tr>
</tbody>
</table>

Ovarian cancer researchers and clinicians have yet to agree on a definitive group of biomarkers that complement CA125; however, HE4 is now included in the vast majority of new multiplex ovarian cancer studies. The biomarker HE4, a whey acidic protein, is upregulated in ovarian cancers\(^6\) and is the only ovarian cancer biomarker other than CA125 to be FDA-approved. Significantly, HE4 is elevated in >50% of the 20% of ovarian cancers that CA125 is not elevated in.\(^5\) HE4 has been found to be overall less sensitive than CA125, but more effective in distinguishing malignant from benign pelvic masses.\(^4\) HE4 has also been shown to have greater sensitivity than CA125 in early stages of ovarian cancer.\(^6\) Additionally, HE4 is elevated in fewer non-ovarian cancer related diseases than CA125, making it more specific.\(^4,6\)
Expanding upon ROCA, a Risk of Ovarian Malignancy Algorithm (ROMA) has been developed and has received FDA approval (but not as a screening method) for combining longitudinal data of CA125 and HE4 values to classify women into high- and low-risk groups of developing ovarian cancer. The ROMA has shown higher sensitivity and specificity than a combination of CA125 and TVS, but this study did not utilize longitudinal data of CA125 in the comparison and used CT and MRI scans, which are not practical for a screening trial. Studies utilizing ROMA with additional biomarkers, in combination with a referral to TVS are still ongoing.

Instead of combining CA125 and HE4 into a single risk calculation, a follow-up study in the PLCO trial replaced TVS with HE4 as a second line screen to CA125 measurement in order to improve specificity. Of 39 cancers, HE4 was found to detect 27, compared to only 17 confirmed by TVS. However, TVS was more effective at confirming stage I cancers and no benefit of health outcomes for either method was demonstrated. The combination of CA125 as the first line and HE4 as the second line screen did in fact increase specificity but reduced sensitivity to the point that 10% PPV was not achieved.

While the addition of HE4 to CA125 looks promising in helping sensitivity and specificity, no multimodal, multimarker study has determined a method of screening that can be recommended for the general population. Expanding upon the work done combining CA125 and HE4, additional biomarkers could serve to improve sensitivity and specificity to the point of achieving the required 10% PPV.
1.6 Point-of-Care Platforms Utilizing Biomarkers for Screening

1.6.1 POC Diagnostic Platforms

Point-of-care (POC) systems integrate technologies to allow for clinical sample analysis in underserved populations and in settings closer to the point of care, such as clinical laboratories, hospitals, doctors’ offices, community centers and patients’ homes.\(^{71}\) Designed to bypass the need for large and expensive equipment by leveraging technological improvements and miniaturization, POC diagnostic systems aim to remove the need for separate, specialized clinical laboratories. POC platforms also typically increase assay speed and automation, provide multi-target analysis capabilities and reduce costs of traditional diagnostic methods.\(^{72}\)

POC methods have the potential to greatly improve the feasibility of general population screening methods. In addition to shorter assay times, relying on localized POC systems rather than transferring to centralized clinical laboratories significantly reduces wait-times for results. This timesaving can be even further utilized if a POC measurement can be returned within a patient visit, allowing for treatment or second line screening in the same visit. Reduced time is typically associated with reduced costs, in addition to reducing patient anxiety and discomfort.\(^{73}\)

1.6.2 Requirements of POC Diagnostic Platforms

While POC systems have the potential to improve analytical performance, shorten analysis times and reduce cost, a significant engineering effort is required to simplify clinical laboratories into a single device at the point-of-care.
For CA125 measurement, hospitals and clinics currently use one of many commercially available platforms, all of which require large equipment and the transfer of samples from the point of collection to the laboratory.\textsuperscript{74} Multiplexing strategies for protein biomarkers have been created and commercialized with success, the most widely used method of which is currently the Luminex\textsuperscript{®} flow cytometric bead-based system.\textsuperscript{75} While currently available multiplexing strategies are capable of analyzing multiple biomarkers concurrently and have been used for biomarker discovery,\textsuperscript{8} they also require significant laboratory infrastructure and in the case of ovarian cancer-specific multimarker assays require an overnight incubation.

A POC diagnostic platform could overcome these limitations by utilizing the inherent advantages of microfluidic systems to reduce equipment size, decrease assay time, and reduce sample and reagent volumes.\textsuperscript{71,76} In designing a device, limitations in equipment size, reagents and hands-on work required by a technician are determined by what is available at the POC.\textsuperscript{77} For typical POC use, a device with a small footprint that is capable of fully analyzing the sample without the need of a skilled technician is required.

Because no general screening method for ovarian cancer has been approved, research on ovarian cancer-specific POC systems has been limited. Electrochemical immunoassay strategies in the microfluidic regime have been applied to multiplexing ovarian cancer biomarkers and have demonstrated good analytical performance,\textsuperscript{78} but long incubation times (10 hours) preclude the use of this method at the POC. Another proposed electrochemical POC platform for ovarian cancer by Wang et al. has achieved 1 hour analysis times, but is incapable of multiplexing and suffers from a limited dynamic range.\textsuperscript{79} A cellphone-based
ELISA strategy was successfully used to measure HE4 values in urine, but several hours of incubation were required, preventing its use at the POC.\(^80\)

While some devices have claimed the ability to measure ovarian cancer biomarkers at the POC, no multiplexable device has been published that utilizes small sample volumes, has short analysis times (<1 hour) and achieves adequate analytical performance.

1.7 p-BNC as a POC Device for Early Detection and Screening

Multiplexing biomarkers in a rapid, POC diagnostic test that can quantitate the clinically relevant range of values has the potential to revolutionize the use of biomarkers in clinical practice. In the last decade, the McDevitt laboratory has continued to evolve the programmable bio-nano-chip (p-BNC) for POC diagnostics.\(^81\)-\(^84\) Two approaches have been utilized with the p-BNC, one utilizing a membrane for cell capture and analysis, and the other utilizing an agarose bead-based sensor for quantitating circulating analytes. The membrane system utilizes a size-selective membrane as a micro-sieve to capture cells, which can then be counted or analyzed for proteins and nucleic acids using fluorescent labeling. This approach has been used to count CD4 cells in HIV patients,\(^85\) to screen for oral cancer with cells collected from mouth swabs\(^86\) and to identify and count bacteria and spores in bio-warfare applications.\(^87\) The bead-based approach uses porous micro-beads, made in-house, that are functionalized with capturing molecules (usually antibodies) to measure analytes with fluorescence-based immunological reactions. An array of wells is used to hold the beads in place while allowing for fluid flow through the porous beads to enable analyte capture in the bead. A different bead-type can be placed in each well, allowing for the multiplexed analysis of a variety of analytes. The p-BNC agarose bead system has been used for a wide
array of diagnostic applications, most notably for cancer and cardiac-related diseases.\textsuperscript{88-92} Both the membrane- and bead-based diagnostic systems have been developed alongside credit-card sized disposable cards that integrate multiple on-card processes required for analysis, including waste-containment. The cards can be utilized for both systems by replacing the bead array with the cell capture membrane. The agarose bead-based system was used in this thesis, and henceforth “p-BNC” will refer specifically to the bead-based system.

The p-BNC assay card (Fig. 1-5) is a lab-on-a-chip platform that features a fully integrated microfluidic network that facilitates on-card sample preparation and metering, reagent storage, mixing, bubble and debris removal, and secure waste containment. By design, the p-BNC assay card minimizes benchtop sample and reagent preparation steps and associated laboratory tools and infrastructure, which is critical for POC analysis. Agarose beads represent the sensor core on which the sandwich immunoassays occur for quantification of biomarker analytes (Fig. 1-5 B). The supporting microfluidic environment, coupled with the 3-D structure of the agarose beads permit optimal analyte capture and detection to achieve low limits of detection and short analysis times. Further, due to the pre-defined spatial arrangement of the beads on the chip (Fig. 1-5 C), the p-BNC permits multiplexing of several biomarkers together. Following sample input, the sample is automatically metered on the card and processed to remove bubbles and debris. The buffer reagents push the sample to the beads, permitting analyte molecules to be captured on the immunosensor (antigen delivery) followed by removal of unbound analytes (wash step). The detecting antibody stored on the glass fiber pad is then eluted by the buffer and delivered to the beads to complete the immunoassay sandwich, followed by a final wash step prior to signal capture. All elements of the card, including the footprint were designed in parallel with
a portable optical analyzer to ensure rapid translation of the device to a fully portable POC assay system.

**Figure 1-5.** Illustration of [A] disposable p-BNC card with [i] syringe pump flow adapters, [ii] sample entry port and [iii] bead array holder. The [iv] sample loop contains an [v] overflow chamber, ensuring a 100μL dose of sample for each card. The right pump adapter flows buffer into the sample channel, displacing the sample into the bead-holding chip and through the beads into the [vi] waste. The second pump washes stored detecting antibody off the [vii] glass fiber pad and into the chip. The flow rate of the second pump is increased to perform the final rinse for imaging. All fluid pumped into the card passes through [viii] bubble traps to remove bubbles and an 8μM filter to prevent debris from reaching the bead sensors. A molecular schematic of the agarose bead is shown in [B], demonstrating a completed sandwich immunoassay. [C] shows a zoomed-in illustration of the bead array holder with different colors indicating different bead sensor types.

Building upon previous work on the development of the bead-based system and p-BNC card, this work focuses on translating and adapting this technology to a multiplexed ovarian cancer diagnostic test to be used as a screening tool at the POC.
1.8 Summary and Dissertation Overview

This chapter details the unmet need of an ovarian cancer screening strategy that is capable of detecting early stage ovarian cancer without referring a significant proportion of healthy patients in the screening population to unneeded surgery. Despite ovarian cancer’s low prevalence, a strong case has been made for a general population screening method has been made due to the low percentage of diagnoses occurring while the disease is still in early stages, combined with the significant survival benefits realized from early detection. As detailed above, by combining recent biomarker discoveries with the inherent advantages of microfluidic diagnostic platforms, this work aims to overcome the challenges associated with detecting a relatively rare and extremely heterogeneous disease. Further, this work aims to translate ovarian cancer screening to the POC, bypassing the need for expensive clinical laboratory equipment with long turnaround times for results. By combining significantly shorter assay times with eliminating the need to transfer samples to specialized labs, a patient can be referred to secondary screening and confirmation in the same visit, reducing patient anxiety and travel while ultimately permitting quicker treatment.

Overcoming the heterogeneity of different ovarian cancer types while differentiating benign tumors from malignant ones has proven difficult for potential screening methods, demonstrating the likely need for a multimodal effort for screening. In this work, we aim to multiplex biomarkers to improve the sensitivity and specificity of a screening method, while easing the burden and cost of a multimodal screening method (second stage referral to TVS) by translating this work to a POC platform. The use of multiple reagents and the co-incubation of multiple analytes in multiplexed immunoassay platforms make achieving the required analytical performance and validation challenging. Overcoming this potential cross-
reactivity and achieving limits of detection low enough for measuring healthy patients are significant challenges to multiplexed diagnostic tests, which are compounded in microfluidic POC systems. Further, the added complexity of longitudinal monitoring is introduced in this work, demonstrating the necessity of high precision for day-to-day variability, in order to create personalized baselines for patients. Significant analytical validation and comparison to gold standard methods is required to demonstrate performance of the proposed multiplexed POC screening method and carefully designed clinical studies are necessary to demonstrate potential clinical application of the platform.

The ultimate vision for this work is to create an ovarian cancer screening test that utilizes a fingerstick blood sample and returns results at the point-of-care to refer patients to a second-line screening of TVS in the same visit. The multiplexing capability of the p-BNC is leveraged to improve sensitivity and specificity, potentially achieving the necessary performance desired from clinicians for a general population screening test. Though it is beyond the scope of this dissertation to determine actual clinical utility of the selected multiplexed panel or to develop a final-form POC product, this dissertation will seek to address the following points of focus:

- Which biomarkers are most promising for an ovarian cancer screening method?
- Can these biomarkers be adapted to the p-BNC with the required analytical performance needed to measure the levels of healthy patients?
- What are the challenges in multiplexing, reducing sample volume 10-fold and transitioning to the p-BNC device from the flow cell used in previously published work on CA125?
• What is the effect of multiplexing on analytical performance compared to individual singleplex assays on the p-BNC?
• What is the degree of cross-reactivity for ovarian cancer biomarkers in the p-BNC?
• Is the p-BNC capable of measuring patient clinical samples and do the values of the multiplexed biomarkers correlate to gold standard diagnostic methods?
• What is the day-to-day precision of the biomarker panel on the p-BNC?
• Can the p-BNC biomarker panel differentiate between healthy, benign, early stage ovarian cancer and late stage ovarian cancer patients?
• Is the precision of the p-BNC adequate enough to longitudinally monitor multiplexed biomarker values of patients?
• Does the longitudinal data of multiplexed biomarkers demonstrate benefit over CA125 alone?
• Can the p-BNC ovarian cancer biomarker panel be translated to a manufactured format of the card with on-board fluid blister packs controlled by actuators?
• How can the length of the assay be shortened to less than 20 minutes to truly allow for same-visit results?
• Is the p-BNC ovarian cancer test capable of being used with an all-in-one analyzer, replacing fluid control, optics and image analysis?
• Are serum, plasma and blood capable of being analyzed on the p-BNC and how do biomarker values in the three fluids compare?
• Can biomarker levels be quantified on the p-BNC using fingerstick quantities of blood?
The remaining sections of this dissertation are organized in the following chapters. **Chapter 2** details the development of the multiplexed p-BNC ovarian cancer panel. The biology of each biomarker is described, in addition to the degree of previous use in research. The adaptation of the four biomarkers on the p-BNC involved extensive optimization to overcome cross-reactivity concerns and to achieve the analytical performance required for the early detection of ovarian cancer. After determining optimal matched pairs for capturing and detecting antibodies, antibody concentration, conjugation methods, buffer, fluorophore, optics and image analysis were optimized to attain limits of detection low enough to measure healthy samples for all four biomarker assays in the p-BNC card. Dose response curves with known concentrations were performed for singleplexed and multiplexed assays to determine analytical performance and measure degree of cross-reactivity for each biomarker. The day-to-day precision values of the multiplexed assays were also measured to demonstrate the ability for the p-BNC ovarian cancer panel to be used for longitudinally monitoring biomarkers.

Following development and analytical validation of the multiplexed biomarker panel, over one hundred clinical samples were analyzed to demonstrate real-world application of the p-BNC ovarian cancer diagnostic test, as described in **Chapter 3**. A set of late-stage ovarian cancer clinical samples was measured on both the p-BNC and the gold standard method for multiplexing in order to validate against an FDA-approved system. Additionally, a set of healthy, benign, early stage ovarian cancer and late stage ovarian cancer samples was measured on the p-BNC. These measurements were used to determine if the p-BNC can differentiate healthy from diseased samples in a pilot-scale clinical trial, and also to distinguish the healthy cut-off value for each biomarker in this small sample set. Finally, the
p-BNC was used to serially measure seven patients with multiple longitudinal time points, two of which developed ovarian cancer. These samples were used to evaluate if the precision of the p-BNC was adequate to create baselines for patients and also to determine if additional biomarker information might provide improved lead time over CA125, which could make earlier detection for a larger variety of ovarian cancers possible.

Following the demonstration of the ability to measure serum clinical samples, Chapter 4 explores the potential of the p-BNC as a fingerstick POC screening tool. The length of the assay was shortened by over half the time by reducing the number of beads analyzed per assay, which enables a more realistic single-visit approach for referral to secondary screening. Additionally, to improve quality and reliability of the cards and to advance towards a more scalable solution, the multiplex assay was transitioned to quality-controlled third-party manufactured cards using injected molded plastics rather than the in-house, handmade xurography-based manufactured cards. This manufactured card also enabled the transition to fluid blister packs, which self-contain all necessary buffers on the card. The blister packs, combined with an in-house developed actuator platform that controls fluid flow, replaced separate syringes and syringe pumps to further advance the p-BNC to a more POC system. On this next generation platform, clinical samples consisting of sets serum, plasma and blood from each patient were measured to determine the feasibility of using blood in the p-BNC.

Collectively, this body of work significantly advances the p-BNC as a POC screening tool for ovarian cancer. This thesis explores the challenges and advantages of using a microfluidic system for the multiplexed analysis of protein biomarkers with the limitations of POC applications. A novel panel of ovarian cancer biomarkers was used that shows promise
in diagnosing ovarian cancer in clinical samples, but this thesis also demonstrates the capability of the p-BNC as a platform to screen and evaluate newly discovered biomarkers that can quickly be adapted to a POC format. Preliminary longitudinal data of multiple biomarkers showed promising results over CA125 alone, laying a foundation for validation of multiplexed longitudinal biomarkers in a larger clinical trial. Finally, serum, plasma and blood were compared on the p-BNC, demonstrating fingerstick analysis capabilities of the p-BNC and showing that blood can be used to measure ovarian cancer-specific biomarkers. Such POC diagnostic systems have the capability to enable low-cost general population screening methods that could improve survival rates of ovarian cancer patients.
Chapter 2: Development and Characterization of Multiplexed Assays on the p-BNC for Ovarian Cancer Biomarkers

2.1 Chapter Overview

In this chapter, four ovarian cancer biomarker assays are developed individually and multiplexed together on the p-BNC. Based on the analytical requirements of measuring early stage disease and healthy patients’ levels, the four assays were optimized to achieve low limits of detection, have a dynamic range of measurement and limit cross-reactivity between markers. Pertinent to accomplishing these objectives on the p-BNC card, biology and possible functions of the four biomarkers in the human body are discussed. Following the description of the biomarkers used in this study, the challenges associated with achieving adequate analytical performance while multiplexing without significant cross-reactivity are surveyed. The optimization required for meeting these performance metrics is examined, followed by the characterization of the fully optimized assay. The characterization includes dose response curves that demonstrate limits of detection and dynamic range, a precision study that establishes day-to-day variability and a cross-reactivity study. The cross-reactivity study compares each of the singleplexed assays with its multiplexed counterpart and demonstrates limited undesirable interactions between non-corresponding antibodies and antigens.

2.2 Introduction

In this introduction, molecular characterization is provided of the four ovarian cancer biomarkers used in this study. Information about each biomarker’s discovery and current use in diagnostics is discussed; however, it is important to note that many biomarkers’ structure and roles in cancer are not well understood. This lack of understanding is largely due to the
fact that biomarker research labs screen for hundreds of biomarkers elevated in cancer samples compared to healthy samples, without differentiating function, size or shape of the biomarkers. Some biomarkers are characterized separately, but the understanding of function and classification of biomarkers is not necessarily required for the development of an effective diagnostic test for research use. However, known biology and function are discussed here to provide insight into issues and optimization efforts regarding assay development on the p-BNC.

2.3 Ovarian Cancer Biomarkers

The four biomarkers used in this study, CA125, HE4, MMP-7 and CA72-4, were chosen based on unpublished data from the Robert Bast ovarian cancer biomarker research group at M.D. Anderson Cancer Center (Table 2-1). The Bast research group chose the top 8 markers from a study that identified several multimarker panels for the early detection of ovarian cancer by assessing 96 ovarian cancer biomarkers utilizing xMAP® bead-based immunoassay technology. These top 8 biomarkers CA125, HE4, s-VCAM, CEA, IGFBP-1, CA72-4, MMP-7 and EGFR, were evaluated further as potential indicators of early stage ovarian cancer using ELISA and after assessing sensitivity, specificity and biological variability, a combination of CA125, HE4, MMP-7 and CA72-4 was chosen as the most promising panel with the highest sensitivity and specificity (unpublished data). Panels with S-VCAM were excluded due to previous difficulties with reagents and assay development.
Table 2-1. Performance of five best multimarker panels in training and validation sets at 98% specificity (unpublished data).

<table>
<thead>
<tr>
<th>Biomarker Panel</th>
<th>Training Sensitivity</th>
<th>Validation Sensitivity</th>
<th>Training Std Dev</th>
<th>Validation Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA125, CA72-4, HE4, MMP-7</td>
<td>82.1%</td>
<td>83.2%</td>
<td>0.034</td>
<td>0.044</td>
</tr>
<tr>
<td>CA125, CA72-4, HE4</td>
<td>82.9%</td>
<td>82.2%</td>
<td>0.036</td>
<td>0.051</td>
</tr>
<tr>
<td>CA125, CA19-9, CA72-4, HE4</td>
<td>83.4%</td>
<td>81.8%</td>
<td>0.03</td>
<td>0.046</td>
</tr>
<tr>
<td>CA125, CA72-4, HE4, CEA</td>
<td>84.0%</td>
<td>81.3%</td>
<td>0.044</td>
<td>0.066</td>
</tr>
<tr>
<td>CA15-3, CA125, CA72-4, HE4</td>
<td>83.3%</td>
<td>80.7%</td>
<td>0.04</td>
<td>0.064</td>
</tr>
</tbody>
</table>

2.3.1 Cancer Antigen 125 (CA125)

The biomarker CA125 is considered the “gold standard” of ovarian cancer biomarkers. Identified in 1981 by Bast et al., CA125 is an FDA-approved biomarker that is upregulated in many ovarian cancers. CA125 has been used in clinics for more than three decades to monitor disease response, evaluate pelvic masses, estimate prognosis, and improve clinical trial design.

2.3.1.1 CA125 Structure and Function

Despite being elucidated in 1981, relatively little is known of the molecular nature of CA125. It is understood, however, that CA125 is a heavily glycosylated transmembrane protein with a high molecular weight and three domains: the carboxy terminal domain, the amino terminal domain, and the extracellular domain. The biochemical analysis of CA125 has shown conflicting data, and as a result has been described as a protein, a mucin, a carbohydrate, and a membrane-associated glycoprotein. Similarly, the reported size of CA125, although agreed to be large, is controversial and ranges from 200-10,000 kDa, depending on source of extraction and number of repeat domains. The epitopes of CA125
(portions of the protein recognized by antibodies) have been mapped as 3 major antigenic domains, OC125, M11 and OV197. A proposed structure of CA125 is shown in Fig. 2-1.

Figure 2-1. Proposed structure of CA125 by O’Brien et al. Reprinted with permission from Karger Publishers, A.G. Basel, Tumor Biology.

The extracellular domain, which is characterized by 7, 20 or 60 repeat domains, is released from the cell when cleavage occurs. These repeats contain SEA (sea urchin, enterokinase, and agrin) domains, which are where the OC125 and M11 antigenic sites are
found.\textsuperscript{99} The fact that the antigenic sites are within the extracellular domain is significant, as antibodies for these sites can be used to detect cleaved CA125 molecules in circulating blood. The variable number of extracellular repeat domains are also highly glycosylated with O-linked and N-linked oligosaccharides, likely causing the large variability in size of CA125.\textsuperscript{94} Furthermore, continuous deglycosylation of oligosaccharides in body fluids, endogenous protease activity, alternative splicing of tandem repeats, variation in expression of different biological sources, and differences in cancer versus normal forms of the protein could all play roles in the heterogeneity and size variation of CA125.\textsuperscript{94,99,100}

From cloning the partial cDNA, a new mucin known as MUC16 was matched to the CA125 antigen.\textsuperscript{100} The MUC16 gene is located on 19q13.2 chromosome\textsuperscript{95} and is expressed in normal tissues, specifically in surfaces of respiratory tract, ocular tract and female reproductive tract epithelia.\textsuperscript{101} This expression in normal tissues results in measurable CA125 levels in healthy patients, though the function in healthy (or diseased) tissues is not completely understood. Suggested functions in the ocular tract include protecting surfaces from pathogen adhesion\textsuperscript{101} and altering the precorneal tear film,\textsuperscript{102} while the respiratory and reproductive tracts could make use of CA125 as a cellular adhesion molecule.\textsuperscript{99} In ovarian cancer, MUC16 has been demonstrated to inhibit human natural killer cells\textsuperscript{103} and bind to galectin-1, which is found on immune cells,\textsuperscript{104} suggesting that MUC16 has a role in preventing anti-tumor immune responses.\textsuperscript{105} The large amount of N-glycans on CA125 has also been shown to allow binding to both glycosylated and non-glycosylated mesothelin,\textsuperscript{105} which are highly expressed in ovarian tumors.\textsuperscript{106,107} The binding of mesothelin could facilitate cell-to-cell interactions, and thereby play a role in metastasis of ovarian cancer tumors.\textsuperscript{105} The
knockdown of MUC16 has also been shown to affect the morphology and motility of ovarian tumor cells, in addition to promoting cell migration and invasion of tumor cells.\textsuperscript{108,109}

\subsection*{2.3.1.2 CA125 Use in Diagnostics}

The biomarker CA125 has been found to be elevated in 90\% of advanced stage ovarian cancers\textsuperscript{43} and 80\% of all ovarian cancers.\textsuperscript{44} Antibodies against CA125 were developed in 1983 by Bast et al.,\textsuperscript{46} allowing for the development of assays that can quantify levels of CA125. Fortunately, the variation in size of CA125 was not found to have a significant impact on antibody-antigen binding,\textsuperscript{97} however, the uncertainty in molecular weight has resulted in the use of U/mL for reporting concentrations of CA125. The original assay for CA125 measurement was a radioimmunometric assay that utilized OC125 antibodies for capture and detection,\textsuperscript{110} but M11 and OV197 antibodies have since been used in various assays.\textsuperscript{96} The second and most commonly used CA125 assay is known as the CA125-II assay, which uses both OC125 and M11 antibodies.\textsuperscript{74} Several other commercially available immunoanalyzers are capable of measuring levels of CA125, including the Access 2 (Becman Coulter, Brea, CA), ADVIA Centaur (Bayer Diagnostics, Tarrytown, NY), ARCHITECT i2000 (Abbott Diagnostics, Abbott Park, IL), AxSYM (Abbott Diagnostics, Abbott Park, IL), Elecsys 2010 (Roche Diagnostics, Indianapolis, IN), IMMULITE 2000 (Diagnostic Products, Los Angeles, CA) and VITROS ECi (Ortho Clinical Diagnostics, Raritan, NJ).\textsuperscript{74} Limits of detection of these systems range from 0.05-1.45 U/mL.\textsuperscript{74}

Previously, a CA125 assay was developed on an early version of the p-BNC that used a flow cell to allow fluids to interact with the beads (Fig. 2-2),\textsuperscript{84} rather than the integrated, disposable card described in this dissertation. After a significant study on the
effect of 30 different matched pair combinations, the extensive optimization on flow rates, incubation times and reagent concentrations resulted in a limit of detection of 1.0 U/mL with inter- and intra-assay precision of 1.2% and 1.9% respectively. However, this study required the usage of large volumes of reagents both for detecting antibody delivery and for washing, in addition to needing 1mL of sample volume per assay, which was recirculated through the beads over the entire sample incubation stage.

Figure 2-2: The flow cell structure that facilitates the interaction between fluids and agarose beads in Raamanthan et al. Figure [A] demonstrates the use of the flow cell and the [A-I] silicon bead holding chip [A-II] as an analog of the fully integrated p-BNC card [A-III]. [B] shows the schematic of the assembled flow cell [B-I], with the beads positioned in the silicon-etched wells [B-II] and the interaction of the antigen and detection antibodies in the agarose bead [B-III]. Reprinted from Cancer Prevention Research with permission from AACR. 84

2.3.2 Human Epididymis Protein 4 (HE4)

HE4 is a whey acidic protein that has been found to be upregulated in ovarian cancers and is now included in the vast majority of new multiplex ovarian cancer studies.
Additionally, HE4 is the only ovarian cancer biomarker other than CA125 to be FDA-approved for certain uses in ovarian cancer.

### 2.3.2.1 HE4 Structure and Function

The biomarker HE4 was discovered by Kirchloff et al. in 1990 in a cDNA screening.\(^{111}\) Interestingly, it was originally identified to be epididymis-specific, which is part of the male reproductive system. The gene that encodes for HE4 is known as WFDC2\(^{112}\) and is located on chromosome 20q12-13.1.\(^{113}\) This gene encodes for a 13 kDa protein that is ultimately glycosylated, resulting in a ~25 kDa protein.\(^{112}\) The total protein consists of a single peptide with two whey acidic protein (WAP) domains that have a ‘four disulfide core’ made from eight cysteine residues.\(^{113}\) Hypotheses of the functional roles of HE4 are largely based on the functions of other proteins containing WAP domains, with SLPI and elafin being the best-studied.\(^{112,114}\) The WAP family proteins have been associated with anti-proteinase, anti-microbial and anti-inflammatory activity, possibly indicating their role in host defense and sperm maturation,\(^{114-116}\) but it is speculation as to whether these functions also apply to HE4. HE4 is also expressed in other normal tissues than the epididymis, including the respiratory tract and nasopharynx.\(^{117}\)

In 1999, Schummer et al. first associated HE4 with ovarian cancer by using comparative cDNA hybridization to find levels of HE4 higher in ovarian cancer patients than in healthy patients.\(^{118}\) Immunohistochemical assessment confirmed the elevation in ovarian cancer by demonstrating that normal surface epithelium does not express HE4, while tumorous epithelium abundantly expressed the biomarker, though the degree of expression was ovarian cancer subtype-dependent.\(^{119}\) More specifically, HE4 was overexpressed in
100% of endometriod, 93% of serous, 50% of clear cell, but 0% of mucinous or germ-cell ovarian cancers.\textsuperscript{119} Other WAP proteins have also been linked to a variety of cancers, indicating a likely role in tumor progression or carcinogenesis for this family of proteins.\textsuperscript{120} Recently, the knockdown of HE4 was found to inhibit the proliferation and spreading ability of tumor cells,\textsuperscript{121} while overexpressing HE4 in tumor cells was found to promote tumor growth.\textsuperscript{122} These findings indicate a role in cancer cell adhesion, migration and tumor growth, although the function of HE4 in ovarian cancer is still not completely understood.

\subsection{2.3.2.2 HE4 Use in Diagnostics}

The biomarker HE4 was first used as a serum biomarker in ovarian cancer diagnostics in 2003, when Hellstrom et al. developed antibodies against expressed HE4.\textsuperscript{66} Using 2H5 and 3D8 mouse monoclonal antibodies, each of which binds to distinct epitopes of HE4, a double determinant ELISA was developed. Consistent with histology of ovarian cancer cell lines, 90\% of women with ovarian cancer had elevated HE4 serum levels at diagnosis.\textsuperscript{66} Though similar sensitivity and specificity values to CA125 were found, fewer false positives were detected.\textsuperscript{66} Similarly, HE4 serum levels of women with benign gynecologic disorders have been found elevated less frequently than CA125,\textsuperscript{2} a promising result for reducing the number of biopsy surgeries needed per correct diagnosis. Measuring levels of HE4 has also been suggested for monitoring ovarian cancer recurrence, with some studies suggesting an improvement over the use of CA125 for the same purpose.\textsuperscript{123,124} Adding HE4 to CA125 has been shown to elevate the sensitivity and specificity of CA125 alone,\textsuperscript{59,64} and, as a result, the combination of CA125 and HE4 has been FDA-approved to monitor recurrence or progression of disease.\textsuperscript{124}
2.3.3 Matrix Metalloproteinase-7 (MMP-7)

The matrix metalloproteinase family has been implicated in cancer for over 30 years, but despite extensive research, its role is still not completely understood.\textsuperscript{125} The biomarker MMP-7 has been researched as an ovarian cancer biomarker for the last 15 years, but its effectiveness, especially compared to CA125 and HE4, has remained unclear.

2.3.3.1 MMP-7 Structure and Function

MMP-7, also known as matrilysin or Pump-1, is a member of the matrix metalloproteinase family, which consists of 23 different proteinases in humans.\textsuperscript{126} The MMP gene family was first identified in 1962,\textsuperscript{127} and its complete function \textit{in vivo} is still not completely understood.\textsuperscript{126} It has, however, been shown to have proteolytic activity, specifically of the extracellular matrix, which affects tissue remodeling, organ development, innate immunity, and regulation of inflammatory processes,\textsuperscript{128} though its complete function \textit{in vivo} is not completely understood.\textsuperscript{126}

The MMPs typically have three domains – the pro-domain, the catalytic domain, and the hemopexin-like C-terminal domain.\textsuperscript{129} MMP-7, identified in 1988 from a rat uterus,\textsuperscript{130} is the only MMP without the hemopexin-like C-terminal domain\textsuperscript{131} and, as a result, is only 28 kDa.\textsuperscript{132} The catalytic domain of MMPs contains a zinc ion in the active site, which, when bound to a cysteine residue in the pro-domain, forms the inactive form of MMPs, known as proMMPs.\textsuperscript{128}

MMP-7 is not found in high levels during embryonic development and does not have a known developmental role in humans.\textsuperscript{126} In healthy adults, MMP-7 is only expressed in mucosal epithelia and has been linked to response to bacterial exposure and wound
healing. In cancer, MMP-7 has been implicated in multiple aspects of tumor growth, invasion and metastasis, and has been linked to cancer of almost every organ in the body, including ovarian cancer. MMPs have been found in virtually all stages of cancer progression, but their exact role is still not well understood.

### 2.3.3.2 MMP-7 Use in Diagnostics

Recombinant human MMP-7 was first produced in 1996 by Kihira et al., which allowed for the production of monoclonal antibodies against MMP-7. These antibodies were used in a sandwich ELISA, intended to measure protein levels related to colorectal cancer. Importantly, these antibodies were able to bind to both pro- and active forms of MMP-7. In relation to ovarian cancer, Tanimoto et al. first reported MMP-7 as a potential ovarian cancer marker in 1999, using quantitative PCR, northern blots and immunohistochemistry to identify elevated MMP-7. Several studies have found increased MMP-7 levels in ovarian cancer patients, however, reliable sensitivity and specificity values have not been determined from a large clinical study. While MMP-7 has compared favorably to CA125 in one study, two others report around half of the sensitivity of CA125. MMP-7 has also not yet been tested in a large trial with a variety of subtypes of ovarian cancer, where MMP-7 might improve the sensitivity and/or specificity of CA125 by improving detection lead-time or detecting cancers that are not detectable by CA125.

### 2.3.4 CA72-4

Though ‘CA72-4’ and ‘TAG-72’ are used interchangeably now, CA72-4 originally referred to the assay that measured the TAG-72 antigen. As it is now more common, CA72-4
will be used in this dissertation to refer to the biomarker. Levels of CA72-4 have been found elevated in several forms of cancer, including ovarian, but its effectiveness in detecting ovarian cancer is still unclear.

2.3.4.1 CA72-4 Structure and Function

The biomarker CA72-4 is a tumor-associated glycoprotein with a large size of 200 to 1,000 kDa.\textsuperscript{141} This large variation in reported size could be due to shearing, which is commonly found in large molecules like mucins, and from the difficulty in assigning molecular weights to heavily glycosylated proteins.\textsuperscript{141} The structure of CA72-4 has not been well elucidated, possibly due to the difficulty in purifying mucins.\textsuperscript{141,142} The function of CA72-4 in healthy or cancerous tissue is also not well understood, as most CA72-4 research has focused on circulating biomarker levels in various diseases. However, if the hypothesis that CA72-4 is a mucin is correct, it likely has a role in lubrication and protection.\textsuperscript{141}

2.3.4.2 CA72-4 Use in Diagnostics

The antibody B72.3 was first reported in 1981 by Colcher et al. in a study testing the reactivity of a panel of antibodies to breast cancer tumor cells,\textsuperscript{143} and was later found to target the antigen TAG-72.\textsuperscript{144,145} A second antibody, CC49, with higher affinity to TAG-72 than B72.3 was identified in 1988,\textsuperscript{146} and the combination of the two antibodies enabled the development of a double determinant assay, named CA72-4, for quantitating TAG-72.\textsuperscript{147}

As CA72-4 was discovered from cancer cells, the biomarker has always been associated with cancer, most commonly in gastric-\textsuperscript{148-150} and ovarian-related cancers.\textsuperscript{151-154} Thor et al. first showed elevated CA72-4 in ovarian tumors in 1986,\textsuperscript{151} and several studies
have since confirmed elevated levels in ovarian cancer patients.\textsuperscript{152,154,155} Two studies have shown lower sensitivity and specificity than CA125,\textsuperscript{8,156} but similarly to MMP-7, it is unknown whether CA72-4 can provide additional diagnostic value or not.

### 2.4 Assay Requirements for Early Detection and Longitudinal Monitoring

The longitudinal monitoring of ovarian cancer biomarkers has shown promise in improving diagnostic performance over using a single cut-off value. By taking serial measurements yearly or bi-yearly, a personalized baseline for each patient can be created, effectively generating their own individualized cut-off value. In order to create an effective baseline, two analytical performance metrics are critical: low limits of detection and system precision (of day-to-day measurements) that is less variable than the biological variation of the biomarker.

While traditionally a limit of detection at the classical cut-off value would have sufficed, longitudinal monitoring requires low limits of detection in order to reliably measure healthy patient values, which are used to create an individual’s baseline. For CA125, 35, 65 or 100 U/mL has most commonly been used as the cut-off between healthy and ovarian cancer patients,\textsuperscript{157} however, values of both healthy and diseased patients under 10 U/mL have been reported.\textsuperscript{65} 40-85 pM have often been used as cut-off values for HE4, but it is important to note that HE4 has recently been shown to be age-dependent, necessitating different cut-off values for different age groups.\textsuperscript{158,159} Healthy patients have average HE4 levels of around 30 pM.\textsuperscript{65} For MMP-7, a cut-off value of 7.4 ng/mL has been used, although this value was developed from cholangiocarcinoma patients and would like vary for ovarian cancer
patients. A cut-off value of 8.5 U/mL has been used for CA72-4, with the majority of healthy patient values not being measureable.

For the second critical performance metric – precision – day-to-day variability of biomarker measurements needs to be low in order to develop a flat baseline that can be used to recognize an increasing concentration. While longitudinally monitoring biomarkers, both the analytical imprecision and the biological variation of samples need to be considered when developing the methodology for differentiating between a natural fluctuation and an at-risk result. The development of the longitudinal monitoring algorithm is beyond the scope of this dissertation, however, the amount of imprecision of the diagnostic assay is critical to an effective algorithm, as it is added to the biological variation of biomarkers in creating fluctuations in values while longitudinal monitoring. The biological variation for these four ovarian cancer biomarkers have been determined by our collaborators at MD Anderson and are shown in Table 2-2 (paper in review). These values demonstrate a significant amount of variation for healthy patients from measurement to measurement, and reinforce the necessity of low imprecision contributed by the assay platform.

**Table 2-2.** Biological variation of CA125, HE4, MMP-7 and CA72-4 assessed in longitudinal healthy controls (paper in review).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Mean</th>
<th>25% Qtl</th>
<th>Median</th>
<th>75% Qtl</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA125</td>
<td>16.7%</td>
<td>10.2%</td>
<td>14.9%</td>
<td>20.1%</td>
</tr>
<tr>
<td>HE4</td>
<td>26.5%</td>
<td>19.3%</td>
<td>24.9%</td>
<td>31.4%</td>
</tr>
<tr>
<td>MMP-7</td>
<td>21.5%</td>
<td>13.0%</td>
<td>20.2%</td>
<td>27.7%</td>
</tr>
<tr>
<td>CA72-4</td>
<td>30.6%</td>
<td>8.6%</td>
<td>21.0%</td>
<td>46.2%</td>
</tr>
</tbody>
</table>
2.5 Challenges with Multiplexed Immunoassays

Multiplexed assays offer significant reagent-, cost- and time-savings by reducing multiple individual assays to a single combined assay. Significantly, multiplexed immunoassays reduce sample requirements and enable larger data collection from the same number of patient samples.\textsuperscript{163} While multiplexed assays have increasingly gained in popularity in protein quantitation studies, the methods for adequate analytical validation and steps for FDA approval are still being investigated, with no consensus yet agreed upon.\textsuperscript{164} The lack of guidance for \textit{in vitro} diagnostic development reinforces the importance of establishing the challenges associated with multiplexed immunoassays and the related validation steps to ensure adequate performance before evaluating clinical samples.

In multiplexing systems that utilize antibody-protein interactions, selection and optimization of capture ligands and the evaluation of their ability to immobilize target proteins are critical to optimize diagnostic performance.\textsuperscript{165} Capture ligands with high affinity for their target molecules need to be selected in order to achieve necessary detection limits, however, their degree of specificity for their target molecule is as important in order to prevent cross-reactivity between the antibodies and proteins associated with the other multiplexed biomarkers.\textsuperscript{165} Due to the heterogeneity in size, hydrophobicity of domains, and dependence on 3-D structure for activity, it is difficult to predict the activity of antibody-protein interactions.\textsuperscript{166} Further, different environments created by the various approaches used in different immunoassay systems, for instance binding to 3D agarose mesh networks rather than 2D plastic surfaces, alters the binding ability and exacerbates the difficulty in predicting effective affinity.\textsuperscript{167}
In order to determine the efficacy of binding, which defines the limits of detection, dilution linearity, and measurable range, calibration curves are performed using a series of known protein concentrations that are plotted against the signal of the assay. A calibration curve is fit to the data using a regression model, which is used to calculate unknowns and determine the lowest quantifiable protein concentration.\textsuperscript{165} While the objective is to have the multiplex performance match that of the corresponding singleplex assays, some degree of cross-reactivity is expected.\textsuperscript{165} Several different interactions between antibodies and proteins can contribute to cross-reactivity, as shown in Fig. 2-3, which limits the number of proteins that can effectively be used in an assay and prevents certain protein combinations from being used in the same panel. Avoiding these interactions involves selectively choosing compatible combinations of antibodies and the optimization of assay parameters that limit cross-reactivity, such as type of buffer used and contact time for various reagents. Singleplex and multiplex calibration curves should be compared to rule out significant cross-reactivity and demonstrate similar analytical validation.\textsuperscript{165} The considerations of assay dilution and linear range are also critical for multiplexed assays, as all simultaneous assays need to utilize the same sample conditions.

\textbf{Figure 2-3:} Cross-reactivity interactions between capturing antibodies (black), detecting antibodies (grey/blue with yellow fluorophore), and antigens (green/red). Reprinted with permission from Journal of Proteome Research. Copyright 2008 American Chemical Society.\textsuperscript{168}
2.6 Materials and Methods

2.6.1 Immunoreagents and Buffers

All reagents (capture and detection antibodies, antigen) utilized in p-BNC immunoassays were prepared with SuperBlock (PBS) Blocking Buffer (Thermo Fisher Scientific Inc., Waltham, MA) to limit nonspecific binding and enhance reagent stability. To develop heterologous double determinant immunoassays in the p-BNC format, murine monoclonal anti-human CA125 (clone M11), anti-human HE4 (clone 2H5) and anti-human CA72-4 (clone CC49) antibodies were utilized to capture CA125, HE4, and CA72-4 respectively. For detection of captured ligands, anti-CA125 (clone OC125), anti-HE4 (clone 3D8) and anti-CA72-4 (clone B72.3) murine monoclonal antibodies were employed. Each of these reagents was generously supplied by Fujirebio Diagnostics, Inc., Malvern, PA. To measure MMP-7, murine monoclonal anti-MMP-7 (clone 111433) was used to capture the ligand and goat anti-MMP-7 polyclonal antibody was used for detection. Anti-MMP-7 antibodies were purchased from R&D Systems, Inc., Minneapolis, MN. Capture antibodies were anchored on glyoxylated agarose microspheres manufactured in our laboratory using reductive amination protocols at a concentration of 320ng per agarose bead sensor, as described below. Primary amino groups of detecting antibodies were conjugated to TFP ester moieties of appropriate fluorophores (Scheme 2-1) – AlexaFluor® 488 (for HE4, MMP-7 and CA72-4 antibodies) and Oregon Green® 488 (for CA125 antibody) to form stable dye-antibody conjugates (Thermo Fisher Scientific Inc., Waltham, MA).
**Scheme 2-1:** Conjugation of antibodies ("L") to AlexaFluor® 488

![Scheme 2-1](image)

Imunoassay standards for calibration curves were prepared by generating appropriate dilutions using purified protein antigen stocks for CA125, HE4 and CA72-4 (Fujirebio, Diagnostics Inc., Malvern, PA) and EIA antigen standard for MMP-7 (R&D Systems Inc., Minneapolis, MN).

### 2.6.2 Bead Fabrication and Conjugation

Bead preparation has been extensively described elsewhere. Briefly, porous agarose beads were prepared by emulsifying a 2% agarose solution at 61°C with a suspending solution made of Span 85 diluted in n-heptane at 58°C. The mixture was stirred at 550 RPM using an RW20 DzM.n overhead stirrer (IKA Works Inc., Wilmington, NC) until the temperature dropped to 24°C, after which the beads were collected on a set of metal screen sieves and washed extensively with deionized water. The 250-280μm bead fraction was collected and rinsed with 50/50 ethanol water for surfactant and organic solvent removal. The beads were then rinsed with water and stored in water at 4°C. On the following day, the beads were cross-linked to create a porous structure and provide rigidity. The beads were removed from the water and resuspended in K₂PO₄, which was then transferred to a conical tube with divinyl sulfone and NaBH₄. The vial was shaken overnight and then washed three times in a NaBH₄ and NaOH solution. A solution of NaBH₄, NaOH and D-mannitol was
added to the beads, rotated for 4 hours to quench unreacted vinyl groups, and washed extensively with water. For glyoxal activation of cross-linked beads, a solution of NaOH, NaBH₄, and glycidol was added to the beads and shaken overnight. The next day the beads were washed with water and resuspended in water and NaIO₄, which was rotated for 1 hour. The beads were then washed with water and stored in carbonate buffer pH 9 at 4°C.

For use in double-determinant immunoassays, capturing antibodies are conjugated to the glyoxal activated cross-linked beads via reductive amination (Scheme 2-2). After washing an aliquot of beads three times with PBS, a solution of NaCNBH₃ dissolved in PBS and the relevant antibodies are rotated with the beads overnight. The beads were then washed three times and added to a solution of Tris buffer to block active sites that were not bound with antibody. After a 1-hour rotation, the beads were washed three times and stored in PBS buffer at 4°C.

Scheme 2-2: Reductive amination of antibodies (“L”) and agarose beads (in blue)

2.6.3 p-BNC Fabrication

The p-BNC was constructed with alternate layers of 3M™ 9500PC double-sided adhesive (3M Company, St. Paul, MN) and 3M™ AF4300 polyethylene terephthalate (3M Company, St. Paul, MN) (Fig. 2-4), which were patterned xurographically with a SummaCut D75 (Summa Inc., Seattle, WA) and a Graphtec FC2250 plotter cutter (Graphtec America, Inc., Irvine, CA), respectively. The resulting 7-layer card featured a network of microfluidic channels for sample delivery, sample metering, detecting antibody reconstitution, antibody
delivery, and washing. Air vents were built into the card using hydrophobic SurePVDF membranes (EMD Millipore, Billerica, MA) to mitigate bubbles and an 8μm Whatman Nuclepore Track-Etch membrane (GE Healthcare, Fairfield, CT) was used for in-line filtration. Glass fiber conjugate pads (EMD Millipore, Billerica, MA) cut into 2x15mm rectangles were used to store detecting antibody in the card and mixers were patterned into the polyethylene terephthalate layer’s channels with the Graphtec plotter cutter to ensure homogenous mixing and consistent delivery of fluids across bead array. The 4x5 array, designed with 20 hexagon-shaped wells to localize individual agarose bead sensors, was cast in a UV-curable photopolymer (Norland Products Inc., Township, NJ) from a custom machined aluminum mold. The array was embedded in the card and was sealed with a cyclo olefin polymer thermoplastic plastic cover (Zeon Corp., Tokyo, Japan) to permit optical access following functionalization with agarose beads on which the sandwich immunoassays were completed.
Figure 2-4: Layers of plastic (white) and double-sided adhesive (gray) that are stacked together to create the p-BNC. Glass fiber pad, bubble traps and bead array chip are not shown.

2.6.4 p-BNC Assay Execution and Image Analysis

All assays were performed using the in-house xurography-patterned microfluidic card as described in the previous section, using NE-1000 syringe pumps (New Era Pump Systems Inc., Farmingdale, NY) to variably control fluid flow. A volume of 12μL of detecting antibody cocktail containing 0.15μg of CA125, 0.15μg of HE4, 0.6μg of CA72-4 and 0.015μg of MMP-7 detecting antibodies prepared in SuperBlock (PBS) Blocking Buffer was deposited onto the glass fiber pad. Agarose beads functionalized with capture antibody specific to the analyte of interest (CA125, HE4, MMP-7 or CA72-4) were localized to predetermined wells to permit spatial identification and, consequently, multiplexing. Four redundant sensor beads per analyte permitted elimination of agarose beads with visual signal
obstruction and enabled evaluation of within-assay variance. In addition to the bead sensors for analytes, two negative control beads coupled with IgGs for irrelevant analytes (IL1-β/cTnI antibodies) and two positive control beads (goat anti-mouse immunoglobulin G (IgG) antibodies) were localized to the wells. Following localization of functionalized beads and corresponding controls, 100μL of sample was loaded into each card through the sample entry port, which was then sealed using a double-sided adhesive cover. Two syringes of SuperBlock (PBS) Buffer were connected to the two main entry ports of the card to supply pressure driven flow inside the card. By actuating the first pump, the 100μL sample was filtered in-card and passed over and through the beads for a period of 30 minutes. After a 1-minute rinse at 100μL/min to wash unbound antigen, the detecting antibody was eluted from the pad and introduced to the bead array at the rate of 10μL/min with the second syringe. After 7 minutes, a final rinse of 100μL/min for 5 minutes was utilized to wash unbound detecting antibody. The total assay time was 43 minutes with a total assay volume of 770μL.

Images were acquired using 10X magnification on a modified Olympus (Tokyo, Japan) BXFM epifluorescent microscope and analyzed using ImageJ (NIH) with previously described custom image analysis macros to determine the mean fluorescence intensity along the perimeter of the bead. Optical obstruction of the agarose sensor beads via bubbles or sample/reagent debris resulted in the rejection of the bead sensor for quantitative analysis.

Calibration curves were generated and fitted to a standard four-parameter logistic regression with SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA) software. Unknown concentrations for samples were interpreted from the standard curve utilizing the same software. Microsoft Excel (Microsoft Corp., Redmond, WA) was used to analyze precision study data.
2.6.5 Precision Study

Human serum-based Liquichek™ Tumor Marker Controls (Bio-Rad Laboratories, Hercules, CA) of three different concentrations (low, medium and high range of each analyte) were assessed on the p-BNC in triplicate over three days to assess within-day and between-day variation of the multiplexed assay on the p-BNC. CA125 is included in the manufacturer’s preparation of the controls and corresponding antigen levels of HE4, CA72-4 and MMP-7 were spiked in each of the three levels (concentrations reported in Appendix Table A1). The intra-assay variation was defined as the variation between redundant bead sensors in a single assay run, whereas the inter-assay precision was defined as the coefficient of variation between different runs over separate days. The coefficient of variation was averaged across the three concentration levels to report the final inter- and intra-assay precision.

2.7 Results

The p-BNC assay card is a lab-on-a-chip platform that features a bead-based sensor core and a fully integrated microfluidic network that facilitates on-card sample preparation and metering, reagent storage, mixing, bubble and debris removal, and secure waste containment. By design, the p-BNC assay card minimizes benchtop sample and reagent preparation steps and associated laboratory tools and infrastructure, which is critical for POC analysis. Agarose beads represent the sensor core on which the sandwich immunoassays occur for quantification of biomarker analytes. The supporting microfluidic environment, coupled with the 3-D structure of the agarose beads permit optimal analyte capture and detection to achieve low limits of detection and short analysis times. Further, due to the pre-
defined spatial arrangement of the beads on the chip, the p-BNC permits multiplexing of several biomarkers together. Following sample input, the sample is automatically metered on the card and processed to remove bubbles and debris. The buffer reagents push the sample to the beads, permitting analyte molecules to be captured on the immunosensor (antigen delivery) followed by removal of unbound analytes (wash step). The detecting antibody stored on the glass fiber pad is then eluted by the buffer and delivered to the beads to complete the immunoassay sandwich, followed by a wash step prior to signal capture. All elements of the card, including the footprint were designed in parallel with a portable optical analyzer to ensure rapid translation of the device to a fully portable POC assay system.

In this work, we configure a four biomarker panel comprising CA125, HE4, MMP-7 and CA 72-4 to the p-BNC. This biomarker panel has been discovered as an optimal panel in distinguishing early stage ovarian cancer patients from corresponding controls resulting from discovery efforts at MD Anderson Cancer Center. As biomarkers are added or removed, the p-BNC system can rapidly be re-configured by replacing, adding or removing bead-types in the array, and therefore reduce the time from biomarker discovery to endpoint analysis.

2.7.1 Assay Development

To develop POC-suitable assays for the multiplex panel, the assay ranges were defined based on clinical requirements. An assay suitable for early detection biomarkers in a POC setting should be capable of measuring the relevant range of biomarkers in a short period of time. The dynamic range to encompass the early detection and healthy range for individuals for all four biomarkers were defined based on previous work. To function in a POC setting, a total assay time of no longer than one hour was set as the upper limit to
include sample incubation, detecting antibody delivery and wash steps to remove unbound antigen and detecting antibody. To measure the four biomarkers on the p-BNC within one hour over a dynamic range with small volumes of plasma, extensive optimization was required.

The four biomarker assays were developed individually, beginning with the identification of an optimal matched pair. Based on previous work, an optimal concentration of 320 ng/bead of capturing antibody was coupled to the beads to permit high sensitivity assays. A 30 minute sample (antigen) incubation time was chosen for delivery to permit adequate analyte capture while maintaining the short assay times required for a POC assay. A 7-minute incubation with detecting antibody was chosen to permit sufficient time to complete the sandwich immunoassay without increasing background. Flow rates were also optimized to permit proper antigen interaction with the capture antibodies (3.3 μL/min), adequate delivery time and consistent dosing of detecting antibody (10 μL/min), and to assure sufficient washing without dislodging or destroying bead sensors (100 μL/min). Following initial development of these assay parameters, extensive optimization was required to achieve necessary assay performance.

2.7.2 Assay Optimization

In general, optimization for HE4 and MMP-7 proved simpler than CA125 and CA72-4. Fluorescent signal on CA125 and CA72-4 beads were found to be exclusively on the perimeter of the bead, whereas HE4 and MMP-7 allowed significantly more penetration, resulting in a brighter signal (Fig. 2-5). It is hypothesized that this is due to the size of the molecules, as HE4 and MMP-7 are under 30kDa, while CA125 and CA72-4 are at least
200kDa, and possibly as large as 10,000kDa. Agarose beads are often used in size-exclusion chromatography and 2% agarose has been used for a protein fractionation range of 80 to 40,000kDa, which would trap molecules in that size range resulting in different elution times, but allow molecules smaller than 80kDa to pass through. Beads made from 1% agarose were produced in order to create larger pores and hence allow for better penetration of larger molecules (protein fractionation range of 1,000 to 150,000kDa), however, these beads were found to lack the structural support needed for use in the p-BNC.

![Figure 2-5](image)

**Figure 2-5**: Representative image of penetration difference between larger molecules (CA125) and smaller molecules (HE4). For CA125, signal is concentrated exclusively on the perimeter of the bead while HE4 has deeper penetration into the bead.

### 2.7.2.1 Competitive Assay Format

Another attempted strategy to overcome the size exclusion issues with large biomarkers was to convert the assay format from non-competitive to competitive. Briefly, rather than conjugating capturing antibodies to the beads, the target analyte is conjugated to the beads. The sample is incubated with the detecting antibody solution before passing through the beads to allow the detecting antibody to bind any antigen in the sample. Depending on the concentration of antigen in the sample, a portion of the detecting antibody is bound and therefore prevented from binding to the antigen attached to the bead. As a result, a low signal indicates a high concentration of the analyte, as most of the detecting
antibody was bound to the analyte in solution. A high signal indicates a low concentration of the analyte, as most of the detecting antibody was left free to bind to the analyte on the bead. Rather than requiring the large protein complex to penetrate the bead, the significantly smaller detecting antibody is the only molecule that would need to fit through the porous agarose. While the large protein still needs to be conjugated in the pores to capture the detecting antibody, this could be done at equilibrium over a longer period of time rather than a quick flow-throw as in the p-BNC assay. A competitive assay was created for CA125, where purified CA125 antigen was conjugated to the beads and the concentration of detecting antibody for pre-incubation with the sample was optimized. The CA125 competitive assay achieved a better dose response than the non-competitive assay (Fig. 2-6).

Figure 2-6: Dose response curve of CA125 in competitive assay format.

Unfortunately, the precision of the competitive assay was significantly worse than the non-competitive format, an important factor when calculating the limit of detection from the
standard deviation of three zero concentrations. Additionally, the fluorescent intensity values began to saturate at concentrations much lower than the non-competitive assay, effectively reducing its dynamic analysis range. Further, pre-incubation adds time to the assay and combining non-competitive and competitive formats in the same multiplexed assay is likely detrimental for cross-reactivity, as there is significant contact time between the detecting antibody and non-corresponding antigens during pre-incubation and sample delivery.

2.7.2.2 Buffer Optimization

Choice of buffer in diagnostic assays is critical in stabilizing reagents and minimizing non-ideal interactions. In multiplexing assays, buffer selection becomes more important as additional reagents are added that can contribute to cross-reactivity. Phosphate buffered saline (PBS), PBS with 1% bovine serum albumin (BSA), PBS with 0.1% BSA, SuperBlock (PBS) Blocking Buffer and Luminex® assay buffer were compared on the p-BNC multiplexed assay to determine which buffer enhances true signal, limits cross-reactivity and effectively rinses unbound detecting antibody. The proprietary SuperBlock (PBS) Blocking Buffer from Thermo Fisher was found to perform best in the p-BNC and was used for diluting standards, clinical samples and detecting antibody before introducing them into the card. SuperBlock buffer was also used as the fluid delivered to the card through the fluid entry ports, which was used to advance the sample, reconstitute the detecting antibody and rinse.
2.7.2.3 Detecting Antibody Optimization

Although the competitive assay showed promise, optimizing other aspects of the assays continued in order to determine if the necessary analytical performance could be achieved without fundamentally changing the system and causing the previously mentioned downsides. Detection antibody optimization involved evaluation of different dilutions from undiluted to a 1:40 dilution. While standard protocols for detecting antibody conjugation were effective for HE4 and MMP-7, CA125 and CA72-4 required further optimization, possibly due to their larger size. Higher dye loading of the detecting antibody than recommended by manufacturer’s protocols (achieved by 24-hour incubation rather than 1-hour incubation) resulted in significantly increased signal without a corresponding increase in background noise for detection antibodies. This strategy increased the number of fluorophore moieties per antibody and was found to be suitable for CA125 and CA72-4 assays (Fig. 2-7). In an attempt to add even more fluorophores to the antibodies, the detecting antibody solution was conjugated a second time, but the slight increase in degree of loading was not significant enough to offset the cost of the second conjugation. Similarly, using 2mg of antibodies (over 1mg standard protocol) increased the protein concentration and slightly improved the degree of labeling, however, the cost increase from using double the amount of antibody was not worth the marginal improvements in signal.
Figure 2-7: A comparison of the degree of labeling (moles dye/moles protein) for different detecting antibody labeling methods of CA125. The first four bars refer to AlexaFluor® 488, while the last used Oregon Green® 488 (“OG488”).

Although AlexaFluor® 488 has been the standard dye molecule used for detecting antibody in the McDevitt lab, alternative dye molecules were evaluated for CA125 due to the limited sensitivity of the assay, even with a significantly optimized AlexaFluor® 488 conjugation procedure. Adequate fluorescent intensity at a given signal was achieved with AlexaFluor® 488 for HE4, MMP-7 and CA72-4 antibodies, whereas Oregon Green® 488 outperformed AlexaFluor® 488 for CA125. The increase in degree loading seen with Oregon Green® 488 is possibly due to its size, as demonstrated in Fig. 2-8. At just over half the size of AlexaFluor® 488, it is hypothesized that more dye molecules can fit on each antibody, thus increasing its degree of loading and performance in the assay.
A detection amplification strategy was also attempted to increase signal without significantly increasing the background noise. A commercially available fluorescence amplification kit (Thermo Fisher Scientific Inc., Waltham, MA) uses a 2-stage strategy to increase signal. First, an anti-fluorescein rabbit antibody bound with AlexaFluor® 488 is passed into the beads, which binds to the Oregon Green® 488 that is attached to the detecting antibody bound in the sandwich assay. A secondary amplification can also be performed by passing in goat anti-rabbit antibodies bound with AlexaFluor® 488, significantly increasing the total number of fluorophore molecules per sandwich. Unfortunately, all attempts at amplification, 1- or 2-stage at a variety of concentrations, significantly increased the signal found on zero concentration assays and hence hurt the performance of the system. Limits of detection and precision both worsened, and adding amplification steps would have been difficult to implement in the microfluidic strategy without increasing the number of fluid entry points.

**Figure 2-8:** Molecular structure and sizes of Oregon Green® 488 and AlexaFluor® 488.\textsuperscript{169}
2.7.2.4 Imaging and Analysis Method Optimization

The optimization of the imaging and analysis methods significantly improved the assay characteristics of the ovarian cancer panel, especially for CA125, where desired detection limits were initially difficult to achieve. In order to fit the entire 20-well chip of the p-BNC in the field of view, a 4X microscope objective has typically been used. However, a 10X magnification was found to significantly improve signal-to-noise ratio (SNR) over the 4X, due to the advantage derived from the higher numerical aperture. A rastering protocol was developed with an automated stage to manually take four individual images at higher magnification and combine them in ImageJ. A 20X objective was also attempted, but only 1 bead fit in the field of view at a time, necessitating 20 images per assay, and signal was found to be slightly diminished compared to 10X, possibly due to enhanced photobleaching of the fluorophore from concentrating the fluorescent beam at high magnification (data in Appendix Figure A1).

A background subtraction method was employed in ImageJ to reduce background noise and further improve the SNR. Additionally, a comparison of image analysis methods was performed, where using a custom macro to measure signal around the periphery of the bead was found more effective than measuring average intensity of the entire bead for all four bead-types. Taken together, these optimization steps (Fig. 2-9) were necessary to achieve the performance required for the early detection assay ranges of the individual immunoassays under POC time constraints.
Figure 2-9: Comparison of 4x and 10x microscope objectives, background subtraction (BS) and analysis methods (circular area of interest (CAOI) and line profile (LP)) on the signal-to-noise ratio of the CA125 assay at 50U/mL.

2.7.3 Analytical Validation

2.7.3.1 Dose Response Curves

Following optimization as described above, the dose-response curves for the resulting multiplexed immunoassays for each of the four individual markers were performed and fitted to a four-parameter logistic equation (Fig. 2-10). Standard curves were generated by serial dilution of a standard concentration of a multiplex cocktail of the four antigen standards, encompassing ranges required to cover the biomarker range of interest (healthy women and patients with early stage ovarian cancers): for CA125 0-400U/mL, HE4 0-200pM, CA72-4 0-50U/mL and MMP-7 0-10ng/mL.
Figure 2-10: Calibration curves showing variation of mean fluorescent intensity (MFI) across concentration ranges tested for [A] CA125, [B] HE4, [C] MMP-7 and [D] CA72-4 obtained on the p-BNC in a multiplexed format and fitted to a four-parameter logistic regression curve. The error bars indicate intra-assay variation measurement between analyte-specific beads. The calibration curves indicate suitability for measurement for low concentrations of biomarkers found in healthy and early disease states with low intra-assay variation across the entire range of measured concentrations.

The multiplexed limits of detection for the four biomarkers, evaluated as the concentration on the 4-parameter logistical equation corresponding to three standard deviations above zero, are shown in Table 2-3 and displayed in the lower range of each dose response curve in Fig. 2-11. All four assays are capable of reliable measurements below the cut-off values established for healthy individuals and reported in literature: CA125 35U/mL,\(^{57}\) HE4 40-85pM (age-dependent),\(^{159}\) CA72-4 8.5U/mL\(^{37}\) and MMP-7 7.4ng/mL\(^{160}\).
These reliable measurements of biomarker concentrations below cut-off levels are essential for establishing baselines for longitudinal biomarker monitoring.

**Table 2-3**: Limits of detection (LOD) for singleplex and multiplex dose response curves of CA125, HE4, MMP-7 and CA72-4 on the p-BNC.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Singleplex LOD</th>
<th>Multiplex LOD</th>
<th>Literature Cut-Off Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA125</td>
<td>1.3 U/mL</td>
<td>1.8 U/mL</td>
<td>35 U/mL</td>
</tr>
<tr>
<td>HE4</td>
<td>2.2 pM</td>
<td>2.3 pM</td>
<td>40-85 pM</td>
</tr>
<tr>
<td>MMP-7</td>
<td>0.3 ng/mL</td>
<td>0.2 ng/mL</td>
<td>7.4 ng/mL</td>
</tr>
<tr>
<td>CA72-4</td>
<td>1.1 U/mL</td>
<td>1.7 U/mL</td>
<td>8.5 U/mL</td>
</tr>
</tbody>
</table>

**Figure 2-11**: Isolation of lower portion of [A] CA125, [B] HE4, [C] MMP-7, and [D] CA72-4 multiplexed dose response curves with a linear fit to demonstrate the region of the limit of detection for each biomarker on the p-BNC.
2.7.3.2 Cross-Reactivity

Arguably the most significant challenge with multiplex immunoassays is the presence of cross-reactivity between the detecting antibodies and capturing antibodies for the different analytes. In an ideal multiplexed assay, only the capturing antibody and detecting antibody specific to the analyte of interest will form an immunoassay sandwich, while interactions with non-analyte specific antibodies and antigen will be minimal. However, in reality, several non-ideal interactions are possible between the reagent components of the various multiplex assays that in turn can lead to decrease or increase in specific signal, which may ultimately impact the analytical performance of the multiplexed immunoassay in comparison to the singleplex assay. Additionally, cross-reactivity can also occur over the entire dynamic range, precluding multiplexing of the candidates of interest. In order to minimize cross-reactivity, all multiplex reagents were evaluated per analyte by using the other three analytes as negative controls. If any signal was observed on the bead-sensors for the non-specific analyte, other matched pair antibody configurations were explored.

Additionally, while optimizing variables such as flow rates, volumes and detecting antibody dilutions, all four bead-sensors were used to select assay conditions that minimized cross-reactivity. To explore the cross-reactivity across the entire range of analyte concentrations, both the singleplex assays (with the analyte, capturing antibody and detecting antibody of interest) and multiplex assays (with all four analytes and their corresponding capture and detecting antibodies) were completed. The calibration curves for the singleplex and multiplex assays obtained are shown in Fig. 2-12. The curves obtained from both methods overlap significantly, demonstrating that the signals measured at a given concentration are similar for both singleplex and multiple assays. Less than 10% variation
was noted between the singleplex and multiplex dose responses demonstrating negligible cross-reactivity. The four detecting antibodies were combined on a single reagent pad during all multiplexed assays, demonstrating initial compatibility for long-term, in-card storage. Notably, the LODs observed post-multiplexing were comparable to the individual assay LODs, demonstrating that the assay performance was also retained upon multiplexing. Thus, reliable measurements may be obtained upon multiplexing without loss of assay performance, with comparable signal intensities and with minimal cross-reactivity.
Figure 2-12: Calibration curves obtained for [A] CA125, [B] HE4, [C] MMP-7 and [D] CA72-4 in a singleplex (black circles) and multiplex (white circles) format. Akin to ELISA, the singleplex experiments only employed the antigen of interest and the corresponding antibody for the sandwich immunoassay, whereas the multiplex experiments assayed a cocktail of all four antigens with a cocktail of all four detection antibodies. The multiplex assays for each of the four analytes are superimposable with the individual singleplex assays demonstrating negligible cross-reactivity from the introduction of other reagents due to multiplexing. Representative images (contrast enhanced) of a singleplex assay [E] and a multiplex assay [F] are shown, where the singleplex demonstrates specific signal for the analyte of interest (CA125) and the multiplex demonstrates specific signals for a cocktail of analytes.
2.7.3.3 Intra- and Inter-Assay Precision of Multiplexed Biomarkers

The intra-assay precision that measures variation between bead-sensors for a given analyte and the inter-assay precision that measures the variation between independent assays for a given analyte were evaluated for three different concentrations encompassing the dynamic range of the assay and were averaged (Table 1). Intra-assay precision ranged from 5.0% to 9.4%, whereas the inter-assay (conducted on three consecutive days) precision ranged from 5.4% to 10.5% and demonstrate low assay-specific variability. Longitudinal algorithms for biomarkers rely on within-person and between-person biological variation in addition to the rise in biomarkers levels from the baseline.\(^{54}\) The within- and between-assay precision were both well below the biological variation of the individual markers, determined previously through ELISA (manuscript in review).

**Table 2-4:** Intra- and inter-assay precision values for CA125, HE4, MMP-7 and CA72-4 assays on the p-BNC.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Inter-assay Precision</th>
<th>Intra-assay Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA125</td>
<td>10.5%</td>
<td>9.4%</td>
</tr>
<tr>
<td>HE4</td>
<td>5.4%</td>
<td>5.0%</td>
</tr>
<tr>
<td>MMP-7</td>
<td>8.5%</td>
<td>8.7%</td>
</tr>
<tr>
<td>CA72-4</td>
<td>8.3%</td>
<td>9.1%</td>
</tr>
</tbody>
</table>

2.8 Conclusions

POC diagnostic platforms have the potential to transform clinical diagnostics by significantly reducing the turn-around time between sample collection and biomarker results that can be interpreted by the clinician. Microfluidic-based POC systems are inherently associated with low sample volumes and short analysis times, in addition to, in the case of a system such as the p-BNC, permitting multiplexing. Unlike traditional ELISAs, where only one biomarker may be assessed, multiplexing offers additional advantages of assessing
multiple biomarkers simultaneously, thus reducing required sample volumes and analysis times. Particularly in the case of ovarian cancer detection, this additional biomarker information has the potential to improve sensitivity while maintaining specificity.

Previously the bead-based p-BNC platform was adapted for measurements of CA125 in sera with analytical performance comparable to ELISAs, but with a shortened analysis time of 43 minutes.\textsuperscript{64} In this work, the sample volume was reduced 10-fold and additional biomarkers from a novel ovarian cancer multimarker panel were incorporated on the p-BNC to permit multiplexing. This multimarker panel, including CA125, HE4, MMP-7 and CA72-4, was chosen from a collaborative study of 96 potential ovarian cancer biomarkers. The p-BNC leverages the microfluidic regime to reduce assay time and cost while enabling multiplexing and providing a path for a POC ovarian cancer screening test. The four assays were successfully adapted to the p-BNC in a 43-minute assay that only requires 100μL total of sample volume. Previously published work with CA125 was performed on a flow cell, a simplified analog of the p-BNC card that has no fluid limitations, allows for recirculation and can tolerate significantly faster fluid rates. Transitioning to the disposable microfluidic card proved to be a significant challenge in several aspects. First, sample volume was reduced 10-fold, effectively lowering the signal by a factor of 10 initially. Additionally the sample was not recirculated through the beads during incubation, which previously allowed for more complete binding of the capturing antibody and antigen during the sample incubation period rather than having a single chance in a flow-through method. Similarly, large volumes could be used for detecting antibody delivery, which allowed for a less concentrated detecting antibody solution to be recirculated through the beads over multiple passes, effectively reducing background noise of the fluorophore. In the p-BNC, the detecting antibody is stored
on a glass fiber pad at high concentration, which results in a plug of highly concentrated fluorophore being delivered to the beads, which was found to increase background noise via nonspecific binding. Finally, the wash steps in the flow cell were significantly more effective at reducing nonspecific signal, as volume of buffer was not restricted and faster flow rates could be used with fewer issues associated with microfluidic regime, such as bubbles and leaks.

Overcoming the challenges associated with the transition to an actual microfluidic platform, in addition to multiplexing biomarkers, proved to require significant optimization to achieve the necessary analytical performance. Reagent optimization involved choosing antibodies, buffers, antigen standards, and fluorophores that provided the best signal to noise ratio. Effective matched antibody pairs for all four biomarkers were identified that created the most successful immunosandwich. A buffer was selected that limited cross-reactivity via blocking, enabled binding of corresponding constituents and washed nonspecific signal. Recombinant or purified antigens were chosen as standards to correspond to levels found in commercially available systems in order to improve correlation when measuring clinical samples. Finally, fluorophores were elected to provide a strong signal with little background noise. Concentrations and conjugation protocols were individually optimized for each of the four biomarkers to further improve assay performance. CA125 and CA72-4, both significantly larger than HE4 and MMP-7, were found to require further optimization due to the lack of penetration of the antigen into the beads. A competitive assay format was used to overcome this challenge, however, lower precision and complications from having non-competitive and competitive assays in the same diagnostic test prevented this strategy from being implemented. Strategies of amplifying detecting antibody and using beads with larger
pores were also attempted to solve the lack of signal, but neither proved to be effective. Ultimately, a combination of detecting antibody optimization, more effective imaging and improved analysis methods were found to achieve the analytical performance necessary for all four biomarkers.

The analytical validation of the multiplexed assay on the p-BNC demonstrated the ability of the platform to measure a wide range of concentrations of CA125, HE4, MMP-7 and CA72-4, including values found in most healthy patient samples. Cross-reactivity between the four assays was found to be minimal, assuring comparable performance between the singleplex and multiplex assays for the individual analytes. Finally, the intra- and inter-assay precision for the p-BNC multiplexed ovarian cancer assay demonstrated the capability of monitoring values of patients over time. Taken together, this multiplexed assay shows promise towards the development of a point-of-care diagnostic method that could be used in a general population screening method.
Chapter 3: Method Validation and Pilot Studies to Identify Clinical Utility of p-BNC for Detection of Ovarian Cancer

3.1 Chapter Overview

In the previous chapter, a panel of ovarian cancer biomarkers was adapted to the p-BNC for rapid and precise quantitative measurement of CA125, HE4, MMP-7 and CA72-4 in a POC-amenable format. In this chapter, the four biomarker assays are used to measure clinical samples from healthy patients, patients with benign pelvic masses, early stage ovarian cancer patients, and late stage ovarian cancer patients. Method validation is performed by comparing measurements of 31 late stage samples by the p-BNC to measurements performed on the Luminex MAGPIX® instrument. Additionally, a small pilot-scale study of 31 patients with various stages of ovarian cancer was analyzed to determine preliminary sensitivity and specificity values and to demonstrate the added value of additional biomarker information. The diagnostic performance of the biomarker panel will be compared to that of the individual biomarkers by utilizing area under the curve (AUC) values of the receiver operating characteristic curves (ROC). Further, biomarker cut-off values for ovarian cancer will be determined for this proof-of-concept clinical study. Finally, the longitudinal monitoring of these four ovarian cancer biomarkers will be evaluated with a set of seven samples, two of which developed ovarian cancer during the monitoring period. This set of data will be used to demonstrate the benefit of using additional biomarkers in monitoring patients over time, and to show how different biomarkers can potentially improve diagnostic lead times over CA125 monitoring alone.
3.2 Introduction

3.2.1 Cross-Platform Validation

In order to determine the value of a new diagnostic assay, a test must be evaluated to conclude if it is capable of measuring the analyte in question effectively. In the previous chapter, the p-BNC was demonstrated to successfully measure known concentrations from a set of standards spiked with varying amounts of each analyte. However, the actual utility of a diagnostic method is determined from its ability to measure clinical samples, typically sera, which contains thousands of different proteins. The complexity of human fluids creates a significant challenge over measuring an analyte spiked in buffer, and as a result additional validation is required to demonstrate effectiveness in human sera.

Biomarker levels measured in clinical samples on platforms used for biomarker discovery may vary significantly from measurements in platforms used in clinical validation, indicating the difficulty in measuring clinical samples consistently. Several other technical and operational challenges impede the frequent use of multiplex assays in clinical settings, and as a result significant validation is required to demonstrate efficacy. In this chapter, the p-BNC ovarian cancer multiplexed assay is validated against the commercially available Luminex® platform, a quality reference method used in multi-institutional collaborative efforts for biomarker discovery of ovarian cancer multiplex panels.

The Luminex® assay platform utilizes 5.6µm polystyrene microspheres as the solid support in a process similar to flow cytometry (www.luminexcorp.com). Capturing antibody for the desired analyte is coupled to microspheres and multiplexing is achieved by including a variety of microsphere types in the assay. Each type of microsphere is dyed with a unique
ratio of a red and orange fluorescent dye, which allows for spectral identification of the different microsphere types during analysis. Similar to ELISA, a sandwich immunoassay is formed on the microspheres after incubation with the sample in a membrane-equipped microtiter plate. Following washing, a detecting antibody solution is added to complete the sandwich. A flow cytometric approach is used to read the individual beads, with the red/orange fluorescent dye ratio indicating bead type and green fluorescence indicating signal of the analyte. While the p-BNC and Luminex® are significantly different platforms, as shown in Table 3-1, they are both bead-based platforms that utilize the 3-D structure of microspheres in an immunoassay sandwich format. These similarities partially bridge the aforementioned gap between discovery (Luminex®) and clinical (p-BNC) platforms, which may improve correlation between the two techniques compared to platforms that use completely different principles.

Table 3-1: Comparison of ELISA (CA125 test from Calbiotech, Spring Valley, CA), Luminex® and p-BNC assay platforms.

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th>Luminex®</th>
<th>p-BNC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Multiplex</strong></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Format</strong></td>
<td>Microtiter plate</td>
<td>Microtiter plate</td>
<td>Microfluidic</td>
</tr>
<tr>
<td><strong>Bead Material</strong></td>
<td>-</td>
<td>Polystyrene</td>
<td>Agarose</td>
</tr>
<tr>
<td><strong>Bead Size</strong></td>
<td>-</td>
<td>5.6μm</td>
<td>250-280μm</td>
</tr>
<tr>
<td><strong>Assay Type</strong></td>
<td>Equilibrium</td>
<td>Equilibrium</td>
<td>Dynamic</td>
</tr>
<tr>
<td><strong>Sample Incubation Time</strong></td>
<td>90 Minutes</td>
<td>Overnight</td>
<td>30 Minutes</td>
</tr>
<tr>
<td><strong>Detection Scheme</strong></td>
<td>Indirect</td>
<td>Indirect</td>
<td>Direct</td>
</tr>
<tr>
<td><strong>Fluorophore</strong></td>
<td>HRP/TMB</td>
<td>Streptavidin/ Phycoerythrin</td>
<td>Alexafluor® 488 / Oregon Green® 488</td>
</tr>
</tbody>
</table>
3.2.2 Clinical Sample Measurement

The analysis of biological fluids (compared to running standard concentrations diluted in buffer) significantly complicates analysis in microfluidic diagnostic devices. While analyzing blood is more desirable to avoid upstream sample processing, serum or plasma is substantially more straightforward.\textsuperscript{173} The varying viscosity of blood (hemorheology) from fluctuating hematocrit and hemolysis makes it difficult to use in microfluidic channels and features.\textsuperscript{174} Additionally, storage of blood leads to clotting and hemolysis, whereas serum and plasma can be stored for long periods of time. Here, serum and plasma are used to initially test the performance of the ovarian cancer p-BNC panel in bodily fluids without the added complications of measuring blood.

3.3 Materials and Methods

3.3.1 Sample Population and Collection

Plasma and serum samples were collected and stored at -80°C at the MD Anderson Cancer Center (Houston, TX) gynecologic oncology tumor bank following standard IRB-approved protocols. Three independent banked sample sets were used in this study, totaling 95 serum samples from 68 patients. For method validation, sera from 31 advanced stage (III-IV) patients were assayed. To evaluate the clinical performance of the p-BNC multiplexed system, sera from healthy individuals (n=7), patients with benign gynecological conditions (n=8), patients with early-stage ovarian cancer (n=7), and patients with late-stage ovarian cancer (n=9) were assayed. To test the potential of the p-BNC to assay sequential samples from the same women, plasma samples were assayed that had been collected at multiple (2-12) time points from women who had remained healthy (n=4) and who had developed
ovarian cancer (n=2) during the course of the study. Samples were acquired from the MD Anderson Normal Risk Ovarian Cancer Study (NROS) Serum Bank. All samples were thawed at 4°C before use and diluted two-fold in SuperBlock (PBS) Buffer prior to analysis on the p-BNC. Samples were diluted to use quantities of sample closer to that of fingersticks, the ultimate volume goal of the p-BNC platform.

3.3.2 p-BNC Analysis

Description of p-BNC fabrication and assay sequence was described previously in section 2.6. The same beads, detecting antibody, p-BNC cards, fluidic pumps and imaging methods were used for clinical measurements. For clinical samples, 50μL of sample was diluted with 50μL of SuperBlock (PBS) Buffer and the subsequent 100μL was loaded into the card for analysis.

3.3.3 Luminex® MAGPIX® Analysis for Cross-Platform Validation

For method validation, sera were measured on custom multiplex assays for CA125, HE4, MMP-7 and CA72-4 (with CA125 and MMP-7 assays from EMD Millipore (Billerica, MA) multiplexed with CA72-4 and HE4 assays developed in-house) at MD Anderson Cancer Center on the Luminex MAGPIX® magnetic bead-based immunoassay system (Austin, TX). Briefly, 16μL of sample was added to the polystyrene beads in a well of a microtiter plate and incubated overnight. After washing, the detecting antibody solution and streptavidin-phycoerythrin solutions were allowed to independently react with the beads followed by a final washing. The beads were analyzed in the MAGPIX® analyzer, where individual bead
type was determined and fluorescent signal of the immunoassay sandwich was measured, which was correlated to a set of standards measured on the Luminex®.

3.3.4 Statistical Analysis

For p-BNC clinical sample analysis, images were analyzed as described in section 2.6.4. Biomarker values of clinical samples were determined in Microsoft Excel (Microsoft Corp., Redmond, WA) from the fit equations of the dose response curves. MedCalc (MedCalc Software, Ostend Belgium) was used to generate receiver operating characteristics curves (ROC) and box plots for clinical sample concentrations. For Luminex®, Bio-Rad (Hercules, CA) Bioplex Data Pro software was used to generate dose response curves and determine biomarker values of clinical samples. SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA) was used to create correlation plots between Luminex® and p-BNC clinical sample values.

3.4 Results

3.4.1 Method Validation

To compare the clinical measurements obtained on the multiplexed p-BNC to laboratory standard multiplex measurements on the Luminex MAGPIX® instrument, sera obtained from 31 late-stage ovarian cancer patients were measured using both methodologies. The method comparison plot (Fig. 3-1) shows the concentrations obtained from both methods for a given sample, plotted against each other. The correlation coefficient obtained for all four biomarkers ranged from 0.71 to 0.93, demonstrating good correlation between the two methods.
Figure 3-1: Correlation between the concentrations obtained on the p-BNC multiplex assays and Luminex® MagPix® multiplex assays for [A] CA125, [B] HE4, [C] MMP-7 and [D] CA72-4 for measurements in the 31 advanced-stage ovarian cancer sera. Plots demonstrate good correlation between the two methods. Plots omitting higher value points and focusing on the lower range of values is included in Appendix Figure A2.

3.4.2 Pilot Study

Following method validation, we chose to explore the ability of the multiplexed biomarker panel assessed on the p-BNC to distinguish ovarian cancer patients from patients with non-malignant gynecological conditions and from healthy women. For this purpose, we assayed 31 sera from: 7 healthy women, 8 patients with benign gynecological conditions, 7 patients with early-stage ovarian cancer and 9 patients with late-stage ovarian cancer. The results obtained are shown as dot plots in Fig. 3-2, displaying the concentrations of
individual biomarkers in cases and controls along with suggested cut-off levels for the given cohort of samples. The specificity and sensitivity derived from utilizing these cut-offs are indicated beside the dot plots. The multiplexed concentrations obtained on the p-BNC are capable of distinguishing between cases and controls. Also, the cut-off concentrations for these individual biomarkers obtained on the p-BNC are comparable with previously reported values for these markers. In addition, the specificities and sensitivities reported for the individual biomarkers for ovarian cancer are concordant with values reported in the literature.

**Figure 3-2:** Dot plots of [A] CA125, [B] HE4, [C] MMP-7 and [D] CA72-4 measured from a set of clinical samples containing healthy and benign patients (Controls, n=15) and early-stage and late-stage ovarian cancer patients (Cases, n=16). Cut-off levels for each biomarker were determined in MedCalc by achieving highest sensitivity (including as many cases above cut-off as possible) without sacrificing significant specificity (including as many controls below cut-off as possible) and are shown by the horizontal line in each plot demonstrating the clinical performance of the p-BNC to distinguish between various disease states.
The values of all four biomarkers were combined utilizing a logistic regression model utilizing the MedCalc statistical software and the ROC curves for the multi-marker combination and the individual biomarkers are shown in Fig. 3-3. The multi-marker combination shows improved performance over individual biomarkers alone, performing with a sensitivity of 68.7% at 80.0% specificity, demonstrating the improved sensitivity from using multi-marker panels. Areas under the curve (AUC) were 0.77 (CA125), 0.70 (HE4), 0.55 (MMP-7), 0.71 (CA72-4) and 0.88 (combined) indicating that the multiplex panel outperformed the individual markers as assessed on the p-BNC.
Figure 3-3: Receiver operating characteristic (ROC) curves for CA125, HE4, MMP-7, CA72-4 and a combination of the four using a logistic regression model. ROC curves were determined from a clinical sample cohort of 31 patients comprised of healthy, benign, early-stage and late-stage ovarian cancer patients.

For this sample set, mean values for each stage of disease for each biomarker are shown in Table 3-2, which were comparable to previously reported values. The average of healthy patient values for HE4 was higher than expected, likely due to two abnormally high healthy values in the small sample set. In a trial with a larger sample size it is expected for this average to revert to the expected mean. All four values for each sample are shown in Fig.
in order to demonstrate the benefit of the multimarker panel. For example, one late stage patient sample, “LS7”, has CA125, HE4 and MMP-7 values in their normal ranges, but CA72-4 is elevated significantly. Similarly, HE4 is in the normal range for “LS1” and “LS2”, two other late-stage patient samples, while MMP-7 and CA72-4 are both significantly elevated, confirming the elevated CA125 readings.

Table 3-2: Mean values of CA125, HE4, MMP-7 and CA72-4 from clinical samples for healthy patients, patients with benign masses, early-stage ovarian cancer patients and late-stage ovarian cancer patients.

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Benign</th>
<th>Early-Stage</th>
<th>Late-Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA125</td>
<td>19.2 U/mL</td>
<td>9.7 U/mL</td>
<td>64.7 U/mL</td>
<td>79.2 U/mL</td>
</tr>
<tr>
<td>HE4</td>
<td>204.9 pM</td>
<td>105.9 pM</td>
<td>451.0 pM</td>
<td>487.4 pM</td>
</tr>
<tr>
<td>MMP-7</td>
<td>2.8 ng/mL</td>
<td>2.5 ng/mL</td>
<td>4.4 ng/mL</td>
<td>3.9 ng/mL</td>
</tr>
<tr>
<td>CA72-4</td>
<td>8.3 U/mL</td>
<td>3.3 U/mL</td>
<td>16.2 U/mL</td>
<td>41.4 U/mL</td>
</tr>
</tbody>
</table>
Figure 3-4: Biomarker values of healthy (“H#”), benign (“B#”), early stage (“ES#”) and late stage (“LS#”) patients for all four biomarkers plotted in a 3D bar graph to demonstrate the added benefit of multiple biomarkers. Values are normalized to largest value for each biomarker.

3.4.3 Longitudinal Monitoring Clinical Samples

To explore the ability of the p-BNC to perform serial measurements of biomarkers over time, a cohort of six individuals was chosen whose sera were collected annually over multiple years. Of these six women, two developed ovarian cancer (“Case 1” and “Case 2”) during the study period while the other four did not develop cancer and were hence deemed healthy. The biomarker profiles of these individuals as obtained on the p-BNC are shown in Fig. 3-5. For each of these individual biomarkers, the healthy individuals have their own baseline that remained flat during the course of the study. In Case 1, all four biomarker concentrations rose sharply in comparison to the horizontal plots for the controls. In Case 2 (isolated in Fig. 3-6), CA125 levels did not increase substantially while the other three
biomarkers rose prior to diagnosis, demonstrating the importance of additional biomarker information.

**Figure 3-5**: Longitudinal plasma from six women with multiple time-points of sample collection for each patient were measured on the p-BNC and values for [A] CA125, [B] HE4, [C] MMP-7 and [D] CA72-4 are shown over time. Two women (Cases 1 and 2) developed ovarian cancer and four other women (Controls 1-4) did not. The longitudinal profiles show rise in biomarker concentrations for cases in comparison to flat profiles for controls. The time point in years of each measurement is listed in Appendix Table A2.
Figure 3-6: Isolation of longitudinal plasma data from Case 2. CA125 levels did not rise substantially while the other three biomarkers rose prior to diagnosis, demonstrating a case where multiplex information was beneficial to diagnosis.

3.5 Conclusions

Microfluidic-based POC diagnostic platforms have the potential to revolutionize healthcare by reducing costs and significantly shortening timeframes associated with receiving results. The p-BNC utilizes the inherent advantages of the microfluidic regime to achieve the performance necessary for biomarker diagnostic assays using low sample and reagent volumes. The p-BNC has been designed for multiplexed analysis of biomarkers, further enhancing the time- and cost-savings by reducing several assays into one. In the previous chapter, the analytical performance of the multiplexed ovarian cancer panel
measured on the p-BNC was assessed and determined to have detection limits and assay ranges necessary for measuring CA125, HE4, MMP-7 and CA72-4 in healthy and diseased patients in just 43 minutes with 100μL of sample. Cross-reactivity between the four biomarker assays was determined to be minimal and the precision of the system was measured to ensure reliability of the multiplexed assay.

Here, the four multiplexed assays were used to measure clinical samples in three unique studies – method validation against Luminex®, a pilot study comparing cases and controls from different stages of ovarian cancer, and a longitudinal study measuring patients’ biomarker values over time. In measuring clinical samples, all patient samples required only 50μL of sample per assay or 12.5μL per analyte. Additionally, the p-BNC was designed to capture all medical waste on card, a significant advantage when measuring patient samples and having to dispose of biohazard waste.

The multiplexed assays were compared to gold standard multiplex methods to ensure similar performance to a commercially available and FDA-approved platform. This method validation was performed using a cohort of 31 late-stage ovarian cancer patients, which were measured on both the p-BNC and the flow-cytometric bead-based immunoassay system known as Luminex®. This cohort showed good correlation for all four markers (R² > 0.71) with Luminex®. A pilot study cohort of healthy, benign, early-stage and late-stage patients was also assessed on the p-BNC to demonstrate the ability of the p-BNC to differentiate cases from controls, which is the main objective of this ovarian cancer panel. ROC curves were created from the data, which showed the advantage of the multiplex panel, rather than relying on CA125 or HE4 alone.
The longitudinal monitoring of biomarkers could significantly improve diagnostic performance of biomarkers by creating personalized baselines rather than utilizing arbitrary cut-offs that could result in higher false positive rates. The cost- and time-reduction of POC tests could benefit from a longitudinal monitoring strategy such as ROCA by reducing two patient visits (sample collection and TVS referral) to one and therefore make three month screenings more feasible for patients with rising levels of biomarkers. This strategy can also be applied to high risk patients with familial history of ovarian or breast cancer or with predisposing mutations such as BRCA1/2, demonstrated to need more frequent screenings in the United Kingdom Familial Ovarian Cancer Screening Study. The ability to process the patient sample and obtain clinically interpretable results within a single visit will permit TVS on the same visit, avoiding anxiety and additional travel time. A POC test used for longitudinal monitoring requires analytical precision (within- and between-assay) that is much lower than the biological variation of patient samples. Indeed, the p-BNC precision study in the previous chapter demonstrated lower analytical %CVs in comparison to the biological variation in healthy individuals for each of these biomarkers. In addition, assessment of longitudinal sera from 2 ovarian cancer patients and 4 healthy controls demonstrated the use of the p-BNC for longitudinal monitoring. Clearly, all four individual biomarkers showed flat baselines in healthy patients and elevated significantly following a period of flat levels in the first case. In the second case with a low CA125 value, HE4, MMP-7 and CA72-4 rose, indicating the potential of biomarker complementarity permitted by use of a multimarker panel.

While it is premature to determine clinical utility with the small sample cohorts assessed, these studies nonetheless demonstrate the ability of the p-BNC to reliably measure
biomarkers to distinguish disease states based on a multiplex panel and also shows strong promise for serial measurements of longitudinal biomarkers in a POC-amenable format. The four-biomarker panel is being validated further with Luminex® in 88 cases and 538 controls from the UKCTOCS trial and will be reported elsewhere. While this panel has shown promise in this study, the p-BNC is capable of being quickly reconfigured to add, remove or replace biomarkers if alternative panels are found more promising.
Chapter 4: Translation to the Point-of-Care

4.1 Chapter Overview

Previously, an ovarian cancer biomarker panel comprising CA125, HE4, MMP-7 and CA72-4 was adapted and optimized to the p-BNC assay platform. Analytical performance was demonstrated to be suitable for the measurement of all four biomarkers in healthy and ovarian cancer patients. Three small-scale clinical studies verified the ability to measure these biomarker levels in human fluids on the p-BNC and the system was validated against the gold standard in multiplexing biomarkers. All of these studies were performed on the handmade, disposable p-BNC card made of alternating layers of double sided adhesives and plastics with microfluidic features xurographically patterned on them. While the p-BNC card has been designed towards use at the POC, several aspects still rely on laboratory-grade equipment.

In this chapter, the ovarian cancer p-BNC diagnostic test is fully amended for biomarker quantitation at the POC. The assay is transitioned to a manufactured card using injection-molded plastics and good manufacturing practices, rather than the in-house cards previously described. The multiplexed assays are re-optimized for use with blister packs containing PBS, replacing the fluid adapters on the card and syringes. The syringe pumps were replaced with a custom-built actuator capable of the various flow rates needed for the assays. Additionally, a custom-built, compact optical microscope that integrates with the actuator platform was developed in order to replace the tabletop microscope and complete the transition to a system with a small footprint that is truly capable of protein measurement at the POC.
The time required for the ovarian cancer assay was also reduced by more than half in order to return results within the timeframe of an average patient visit. Using the CA125 assay in a proof-of-concept study, the number of beads included in each assay was reduced from 20 to 4, which was used to achieve the necessary limits of detection in an ultrafast diagnostic method. In order to determine whether the ultimate vision of a blood fingerstick diagnostic test was possible, serum, plasma and blood from the same patient were compared on the p-BNC for 22 samples. Finally, dose response curves for the multiplexed assay in the ultrafast format were performed completely on the fully integrated POC analyzer.

4.2 Introduction

4.2.1 p-BNC as a Point-of-Care Device

There is a global need for simple, low-cost, accurate diagnostic methods that can be applied to a variety of diseases. Although infectious disease is the main cause of concern in resource-poor countries, the same technological advances that can address these concerns can also be utilized in developed countries for a variety of diseases that achieve improved survival rates with earlier diagnosis, including cancer.176 While reducing costs is one of the biggest advantages of microfluidic POC diagnostic devices, another major benefit is that ease of access can make a significant impact on survival by encouraging more frequent measurements. Additionally, for developing countries, portable and rugged analyzers that contain all medical waste and do not require expert users are necessary for effective clinical diagnostics without the use of a clinical laboratory. Meanwhile, this same technology can be applied towards diagnostics at the POC in developed countries by replacing the traditional
hospital-clinical laboratory relationship with robust analyzers capable of immediate measurement near the site of patient care.

In the case of the p-BNC diagnostic platform, research on a variety of diseases has been underway for the last two decades using technology designed for translation to the point-of-care. In the previous chapters of this dissertation, this technology has been applied to diagnosing ovarian cancer with a novel panel of biomarkers. While this work has made significant steps towards use at the POC by using disposable cards that contain all of the necessary microfluidic features needed for analysis, several aspects of the research were still not ready for POC use. Syringes and syringe pumps were used as an analog to blisters and fluid-controlling actuators, while a tabletop epifluorescent microscope was used for imaging in place of the final form optics built into the analyzer. These limitations require laboratory-grade equipment, and as a result, preclude the real world use at the POC. Another limitation of the work described in the previous chapters was the fabrication method of the p-BNC cards. All of the cards were built by hand and required one hour per card on average due to the manual process of cutting, peeling and combining layers. The excessive amount of manual hours needed would be a significant bottleneck in using the system at the POC in a large clinical trial or as a commercial product.

In this chapter, the p-BNC is fully amended to the POC. Through a collaborative effort with MiniFab Pty Ltd (Victoria, Australia), the p-BNC lab card was redesigned to improve manufacturing methods and scalability. A custom-made actuator platform was developed to control the fluid flow from blister packs that store PBS on-card. A custom microscope was built to integrate with the actuator platform, completing a backpack-sized analyzer capable of meeting the demands of a POC system. Further, the test was re-optimized
for this new platform and improved to work in an ultrafast format, only requiring 17 minutes to complete the measurement.

4.2.2 Fingerstick Blood Analysis

In creating a POC diagnostic test for ovarian cancer, a rapid and accurate whole blood fingerstick test is desired that can circumvent the need for preprocessing samples, effectively shortening the total analysis time of the test and removing the need for a centrifuge. CA125 has been studied in serum for decades and several commercial products are available for measurement, however, no studies have used whole blood. Although many microfluidic platforms designed for POC use have been tested with blood, no tests specific to ovarian cancer have been used in testing fingerstick quantities of blood.

While CA125 levels in serum from peripheral blood has been found to correlate well to serum from blood taken directly from the ovaries, it is unclear how measuring actual blood will affect CA125 levels compared to plasma or serum, as blood cells and hemoglobin are known to interfere with detection schemes that use optical measurements. Plasma and serum are isolated from blood by centrifugation or sedimentation, separating the heavier blood cells (red and white) from the remaining fluid in blood. Plasma and serum vary by method of blood cell separation. For serum, the blood is allowed to clot in the collection tube and the clot is removed, normally by centrifugation. For plasma, EDTA, heparin or sodium citrate is used to prevent clotting and a centrifuge is used to remove the non-clotted cells from the suspension liquid. As a result, plasma contains clotting factors such as fibrinogen that are removed in the clot for serum. Additionally, the platelet activation from clotting releases proteins that contribute to altering the composition of the matrix. While the
clotting factors found in plasma are not expected to significantly affect the concentration of biomarkers compared to serum, blood cells often make up 30-50% of the volume of blood, which considerably dilutes the concentration of protein biomarkers.

One comparison of pulmonary disorder-related biomarker values in serum and plasma, performed on Luminex®, found the levels to be similar, though not for all analytes. For 16% of the samples, serum produced higher biomarker values than plasma compared to 3% of samples having higher plasma levels. However, the analytes measured in this study relate to pulmonary disease and are therefore more likely to be affected by the cytokine and metabolite release from platelet activation than ovarian cancer biomarkers. A comparison of CRP levels in serum, plasma, venous blood and fingerstick blood was performed by Brindle et al. Compared to serum, plasma values were 7% lower on average, while fingerstick blood values were 31% lower and venous blood values were 33% lower on average. However, both blood measurements were performed with dried blood and the effect of using dried versus fresh blood is not clear. Another study compared perfluorinated acid levels in blood, serum and plasma. No difference was found between serum and plasma levels, and blood was found to have concentrations corresponding to the volume displacement by blood cells. In measuring ethanol, serum and plasma levels have been found similar while blood is 9-18% lower. Similarly, theophylline concentrations in serum and plasma were found to be 16-26% higher than in blood. However, it is important to note that chemical analyses in blood are likely more straightforward than protein analysis in blood due to the lack of interactions with other proteins and protein-binding compounds.

The percentage of blood cells in whole blood, known as hematocrit, varies from patient to patient. Not only does this variably dilute protein concentration in blood, but
hematocrit levels also significantly affect the rheological properties of blood,\(^{186}\) a noteworthy consideration for use in microfluidics. The variation in hematocrit is not easily predicted, but age, smoking and overall health have been linked.\(^ {187,188}\) In the p-BNC, blood has not been evaluated extensively as a sample matrix. How blood affects the binding of the immunoassay sandwich and readings of fluorescent signal in the p-BNC is unknown. Potentially more inhibiting, it is not clear how blood affects the porous agarose beads and whether increased shear force will damage the beads or if the higher viscosity will result in frequent clogging. Flow rates also have an affect on the viscosity of blood,\(^ {189}\) which will warrant consideration during assay development in order to avoid pressure failures in the cards.

Many microfluidic diagnostic strategies have attempted to avoid preprocessing and overcome blood issues with in-system separation of red blood cells from plasma\(^ {173,178,190-192}\). Size exclusion, magnetic, dielectrophoretic, deterministic hydrodynamic and several other separation techniques have been successfully implemented in microfluidic devices\(^ {193}\) that can be applied to the microfluidic channels in the p-BNC. However, in this study whole blood will be used without filtering to determine if there is a need for in-card processing.

In this chapter, the ability of the p-BNC to measure protein concentration in blood samples is determined. Additionally, the correlation between CA125 values of blood, serum and plasma from the same patient are evaluated in order to determine the capability of ovarian cancer fingerstick blood tests on the p-BNC.
4.3 Materials and Methods

4.3.1 MiniFAB p-BNC Analysis at the POC

MiniFAB p-BNC cards (Fig. 4-1) were manufactured at MiniFAB (Victoria, Australia), which included the blister fluid packs, 20-well array and array covers. These cards were composed of an injection-molded body with three adhesive capping layers. The lower-capping layer is made from heat seal laminate and is used to close the bottom of the channels formed in the injection-molded layers. Vent materials, blister packs and the sample port are bonded to the middle-capping layer, which is made from a double-sided pressure sensitive material. The upper-capping layer covers the remaining exposed adhesive. Finally, a protective shell is used to prevent damage to blisters and vent materials. The manufactured cards were modified in-house to only use 4 of the 20 wells by masking off the extra wells with a custom double-sided adhesive layer. A modified array cover was also used that included a bubble trap membrane before the array in order to minimize bubbles during wetting. An adapted procedure for loading the beads was developed for the 4-well array to ensure necessary alignment of layers and to prevent adhesive layers from getting wet in order to prevent leaks.
Figure 4-1: Schematic and image of the p-BNC card manufactured by MiniFAB. The card consists of a main injection-molded cartridge body that contains channels and bead array. Three capping layers enclose the channels and allow for the attachment of the blister packs and vent membranes. The top layer is a protective shell (not pictured in image), used to protect accidental puncture of blister packs or vent membranes.

For the 4-well assay, 1 bead of CA125, HE4, CA72-4 and PSA (negative control) were used. All capture and detecting antibodies used in the MiniFAB p-BNC card were described previously (section 2.6.1), however the dilution of the detecting antibody was adjusted for the new card. 0.038g of CA125, 0.019g HE4 and 0.15g CA72-4 of detecting antibody were diluted in 6μL of SuperBlock (PBS) Blocking Buffer (Thermo Fisher Scientific Inc., Waltham, MA) and deposited onto a glass fiber pad in the MiniFAB p-BNC
Following deposition, the plastic that coats the adhesive of the detecting antibody pad cover was removed and the detecting antibody chamber was sealed. Immunoassay standards for calibration curves were prepared by generating appropriate dilutions using purified protein antigen stocks for CA125, HE4 and CA72-4 (Fujirebio, Diagnostics Inc., Malvern, PA) in goat serum. 100μL of the sample was loaded into the sample entry port of the card and allowed to fill the sample channel via capillary action. For blood, serum and plasma, 50μL of sample was added to 50μL of SuperBlock buffer and the total 100μL was added to the card. Following sample loading, the adhesive backing of the sample port cover was removed and the cap was sealed.

For the sample incubation time study and the blood, serum and plasma comparison, only CA125 was measured. Three CA125 beads and one PSA (negative control) bead were used in the four-well array. Only CA125 antigen was used in the standards and 0.038g of CA125 detecting antibody diluted in 6μL of SuperBlock buffer was included on the detecting antibody pad. For whole blood clinical samples, gravity separated blood was mixed gently with a pipette tip before transferring to the card in order to avoid lysing red blood cells.

Following sample input and bead loading, the card was inserted into a custom-made actuation device (Fig. 4-2) to perform the assay. Using force sensitive resistors and mechanically actuated arms with step motors, variable pressures were applied to the blister packs attached to the cards to control fluid flow rates of the assay. This actuation device was developed with a small profile and was designed for straightforward reconfiguration for use in the final analyzer. A custom MATLAB (MathWorks, Natick, MA) program was used to control the actuation system and to input desired flow rates and delivery times. For the shortened assay, following initial burst of the right blister to release fluid into the card, a 10-
minute sample delivery stage was performed using a flow rate of 10μL/min. A 1-minute wash step at 100μL/min was used to clear unbound antigen. The left blister was then burst and used to reconstitute the detecting antibody stored on the glass fiber pad, which was delivered to the beads at 10μL/min for 3 minutes. A final wash was performed for 3 minutes at 200μL/min to clear unbound detecting antibody and the final image was taken.

![Custom made actuator designed to burst blister packs of fluid attached to MiniFAB p-BNC cards. Using step motors and force sensitive resistors the actuator can control the flow rates of buffer delivery into the card. Custom software controls the pressure of the actuation arms on the blister packs, which controls fluid flow.](image)

**Figure 4-2:** Custom made actuator designed to burst blister packs of fluid attached to MiniFAB p-BNC cards. Using step motors and force sensitive resistors the actuator can control the flow rates of buffer delivery into the card. Custom software controls the pressure of the actuation arms on the blister packs, which controls fluid flow.

### 4.3.2 Patient Population and Sample Collection

Blood (500μL), serum (250μL) and plasma (250μL) from 22 patients were collected at the MD Anderson Cancer Center (Houston, TX) gynecologic oncology tumor bank following standard IRB-approved protocols. All samples were transferred to Rice on the day of their collection and stored at 4°C before use. All three fluids were diluted two-fold in
SuperBlock (PBS) Buffer prior to analysis on the p-BNC. Following analysis on the p-BNC, serum samples were stored at -80°C for the Luminex® correlation at the end of the study.

4.3.3 Image and Statistical Analysis

Images were analyzed with a custom MATLAB (MathWorks, Natick, MA) program. Following background subtraction of each image in ImageJ (NIH), beads were manually identified in the MATLAB program, which was then used to calculate the mean fluorescent intensity inside of a donut-shaped area around the periphery of the bead. The donut consisted of the outer 20% of the bead to focus on peripheral signal and ignore nonspecific binding in the center of the bead. For dose response curves, SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA) was used to fit four parameter regression curves in order to determine limits of detection and unknown concentrations. Microsoft Excel (Microsoft Corp., Redmond, WA) was used to compare sample incubation times and to compile data.

4.3.4 Luminex® Analysis

For the blood, serum and plasma comparison, only CA125 values were measured in the p-BNC. In order to validate these results on a commercially available system, CA125 levels were measured in the serum samples using a Luminex® kit from EMD Millipore (Billerica, MA). After the antibody-immobilized beads, quality controls and standards were prepared, the wells of the microtiter plate were washed with assay buffer. 25μL of assay buffer, 25μL of sample and 25μL of beads were added to each well and incubated overnight. The plates were then washed 3 times and 25μL of detection antibodies were added and incubated for 1 hour. 25μL of streptavidin-phycoerythrin was added to each well and after 30
minutes, the plate was washed 3 more times. The fluorescent intensity of the beads was measured using a MAGPIX® analyzer with Xponent software and Bio-Rad (Hercules, CA). Bioplex Data Pro software was used to generate dose response curves and determine biomarker values of clinical samples. SigmaPlot was used to create the method validation plot between p-BNC and Luminex®.

4.4 Results
4.4.1 Assay Time Reduction

In the previous chapters, a sample incubation period of 30 minutes was used in order to allow for adequate binding of antigen to capture antibodies on the bead. From the recommendation of clinicians, a shorter assay was desired to ensure the return of biomarker values during an average patient visit. Fig. 4-3 demonstrates the challenges associated with reducing the incubation time for the capturing antibody-antigen interaction. At higher concentrations, shortening the delivery time of the 100μL sample does not diminish signal. In fact, the faster flow rates appear to slightly improve the signal, possibly due to increased penetration into the bead due to the higher pressure associated with the increased flow rate. On the other hand, signal from lower concentrations closer to the limit of detection is significantly lower with shorter sample incubation times. At 50U/mL, the signal was almost 2.5 times higher than the zero for a 30-minute incubation time, allowing for a sub 2U/mL limit of detection. For a 10-minute incubation time, 50U/mL is nearly undifferentiable from the zero, preventing the measurement of the vast majority of healthy patients’ values.
Figure 4-3: Comparison of sample incubation times for a 100μL sample delivery of high concentration (200U/mL) and low concentration (50U/mL) CA125 standards. Sample volume was kept constant, resulting in varying flow rates for the delivery – 3.3μL/min for 30 minutes, 10μL/min for 10 minutes and 20μL/min for 5 minutes. Signal to noise was calculated by dividing the signal of positive CA125 beads by the signal of negative control beads.

In order to reduce the time of the assay and overcome the associated worsening in limit of detection, the number of wells utilized in a single assay was reduced from 20 to 4. In a 20-well assay, each bead effectively receives 5μL of the 100μL sample. Reducing the wells to 4 increases the amount of sample per bead to 25μL, a 5-fold increase of antigen available to bind to the capturing antibodies on each bead. Fig. 4-4 demonstrates the analytical performance improvements seen from the increase in sample delivered per bead for a 10-minute sample delivery. For the 20-well assay, 200 U/mL is the first clearly detectable point on the dose curve. For the 4-well, 10 U/mL was clearly differentiable from the zero concentrations. Limits of detection were calculated to be 1.5U/mL for the 4-well assay compared to 155.5U/mL for the 20-well assay.
Figure 4-4: Comparison of 4-well and 20-well CA125 dose response curves with a 10-minute sample delivery stage. By reducing the number of wells, more antigen is delivered to each bead, increasing its signal.

Total assay time was also reduced with the transition to the MiniFAB card and actuator platform. The reconstituted detecting antibody arrived at the array significantly quicker in the MiniFAB card compared to the p-BNC lab card due to shorter channel length, enabling a reduction in detecting antibody delivery time from 7 minutes to 3 minutes. The MiniFAB card also required less concentrated detecting antibody, possibly due to the shorter distance from the detecting antibody pad to the beads, which results in less dilution in card. Using more dilute detecting antibody also requires less rinsing to effectively wash nonspecific binding from the beads. Due to the significantly higher construction quality of the MiniFAB cards over the lab-made p-BNC cards, leaks were less of a concern. As a result, faster flow rates could be used for rinsing, which enabled a reduction in the rinse time from 5
minutes to 3 minutes. Taken together, these improvements reduced the total assay time for CA125 measurement from 43 minutes to 17 minutes without sacrificing analytical performance.

4.4.2 Blood, Plasma and Serum Comparison

Following the reduction in assay time of over 60%, the ability of the p-BNC platform to measure CA125 values in various sample matrices was evaluated. As the end goal of the device is for a fingerstick blood test, whole blood, plasma and serum were compared to determine whether measuring CA125 from whole blood in the p-BNC was feasible. The dose response curve used to evaluate the clinical samples is shown in Fig. 4-5, which had a limit of detection of 1.5U/mL, low enough for the vast majority of healthy patients’ values of CA125.

![Figure 4-5: CA125 dose response curve used in sample matrix comparison. The four-parameter equation fit to the data was used to calculate concentrations from signal intensity.](image)
A total of 22 samples (9 healthy and 13 with ovarian cancer) were obtained from MD Anderson and analyzed on the day that they were collected without being frozen. For each patient, whole blood, serum and plasma were evaluated on the p-BNC. Additionally, serum samples were evaluated on Luminex® for method validation. Correlation plots for the three matrices and to Luminex® are shown in Fig. 4-6. Serum CA125 measurement on the p-BNC correlated well to serum measurements in Luminex® ($R^2 = 0.97$). Unsurprisingly, plasma and serum measured on the p-BNC correlated extremely well ($R^2 = 0.98$), demonstrating that the clotting factors found in plasma did not significantly affect CA125 measurement. Serum and plasma correlated to blood on the p-BNC with $R^2$ values of 0.91 and 0.91, respectively.

**Figure 4-6:** Correlation of [A] p-BNC serum to Luminex® serum, [B] p-BNC serum to p-BNC plasma, [C] p-BNC serum to p-BNC blood and [D] p-BNC plasma to p-BNC blood for a set of 22 clinical samples.
The differences in CA125 concentrations measured from the three matrices are shown in Table 4-1. CA125 values measured from plasma were on average 8.7% less than from serum. When measured from blood, CA125 values were 40.8% lower than plasma and 45.9% lower than serum. These values agree well with the average hematocrit values of women, which describe the percentage of blood comprised of blood cells.

**Table 4-1:** Average values for blood, plasma and serum on the p-BNC and serum on Luminex® for the 22 patient cohort, 9 of which were healthy and 13 of which had ovarian cancer.

<table>
<thead>
<tr>
<th></th>
<th>p-BNC Serum</th>
<th>p-BNC Plasma</th>
<th>p-BNC Blood</th>
<th>Luminex®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Patients (n=9)</td>
<td>5.5 U/mL</td>
<td>6.3 U/mL</td>
<td>5.0 U/mL</td>
<td>4.6 U/mL</td>
</tr>
<tr>
<td>Ovarian Cancer Patients (n=13)</td>
<td>162.4 U/mL</td>
<td>148.4 U/mL</td>
<td>75.1 U/mL</td>
<td>188.0 U/mL</td>
</tr>
</tbody>
</table>

### 4.4.3 Fully Integrated POC Multiplexed Assay

The 17-minute long CA125 assay and blood, plasma and serum comparisons were performed on the MiniFAB p-BNC card with the custom-built actuation platform. All buffer introduced to the card to deliver the sample, reconstitute the detecting antibody and wash the beads came from the blister packs attached to the cards, and all waste was contained within the cards’ waste chambers. The only remaining pieces left to be adapted to fully integrated measurement at the point-of-care were the epifluorescent lab microscope and the computer used to control the actuator and take images. Both of these were replaced in the final form p-BNC analyzer, shown in Fig. 4-7. This sub-22lb analyzer contains the actuator elements to control fluid flow, optics for bead imaging and the computer for analysis of images in a single package for POC analysis. The optical components consist of an objective lens, CCD camera and a motorized stage for automated camera focus. The computer is controlled with a
touchscreen interface and controls the assay length and flow rates in addition to obtaining and analyzing images. This combined device is approximately 9” (L) x 9” (W) x 12” (H), requiring minimal counter space, and can fit inside of a backpack for easy transport.

![Image of analyzer](image)

**Figure 4-7**: Final form p-BNC analyzer and MiniFAB p-BNC card. An injection molded plastic casing contains all actuating, imaging and analysis components.

This analyzer was used to perform multiplexed dose curves of ovarian cancer biomarkers in a truly point-of-care system. Assays for CA125, HE4 and CA72-4 were performed in the 4-well, 17-minute long format and their dose curves are shown in **Fig. 4-8**. Good limits of detection were achieved for all three, though these dose curves were obtained using the 10X lab microscope after analysis in the analyzer, as the current optics setup in the analyzer is not powerful enough to quantify signal on the lower concentrations of CA125 and CA72-4 (discussed further in conclusions).
Figure 4-8: Dose response curves of [A] CA125, [B] HE4 and [C] CA72-4 performed on the fully integrated p-BNC analyzer for use at the POC. The three assays were multiplexed together in a 17-minute assay.
4.5 Conclusions

In order to make the transition to a true POC device that can be used in large clinical trials and ultimately become a commercial product, the manufacturing methods of cards required improvement. Through collaboration with MiniFAB, the card was adapted and features were redesigned in order to reduce the number of layers from 7 to 3, significantly improving manufacturability. Transitioning from handmade cards to best practice manufacturing methods improved the reliability of the cards and enabled larger-scale construction of cards. The MiniFAB cards also permitted the use of blister packs attached to the cards that removed the need for external buffer input and replaced the lab bench syringe pumps with a custom-built actuation device. While the MiniFAB card design was based on the p-BNC lab card and shares many of the same features, the changes were significant enough to warrant re-optimization of detecting antibody concentrations and flow rates.

Another significant factor to consider in the transition to true point-of-care analysis was the length of the assay. While 43 minutes is a significant improvement from currently available methods, the ultimate vision of this platform is to obtain results during a normal patient visit.

In this chapter, a 17-minute version of the ovarian cancer p-BNC multiplexed assays was developed. Signal in the lower range of the dose curve was shown to be extremely dependent on the amount of time that the sample was allowed to interact with the beads, a significant challenge when reducing the sample delivery time by a factor of 3. A 4-well array was configured to increase the amount of sample delivered to each bead from 5μL (20-well array) to 25μL. This reduction was found to substantially increase signal, which was required to match limits of detection and assay performance previously established on the p-BNC. The CA125 assay, measuring the most critical biomarker for ovarian cancer diagnosis, was used
as a proof-of-concept assay for the development of the shorter test. The 4-well assay lowered the limit of detection of CA125 by a factor of 100 compared to the 20-well assay with the same time constraints.

Another factor in the evolution of analysis near the patient was overcoming significant preprocessing of sample fluids. The ability to use a fingerstick quantity of blood is a significant advantage for a POC analysis test, as it removes the necessity of having a laboratory centrifuge. Here, a comparison of CA125 values measured from 50μL of blood, plasma and serum was performed in order to determine what level of processing is required. Whole blood from a patient was processed into serum and plasma and all three fluids were analyzed on the p-BNC. Blood, plasma and serum were found to correlate well ($R^2 > 0.90$), however, values in blood were found to be around 40% lower than values in plasma and serum. These results confirmed expectations, as hematocrit (percentage of blood cells in whole blood) in women averages around 40%, which reduces the concentration of proteins in the sample via dilution. Quantitation of compounds in blood have been adjusted for hematocrit percentage in previous studies, which could compensate for decreased concentrations in blood and improve correlation to serum by overcoming the variation in whole blood composition.

Serum and plasma were found to correlate better to each other than to blood, which was unsurprising due to the level of complexity added from whole blood analysis. First, it was unclear whether red blood cells and white blood cells would interfere with the binding of the immunoassay sandwich and if the darker color of blood would affect optical measurements if not rinsed completely. The additional pressure created from passing a more viscous fluid through the beads was also cause for concern. Measuring blood did in fact
result in more card failures, mostly through clogging of the beads, resulting in a strong backpressure and subsequent leak at weak points of the card such as bubble traps or sample port. However, successful measurements of all 22 samples were performed with 50μL of blood and as previously discussed, were found to correlate well to serum and plasma CA125 values. While it was determined that the p-BNC platform can measure biomarker values in whole blood that can correlate well to serum, in-card separation techniques warrant further investigation. Although this study uses venous blood in a fingerstick format, the two methods of collection have shown 99% correlation in previous studies. Nonetheless, correlation for ovarian cancer biomarkers in fingerstick blood compared to venous blood will need to be confirmed.

Following the matrix comparison, the ovarian cancer multiplexed assay was adapted to a fully integrated POC format. A custom-built analyzer was developed that replaced the lab microscope, fluid delivery system and computer. The device can fit inside of a backpack for easy transportation and requires minimal counter space in a doctor’s office or clinic where biomarker levels can be measured. CA125, HE4 and CA72-4 were multiplexed in the 4-well, 17-minute assay with good results. MMP-7 was not included because our collaborators at MD Anderson concluded that it did not provide additional benefit over the other three markers in a large validation study. As a result, including a negative control bead (PSA) was determined to be more valuable than including MMP-7. Although no cross-reactivity was noted in this study, the blocking buffer used in previous chapters can replace the PBS in the blister packs of the MiniFAB cards to potentially improve the diagnostic performance of these multiplexed assays. While the dose response curves obtained were completely performed on the final form analyzer, the optics proved to be inadequate for low
concentration measurements of CA125 and CA72-4. As a result, images were also taken on the 10X lab microscope in order to achieve better assay performance. The analyzer uses an LED light source in an oblique illumination format compared to a mercury burner light source in an epifluorescent format for the lab microscope. Although the analyzer format with off-the-shelf parts currently has lower system numerical aperture than the lab microscope format, it can be improved with custom-made parts, which are currently being designed. While the optics of the analyzer need to be upgraded, the actuation method and associated software were found to be reliable.

Taken together, this work has detailed the development of a truly point-of-care, fully integrated diagnostic platform for the multiplexed measurement of ovarian cancer biomarkers. Strong analytical performance capable of measuring healthy and diseased clinical samples has been demonstrated in an ultrafast 17-minute format that has been shown to be compatible with fingerstick quantities of whole blood. The ability to multiplex with limited cross-reactivity was verified and the specific panel used in this study was shown to have more promise in detecting ovarian cancer than singleplexed biomarkers. This work also demonstrates the programmability of the p-BNC and lays a foundation for development and optimization of future assays for novel ovarian cancer biomarkers and for other diseases.
Thesis Summary and Future Perspective

This dissertation has characterized the development of a fully integrated point-of-care ovarian cancer diagnostic test. A novel panel of four ovarian cancer biomarkers – CA125, HE4, CA72-4 and MMP-7 – was adapted to the p-BNC platform in a multiplexed format that required less than 45 minutes for analysis. Despite this significant reduction in analysis time compared to commercially available systems, the analytical performance of the p-BNC correlated well to gold standard methods and achieved similar limits of detection. This test was further improved by adapting it to a disposable card capable of being mass manufactured that was designed to work with a custom-made analyzer. The card contains all fluid necessary for the assay and has a built-in waste chamber to enclose medical waste. The analyzer automatically performs the flow protocol necessary for the test and is capable of taking and analyzing images. Designed for use at the point-of-care, the analyzer can fit inside of a backpack and only requires a small amount of counter space to perform the multiplexed analysis of protein biomarkers. For true analysis during a single patient visit, the analysis time was further reduced to less than 20 minutes without sacrificing analytical performance by utilizing the custom configurability of the p-BNC platform. In terms of readiness for actual use at the point-of-care, the p-BNC platform is completely prepared for near patient analysis, with the exception of the performance of the microscope built into the analyzer. However, with a larger objective and a more powerful light source the miniaturized optics should be able to approach the performance of the laboratory epifluorescent microscope used in this study.

This ovarian cancer diagnostic test was used to measure values of over 130 clinical samples, which demonstrated strong promise in being used as a clinical tool. From the
promising results of this study, the p-BNC has been invited to take part in the largest pre-clinical ovarian cancer clinical trial. The United Kingdom Collaborative Trial of Ovarian Cancer Screening has collected longitudinal samples of over 200,000 postmenopausal women. For the portion of these women that develop ovarian cancer, having a history of samples leading up to the diagnosis is extremely useful for biomarker research. Longitudinal sera from 88 patients who develop ovarian cancer and 5 annual serum samples from 538 healthy post-menopausal women (2,690 healthy samples) have been made available for measurement on the p-BNC, in order to validate both the multiplex panel and the p-BNC as a clinical tool for the early diagnosis of ovarian cancer.
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**APPENDIX**

**Table A1.** Biomarker concentrations for each level of tumor marker control (TMC) used in precision study. CA125 concentration is averaged from reported ranges from Bio-Rad Laboratories, Hercules, CA.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>TMC Level 1</th>
<th>TMC Level 2</th>
<th>TMC Level 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA125</td>
<td>35U/mL</td>
<td>90U/mL</td>
<td>275U/mL</td>
</tr>
<tr>
<td>HE4</td>
<td>20pM</td>
<td>100pM</td>
<td>200pM</td>
</tr>
<tr>
<td>MMP-7</td>
<td>1.5ng/mL</td>
<td>5ng/mL</td>
<td>10ng/mL</td>
</tr>
<tr>
<td>CA72-4</td>
<td>10U/mL</td>
<td>25U/mL</td>
<td>50U/mL</td>
</tr>
</tbody>
</table>
Figure A1: A comparison of the effect of microscope objective magnification on signal intensity at various concentrations of CA125.
Figure A2: Isolation of the lower range of values for the correlation of the four multiplexed assays on the p-BNC to the four multiplexed assays on the Luminex. $R^2$ values and linear fits were used from Figure 4, which includes the full range of values.
Table A2. Dates of plasma collection for longitudinal clinical samples.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Case 1</th>
<th>Case 2</th>
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