UNIVERSITY OF CALIFORNIA SAN DIEGO

Protease Activity Detection from Dried Blood Spot Cards in Pancreatic Ductal Adenocarcinoma: Feasibility and Storage Stability of the RAPD Assay Platform

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Bioengineering

by

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THESIS APPROVAL PAGE

The Thesis of Megna Gopalkrishna Srinivas is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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PDAC	Pancreatic Ductal Adenocarcinoma
RAPD	Rapid Assay for Protease Detection
BSC	Blood Spot Card
IPMN	Intraductal Papillary Mucinous Neoplasm

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ABSTRACT OF THE THESIS

Protease Activity Detection from Dried Blood Spot Cards in Pancreatic Ductal Adenocarcinoma: Feasibility and Storage Stability of the RAPD Assay Platform

by

Megna Gopalkrishna Srinivas

Master of Science in Bioengineering

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Professor Geert Schmid-Schoenbein, Chair
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Background: Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal malignancies worldwide, with a five-year survival rate of approximately 12–13% due to the absence of reliable early detection tools. Elevated circulating protease activity, including cathepsin S and chymotrypsin, has been demonstrated in PDAC patient blood using the Rapid Assay for Protease Detection (RAPD), a charge-changing peptide (CCP)-based gel electrophoresis platform developed at UC San Diego. Dried blood spot (DBS) cards offer a cold-chain-independent sample blood sample collection from a finger prick that could enable home-based PDAC screening; however, no validated method exists for protease activity detection from DBS matrices.

Result: This thesis tests the feasibility and storage stability of CCP-based protease activity detection from PDAC patient whole blood spotted onto blood spot cards (BSCs;

Whatman 903 protein saver cards). Whole blood spotted onto BSCs produced significantly higher normalized fluorescent intensity than matched plasma for both cathepsin S (2.7-fold; p=0.030) and chymotrypsin (2.3-fold; p=0.003) substrates, consistent with the hypothesis that significant protease activity is present in the cellular fraction of whole blood. BSC storage temperatures between 4°C, –20°C, and –80°C revealed no statistically significant differences in protease activity for either substrate (cathepsin S: p=0.503; chymotrypsin: p=0.182 by one-way ANOVA), suggesting that refrigerator-temperature storage may be sufficient for clinical deployment.

Conclusion: These findings provide a foundational pre-clinical validation for a BSC-based PDAC diagnostic system in which fingerstick blood samples can be mailed as cards into a centralized RAPD analysis, without phlebotomy or cold-chain logistics.

INTRODUCTION

CHAPTER 1: BACKGROUND AND SIGNIFICANCE

1.1 Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal malignancies worldwide, accounting for an estimated 66,440 new cases and approximately 51,750 deaths in the United States in 2024 alone [1]. PDAC constitutes more than 90% of all pancreatic cancers and currently ranks as the third leading cause of cancer-related mortality in the United States, with projections indicating it may surpass breast cancer to become the second leading cause by 2030 [1,2]. Despite advances in oncology over the past four decades, the overall five-year survival rate for PDAC remains approximately 12–13% across all stages, and falls to just 3% for metastatic disease [1,3]. This dismal prognosis is largely attributable to the absence of reliable screening tools and the non-specific nature of early symptoms, including weight loss, abdominal discomfort, nausea, and jaundice, which frequently result in diagnosis at an advanced, unresectable stage [3,5].

The disease predominantly affects older adults, with a median age at diagnosis of approximately 72 years, and is slightly more common in men than women [2,4]. Established risk factors include chronic pancreatitis, type 2 diabetes mellitus, obesity, tobacco use, and a family history of pancreatic cancer [2,5]. Approximately 85% of PDAC cases arise from the exocrine tissue of the pancreas, with the liver serving as the most common site of metastasis [2,4]. A small subset of PDAC cases arise from identifiable precursor lesions, including pancreatic intraepithelial neoplasias (PanINs) and intraductal papillary mucinous neoplasms (IPMNs), which in principle offer an opportunity for early surgical intervention before malignant

transformation [5]. However, the vast majority of patients are diagnosed at a stage when curative resection is no longer feasible, underscoring the urgent clinical need for improved diagnostic strategies that enable detection at earlier, more treatable stages [3,5].

1.2 Current Treatment for PDAC

The management of PDAC remains profoundly limited by the advanced stage at which most patients are diagnosed. Surgical resection offers the only potentially curative treatment; however, due to widespread metastatic or locally advanced disease at presentation, only 15–20% of patients are candidates for upfront resection [6]. Among those who undergo surgery, adjuvant chemotherapy is recommended to reduce recurrence risk. The landmark PRODIGE 24 trial established modified FOLFIRINOX, a four-drug regimen comprising fluorouracil, leucovorin, irinotecan, and oxaliplatin, as the preferred adjuvant regimen, demonstrating a significant improvement in disease-free survival over gemcitabine alone [7]. For patients with metastatic disease, the MPACT trial established the combination of gemcitabine and albumin-bound paclitaxel (nab-paclitaxel) as a standard first-line option [8]. More recently, the NAPOLI-3 trial demonstrated superiority of NALIRIFOX over gemcitabine/nab-paclitaxel in the first-line metastatic setting, leading to FDA approval in 2024 [9].

Targeted therapies have yielded benefit only in select genomic subgroups. Patients harboring germline BRCA1/2 or PALB2 mutations, present in approximately 5–17% of cases, may benefit from PARP inhibition with olaparib as maintenance therapy, while targetable KRAS mutations remain a major unmet need [6,9]. Immunotherapy has demonstrated the most limited success in PDAC compared to other solid tumors. The only currently approved immunotherapy agent for PDAC is pembrolizumab, and its use is restricted to the small minority of patients (~2%) with microsatellite instability-high or mismatch repair-deficient tumors [10]. The dense,

desmoplastic, and immunosuppressive tumor microenvironment (TME) of PDAC, characterized by abundant cancer-associated fibroblasts, tumor-associated macrophages, and regulatory T cells, renders the tumor largely impenetrable to immune effector cells and represents a fundamental barrier to immunotherapy efficacy [3,6]. These collective limitations underscore the critical need for novel diagnostic and therapeutic strategies, including early biomarker detection approaches that could shift the window of diagnosis to earlier, more treatable stages.

1.3 Background on Proteases

Proteases are proteolytic enzymes that catalyze the hydrolysis of peptide bonds within proteins, and they play indispensable roles across virtually every aspect of human physiology [11]. In humans, approximately 569 known proteases have been identified, classified into five mechanistic families based on the chemistry of their active sites: serine, cysteine, metalloprotease (including matrix metalloproteinases), aspartate, and threonine proteases [11,12]. Digestive proteases, including trypsin, chymotrypsin, elastase, carboxypeptidase, and pepsin, are secreted by the exocrine pancreas and activated in the duodenum to facilitate protein digestion. Secondary proteases, such as matrix metalloproteinases (MMPs), cathepsins, and kallikrein-related peptidases (KLKs), are expressed by multiple cell types and participate in processes including extracellular matrix (ECM) remodeling, immune regulation, angiogenesis, and tissue repair [11,12].

Under normal physiological conditions, proteolytic activity is tightly regulated through two principal mechanisms: the secretion of proteases as catalytically inactive precursors (zymogens), which require specific cleavage events for activation, and the maintenance of a precise balance between active proteases and their endogenous inhibitors [11,13]. Disruption of this

regulatory equilibrium, whether through mutation, inflammation, or oncogenic transformation, results in aberrant proteolytic activity that has been linked to the pathogenesis of numerous diseases. Elevated protease activity has been documented in conditions including cancer, chronic inflammation, type 2 diabetes mellitus, hemorrhagic shock, and coagulation disorders [13,14], establishing proteases as both mechanistic drivers of disease and measurable biomarker candidates in blood.

1.4 The Role of Proteases in Cancer Pathophysiology

Aberrant expression or activity of proteases is now recognized as a hallmark of malignant disease, with dysregulated proteolytic networks contributing directly to multiple stages of tumor development and progression [15]. In PDAC specifically, elevated levels of MMPs, cathepsins, and kallikreins have been documented in tumor tissue, plasma, and serum, where they collectively promote cell proliferation, invasion, angiogenesis, immune evasion, drug resistance, and metastasis [13,16]. MMPs, including MMP-2 and MMP-9, degrade basement membrane components and release sequestered growth factors from the ECM, facilitating both local invasion and distant metastasis [13]. Inhibition of MMP-2 has been shown to reduce tumor growth and invasion in preclinical PDAC models, supporting the functional importance of this protease family in disease progression [17]. Cathepsins contribute to ECM remodeling and tumor cell survival through lysosomal membrane permeabilization and extracellular degradation of structural proteins, while kallikrein-related peptidases regulate growth factor bioavailability and have been correlated with tumor staging in multiple cancer types [13,16].

A particularly important mechanism in PDAC is the cascade that leads to activation of secondary proteases by digestive enzymes. Premature activation of trypsinogen within pancreatic acinar

cells triggered by inflammation, ductal obstruction, or oncogenic signaling may generate active trypsin that can subsequently activate MMPs, cathepsins, and other downstream proteolytic effectors [14,15]. This amplification cascade can not only drive tissue damage and tumor progression but also result in measurably elevated circulating protease activity in the blood of PDAC patients [14,16]. Elevated Cathepsin-S and chymotrypsin activity in PDAC patient plasma, as well as elevated MMP-2 activity, have been demonstrated by our laboratory using the RAPD assay, suggesting that the protease activity profile of blood may serve as a composite, multiplexed biomarker signature for disease detection and monitoring [37]. The present study builds on this principle, investigating whether such protease activity signatures can be preserved and reliably detected from blood spotted onto filter paper cards.

1.5 Pancreatic Trypsin and its Role in Cancer

Pancreatic trypsin is a serine protease produced by the exocrine pancreas in its inactive zymogen form, trypsinogen, and is normally activated in the duodenum by enteropeptidase to facilitate protein digestion [19]. Under pathological conditions, including pancreatitis and PDAC, trypsinogen undergoes premature intracellular activation within pancreatic acinar cells, generating active trypsin that initiates a proteolytic cascade with wide-ranging consequences for tumor biology [19]. Somatic mutations in the cationic trypsinogen gene (PRSS1), while most associated with hereditary pancreatitis, serve as a model for understanding the broader role of pathological trypsin activation in PDAC carcinogenesis [19]. Once activated, trypsin can exert its pro-tumorigenic effects through proteinase-activated receptor 2 (PAR-2), a G protein-coupled receptor expressed on pancreatic acinar and ductal cells, stellate cells, and sensory nerve fibers [19,20]. Trypsin-mediated PAR-2 activation could stimulates cancer cell proliferation, promotes

invasion of surrounding stroma, and facilitates early metastatic dissemination, a mechanistic framework described by Søreide et al. as the "Trypsin Trojan Horse" hypothesis, but further analysis is required [19].

Beyond its intratumoral role, trypsin leaks into the peripheral circulation in PDAC patients, providing a rationale for blood-based detection. Using the gel electrophoresis-based RAPD assay developed by the Heller and Schmid-Schönbein laboratories at UCSD, trypsin activity has been measured directly in whole blood and plasma with as little as 5–10 µL of sample [18,21]. Importantly, prior work from this laboratory demonstrated that trypsin activity was approximately 10-fold higher in the whole blood of PDAC patients compared to their matched plasma samples, and was significantly elevated relative to non-symptomatic controls ($p<0.01$) [37]. This finding that trypsin activity is concentrated in the cellular fraction of blood rather than the cell-free plasma has direct implications for the design of blood spot card-based diagnostics, as whole blood spots capture both the plasma and cellular compartments, potentially preserving the full trypsin activity signal without the need for prior centrifugation.

1.6 Cathepsin S and its Role in Cancer

Cathepsin S is a lysosomal cysteine protease with a notably restricted tissue expression profile, predominantly found in professional antigen-presenting cells (APCs) such as B lymphocytes, macrophages, and dendritic cells, as well as certain epithelial and endothelial cell populations [22]. What distinguishes cathepsin-S from other members of the cathepsin family is its ability to remain active and stable across a wide pH range, including neutral pH conditions encountered in the extracellular environment, making it a functionally relevant protease outside the lysosomal compartment [22]. Its principal physiological role involves processing invariant

chain (Ii) during MHC class II antigen presentation, a process critical for the activation of CD4+ T helper cell responses [22]. Overexpression of cathepsin-S has been associated with the pathophysiology of multiple immunological and metabolic diseases, including rheumatoid arthritis, obesity, and type 2 diabetes, in addition to its increasingly recognized role in cancer [22].

In PDAC specifically, cathepsin-S is predominantly secreted by tumor-associated macrophages (TAMs) within the tumor microenvironment, where it contributes to neoangiogenesis, ECM degradation, invasion, and metastatic spread [24]. Cathepsin-S expression has been positively correlated with the infiltration of CD8+ T cells, CD4+ T cells, and macrophages in pan-cancer analyses, suggesting a dual role as both a driver of tumor progression and a modulator of anti-tumor immune responses [25]. Elevated Cathepsin-S activity in the plasma of PDAC patients, approximately 3.8-fold higher than in non-symptomatic controls ($p<0.01$), was recently demonstrated by Iyer et al. using the RAPD assay, with consistent elevation also observed in PDAC mouse plasma and tumor tissue lysates [37]. Given the availability of potent and selective Cathepsin-S inhibitors, including LY3000328, which has demonstrated preclinical efficacy and acceptable tolerability in human subjects [26], cathepsin-S represents both a compelling diagnostic biomarker and an actionable therapeutic target in PDAC, a duality that directly motivates its inclusion in the protease panel assessed in the present study.

1.7 Chymotrypsin and its Role in Cancer

Chymotrypsin is a serine protease secreted by the exocrine pancreas as its inactive precursor, chymotrypsinogen, and activated in the duodenum by trypsin to facilitate the digestion of proteins at aromatic and large hydrophobic amino acid residues (phenylalanine, tyrosine, tryptophan, and leucine) [14]. Under normal conditions, chymotrypsin activity is confined to the gastrointestinal lumen; however, in pathological states characterized by compromised epithelial

barrier integrity such as PDAC and colorectal cancer, digestive enzymes including chymotrypsin can leak into the mesenteric lymphatics and systemic circulation through a process termed autodigestion [19]. This concept, developed by Schmid-Schönbein and colleagues at UCSD, posits that circulating digestive proteases drive systemic pathology by cleaving cell surface receptors, activating downstream protease cascades, and contributing to organ dysfunction beyond the primary tumor site [14].

Evidence for chymotrypsin-like protease elevation in cancer has been demonstrated across multiple tumor types. In a proteomic analysis of colorectal cancer mouse models, chymotrypsinogen B (CTRB1) and several chymotrypsin-like elastases were among the serine proteases most consistently elevated in both tissue interstitial fluid and serum as a function of tumor progression, suggesting potential utility as circulating cancer biomarkers [27]. In the context of PDAC, a charge-changing peptide (CCP) panel study confirmed the detection of chymotrypsin activity in gastrointestinal cancer plasma, validating the assay format used in the current study [28]. Critically, Iyer et al. demonstrated that chymotrypsin was the only digestive protease to reach statistical significance in PDAC plasma compared to non-symptomatic controls using the RAPD assay ($p=0.02$), while peptidomic analysis further revealed a chymotrypsin-consistent cleavage pattern in immunostimulatory receptor CD226 fragments, suggesting active chymotryptic proteolysis of immune regulatory proteins in the PDAC environment [37]. These findings collectively support the inclusion of chymotrypsin in the BSC protease panel evaluated in this thesis.

1.8 Protease Assays as a Diagnostic Tool in Cancer

The development of sensitive, non-invasive tools for early cancer detection remains one of the central challenges in oncology. Liquid biopsy approaches based on circulating tumor DNA

(ctDNA) and circulating tumor cells (CTCs) have advanced considerably; however, both face fundamental sensitivity limitations at early cancer stages (Stage 0–I), when tumor cell numbers are insufficient to generate detectable levels of shed nucleic acids or cells in peripheral blood [35]. Protease activity-based detection offers a mechanistically distinct and potentially more sensitive approach: because proteases function catalytically, a single enzyme molecule can cleave multiple substrate molecules within a defined incubation period, generating an amplified, measurable signal that is not limited by the absolute number of molecules shed from the tumor [29,30]. This enzymatic gain means that even sub-nanomolar concentrations of circulating protease may produce a quantifiable readout, offering particular promise for early-stage applications.

Existing protease detection technologies include fluorescence resonance energy transfer (FRET) substrates, activity-based protein profiling (ABPP), and enzyme-linked immunosorbent assays (ELISAs). While FRET substrates provide high sensitivity, they are typically designed for single-protease detection and require laboratory-grade plate readers. ABPP is powerful for mechanistic research but requires mass spectrometry infrastructure incompatible with clinical or point-of-care deployment. ELISA measures protease protein concentration rather than enzymatic activity, potentially missing the functionally relevant activated fraction of a protease [29,30]. To address these limitations, the Rapid Assay for Protease Detection (RAPD), utilizing charge-changing fluorescent peptide (CCP) substrates, was developed by Heller and Schmid-Schonbein at UCSD [18]. In this system, negatively charged intact substrates undergo a charge reversal upon cleavage by a target protease, generating positively charged fluorescent product fragments that migrate in the opposite direction during gel electrophoresis, enabling simple visual and quantitative detection from as little as 5–10 μL of whole blood, plasma, or serum in 30–60 minutes [18,21]. The assay has been validated for trypsin, chymotrypsin, elastase,

MMP-2, and Cathepsin-S across multiple clinical contexts, including type 2 diabetes, coagulation disorders, and PDAC [14,18,21]. Importantly, an independent research group recently validated the CCP panel approach for the detection and classification of gastrointestinal cancers in a multi-center cohort study, confirming the generalizability of the platform [28]. A US Provisional Patent (US63/386,635) has been filed covering the application of this technology to blood spot card samples, and prototype handheld POC devices are under active development [38].

1.9 Blood Spot Cards

Dried blood spot (DBS) cards, originally developed by Robert Guthrie in 1963 for neonatal phenylketonuria (PKU) screening, represent one of the most established minimally invasive sample collection technologies in clinical medicine [33]. The standard collection procedure involves depositing a small volume of capillary blood (typically 5–10 μL) obtained by fingerstick onto a filter paper card, most commonly Whatman 903 protein saver cards, where the sample dries at ambient temperature within 1–2 hours [32,34]. Once dried, the blood spot stabilizes many analytes against degradation, enabling storage and transport at room temperature without the cold-chain requirements that constrain conventional venipuncture-based sample logistics [31,32]. This collection format offers compelling practical advantages over traditional blood draw tubes: it eliminates the need for phlebotomist administration, centrifugation, and refrigerated sample transport; requires only a fingerstick volume of whole blood; and is inherently compatible with home-based collection and postal shipment to a central analysis facility [31,34].

The analytical utility of DBS cards has been validated across a broad range of biomarker classes. Antibody concentrations measured from DBS eluates correlate strongly with matched serum or plasma values ($r>0.85$ across multiple infectious disease applications), supporting their

use in large-scale epidemiological and vaccine efficacy studies [31]. More recently, DBS-based measurement of neurodegenerative biomarkers including phosphorylated tau 217 (pTau217), GFAP, and neurofilament light chain (NfL) has demonstrated excellent correlation with paired plasma concentrations, illustrating the expanding applicability of this matrix to protein biomarkers relevant to chronic disease [36]. DBS cards have also found application in pharmacokinetic studies, metabolomics, and environmental exposure biomarker research [34]. However, the primary limitations of DBS-based analysis, including the cumbersome nature of paper punch extraction, potential loss of labile analytes during the drying process, hematocrit effects on spot volume, and effective sample dilution in the eluate must be carefully considered in assay development and validation [31,34]. To date, no validated method has been established for the detection of protease enzymatic activity directly from PDAC patient blood spot cards, representing a significant gap in the application of this accessible collection format to cancer diagnostics. The present thesis addresses this gap directly, establishing the feasibility and stability parameters for CCP-based protease activity detection from BSCs across clinically relevant storage temperatures and time points.

1.10 Hypothesis

In this thesis, I hypothesize that cancer-associated protease activity, including Chymotrypsin and Cathepsin-S, is detectable and analytically stable in PDAC patient blood samples collected on dried Blood Spot Cards (BSCs) and stored at 4°C, −20°C, and −80°C for up to 7 days. Specifically, I infer that protease activity measured from BSC-eluted samples will retain the relative activity patterns observed in matched liquid plasma controls analyzed on the

same gel, with no statistically significant loss of detectable activity across the three storage temperatures.

Furthermore, I hypothesize that whole blood and whole blood spotted directly onto BSCs at Day 0 will produce protease activity signals comparable to or greater than those observed in plasma-derived samples, consistent with prior reports of elevated cathepsin S activity within the cellular blood fraction of PDAC patients [37]. Because BSCs retain both plasma and cellular components, I further anticipate that protease activity measured from BSC eluates may exceed that observed in matched plasma samples due to preservation of proteases associated with circulating blood cells.

Demonstration of BSC stability across these parameters would provide a critical pre-clinical validation step for the development of a home-compatible, point-of-care PDAC diagnostic system in which a patient's fingerstick sample is mailed on a BSC to a central laboratory for analysis with an electrophoresis device, without the logistical burden of phlebotomy and cold-chain transport [31,32].

CHAPTER 2: MATERIALS AND METHODS

2.1 Human Blood Sample Collection

De-identified patient blood samples were obtained from the UC San Diego Moores Cancer Center Biorepository under Institutional Review Board protocol #181755. Non-symptomatic volunteer blood was obtained from the UC San Diego Moores Cancer Center Biorepository under Institutional Review Board protocol #100556. Clinical characteristics of the patient cohort used in this study are described in Table 2.1. Blood was collected from PDAC patients in lithium heparin tubes and processed immediately or stored according to study-specific protocols. Batch control plasma was obtained from a single donor normal human plasma source (Innovative Research).

Table 2.1. Patient Sample Characteristics and Study Parameters

Parameter	Details
PDAC Patients (N)	4
Patient IDs	PDAC 149, 168, 173, 174, 175, 176
Sample Collection	UC San Diego Moores Cancer Center Biorepository (IRB #181755, IRB #100556)
Blood Collection Tube	Lithium heparin
Batch Control Plasma	Single donor normal human plasma (Innovative Research)
BSC Card Type	Whatman 903 Protein Saver Card
Storage Temperatures	4°C, –20°C, –80°C

2.2 Plasma Isolation

Heparinized blood was centrifuged at 1,250 × g for 10 minutes at room temperature, after which plasma was removed from the upper layer. The collected plasma was centrifuged again at 1,500 × g for an additional 10 minutes to remove residual cells. Plasma was then aliquoted into

cryovials and stored at –80°C until use. Plasma freeze-thaw cycles were minimized, and plasma was thawed at 4°C prior to experimental use.

2.3 RAPD Protease Assay

The Rapid Assay for Protease Detection (RAPD), developed by Dr. Heller at the University of California, San Diego, was used to measure protease activity in plasma and whole blood samples. The assay employs synthetic fluorescent charge-changing peptide (CCP) substrates. In their intact state, CCP substrates carry a net negative charge. Upon cleavage by the target protease, the C-terminal fragment acquires a net positive charge. This charge reversal forms the basis of the electrophoretic readout. The substrates used in this study, specific for chymotrypsin and cathepsin S, are listed in Table 2.2. Substrates were synthesized by AAPPTec and tagged with the Bodipy-FL-SE fluorophore (Invitrogen). Working solutions were prepared fresh at 5 ppm (5 µL stock + 95 µL PBS) before each experimental session and kept on ice until use.

Table 2.2. RAPD Assay Protease Substrates: Sequence and Charge

Target Protease	Substrate Sequence	Net Charge	Cleaved Peptide Charge
Chymotrypsin	Acetyl-N-D-GD-AAY/AAY/AGA-G-diaminoethyl(Bodipy FL)-CONH2	–2	+1
cathepsin S	Succinyl-N-TVG/LAG-AGK-(Bodipy FL)-CONH2	–1	+1

2.4 Inverted Polarity Gel Electrophoresis and Image Analysis

A critical feature of the RAPD assay is its use of an inverted polarity gel electrophoresis configuration, which is essential for capturing the positively charged cleaved protease product. The gel is oriented with the anode (+) at the top and the cathode (–) at the bottom of the XCell

SureLock Mini-Cell apparatus, which is the reverse of conventional PAGE. Under this configuration, two simultaneous events occur. First, the intact negatively charged uncleaved substrate is repelled upward toward the anode and exits the wells into the upper buffer reservoir, where it is lost. Second, any substrate cleaved by a target protease generates a C-terminal fragment with a net positive charge; this fragment is attracted downward toward the cathode and migrates into the gel matrix, forming a discrete fluorescent band. The presence and intensity of this downward-migrating band is the direct readout of protease activity in the sample. If the gel is run in conventional polarity (cathode at top, anode at bottom), the intact substrate migrates downward and the cleaved fragment is lost upward, rendering the assay uninterpretable. Correct gel polarity must be verified before every run.

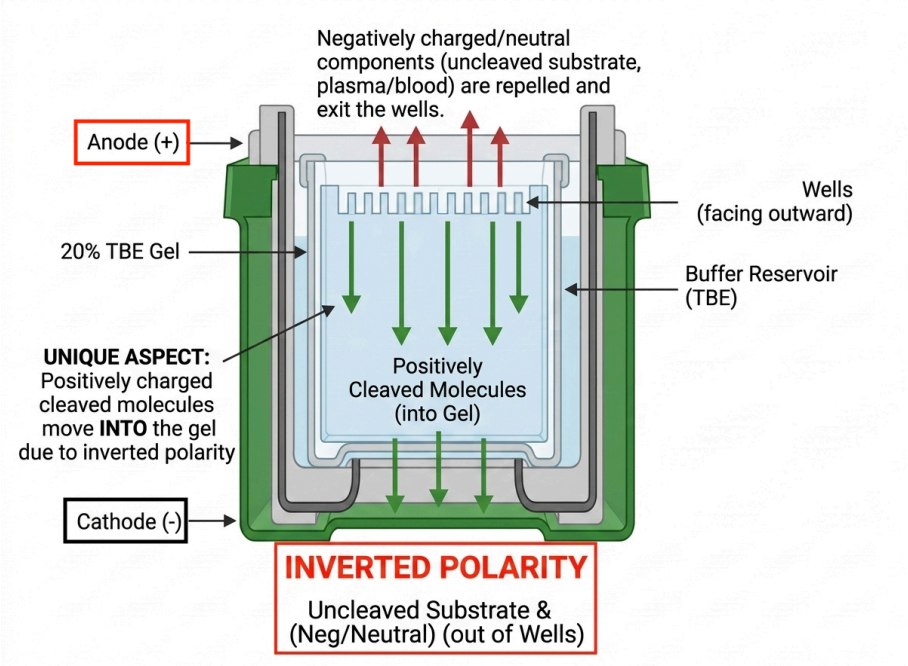

Figure 2.1. Inverted polarity gel electrophoresis configuration for the RAPD assay. Anode (+) is at top; cathode (–) is at bottom. Intact negatively charged CCP substrate (red) exits wells upward. Positively charged cleaved protease product fragment (green) migrates downward into gel matrix and is retained as a quantifiable fluorescent band.

Aliquots of 5–7 µL per sample were loaded onto 20% TBE polyacrylamide gels (15- or 12-well format) in a vertical polyacrylamide gel apparatus (mini-cell tank). Gels were run at a

constant voltage of 225 V for approximately 30–35 minutes in a 0.5× Tris/Borate/EDTA (TBE) running buffer until visible fluorescent bands were observed. Empty wells (marked X) were included between sample groups to prevent well distortion caused by the high viscosity of whole blood and BSC eluates.

Gels were imaged using a ChemDoc Imaging System (Bio-Rad Laboratories Inc.) with SYBR Green settings (590/110 nm UV Trans) at auto-rapid exposure. Images were captured at various exposure times and exported for analysis (Figure 2.2). Fluorescent gel images were analyzed using Fiji (ImageJ, NIH). The mean gray value was calculated for each fluorescent band and for two adjacent background regions per lane. Background values were subtracted from all measurements. All sample values were normalized to a commercial batch control Plasma 45615 (Innovative Research) run on the same gel. For the temperature stability experiments, BSC-derived fluorescent intensities were expressed relative to the batch control plasma value. For the whole blood comparison experiment, whole blood, fresh BSC eluate, and matched PDAC plasma values were each normalized to the batch control plasma from the same gel.

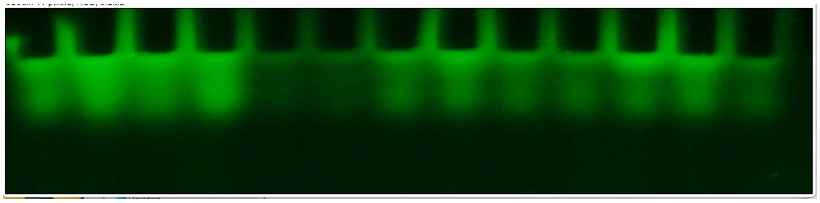

Figure 2.2 Representative RAPD gel image demonstrating inverted polarity electrophoresis of CCP substrate in PDAC patient plasma. Bright upper bands near the wells represent intact, negatively charged substrates. Downward-migrating lower bands of varying intensity reflect positively charged cleavage products retained within the polyacrylamide gel matrix, indicating sample-derived protease activity. Lane intensities were quantified by densitometry in Fiji (ImageJ).

2.5 Blood Spot Card Sample Preparation

Blood spot cards (BSCs) were prepared using Whatman 903 protein saver filter paper cards. Three microliters (3 μL) of PDAC patient whole blood were spotted onto BSC cards and

allowed to dry for 15 minutes at ambient temperature in an airtight container with no moisture exposure. Cards were then stored at –80°C, –20°C, or 4°C as specified for each experimental condition. For elution, 3 mm diameter circular punches were excised from dried blood spots and placed into individual wells of a 96-well plate. Each punch was incubated with 30 µL of 1X PBS buffer and 10 µL of CCP substrate for 60 minutes at room temperature. Following incubation, 12 µL of 70% v/v glycerol was added to each sample. Matched liquid plasma controls (10 µL plasma + 10 µL substrate) and batch control plasma samples were run on every gel for normalization. The full BSC assay workflow is summarized in Table 2.3.

Table 2.3. Blood Spot Card RAPD Assay Workflow

Step	Description
1	3 µL of PDAC patient whole blood spotted onto Whatman 903 protein saver BSC card; allowed to dry 15 minutes at ambient temperature in airtight box with no moisture exposure.
2	3 mm punch used to excise dried blood spot; placed into 96-well plate.
3	BSC punch incubated with 30 µL 1X PBS buffer + 10 µL CCP substrate (cathepsin S, chymotrypsin, or trypsin) for 30 minutes at room temperature.
4	12 µL of 70% v/v glycerol added to each sample.
5	7–13 µL loaded onto 20% TBE PAGE gel; run at 225 V for 30–35 minutes in 0.5× TBE running buffer.
6	Fluorescent bands imaged; mean gray value quantified by ImageJ. Background subtracted; values normalized to batch control plasma.

2.6 Substrate Spiked Dilution Series

To further characterize the substrate-intrinsic reaction in the RAPD assay, a serial dilution series of cathepsin S and chymotrypsin CCP stocks was prepared in PBS and reacted with Subject A (non-symptomatic Control) plasma and analyzed by RAPD gel electrophoresis. For cathepsin S, a 5-fold serial dilution was prepared from 70 ng/µL to 0.0016 ng/µL, described in Table 2.4. For chymotrypsin, a 2-fold serial dilution was prepared from 100 ng/µL to 12.5 ng/µL in both plasma and whole blood matrices, described in Table 2.5. Each dilution (10 µL) was

reacted with 10 µL substrate for 30 minutes at room temperature before electrophoresis. Pearson correlation coefficients were calculated between $\log_{10}$-transformed enzyme concentration and NFI.

Table 2.4. Cathepsin S Substrate Dilution Series

Dilution	Concentration (ng/µL)	Preparation
5x	70	50 µL working stock
1x	14	10 µL stock + 40 µL PBS
0.2x	2.8	10 µL 1x dilution + 40 µL PBS
0.04x	0.112	10 µL 0.2x dilution + 40 µL PBS
0.008x	0.0016	10 µL 0.008x dilution + 40 µL PBS

Table 2.5. Chymotrypsin Substrate Dilution Series

Dilution	Concentration (ng/µL)	Preparation
1x	100	50 µL working stock
0.5x	50	10 µL stock + 40 µL PBS
0.025x	25	25 µL 1x dilution + 25 µL PBS
0.012.5x	12.5	25 µL 0.2x dilution + 25 µL PBS

2.7 Whole Blood versus Plasma Comparison

To test the hypothesis that whole blood contains higher protease activity than matched plasma, attributable to the cellular fraction, whole blood and plasma from the same PDAC patients (PDAC 175, 176, 167, 187, Subject B (non-symptomatic Control) were analyzed side by side on the gels using both cathepsin S and chymotrypsin substrates. All values were normalized to the batch control plasma from the same gel. A two-tailed paired t-test was used to compare whole blood versus plasma NFI within matched subjects (n = 5).

2.8 PDAC Plasma Cohort Activity Comparison

PDAC plasma samples (PDAC 50, 59, 60, 62, 105, 108, 110, 112, 116, 117, 164, Subject C (Normal Control)) were run on the same gel to establish that there is variability in proteolytic activity within patients as a consideration. Samples were processed and analyzed as described in sections 2.4 and 2.5. All values were normalized to the batch control plasma from the same gel. A two-tailed paired t-test was used to compare whole blood versus plasma NFI within matched subjects (n = 12).

2.9 Blood Spot Card Temperature Stability Experiment

To evaluate whether whole blood spotted onto dried blood spot cards (BSCs) retains protease activity across storage temperatures, PDAC patient whole blood was spotted onto Whatman 903 protein saver filter paper cards (3 μL per spot) and allowed to dry for 15 minutes at ambient temperature in an airtight container. Cards were stored at 4°C, –20°C, or –80°C. At each time point, a 2 mm punch was excised from the dried blood spot, placed into a 96-well plate, and incubated with 30 μL of 1x PBS buffer and 10 μL of CCP substrate for 60 minutes at room temperature. The sample was then processed identically to liquid samples as described in section 2.4. N=6 samples (PDAC 149, 168, 173, 174, 175, and Subject A (non-symptomatic Control)) were analyzed. Fluorescent intensity was normalized to batch control plasma on the same gel.

2.10 Statistical Analysis for BSC Storage Temperature Analysis

Raw fluorescent intensity values were quantified from gel images using Fiji (ImageJ, NIH). Mean gray values were measured for each band and for two background regions per gel. Background-subtracted fluorescent intensity values were normalized to the average batch control plasma value across two independent gel runs for each substrate (BC Cat-S mean = 5.12 arbitrary

units; BC Chy mean = 11.03 arbitrary units). Group-level descriptive statistics (mean ± standard deviation) were calculated for each storage temperature condition across N=6 samples. To assess whether storage temperature significantly affected retained protease activity in BSC eluates, a one-way analysis of variance (ANOVA) was performed separately for cathepsin S and chymotrypsin, with storage temperature (4°C, –20°C, –80°C) as the independent variable and normalized fluorescent intensity as the dependent variable. To compare matched plasma versus BSC protease activity within each subject, a two-tailed paired t-test was performed using the mean BSC value across all three temperatures for each sample. A significance threshold of $p < 0.05$ was applied to all comparisons. All statistical analyses were performed using SciPy (version 1.9, Python 3).

CHAPTER 3: RESULTS

3.1 The RAPD Assay Detects Dose-Dependent cathepsin S Activity in Spiked Plasma

To establish assay function and sensitivity for cathepsin S, recombinant enzyme was spiked into normal human plasma across a serial dilution series spanning 0.0016 to 14 ng/µL and analyzed by RAPD gel electrophoresis (Figure 3.1). Background-subtracted NFIs (normalized to batch control plasma, BC Cat-S = 2.1) were 1.05, 2.10, 3.19, and 6.19 at 0.0016, 0.112, 2.8, and 14 ng/µL respectively, indicating a monotonically increasing signal with enzyme concentration. Pearson correlation between $\log_{10}$-transformed concentration and NFI confirmed a strong positive trend ($r = 0.893$, $p = 0.107$). While the p-value did not reach significance at $\alpha = 0.05$ due to the limited number of data points ($n = 4$), the clear monotonic increase and $r > 0.89$ demonstrate that the CCP substrate chemistry and gel configuration are functioning correctly and that the downward-migrating cleaved band is proportional to cathepsin S concentration.

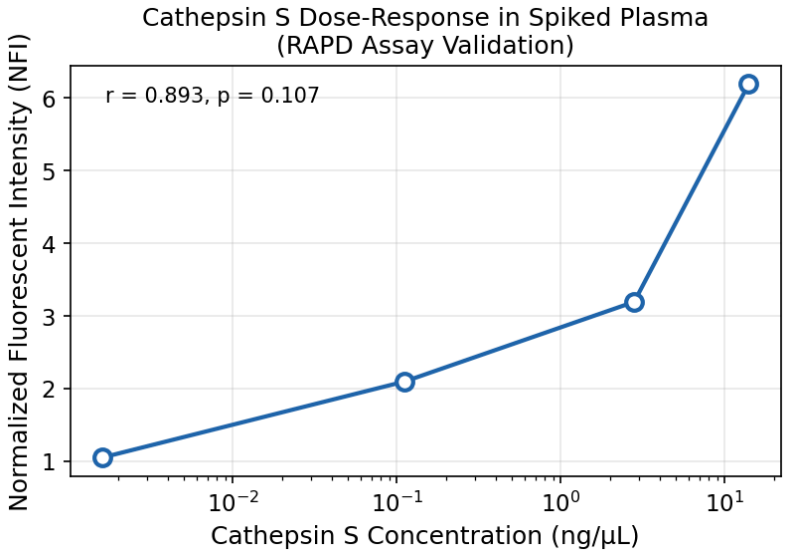

Figure 3.1 Cathepsin S dose-response in spiked normal human plasma (RAPD assay validation). Recombinant cathepsin S was serially diluted (0.0016–70 ng/µL) and incubated with the cathepsin S CCP substrate. NFI values normalized to batch control plasma (BC = 2.1). $r = 0.893$, $p = 0.107$ (Pearson correlation, $\log_{10}$-transformed concentration). Monotonic signal increase confirms functional assay readout.

3.2 Chymotrypsin Dose-Response is Detectable in Both Plasma and Whole Blood Matrices

To validate assay function for chymotrypsin and assess matrix effects on detection, recombinant chymotrypsin was spiked into normal human plasma across a 2-fold dilution series from 12.5 to 100 ng/µL (Figure 3.2, left panel). Background-subtracted NFIs were 2.43, 2.96, 5.10, and 6.17 at 12.5, 25, 50, and 100 ng/µL respectively (normalized to BC Chy = 13.4). Pearson correlation confirmed a significant positive dose-response ($r = 0.977$, $p = 0.023$). The same dilution series was run in parallel in both plasma and whole blood matrices on a second gel (Figure 3.2, right panel), normalized to BC = 13.4. Positive trends were observed in both matrices (plasma: $r = 0.933$, $p = 0.067$; whole blood: $r = 0.885$, $p = 0.115$), though neither reached significance after exclusion of the saturated highest-concentration point. Together, these results confirm that the RAPD assay detects chymotrypsin across a biologically relevant concentration range in both sample types, validating the downward-migrating band as a genuine enzymatic cleavage readout.

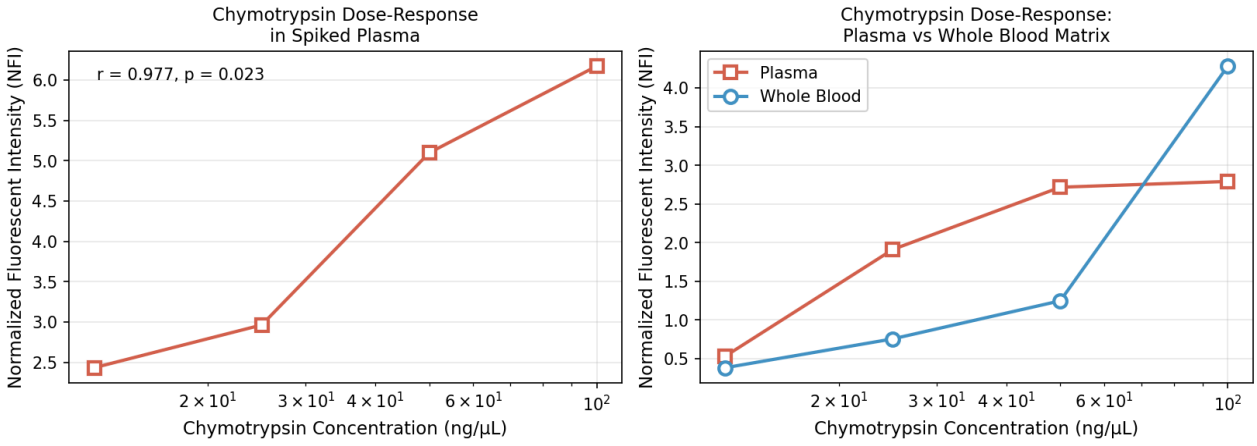

Figure 3.2 Chymotrypsin dose-response in spiked normal human plasma and whole blood.
Left: Serial dilution (12.5–100 ng/µL) in plasma; NFI normalized to BC = 13.4 ($r = 0.977$, $p = 0.023$). Right: Same dilution series in plasma (red) vs. whole blood (blue) on a second gel; positive trends observed in both matrices.

3.3 Cathepsin S and Chymotrypsin Activity are Elevated in PDAC Plasma Compared to Normal Controls

Having validated the RAPD assay with spiked recombinant enzymes, we measured endogenous protease activity directly in a PDAC patient plasma cohort (n = 11 PDAC patients + IPMN (Intraductal Papillary Mucinous Neoplasm) 50; Figure 3.3). All values were normalized to a batch control plasma run on the same gel (BC Cat-S = 1.9; BC Chy = 22.8). The matched normal control (Subject C) produced NFI of 0.89 for cathepsin S and 0.45 for chymotrypsin.Cathepsin S NFI across the PDAC cohort ranged from 1.26 to 31.58, with a mean of 6.93 ± 8.38. A one-sample t-test against the normal control NFI (0.89) confirmed significantly elevated cathepsin S activity in PDAC plasma (t = 2.28, p = 0.046). Chymotrypsin NFI ranged from 0.24 to 1.67, with a mean of 0.95 ± 0.45, also significantly elevated relative to the normal NFI of 0.45 (t = 3.51, p = 0.006). PDAC 105 was a notable outlier for cathepsin S (NFI = 31.58), consistent with prior gel observations of this patient. IPMN 50 also showed elevated activity in both substrates, consistent with reports of elevated protease activity in pancreatic cystic lesions.

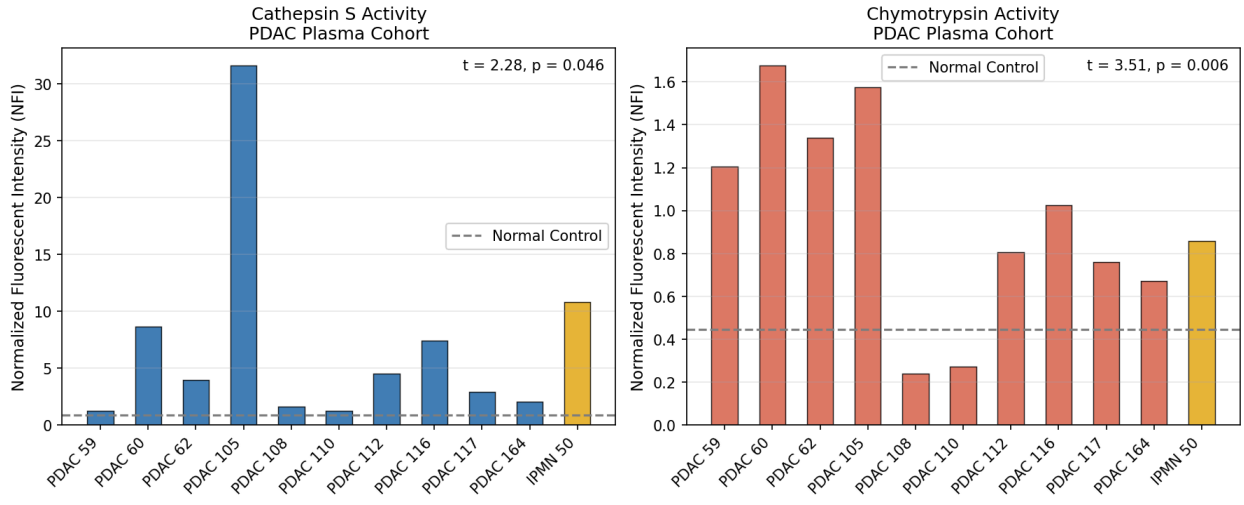

Figure 3.3. Cathepsin S (left) and chymotrypsin (right) normalized fluorescent intensity (NFI) in PDAC patient plasma cohort (n = 11 PDAC + 1 IPMN). All values normalized to batch control plasma (BC Cat-S = 1.9; BC Chy = 22.8). Dashed line indicates normal control (Subject C) NFI. One-sample t-test vs. normal control: Cathepsin S t = 2.28, p = 0.046; Chymotrypsin t = 3.51, p = 0.006.

3.4 Whole Blood Contains Higher Protease Activity Than Matched Plasma in PDAC

To test the hypothesis that whole blood retains higher proteolytic activity than plasma due to protease concentration in the cellular fraction, whole blood and matched plasma from five subjects (PDAC 175, PDAC 176, PDAC 167, PDAC 187, and a non-symptomatic control) were analyzed side by side on the same gel using cathepsin S and chymotrypsin substrates (Figure 3.4). All values were normalized to batch control plasma (BC Cat-S = 2.1; BC Chy = 11.0).For cathepsin S, mean whole blood NFI was 30.77 ± 4.17 versus mean plasma NFI of 10.28 ± 8.40, which is a ~3.0-fold elevation. For chymotrypsin, mean whole blood NFI was 24.28 ± 21.54 versus plasma NFI of 7.85 ± 8.34, which is a ~3.1-fold elevation. Paired t-tests confirmed that whole blood NFI was significantly higher than matched plasma for both cathepsin S ($t = 4.38$, $p = 0.012$) and chymotrypsin ($t = 4.91$, $p = 0.008$). These findings support the premise that centrifugation removes a substantial protease-rich cellular compartment, and that BSCs which preserve whole blood capture this activity.

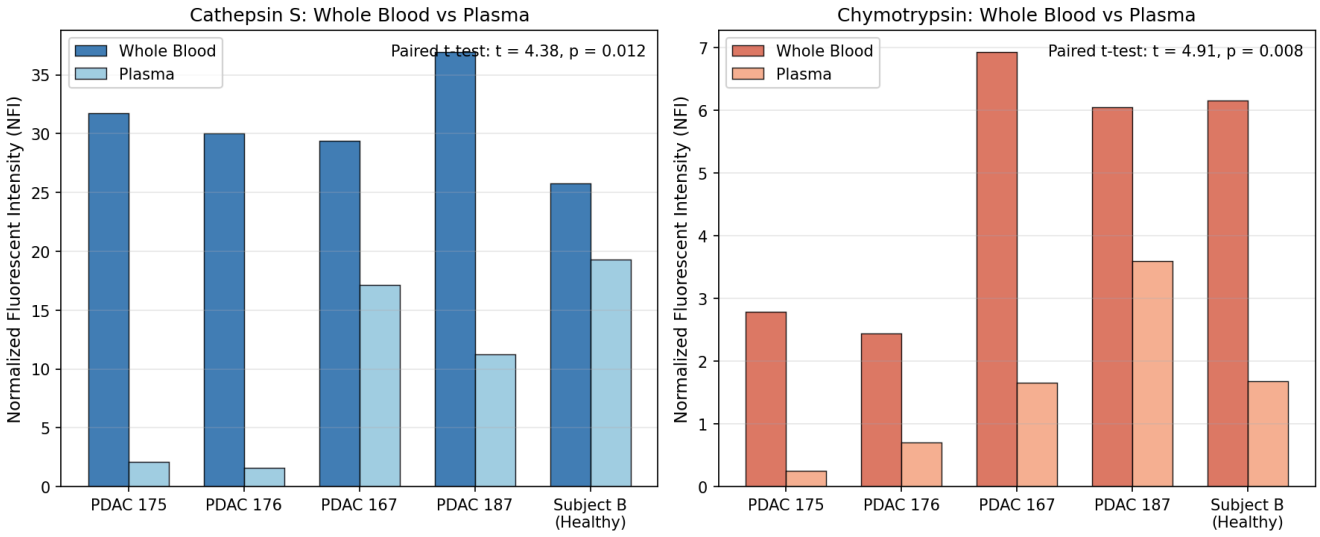

Figure 3.4. Whole blood vs. matched plasma normalized fluorescent intensity for cathepsin S (left) and chymotrypsin (right) across five samples. All values normalized to batch control plasma (BC Cat-S = 2.1; BC Chy = 11.0). Paired t-test: Cathepsin S $t = 4.38$, $p = 0.012$; Chymotrypsin $t = 4.91$, $p = 0.008$. Whole blood consistently produces higher NFI than matched plasma across all subjects and substrates.

3.5 Blood Spot Card Eluates Retain Protease Activity Comparable to Matched Whole Blood

To demonstrate that BSC elution captures the protease activity present in freshly collected whole blood, matched plasma, fresh whole blood, and Day-0 BSC eluate from PDAC 175 were run on the same gel (Figure 3.5). All values were normalized to batch control plasma from the same gel (BC Cat-S = 13.168; BC Chy = 35.84).For cathepsin S, the plasma NFI was 0.87, while both whole blood (NFI = 9.87) and BSC eluate (NFI = 9.57) were approximately 11.3-fold higher than plasma and nearly identical to one another. For chymotrypsin, a similar pattern was observed: plasma NFI = 0.82, whole blood NFI = 2.23, BSC eluate NFI = 2.18. The near-equivalence of whole blood and BSC eluate signals for both substrates indicates that the blood spot card matrix captures and preserves the full proteolytic activity of whole blood with negligible loss during the spotting and drying process.

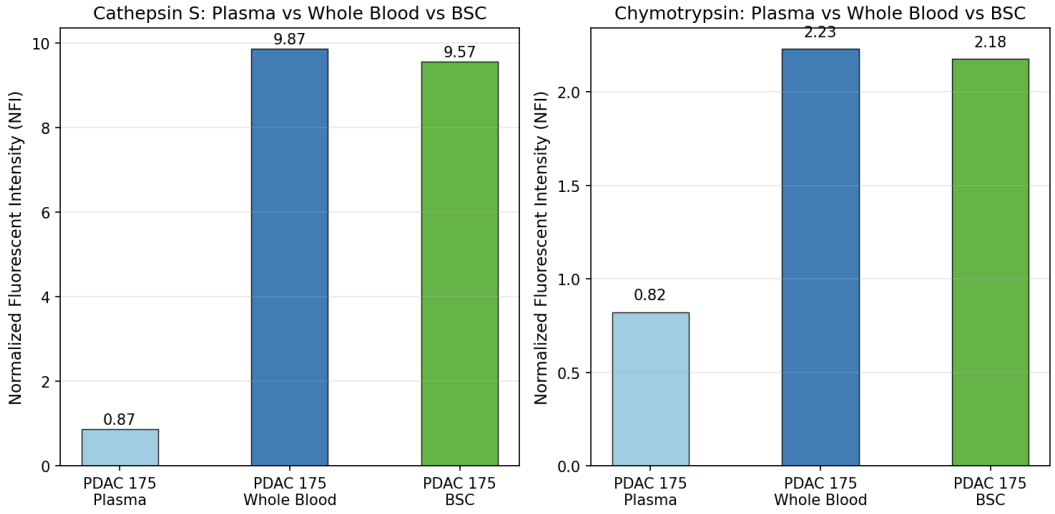

Figure 3.5. Normalized fluorescent intensity (NFI) for cathepsin S (left) and chymotrypsin (right) in matched plasma, whole blood, and Day-0 BSC eluate from PDAC 175. NFI normalized to batch control plasma (BC Cat-S = 13.168; BC Chy = 35.84). Whole blood and BSC eluate produce near-identical signals for both substrates, substantially exceeding matched plasma.

3.6 Storage Temperature Does Not Significantly Affect BSC Protease Activity Retention

To evaluate whether storage temperature influences retained protease activity in BSCs, PDAC patient whole blood was spotted onto Whatman 903 cards from six subjects (PDAC 149, 168, 173, 174, 175, and non-symptomatic Subject B) and stored at 4°C, −20°C, and −80°C. BSC eluate NFI values and matched plasma NFI values were normalized to the mean of two independent batch control measurements (BC Cat-S mean = 5.12; BC Chy mean = 11.03). Note that cards were stored over durations between approximately one week to one month due to experimental troubleshooting. This variability is expected to minimally affect the measurements at different temperatures given the established long-term preservation properties of BSCs [31,32].

BSC eluates across all temperatures consistently produced higher NFI than matched plasma for both substrates. Two-tailed paired t-tests confirmed that mean BSC activity (averaged across all three temperatures per subject) was significantly higher than matched plasma for both cathepsin S (mean BSC NFI = 7.20 ± 2.37 vs plasma NFI = 2.66 ± 1.68; $t = 3.01$, $p = 0.030$) and chymotrypsin (mean BSC NFI = 2.46 ± 0.57 vs plasma NFI = 1.09 ± 0.30; $t = 5.47$, $p = 0.003$). Notably, control subject B exhibited elevated cathepsin S BSC signals (4°C: NFI = 10.42; −20°C: NFI = 11.07; −80°C: NFI = 14.97) exceeding the PDAC sample means, while Subject B's matched plasma cathepsin S NFI was the lowest of all samples (0.75 NFI). This underscores that whole blood BSC cathepsin S signal reflects total cellular and plasma protease content and is not PDAC-specific in isolation, motivating future studies comparing BSC PDAC versus non-symptomatic whole blood in larger cohorts.

Per-subject NFI values grouped by storage temperature and matched plasma are shown in Figure 3.6. For cathepsin S, mean NFI was 6.77 ± 2.07 at 4°C, 6.46 ± 2.73 at −20°C, and 8.36 ± 3.13 at −80°C. For chymotrypsin, corresponding values were 2.26 ± 0.73, 2.12 ± 0.53, and 3.01

± 0.98. The three BSC storage temperature conditions yielded no significant NFI differences for either cathepsin S ($F = 0.72$, $p = 0.503$; One-way ANOVA) or chymotrypsin ($F = 1.91$, $p = 0.182$), indicating that **protease activity is preserved at all three temperatures tested**. Summary mean NFI values with standard deviations across for six subjects are shown in Figure 3.7.

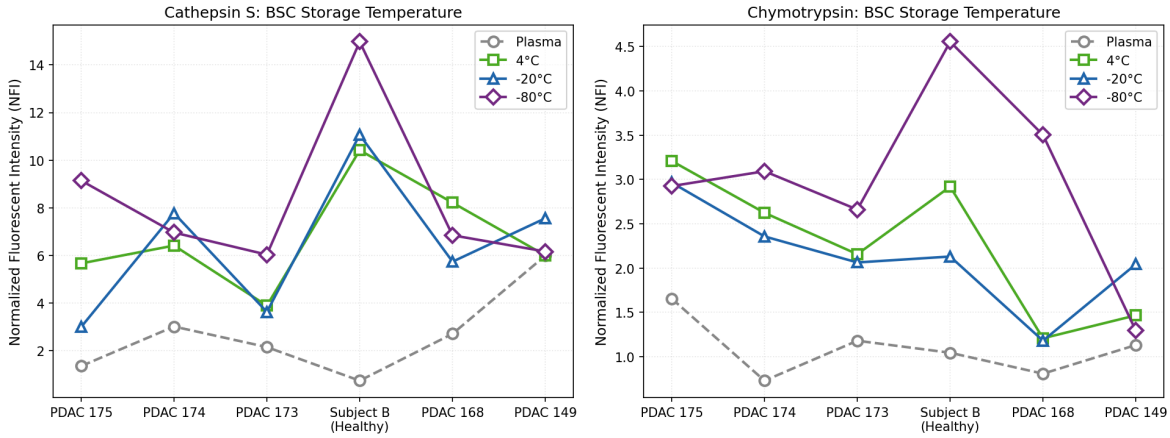

Figure 3.6. Per-subject cathepsin S (left) and chymotrypsin (right) NFI for matched plasma and BSC eluates stored at three temperatures across six subjects (PDAC 149, 168, 173, 174, 175, and Subject B). All values normalized to mean batch control plasma (BC Cat-S mean = 5.12; BC Chy mean = 11.03). One-way ANOVA across storage temperature conditions: Cathepsin S $F = 0.72$, $p = 0.503$; Chymotrypsin $F = 1.91$, $p = 0.182$ (not significant). BSC eluates consistently exceed matched plasma NFI within subjects.

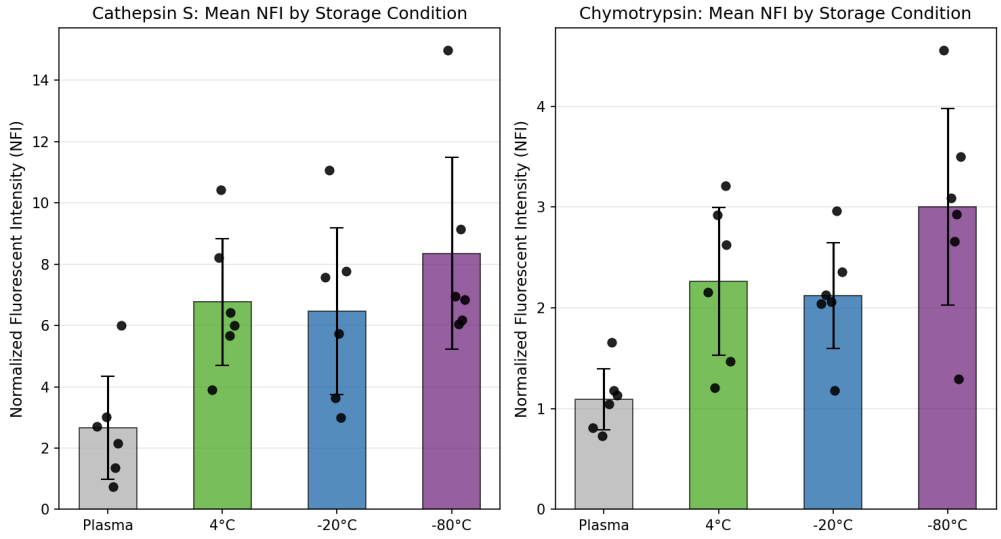

Figure 3.7. Mean (± SD) for cathepsin S (left) and chymotrypsin (right) NFI by storage condition across all six subjects. Plasma (gray), 4°C (green), −20°C (blue), −80°C (purple). Error bars = 1 SD. One-way ANOVA (BSC temperatures only): Cathepsin S $p = 0.503$; Chymotrypsin $p = 0.182$. Two-tailed paired t-test comparing mean BSC vs. matched plasma: Cathepsin S $t = 3.01$, $p = 0.030$; Chymotrypsin $t = 5.47$, $p = 0.003$.

CHAPTER 4: DISCUSSION

4.1 Overview of Findings

The primary objective of this study was to evaluate whether pancreatic ductal adenocarcinoma (PDAC)-associated protease activity could be reliably detected and preserved from whole blood spotted onto dried blood spot cards (BSCs) and whether these samples would remain stable under clinically relevant storage conditions. The RAPD assay detects dose-dependent protease activity in PDAC patient blood; whole blood contains substantially more proteolytic signal than matched plasma; BSC elution captures that signal with negligible loss; and storage temperature, whether 4°C, −20°C, or −80°C, does not significantly alter the retained activity for either cathepsin S or chymotrypsin. Taken together, these findings provide a coherent, data-supported feasibility case for BSC-based protease diagnostics as a minimally invasive alternative to conventional venipuncture and cold-chain sample handling in PDAC.

4.2 Assay Validation: The RAPD Platform Detects Protease Activity in a Dose-Dependent Manner

Initial dose-response experiments confirmed the analytical performance of the assay. The spiked dilution experiments for both cathepsin S and chymotrypsin address this directly. For cathepsin S, a monotonically increasing signal was observed across a 0.0016 to 14 ng/μL range in normal human plasma, with a strong positive Pearson correlation between log10-transformed concentration and NFI ($r = 0.893$). While the p-value ($p = 0.107$) did not meet the conventional $\alpha = 0.05$ threshold; this is attributable to the limited number of dilution points available ($n = 4$) rather than to a weak underlying trend; the effect size and monotonic pattern are biologically meaningful. For chymotrypsin spiked into plasma, the dose-response was both stronger and

statistically significant ($r = 0.977$, $p = 0.023$), confirming that the assay is sensitive across a biologically relevant concentration range.

The parallel chymotrypsin dilution series run in whole blood alongside plasma (3.2, right panel) showed positive trends in both matrices, and the near-equivalent dose-response shapes confirm that the whole blood does not fundamentally compromise assay readout. The observation that both matrices show saturation at the highest spiked concentration (100 ng/μL) is not unexpected and simply reflects the upper limit of gel-based fluorescence detection; within the biologically relevant range of endogenous protease activity, the assay operates in a linear regime. This validation is consistent with prior reports from the Heller laboratory demonstrating RAPD assay linearity for trypsin, elastase, and MMP-2 in whole blood and plasma matrices [18,37], and with an independent multi-center study validating CCP-based panels for gastrointestinal cancer classification [28].

4.3 Elevated Protease Activity in PDAC Plasma

Measurement of endogenous protease activity in the PDAC plasma cohort (n = 11 PDAC patients + IPMN 50) confirmed significantly elevated cathepsin S ($t = 2.28$, $p = 0.046$) and chymotrypsin ($t = 3.51$, $p = 0.006$) activity relative to a matched normal control (Figure 3.3). These findings align directly with the prior work of Iyer et al., who reported approximately 3.8-fold elevated cathepsin S activity in PDAC plasma compared to non-symptomatic controls using the same RAPD platform [37], and with the Suwatthanarak et al. study demonstrating that a CCP panel can classify gastrointestinal cancers with high sensitivity [28]. The current cohort replicates and extends these findings by including a larger patient set (n = 11) analyzed with rigorous batch normalization.

4.4 Whole Blood Displays Elevated Protease Activity versus Plasma

One of the most important findings in this thesis is the consistent and statistically significant elevation of protease activity in whole blood relative to matched plasma for both cathepsin S (paired t-test: $t = 4.38$, $p = 0.012$; ~3.0-fold) and chymotrypsin ($t = 4.91$, $p = 0.008$; ~3.1-fold) across five subjects (Figure 3.4). This result is mechanistically coherent: plasma is the acellular supernatant obtained after centrifugation, and centrifugation removes red blood cells, platelets, leukocytes, and their associated vesicles, all of which carry membrane-associated or intracellular proteases. Cathepsin S in particular is predominantly expressed in macrophages, B lymphocytes, and dendritic cells [22], all of which circulate in whole blood and are discarded in the centrifugation pellet. The consistent whole blood > plasma pattern observed across all five subjects, including a non-PDAC non-symptomatic control, confirms that this is a general property of whole blood rather than a PDAC-specific phenomenon.

This finding has direct design implications for the BSC platform. As the BSCs are spotted with whole blood before any centrifugation step, they capture the full proteolytic complement of both the cellular and plasma fractions simultaneously. This is an inherent advantage over plasma-based assays: BSC eluates effectively represent a "total blood protease" measurement, which the paired comparisons demonstrate, is consistently higher and potentially more sensitive than plasma alone. The tradeoff is that the elevated BSC signal reflects protease activity from all blood cell types, not selectively from cancer-associated cells, which means that absolute threshold-based discrimination between PDAC and non-symptomatic individuals will require either normalization to cellular counts or multiplexed substrate panels rather than a single substrate cutoff. The current data do not resolve this question but motivate its investigation in future studies.

4.5 BSC Elution Preserves Whole Blood Protease Activity

The near-perfect equivalence between fresh whole blood and Day-0 BSC eluate (cathepsin S: NFI 9.87 vs. 9.57; chymotrypsin: NFI 2.23 vs. 2.18 for PDAC 175; Figure 3.5) confirms that the spotting and drying process on Whatman 903 protein saver cards does not measurably reduce protease activity over a timescale of hours at ambient temperature. This is consistent with the established preservation properties of Whatman 903 cards, which are designed to stabilize proteins through low-moisture, fibrous encapsulation that limits protease autodegradation and environmental oxidation [32]. The present results extend this prior characterization to enzymatic activity specifically, demonstrating that the conformational integrity and catalytic function of serine and cysteine proteases are retained through the dry-spot matrix.

It is important to note that the BSC versus whole blood comparison currently rests on data from a single subject (PDAC 175) due to logistical constraints in the experimental workflow. While the concordance is striking and internally consistent with the broader pattern of results, replication across additional subjects is needed before this finding can be generalized with full confidence. This is identified as a priority for future work, particularly for the chymotrypsin substrate, where matrix quality issues were noted in the whole blood spiked gel run, and results should be confirmed with fresh substrate preparations.

4.6 Temperature Stability of BSCs: Key Finding for Clinical Translation

The central translational finding of this thesis is that protease activity in BSC eluates is not significantly affected by storage temperature across the range of 4°C to −80°C, as confirmed by one-way ANOVA for both cathepsin S ($F = 0.72$, $p = 0.503$) and chymotrypsin ($F = 1.91$, $p =$

0.182) across six subjects (Figure 3.6, Figure 3.7). Although the −80°C condition produced numerically higher mean values for both substrates (cathepsin S: 8.36 ± 3.13 vs. 6.77 ± 2.07 at 4°C; chymotrypsin: 3.01 ± 0.98 vs. 2.26 ± 0.73 at 4°C), the absence of statistical significance indicates that this trend is not robust enough to constitute a clinically meaningful difference. These findings suggest that refrigerator-temperature storage at 4°C is sufficient to preserve protease activity in PDAC patient BSCs, at least over the storage durations examined here.

This result is particularly consequential for the proposed point-of-care application: a home-compatible PDAC screening system in which patients collect fingerstick samples, spot blood onto cards, and mail them to a centralized RAPD analysis laboratory. For such a system, the ability to store cards at 4°C eliminates the need for dry ice or −80°C shipping conditions, dramatically simplifying the sample logistics chain and reducing costs. Prior DBS validation studies for protein biomarkers, including immunoglobulin quantification [31] and neurodegenerative markers such as pTau217 and NfL [34], have similarly demonstrated acceptable analyte preservation at ambient or refrigerator temperatures, and the present findings are consistent with this body of evidence. The extension of this stability characterization to enzymatic activity, which is inherently more labile than stable protein concentrations - strengthens the case for BSC-based sample collection in this application.

One important caveat regarding the temperature stability experiment is that BSC storage durations were not identical across subjects due to experimental troubleshooting that required some gels to be rerun. Cards were stored for approximately one week to one month before analysis, which introduces variability in absolute storage time across subjects. While this does not affect the temperature comparison within a given subject (each subject's cards were stored at

all three temperatures for the same duration), it prevents direct time-point analysis and limits the ability to disentangle the effects of temperature from those of absolute storage duration. Future experiments should use a fully factorial design with matched storage times across all subjects at each temperature and time point to address this limitation rigorously.

4.7 Conclusion

This study demonstrates the feasibility of protease activity measurement, including cathepsin S and chymotrypsin activity, in PDAC patients from whole blood spotted onto dried blood spot cards. The activity is preserved without significant degradation across storage temperatures of 4°C, −20°C, and −80°C. Cathepsin S and chymotrypsin exhibited concentration-dependent cleavage of their respective substrates, confirming assay functionality and establishing a reliable basis for biological sample analysis. Elevated protease activity was observed in PDAC patient plasma samples, supporting previous findings that these enzymes may serve as relevant biomarkers of pancreatic disease.

A major finding of this work was that whole blood, including BSC eluates, produced higher protease activity signals than matched plasma samples. These results indicate that the cellular fraction of blood contributes substantially to measurable protease activity and that this information is preserved within the dried blood spot matrix. Furthermore, BSC eluates retain significantly greater protease activity than plasma, demonstrating that dried blood spot collection can effectively capture biologically meaningful enzymatic activity without the need for centrifugation or specialized processing.

Storage studies revealed no significant differences in protease activity among samples stored at 4°C, –20°C, or –80°C for a long-term storage period of 4 weeks). This finding suggests

that dried blood spots provide a stable environment for protease preservation and support the possibility of refrigerator-based storage and transport. Such flexibility could greatly expand the accessibility of protease-based diagnostics by enabling remote and home-based sample collection.

While additional work is needed to validate these findings in larger patient cohorts, optimize assay conditions, and establish diagnostic thresholds, the present study provides the first evidence that PDAC-associated protease activity can be measured from dried blood spot samples. Collectively, these results establish an important foundation for the continued development of BSC-based protease diagnostics and support future efforts aimed at creating a minimally invasive, accessible platform for early PDAC detection.

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