Leveraging biotransport mechanisms in the design of technologies to improve access to blood-based diagnostics

By

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A dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering at Brown University

PROVIDENCE, RHODE ISLAND

MAY 2023

This dissertation by Kiara R. Lee is accepted in its present form by the Biomedical Engineering program, a joint program in the Division of Biology and Medicine and the School of Engineering as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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Curriculum Vitae

EDUCATION

Brown University *PhD candidate in Biomedical Engineering* Mar. 2023
- Joukowsky Family Outstanding Dissertation Award Recipient

Brown University *ScM in Global Public Health* Feb. 2023

University of Pittsburgh *Bachelor of Science in Bioengineering, 2018* Pittsburgh, PA
- Magna Cum Laude
- Minors: Spanish & Chemistry; Certificate: International Engineering Studies

RESEARCH PROJECTS

PhD Advisor: Dr. Anubhav Tripathi, Brown University, Providence, RI
- Project #1: Investigating miniaturization for the extraction of genomic DNA from whole blood for sequence-based diagnostics
  - Created a microfluidic protocol for extraction of high-quality DNA from a very small volume of whole blood, using magnetic bead chemistry
  - Mathematically characterized the movement of DNA through the microchannel
- Project #2: Development of a novel microfluidic chip for RNA extraction
  - Developed a microfluidic method for extraction of high-quality RNA from a very small volume of whole blood, using magnetic bead chemistry
  - Modeled, using COMSOL, electrophoretic and electroosmotic velocities of the microfluidic channels
- Project #3: Design of a device to enhance DNA extraction from dried blood spots using an electric field
  - Created a low-cost, electrical-based device for dried blood spot DNA extraction in 5 minutes
  - Performed an in-depth mechanistic analysis of dried blood spot DNA adsorption and desorption
- Project #4: Lyophilization of DNA/RNA amplification techniques to reduce the need for reagents' cold-chain shipping for HIV diagnosis and monitoring
  - Testing lyophilization of various mixtures of PCR, qPCR, and RT-qPCR reagents
- Project #5: Qualitative needs assessment for clinicians and laboratory personnel working in the Dominican Republic
  - Principal investigator for a study performing qualitative interviews with physicians and laboratory personnel in the Dominican Republic to understand needs around point of care testing implementation

M.S. Advisors: Dr. Shira Dunsiger and Dr. Jennifer Pellowski, Brown University, Providence, RI
- Thesis: "The impact of racism and discrimination in racial and ethnic disparities in hypertensive disorders of pregnancy, a PRAMS analysis"
  - Principal investigator for a study that performed logistic regression analyses of the association between discrimination and racism with hypertensive disorders of pregnancy

PUBLICATIONS

- **Lee, K.,** Arango, D., Rosen, R., Agénor, M., Tripathi, A., Rodriguez, C., and Sanchez, M., "Biological sample analysis technology development for the point of care: a qualitative needs assessment for scientists working in the Dominican Republic" (*in preparation*)
- Campbell, K.M., Jakachira, R., Harling, M., **Lee, K.,** Behar, M., Block, A., Howes, A., Karambizi, D. Krishna, K., Lin, Z., Masri, A., McCall, A., Shah, A., Petrus, A., Sharan, R., Dodson, C., Gray, M., Hilliard, R., Hu, E., Kornegay, K. Mathiowitz, E., Patena, J., Ranney, M.L., Shukla, A., Kingon, A., Mammen, J., Toussaint, K.C. "Exploring opportunities for advancement in home-health technologies: Outcomes of the "Health-Home Technologies in 2032" workshop" (*in preparation)*

- **Lee, K.,** Pellowski, J., and Dunsiger, S., "The association of racism and discrimination in disparities of hypertensive disorders of pregnancy: an analysis of PRAMS data" (*under review*)
- **Lee, K.** and Tripathi, A., "Mechanistic insights into genomic DNA extraction from dried blood spots for the development of standardized nucleic acid extraction protocols". 2022. *Biopreservation and Biobanking*. (*accepted*)
- Brakewood, W.*, **Lee, K.***, Schneider, L., Lawandy, N., and Tripathi, A. "A Capillary Flow-driven Microfluidic Device for Point of Care Blood Plasma Separation". *Front. Lab on a Chip*, 1(November):1-12. 2022.
- **Lee, K.**, Murphy, J., and Tripathi, A., "Electro-DBS: A Simple Method to Rapidly Extract Genomic DNA from Dried Blood Spots". *Anal. Chem*. 2022.
- **Lee, K.** and Tripathi, A., "An investigation into improving RNA integrity for transcriptomics with an electrokinetically-driven microfluidic protocol". *Biomicrofluidics*, vol 16, no. 044107, pp. 1-13, 2022.
- **Lee, K**., Brayboy, L. and Tripathi, A. Pre-eclampsia: a Scoping Review of Risk Factors and Suggestions for Future Research Direction. *Regen. Eng. Transl. Med.,* 2022.
- **Lee, K.** and Tripathi, A., "Parallel DNA extraction from whole blood for rapid sample generation in genetic epidemiological studies," *Front. Genet.,* vol. 11, no. 374, pp. 1–16, Apr. 2020.

FELLOWSHIPS

- NIH Fogarty LAUNCH Fellowship Jul. 2023 – Jul. 2024
- National Science Foundation (NSF) Graduate Research Fello Aug. 2020 – Aug. 2023
- Ford Foundation Predoctoral Fellowship, offered (declined) Apr. 2020
- Ford Foundation Predoctoral Fellowship Honorable Mention Apr. 2019
- Brown University Initiative to Maximize Student Development (IMSD) Fellow 2019-2020
- GEM Associate Fellow 2019-Present
- Donald Henderson Scholar: University of Pittsburgh full tuition, room and board scholarship (2013-2018)
- Eaton Multicultural Scholarship May 2015

TEACHING & MENTORSHIP

Student Mentorship:

- Mentorship of two undergraduate students with wet lab experiments toward undergraduate theses
- Pitt EXCEL Graduate School Seminar Series Mentor (2020-2021)
    - A series dedicated to exposing undergraduate students to graduate school and aiding those applying with all aspects of the applications process

Teaching Assistant: School of Engineering, Biomedical Engineering Capstone, Brown University, *Fall 2021*

Teaching Assistant: School of Engineering, Topics in Translational Medicine, Brown University, *Spring 2020, Spring 2021, Spring 2022*

Summer @ Brown Instructor: Engineering for Biomedical Systems, Brown University, *July 2019*

- Designed and taught a course for high school students focused on diagnostic devices
- Designed and led laboratory experiments regarding microfluidics

Teaching Assistant: Summer @ Brown, Brown University, *Summer 2019 & Spring 2020*

Teaching Assistant: School of Engineering, Biomaterials, Brown University, *Fall 2018 & Fall 2019*

- Held weekly office hours and graded student assignments and exams
- Worked with a team of teaching assistants and lead review sessions regarding course material

LEADERSHIP

Initiative to Maximize Student Development Senior Scholar September 2021

- Teaching assistant for the Demystifying the PhD Module, in which we discussed with students the PhD process and mentored them through creating their PhD development plan

Graduate Community Fellow Application Reviewer Sep. 2020

- Review applications of students applying to be Graduate Community Fellows

Brown University Resuming Research Committee Member Jun. 2020 – Jan. 2022

- Committee developed to develop reopening plans to resume research on Brown's campus after COVID-19 closures
- Offer suggestions from the graduate student perspective and critically think about issues presented to the committee

Graduate Community Fellow – DiversiTEAs Aug. 2019 – May 2020

- An award to groups of graduate students for the design and implementation of community-building initiatives for the Brown community
- As co-president of Graduate Students of Color in STEM (GSOCnSTEM) collaborated with leaders of out in STEM (oSTEM) and Society for Advancement of Chicanos/Hispanics and Native Americans in Science (SACNAS)
- DiversiTeas is a community series developed to connect students, faculty, and staff within the STEM community at Brown under the umbrella of "Identity".

Womxn in STEM Symposium Planning Committee Member Mar. – Oct. 2019

- The WiSTEM Symposium is a student-organized conference for womxn completing their masters, doctoral, and postdoctoral training
- Solicited faculty volunteers and career panelists, evaluated attendee applications, and planned logistical items

Brown Biomedical Engineering and Biotechnology Graduate Advisory Board – Invited Speakers Committee Sep. 2019 - Present

- Poll students to choose external speakers to invite on behalf of students to our department's seminar series
- Communicate with faculty members and administrators to organize small student group meetings with the invited speakers

Co-founder: Graduate Students of Color in STEM (GSOCnSTEM) at Brown University (Jan. 2019)

- Co-president (Jan. 2019 – Aug. 2020)
    - Organize the executive board, plan events, and solicit funds
- School of Engineering Student Advisory Board Representative (Aug. 2020-Aug. 2022)

Biomedical Engineering Society (BMES) Aug. 2015 -- Present

- International Affairs Subcommittee Member (Jan. 1, 2021 – Dec. 2023)

National Society of Black Engineers (NSBE) Aug. 2013 - Present

- Initiation of and participation in Brown University graduate student recruitment and representative at local and national conferences (November 2018, March 2019, November 2019, August 2020)
- Delivered a talk titled "Demystifying the PhD" to introduced undergraduate students to graduate school (November 2018)
- University of Pittsburgh Chapter's Business Diversity Chair (2014-2015)
    - Connected students with industry contacts and resources

CONFERENCES

Keystone Symposium - COVID and Beyond: Novel Approaches to Global Infectious Diseases – Poster Presentation Oct. 2022

Recent Advances in Biomedical Diagnostic Platforms, Institute of Medical Science - Mongolia – Virtual Oral Presentation Sep. 2022

New England Science Symposium – Virtual Poster Presentation Apr. 2022

Biomedical Engineering Society Annual Meeting – Virtual Poster Presentation Oct. 2020

Global Health Innovation Conference – Virtual Poster Presentation Apr. 2020

Womxn in STEM Symposium – Oral Presentation Oct. 2019
AfroBiotech 2019 – Poster Presentation Oct. 2019
Biomedical Engineering Society Annual Meeting – Poster Presentation Oct. 2017
Biomedical Engineering Society Annual Meeting – Poster Presentation Oct. 2015

MEETING ABSTRACTS

- **Lee, K.,** Murphy, J., and Tripathi, A. "Electro-DBS: A Simple Method to Rapidly Extract Genomic DNA from Dried Blood Spots". Keystone Symposium - COVID and Beyond: Novel Approaches to Global Infectious Diseases, Brussels, Belgium, October 2022 (*Poster presentation*)
- **Lee, K.,** Murphy, J., and Tripathi, A. "Electro-DBS: A Simple Method to Rapidly Extract Genomic DNA from Dried Blood Spots". Recent Advances in Biomedical Diagnostic Platforms, Institute of Medical Science – Mongolia, Virtual Conference, September 2022 (*Oral presentation*)
- **Lee, K.,** and Tripathi, A. "An investigation into improving RNA integrity for transcriptomics with an electrokinetically-driven microfluidic protocol". New England Science Symposium, Virtual Conference, April 2022 (*Poster presentation*)
- **Lee, K.**, Tripathi, A. "Parallel DNA Extraction from Whole Blood for Rapid Sample Analysis in Genetic Epidemiological Studies", BMES 2020 Annual Meeting, Virtual Conference. April 2020. (*Poster Presentation)*
- **Lee, K.**, Tripathi, A. "Parallel DNA Extraction from Whole Blood for Rapid Sample Analysis in Genetic Epidemiological Studies", Global Health & Innovation Conference, Virtual Conference. April 2020. (*Poster Presentation)*
- **Lee, K.**, Tripathi, A. "Parallel DNA Extraction from Whole Blood for Rapid Sample Analysis in Genetic Epidemiological Studies", Womxn in STEM Symposium, Providence, RI. October 2019. (*Oral Presentation)*
- **Lee, K.**, Tripathi, A. "Parallel DNA Extraction from Whole Blood for Rapid Sample Analysis in Genetic Epidemiological Studies", AfroBiotech 2019, Atlanta, GA. October 2019. (*Poster Presentation)*
- **Lee, K**., Lampejo, A., Hernadez-Gordillo, V., Griffith, L., "Assessing Intestinal Organoids Drug Response in A Well-Defined Synthetic Basement Microenvironment", BMES 2017 Annual Meeting, Phoenix, AZ. October 2017. (*Poster Presentation*)
- **Lee, K**. McDonald, I. Graves, L., "CRISPR-Cas9 Mediated Knockout of MELK in Panc-1 Cells", BMES 2015 Annual Meeting, Tampa, FL. October 2015. (*Poster Presentation*)

OTHER RESEARCH EXPERIENCE

Drug Discovery Institute, Student Researcher, Pittsburgh, PA Oct. 2017-Apr. 2018
- Comparison of effects of gene knockout via CRISPR in diseased hepatocytes

Amgen Scholars Program – MIT, Cambridge, MA Jun. 2017-Aug. 2017
- Assessed intestinal organoids drug response in a well-defined synthetic basement membrane microenvironment

Baxter Healthcare, Pharma Solutions Engineering Co-op, Round Lake, IL
Jan. 2017-May 2017
- Wet bench chemistry, technical writing and presentation skills, lyophilization, non-isothermal kinetics modeling, solubility screening by UV spectroscopy, and gas permeation modeling (with Berkeley-Madonna).

Baxter Healthcare, Renal Systems Engineer Co-op, Round Lake, IL 2015 - 2016
- Technical writing & experimental design: class B protocol and report writing
- Document management: organizing documents within various document management systems
- Compliance: familiarity with REACH compliance

University of North Carolina, Chapel Hill Summer Research Program (SURE)
May 2015-Jul. 2015
- Investigated the kinome responses in pancreatic cancer to targeted therapeutics

ACTIVITIES

Reviewer: *Clinical Chemistry* Sep. 2022

Rhode Island Free Clinic Volunteer May 2021 – Aug. 2021

- COVID-19 Vaccination Clinic: Helped check-in and check-out mostly Spanish-speaking members of the Providence community during their visit to receive their COVID-19 vaccine

Brown Effective Performance Workshop Participant Jan. 2020 – Feb. 2020

- Applied and selected to participate in a workshop led by professional actors to give graduate students performance and improvisation techniques to improve presentation and storytelling skills

Emory Global Health Case Competition Participant Mar. 2019

- Competition to plan and present a case to a specific global health issue
- Nominated by Brown University to attend with five other students

Pitt Study Abroad Programs

- Plus3 Chile (May 2014): Participated in site visits at various companies during a two-week study abroad experience for business and engineering students
- Engineering the Americas, Peru (May 2015): Analyzed the civil engineering methods in the design and construction of major pre-Columbian structures and participated in a volunteer project
- Global E3, Spain (Jan. 2016 – Jun. 2016): Semester spent studying abroad at the Polytechnic University of Madrid taking engineering classes in Spanish

Panel Participant

- Brown Graduate Students of Color (GSOC) Initiative
    - GSOC Orientation, August 2019 and 2021: "You're a WHOLE graduate student: A Conversation on Holistic Graduate Student Life"
    - GSOC Virtual Orientation August 2020: "Applying for (and winning!) Fellowships"
    - GSOC Preview Day, October 2019 and 2020 (Virtual) – Graduate Student Panel
    - Super Monday, March 2019 – 1st year Graduate Student Panel
- Pitt EXCEL
    - DIVA Alumni panel (Virtual), November 2020: "What is success?"
- Pitt Engineering International Programs
    - Global Engineering Engagement Series, October 2020 and January 2023: "Virtual Graduate School Panel"
- Pitt Pink Panthers
    - Young Alumnae Night (April 2021 and 2022)

SKILLS

Conversational in Spanish; Beginner in Portuguese; Proficient in Matlab, R, COMSOL Multiphysics, and Microsoft Excel

Preface and Acknowledgements

There are so many people that were a part of this journey that I would like to thank. Firstly, thank you to my advisor, Dr. Anubhav Tripathi, for allowing me to work with you. Even though I don't always show it, I really have appreciated your guidance and encouragement as I tried to find my voice in this field. I know this is only the beginning of our mentor-mentee relationship.

Thank you to my committee members Dr. Tejal Desai, Dr. Rami Kantor, Dr. Mark Lurie, and Dr. Kimani Toussaint for helping me get to this point. I admire each of your science and am in awe of your powerhouse contributions. I also appreciate your dedication to your fields and for crossing fields to give me fantastic feedback. Thank you to my funding sources, NSF, IMSD, the Open Graduate Education Program and GEM for believing in me and supporting me to do the work I sought out to do. Thank you to Dean Vanessa Ryan for being in my corner and cheering me on. Thank you to Dr. Chris Rose for being you and for always taking the time to listen to me and cheer me on. Thank you to John Murphy for your help in designing the Electro-DBS device, working with you was always so fun.

Thank you to the qualitative researchers that taught me everything I know, Dr. Alex Collins, Dr. Rochelle Rosen, and Dr. Madina Agénor. You really helped me to discover another aspect of public health and science that I am passionate about.

I'd also like to recognize the participants of my studies that I met or did not meet, thank you for trusting me to share your voice or use your samples. This work would be truly impossible without you. Thank you to Vinald Francis for his work to make the illustrations in this dissertation, working with you is always so fun.

Lastly, to my family and friends, thank you for your love, support, and interest in my work; every “like” and “comment” on my social posts about my research means so much to me even if you don’t fully understand it. Thank you to Maria Isabel, who without meeting you through this program introduced me to a dance community I had been searching for at Metamorphosis Dance Company. Thank you to my friends at Brown, you all showed me graduate school could be fun. To my friends outside of Brown and Providence, thank you for sticking with me on this journey, it has been amazing to watch you all grow as well. Thank you to Dr. Sossena Wood for encouraging me to stand up for myself and establish my power in the academy. To my parents, in-laws, siblings, and grandparents, thank you for your endless support and patience. I cannot express in words how much your encouragement has meant to me. To my grandfather, I know how much this would’ve meant to you to be able to see, but I know you are cheering me on from above. You always made sure to (and continue to) remind me that this degree is bigger than me, but a demonstration of the progress we as African Americans have made in this country.

To my boyfriend, turned fiancé, turned husband as I got this degree, thank you for reading over all my work, listening to my presentations, and for picking me up when I was down. Thank you for being my support system as I tried to balance it all.

Thank you all for keeping me afloat. It truly takes a village.

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CHAPTER 1: INTRODUCTION

1.1 Motivation

At least half of the world's population lacks access to essential health services, and point of care testing is one solution that can improve access to healthcare in various settings [1]. Point of care devices developed for use with blood of are of utmost importance as blood is a commonly used sample for monitoring of one's health status and for disease diagnoses. This includes glucose monitoring, diagnosis with a viral or bacterial infection, monitoring of cancer, and more. However, blood-based diagnostics developed for the point of care face challenges as blood-based analyses rely on cold chain shipping, must remove inhibitors of downstream processes, are highly manual, and require large sample quantities. Nucleic acid extraction from blood, which is a large focus of this dissertation, also faces these challenges, as several reagents and techniques are required to extract and purify high quality nucleic acids. Thus, this dissertation focuses on reducing the burden of blood-based diagnostic testing at the point of care and incorporating user voices in the development of these technologies.

1.2 The importance of qualitative needs assessments for point of care device development

The concept of translational research emerged in the 1990s [2] and can be defined as ensuring that new treatments and research knowledge actually reach the populations for whom they are intended and implemented correctly [3]. However, approximately 70% of ventures fail due to the product not meeting the customer's needs [4]. Studies have shown that it is beneficial to have user perspectives when developing devices because users can generate ideas, improve design and user interfaces, aid in the improvement of the functionality, usability, and quality of the developed device [5], [6]. There are challenges to getting user involvement, though, which include lack of resources, inability or

unwillingness of the users to give feedback, ethical concerns, and manufacturers' unwillingness to involve users [5]. A qualitative study by Money et al. interviewed developers of medical device technology and found that they did not see the benefit of engaging with users [7]. This study also showed that the one technology developer out of the eleven interviewed thought engaging users was important and they likely had that notion due to their educational experience involving user-centered design [7].

When developing devices for the point of care (POC), which is a large focus of the World Health Organization (WHO), similar trends are seen, in which there are a few devices that have gone beyond the benchtop [8] and needs assessments are rarely performed [9]. If they are performed, they are focused on clinicians and not with other users [8]. Qualitative methods are recommended when developing and performing a needs assessment [10], and getting a diversity of opinions is essential for developing an impactful product [11]. A qualitative study by Engel et al. showed that POC device developers believe that understanding user needs is insufficiently done, and that this process should be done iteratively [9]. Shah et al. developed a theoretical framework for involving users in the development process, and for the first stage of device development (idea generation) they state that methods such as ethnography, expert user meetings, and interviews are necessary and should be performed iteratively to continually gain feedback [12]. Additionally, Engel et al. highlighted that POC devices are marketed as being able to be used in the absence of laboratory equipment and infrastructure, though this is not often the case [9]. Refrigeration or expensive laboratory items are often still necessary for POC device to function. This is especially an issue when working with blood-based analyses [13].

1.3 The diagnostic value of blood

The use of human whole blood for diagnostics began to expand and advance rapidly in the 17th century, largely due to the invention of the microscope. In the body, blood is responsible for transporting nutrients and waste and for the regulation of body temperature, osmotic pressure, and pH of body fluids [14]. Thus, blood contains several indicators of one's health status. Whole blood is made up of several components, which include red blood cells, white blood cells, and plasma. Plasma (~55%) is the liquid component of blood that contains clotting factors (plasma without clotting factors is called serum [15]), circulating nucleic acids, ions, and other molecules [14], [16]. White blood cells (<1%) are several nucleated cells that contain human genomic DNA and total RNA. These cells lead the body's ability to fight infection [17]. Lastly, red blood cells are non-nucleated cells that have the biggest importance on blood rheology, i.e. red blood cells largely influence blood's viscosity, shearing capabilities, and flow properties. Additionally, red blood cells contain hemoglobin which carry the oxygen that is distributed to tissues throughout the body. Human blood is used for a number of screenings and diagnostics regarding one's health, including coagulation, viral diagnoses, parasite diagnoses, monitoring of therapies, and detecting several other biomarkers [14].

1.4 Blood stored in a vacutainer versus dried blood spots

Blood is collected for screening and diagnostics using two main sample types: vacutainer and dried blood spots. A vacutainer is a tube that contains a vacuum to obtain the proper amount of blood from the patient. These tubes are also able to be centrifuged. Most importantly, they typically contain an anticoagulant such that the red blood cells do not coagulate to interfere with analyses [18]. Dried blood spots are droplets of blood obtained via a finger prick that are placed onto a paper matrix. These methods are compared in Table 1.1.

Table 1.1 Comparison of vacutainer and dried blood spot samples.

Sample type	Vacutainer	Dried Blood Spots (DBSs)
Collection method	Venipuncture	Finger prick
Collection materials	Needle and Vacutainer	Lancet and DBS paper
Volume of sample collected	Typically 25mL [14]	15-200 μL [19]
Length of storage at room temperature	Few days [20]	2 weeks [19]
Optimal storage condition	4°C [21]	-20°C [19]
Time for gDNA viability	>130 days [21]	Decades [22]
Time for total RNA viability	Few days [23]	20 years [22]
Challenges	• Not stable for long times at room temperature • Requires cold chain for shipping • Some diagnostic tests require centrifugation	• Stable for longer times at room temperature • Can be sensitive and difficult to obtain quantification • Low volume • More expensive than tubes • No standardized protocols for nucleic acid extraction • Unable to isolate specific blood components
Benefits	• Gold standard • Many years of use • Able to isolate specific blood components via centrifugation • Well-established protocols for nucleic acid extraction • Commercially-available robots developed for processing from sample to result	• Newer technology • Does not require cold chain for shipping • Smaller sample volume than venipuncture • Commercially-available robots developed for processing from sample to result

Generally, blood obtained via venipuncture and stored in a vacutainer is more commonly used than dried blood spots for several reasons. Venipuncture is a gold standard method that has many established protocols for collection, storage, and sample processing. Additionally, it allows for isolation of specific blood components (i.e., plasma, white blood cells, and red blood cells) via centrifugation. As for dried blood spots, they can be shipped at room temperature, require a smaller volume of sample, and are highly useful for neonatal diagnostics, in which obtaining a blood sample via venipuncture may be difficult. There are several challenges to dried blood spots, though, which include a lack of standardized protocols for nucleic acid extraction and the low abundance of analytes which leads to challenges in sensitivity and quantification of the analytes [24]. The low abundance of analytes is especially a challenge for analyzing nucleic acids for viral infections, genome sequencing, and more.

1.5 Nucleic acids in blood: the process of obtaining them and challenges

Nucleic acids (NAs) in blood are found within white blood cells (genomic DNA and total RNA) and plasma (circulating DNA and RNA, microRNAs, viral particles). This thesis largely focused on genomic DNA and total RNA to eventually be used for whole genome or whole transcriptome sequencing, respectively. For sequencing or other applications, obtaining high-quality nucleic acids, that is, non-degraded, is arguably the most important step to analyzing them. In addition to obtaining non-degraded nucleic acids, inhibitors that may be present in the sample or that are introduced through the purification process should be removed. Blood contains several inhibitors to NA stability and to downstream processing like PCR, which include heme, the heparin anticoagulant, and some hormones [25]. For RNA, RNases found in the blood and the environment degrade RNA [26].

NA extraction has evolved from using harsh chemicals such as chloroform to a method called solid-phase extraction (SPE) [27]. SPE is based on liquid and solid phases,

with NAs being adsorbed onto the solid phase depending on the pH and salt concentrations of the buffers used. After the NAs are adsorbed onto the solid phase, they must be washed and eluted off. Various solids for adsorption have been used in SPE and typically involve centrifugation, vacuum filtration, or column separation during the wash and elution steps [28]. These processes during the wash and elution steps increase experimental time and require additional equipment. Advances in SPE methods have allowed for the use of magnetic beads as the solid phase. NAs bind to the magnetic beads and they are manipulated by a magnet for washing, which removes the need for centrifugation, vacuum filtration, or column separation (Figure 1.1). To elute the NAs off the beads, the NA-bound beads are simply placed into an elution solution, leaving the isolated NAs in the supernatant.

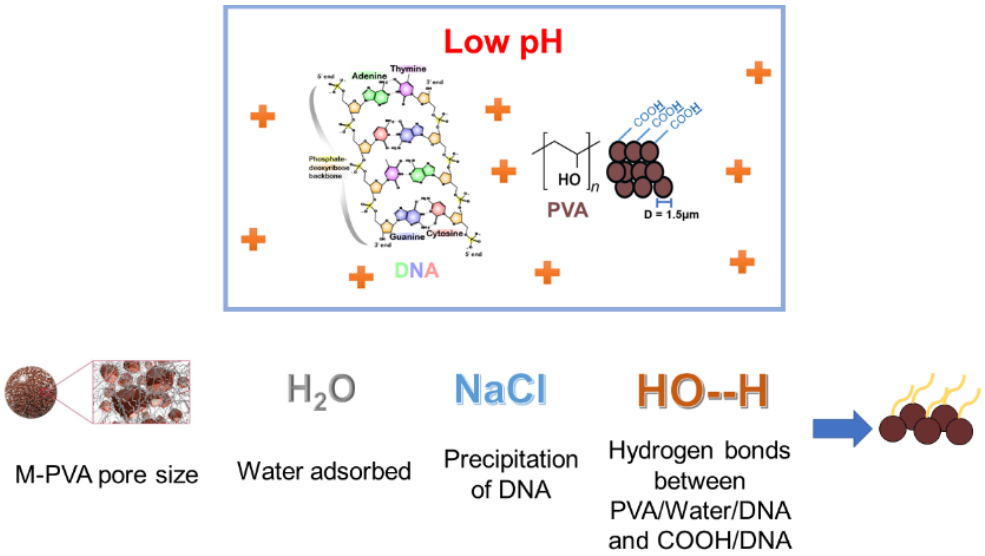

Figure 1.1: Mechanism for DNA binding to M-PVA beads.

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This thesis, in Chapter 4 and Chapter 5, use commercially available polyvinyl alcohol magnetic particles (M-PVA beads) for NA extraction [29]. These beads have low unspecific protein binding properties, high functionalization potential, and high magnetite

content. These factors contribute to maximizing the purity of the NA eluate and the speed of extraction. Additionally, these magnetic beads do not require the use of chaotropic salts for NA binding [30]. Chaotropic salts, which are typically present in lysing and binding buffers during SPE, are polymerase chain reaction (PCR) inhibitors, so the reduction of their presence increases the likelihood of successful PCR [31]. The literature on the exact mechanisms of M-PVA beads and NA binding is limited; however, underlying mechanisms for NA binding to silica beads are well-documented. Based on the available literature [32]–[34] NAs primarily bind to the silanol groups of silica beads via hydrogen bonding. Following the NA-silanol binding mechanism, a simple model for NAs binding to M-PVA beads is proposed here (Figure 1.1). In the case of DNA, it is assumed that M-PVA beads are functionalized with carboxylic acid groups [29], [35]. For RNAs, M-PVA beads are functionalized with oligonucleotides to bind their poly(A) tails [30]. The beads are also fabricated to contain pores to increase surface area for hydrophilic interactions. For binding, the DNA present in the bulk solution is salted out onto the bead surfaces using a salt solution (indicated by '+' in Figure 1.1). At low pH the phosphate groups of DNA are protonated, as are the non-functionalized hydroxyl groups and the carboxylic acid groups of PVA. Then, this pH allows for the adsorbed water to act as a bridge, forming hydrogen bonds between PVA and DNA; hydrogen bonds are also formed between DNA and the carboxylic acid groups. RNA binding likely works similarly, but by binding more specifically via the poly(A) tails. The efficiency of DNA binding can be maximized by optimizing surface functionalization, available surface area of beads, and ionic composition of DNA buffer. This binding mechanism has allowed for more simple formulations of kits that extract nucleic acids using magnetic bead technologies. The utility of magnetic beads for point-of-care devices has been made clear through their incorporation into microfluidic devices.

1.6 The point of care and microfluidic devices

Point of care technologies are technologies and tools developed to perform laboratory-like analyses outside of the laboratory. There are several types of devices that have been developed, some of which are commercially available for various types of blood nucleic acid-based testing like for HIV viral load and circulating DNA [15], [36]. These devices aim to increase access to molecular testing and monitoring and can be liquid handling robots, microfluidic devices, paperfluidic devices, and more.

Microfluidic devices are a great solution to reducing the sample preparation and detection burden [37]. These devices are made in a multitude of ways, including paper-based and polymer-based fabrication systems. They use smaller (on the microscale) sample and reagent volumes and can be automated, meaning few manual steps are needed to be performed by the user. Additionally, lower sample volumes decrease the presence of molecules that will inhibit downstream sequencing processes [38]. For users and patients, this is beneficial because: 1) a significantly lower patient sample volume is needed; this implies potentially smaller amounts of sample to be taken, 2) a lower quantity of reagents is necessary, which reduces shipping costs and user costs, and 3) faster results for diagnostic tests due to the automation of NA extraction protocols and the ability in some systems to run multiple reactions at once. All of these factors make microfluidic devices advantageous at the point of care, which is extremely valuable for low resource areas.

1.7 Summary

This thesis leverages various disciplines in the design of technologies to improve access to blood-based nucleic acid diagnostics. This was approached in three main pathways: qualitative understanding of the multi-faceted needs for obtaining a diagnosis, microfluidic device protocol development to simplify nucleic acid-magnetic bead extraction

and purification methods, and the development of a new tool, Electro-DBS, for faster and more standardized gDNA extraction from DBSs. The innovations, impact, and a map of contributions of each chapter in this dissertation are shown in Figure 1.2.

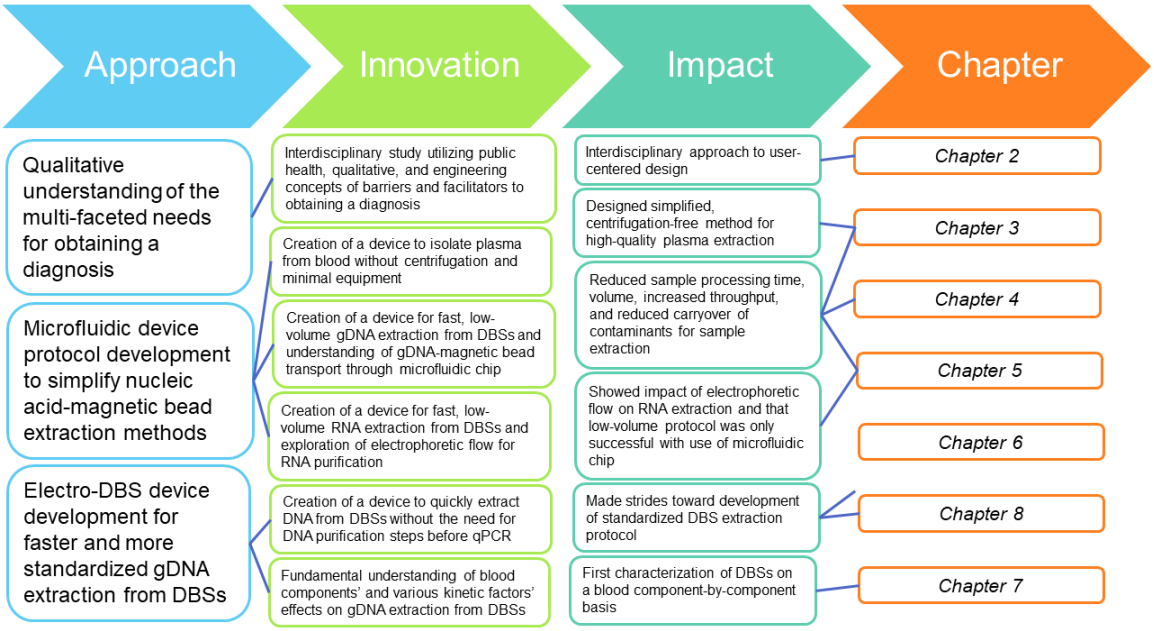

Figure 1.2 Diagram of thesis contributions to the design of technologies to improve access to blood-based nucleic acid diagnostics.

Chapter 2 is a qualitative approach to understanding challenges and opportunities to obtaining a HIV diagnosis in the Dominican Republic. This study involved interviewing 36 lab personnel and clinicians at a large, public hospital in Santiago de Los Caballeros, Dominican Republic. Participants discussed barriers and facilitators to obtaining a diagnosis, as well as characteristics they'd like to see in a new point-of-care device. Themes were developed using the social ecological model and several recommendations were made to device developers based on user input. This chapter demonstrates an interdisciplinary approach to user-centered design to maximize the impact of future device designs. This work provided evidence of the importance of the laboratory-based work in the rest of the chapters.

Chapter 3 describes the design, optimization, and validation of a microfluidic device to isolate plasma from whole blood using capillary flow. A microfluidic device was designed that contained a filter to separate plasma from other blood components and used capillary flow to collect the isolated plasma into a collection well. This chapter investigates the role of hematocrit, various parameters regarding sample input, and lastly quantifies the amount of proteins obtained via the microfluidic device compared to those obtained when plasma is isolated via centrifugation. The device was consistent in isolating plasma in samples with various hematocrit percentages ranging from 25 – 65%. Plasma was produced with a maximum separation of 88.5% and we were able to obtain 14 μL of plasma from a 50 μL whole blood input. This work used knowledge gained from Chapters 4 and 5 to develop a more simplified microfluidic device.

Chapter 4 begins our work with nucleic acid extraction from whole blood, with the development of a microfluidic chip protocol to extract and purify gDNA from diluted whole blood. In this chapter we developed a protocol in which blood lysate with gDNA bound to paramagnetic beads was placed into the input well of the microfluidic chip. Then, the gDNA-magnetic bead complex was pulled, using a magnet, through a central channel containing a wash buffer into the output well, which contained an elution buffer. We used an input of 4 μL of diluted blood and a total of 75 μL of reagents per extraction. Additionally, with the microfluidic design, we could extract gDNA from up to six samples simultaneously. This resulted in a >99% reduction in starting blood volume, 98.6% reduction in volume of reagents used that yielded comparable quality of gDNA for downstream processing (qPCR and preparation for sequencing).

Chapter 5 builds upon the work in Chapter 4 with the design of a microfluidic chip and development of a protocol for total RNA extraction. This microfluidic chip was based on that in Chapter 4, with adjustments to accommodate the larger volumes of samples

needed for this sample and to improve solution loading. The sample input into the chip was white blood cell lysate, enzymatically degraded DNA, and total RNA bound to magnetic beads. The magnetic beads were pulled through the center wash channel with a magnet into a wash buffer and elution was performed off the microfluidic chip. With this microfluidic protocol the sample input volume was reduced by 95.2% and reagent volumes were reduced by 93.6%. We investigated the use of electrokinetic flow to further purify the RNA, and found that electrophoretic transport was the most effective way to purify the RNA. Lastly, this chapter showed that reducing the volumes of reagents and samples was only successful in yielding high quality total RNA when using the microfluidic chip.

Chapter 6 begins our work with dried blood spots by exploring and understanding mechanisms the aid and inhibit gDNA from being extracted from dried blood spots (DBSs), using knowledge gained in Chapters 4 and 5. We aimed to aid in establishing a standardized method for gDNA extraction from DBSs. We explored several protocols for gDNA extraction and compared their yields. Then we explored the role of individual blood components (white blood cells, plasma, and red blood cells) in obtaining gDNA. We found that using a red blood cell lysis in the extraction procedure increased yield 1.5 to 5-fold. Lastly, we tested the importance of several kinetic factors (heat, agitation, and chemical lysis) in extraction yield and found that a chemical lysing agent in combination with either heat or agitation was sufficient to elute PCR amplifiable gDNA in 5 minutes.

Chapter 7 describes our development of a novel device for rapid gDNA extraction from DBSs, using knowledge gained from Chapter 4. This device uses electrophoresis to extract gDNA from DBSs for direct use with qPCR by having a 6 mm punch of a DBS placed between two electrodes that are connected to a DC power supply. We showed that the gDNA obtained using the Electro-DBS device contained less contaminants than with

a manual protocol. The yield was most comparable when using a lysing buffer versus a non-lysing buffer for extraction.

Chapter 8 explores the application of the Electro-DBS method to total RNA and HIV-1 RNA extraction from dried blood spots, building upon the work in Chapter 7. We tested a variety of parameters related to the Electro-DBS system, including length of time the electric field is applied, the 96-well plate, the Electro-DBS components, buffers, and the impact of the electric field on purified luekocyte RNA. We found that this method has potential to yield total RNA, but multiple parameters should be explored to optimize the Electro-DBS for this analyte.

Chapter 9 concludes the thesis with a summary of the work and its impact, limitations of the work, future directions, and recommendations.

CHAPTER 2: BIOLOGICAL SAMPLE ANALYSIS TECHNOLOGY DEVELOPMENT FOR THE POINT OF CARE: A QUALITATIVE NEEDS ASSESSMENT FOR SCIENTISTS WORKING IN THE DOMINICAN REPUBLIC

This chapter will be published as an original research article.

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2.1 Introduction

Point of care technologies are devices that are used at or near the site of care [39]. The World Health Organization recommended in 2003 that these devices should meet the ASSURED criteria: “Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and can be Deliverable to end-users. Recently this has been updated to REASSURED criteria to include Real-time connectivity (in the context of digital technologies) and ease of specimen collection [40], [41]. It is thought, and has been shown, that point of care devices can improve one’s access to healthcare by decentralizing healthcare, lowering cost of healthcare, increasing speed for a person to results, and more. However, developers rarely consider the clinical barriers or factors in the adoption of the technology in early development [11]. There are several barriers to adoption such as regulatory issues, economic constraints, and lack of research to understand the needs of stakeholders [11], [42].

Studies have shown that it is beneficial to have user perspectives when developing devices because users can generate ideas, improve design and user interfaces, aid in the improvement of the functionality, usability, and quality of the developed device [5], [6]. There are challenges to getting user involvement, though, which include lack of resources, inability or unwillingness of the users to give feedback, ethical concerns, and manufacturers’ unwillingness to involve users [5]. A qualitative study by Money et al. interviewed developers of medical device technology and found that they did not see the benefit of engaging with users [7]. Money et al.’s study also showed that the one technology developer out of the eleven interviewed thought engaging users was important and they likely had that notion due to their educational experience involving user-centered design [7].

While beneficial to reducing barriers to care by expanding where laboratory analyses can be performed, point of care tests can be more costly to obtain and maintain, as some devices have several specialized components such as cartridges and reagents that are only made by a single company [43]. This reliance on one company's supply can easily be disrupted supply chain issues such as during the COVID-19 pandemic. This largely affects low- and middle-income countries (LMICSs) who in the current model of the biotechnology industry, rely heavily on biotechnology imports from high-income countries [44].

Point of care technologies are often developed for LMICs, but without input of the people living in those countries or using those devices [10]. It is important to incorporate user voices when developing new technologies not only for successful implementation and adoption, but to understand the context in which they are deployed. Therefore, this study qualitatively assessed the political, social, logistical, economic, and technological contexts for the initiation of a biotechnology collaboration with a hospital in the Dominican Republic (DR). The DR is an upper middle-income country in the Caribbean that shares the island of Hispaniola with Haiti [45]. In the DR, diagnostic testing is mostly supplied by the private sector and a national public laboratory [46]. As an upper-middle income country, the DR's economy is growing, but investment in healthcare is lower than other countries with comparable economies [47]. Like many other LMICs, researchers in the DR have acknowledged how COVID-19 further exposed the inequities in global health, with the perspective of those in the Global South being rarely incorporated into interventions [46].

We focused our questions largely on understanding challenges and successes in obtaining an HIV diagnosis, but also asked about obtaining a diagnosis more generally. We focused on HIV for a few reasons. Firstly, 75% of people living with HIV/AIDS in the

Caribbean are reported by Hispaniola, with most of living in Haiti. However, due to the persistence of *bateyes (*underserved communities of predominantly Haitian sugarcane workers), many Haitians have become some of the most vulnerable groups in the DR. There are no educational materials in Haitians' primary language, Creole, and no efforts targeting these migrants. Adding to the prevalence of HIV in the DR is its large tourism industry, which increases the probability for sex tourism. Additionally, there are some "hidden" populations that are at risk, like men who have sex with men and sex workers. These populations are largely ignored in terms of being provided prevention services due to the stigmatization of homosexuality and prostitution, difficulties in negotiating condom use, and more [48]. Therefore, the quick diagnosis of HIV in the DR is not only important due to the epidemiology of the condition, but also because those who are at highest risk are migratory persons whose location may change rapidly.

Brown University and Rhode Island Hospital in the US and Hospital Regional Universitario Jose Maria Cabral y Báez (HRUJMCB) in the DR have had a long-standing exchange program for medical residents for many years [49]. We hoped to deepen this collaboration by incorporating facets of biomedical engineering. Therefore, this study had several aims: 1) to understand barriers and facilitators to biological sample analysis primarily for HIV, but also more generally, 2) to seek input for the development new biotechnologies for biological sample analysis, and 3) to report recommendations from participants regarding improving access to care for HIV.

2.2 Methods

2.2.1 Study Site

This study is set primarily in Santiago de Los Caballeros, the second largest city in the Dominican Republic, with its largest city being the capital Santo Domingo, where one interview was performed. All interviews were conducted over the span of a month from

late July 2022 to August 2022. In Santiago de Los Caballeros, the interviews were conducted with medical residents, lab personnel, and other stakeholders at the Tercer Nivel de Atención/Third Level of Care public regional hospital, Hospital Regional Universitario Jose Maria Cabral y Báez (HRUJMCB). HRUJMCB is the largest public hospital and most complex in the northern region of the Dominican Republic[50]. A Tercer Nivel de Atención/Third Level of Care establishment in the Dominican Republic has the highest level of services and specialties available (compared to First and Second Levels), with patients from the Primer/First and Segundo/Second Nivel de Atención/Level of Care establishments being referred here[51]. Additionally, the HJMCB is a regional hospital, so it serves the entire region of Santiago, with outpatient and inpatient services [50]. In Santo Domingo, the interview was conducted with a researcher at an institute of a private university whose work relates to HIV.

2.2.2 Sampling and Recruitment

To participate in this study, participants had to be involved in the sample collection, sample analysis, oversight, or any other major roles in HIV surveillance and biological sample analysis in general. At HRUJMCB, purposive sampling was done by initiating contact with two point people: a clinician who worked at the hospital's HIV center, the CEDI (La Clínica de Enfermedades por Daño Inmunológico), of HRUJMCB and the director of the hospital's laboratory. The clinician that worked at the CEDI then performed purposive sampling by connecting the interviewers with a third point person, a third-year internal medicine resident. The internal medicine residents, specifically first-years, typically handled sample collection for their patients and see a variety of patients, which fit the primary criteria of being a participant in the study. The third-year medical resident point person would then ask residents who were available for an interview to meet with the interviewers when the interviewers were at the hospital. The hospital lab director (and

assistant director) similarly performed convenience sampling by also asking the laboratory personnel who were available to meet with the interviewers to participate in the interviews when the interviewers were at the hospital. The laboratory personnel work in three main shifts, the morning, afternoon, and overnight shifts. The interviews were conducted with morning and afternoon shift personnel. In Santo Domingo, expert purposive sampling was done to recruit the researcher who studied HIV at the university institute.

Overall, at HRUJMCB 20 medical residents, 13 lab personnel, and two participants who worked at the CEDI were interviewed in August 2022. In Santo Domingo, one participant was involved in research and treatment of HIV in a university setting was interviewed. We were encouraged to seek out the participant in Santo Domingo to offer a different perspective, though limited about HRUJMCB, their perspective was helpful to understanding the context of HIV in the DR. Participants were recruited by word of mouth except the one in Santo Domingo, who was recruited by email.

2.2.3 Interview guide development

The semi-structured, in-depth interview guides were developed by the lead author, PhD candidate Kiara Lee, of this dissertation and initially written in English. The guide was reviewed by Dr. Rochelle Rosen, a qualitative researcher in the School of Public Health. Three guides were developed, one for the medical residents and clinicians of CEDI, one for the lab personnel, and one for the university researcher. In the US, the study was reviewed by Brown University's IRB who deemed the research not human subjects research, but key informant interviews.

After Brown University's IRB determination, the interviews were translated into Spanish by David Arango, a master's student in Public Health and reviewed by Dr. Martha Sanchez, a clinician at Miriam Hospital and leader of the collaboration between HRUJMCB and Brown University medical residents. Once the interviewers arrived in the DR, the

interview guides were then reviewed by the ethics board of HRUJMCB. The guide contained five topics: role & responsibilities in the workplace, facilitators to biological sample analysis, barriers to biological sample analysis, social and political context related to HIV and healthcare more generally, and the future of developing technologies for biological sample analysis, with probing questions for each question. The future of developing technologies for biological sample analysis section asked participants to describe point of care devices they were familiar with, to imagine and describe characteristics of their ideal device, and to explain what they think the gaps in technology are right now for biological sample analysis. All interviews were conducted in Spanish.

2.2.4 Data collection and analysis

The semi-structured, in-depth interviews were conducted by two interviewers from the US, Kiara Lee and David Arango; they are also the first and second authors on this work. One (David Arango) was a native Spanish speaker (Interviewer/Entrevistador A) and the other (Kiara Lee) was a learned Spanish speaker (Interviewer/Entrevistadora B). Both interviewers studied qualitative methods for a semester as a part of their Masters' in Public Health coursework, in which Kiara developed a pilot version of this study. David had experience interviewing Spanish-speakers in previous work. The interviews were led by Interviewer/Entrevistador A. Interviewer/Entrevistadora B was present for all interviews primarily as a notetaker and would interject when they had a clarifying question or a question in the context of the interview guide. Most interviews were performed in a private room, some (5) were performed in a break/lunchroom and lasted on average, 30 minutes each. Before each interview participants were read a description of the study, an informed consent statement, and asked if they agreed to participate and if we could record them. Once they agreed to be recorded, the audio device began recording and the participants were described by their interview number and asked again if they agree to participate and

to be recorded. Once the interview was concluded, participants were given a pen as a token of our appreciation; they were not aware we would give them a pen until the interview was concluded.

Interviews were audio-recorded, transcribed using HappyScribe [52] (HappyScribe.com, Barcelona, Spain) with their automated transcription software which uses artificial intelligence to transcribe audio files. All data was stored on Google Drive, in folders that only the interviewers have access to. The data was de-identified by removing names and other identifying information. Transcriptions were reviewed first by Interviewer/Entrevistadora B and then cross-checked by Interviewer/Entrevistador A. The transcriptions were uploaded and coded by Interviewer/Entrevistadora B in NVivo (version 1.6.1, QSR International Pty Ltd, Doncaster, Australia). Coding for barriers and facilitators was done using thematic analysis, specifically template-style thematic analysis [53], with deductive codes developed from the interview guide and inductive codes from the transcripts. From there, themes were developed using the social ecological model. For codes involving the characteristics participants would like in a new device, matrix template analysis was done, with deductive codes developed from the interview guide and inductive codes from the transcripts. Themes were developed based on the major categories that participants discussed.

2.3 Results

2.3.1 Participant characteristics

Of the clinicians that were interviewed, 10 (44%) were male and 13 (56%) were female. Of the lab personnel that were interviewed, one participant was male (7.6%) and 12 were female (92.4%). The clinicians were mostly residents that had been at the hospital from one month (first year residents or R1s) to two years (third year residents or R3s). First year residents are those who are closely involved in sample collection as one of their main

duties includes collecting samples from patients and taking them to the laboratory for analysis.

2.3.2 Theme Development: The Social Ecological Model

We aimed to understand the barriers and facilitators of sample collection and analysis by first asking participants about difficulties (barriers) they have had when performing sample collection, analyzing samples, or in receiving results for the diagnosis of a patient. We then asked about facilitators, by asking if participants were satisfied with current processes, what has been essential so that their work is efficient and fluid, and what they would like to implement to make their work easier. To develop themes (Table 2.1), we organized the inductive and deductive barriers and facilitators codes into the five levels of the social ecological model [54]: individual (patients), interpersonal (lab personnel, clinicians, and/or family members), organizational (the hospital, HRUJMCB), community (Santiago de Los Caballeros), and societal (The Dominican Republic).

Table 2.1: Barriers and Facilitators to obtaining a diagnosis, analyzed via the Social Ecological Model.

Level	Barrier	Facilitator
PATIENTS		
Individual	• Lack of education around HIV • Difficulties in accessing care • Non-compliance or non-adherence to care procedures	• Compliance and adherence to care procedures
FAMILY MEMBERS, LAB PERSONNEL, AND CLINICIANS WITH PATIENTS		
Interpersonal	• Cultural and linguistic differences between patient and provider • Inadequate sample collection	• Communication among family members, patients, laboratory personnel, and/or clinicians
THE HOSPITAL (HRUJMCB)		
Organizational	• Inefficient laboratory or hospital workflow and limited lab hours	• A well-resourced and well-managed laboratory

	• Inconsistent access to laboratory supplies • Limited laboratory workforce	• Having a laboratory within the hospital • Psychologists to give HIV diagnosis
SANTIAGO DE LOS CABALLEROS, DOMINICAN REPUBLIC		
Community	• Difficulties sending samples from the hospital to private laboratories in Santiago de Los Caballeros	• Community organizations
THE DOMINICAN REPUBLIC		
Societal	• Stigma • Inconsistent government investment in public health	• Investments in public health (ex. laboratory instruments, disease treatment programs, and affordable health insurance)

2.3.3 Barriers and Facilitators at the Individual Level

2.3.3.1 Patients' lack of education about HIV is a barrier to seeking out and receiving care (Individual Barrier)

Many of the clinicians discussed how many of their patients lack education about HIV which fuels their fear of the virus and misinformation. They stated that many of their patients do not know how one becomes infected with the virus, how to prevent the spread of HIV, or the outlook and treatment options once diagnosed with HIV. Médico/Clinician 122 explained some of the misinformation around how one contracts HIV:

> *"No es una enfermedad que se pega por un contagio de que te salude esa no va a pegar. Entonces es una educación que hay que extenderlo […] Si tú no estás lacerado, tú tampoco te va a contagiar. Entonces eso es una educación de… no es tan grave como tú crees, no se contagia tan fácilmente, ¿entienden? Porque yo te salude, porque por tenga VIH… que se acabó el mundo" (Médico 122)*

> *"*It is not a disease that you get by someone simply greeting you. So, it is an education that must be extended […] If you are not lacerated, you won't be infected either. So that's an education of... It's not as serious as you think, it's not as easily spread, you understand? Because I greeted you, because I have HIV... that the world has ended…" (Clinician 122)

Patients often believe that they will die or have very poor outcomes once diagnosed with HIV and are unaware of the treatment options. This leads to them to avoid seeking care, accepting their diagnosis, and following care procedures. The lack of education also makes Médico/Clinician 107 feel *"un mundo aparte"*, a world apart, from their patients when trying to give care. The lack of education also extends to the laboratory where Personal del Laboratorio/Laboratory Personnel 210 said that:

> *"A veces la paciente, a veces no tan bien orientado y te exigen, si ellos no saben, como, tú sabes, que uno pasa aquí dentro…" (Personal del laboratorio 210)*

> "Sometimes the patient, sometimes they're not so well guided and they demand of you, if they do not know how, you know, what happens in here…" (Laboratory Personnel 210)

2.3.3.2 Some patients have difficulties in accessing care (Individual Barrier)

The clinicians spoke about the general population that receives care at the hospital, which are those who have a low or very low socioeconomic status. Thus, they often do not have resources to get testing done outside of the hospital

(should they need it done if the hospital does not have certain testing available). If they live in more rural areas, they may live far from the hospital or lab they are referred to and may not have access to a method of transportation to receive care or treatment. The difficulty in accessing care was mostly discussed in the context of immigrants, largely Haitian immigrants, though Venezuelan and Colombian immigrants often receive care at the hospital. With immigrants, there is an added layer of care access that is tied to legal residency status, which affects their ability to get insurance and other health services. For Haitian immigrants, since they tend to be more migratory [55], and do not have a constant address or phone number, this makes it difficult for them to receive and maintain constant access to care. Médico/Clinician 123 explained the difficulties one may have in accessing consistent care for multiple reasons:

> *"Porque tienen que trabajar y dejar un día de trabajo para ir al centro [para ir a una cita], representa una pérdida económica. Otros elementos que podrían estar involucrados con eso tiene que ver también con la percepción de riesgo de las personas. O sea, yo entiendo que yo puedo operar porque yo no me siento nada esperar más tarde para ir al centro, porque ahora mismo estoy muy ocupado, […] tengo que trabajar, tengo que buscar dinero, y además soy un inmigrante, tengo que buscar a dónde voy a vivir.*
>
> *Otro factor, además del factor personal, tiene que ver con la legislatura. El no tener documentación hace que las personas no vayan a los servicios de salud. Si yo no tengo papeles, si no tengo un pasaporte, si no tengo un documento, pues muy probablemente, eh, no voy a poder, eh, recibir los servicios. Entonces eso también es otro problema, es otra barrera que se presenta muy a menudo en personas que son migrantes. No tener documentación ya es como dar con problemas para poder acceder a los servicios." (Médico 123)*

> “Because they have to work and leave a day's work to go downtown [to go to an appointment], it represents an economic loss. Other elements that could be involved with that also have to do with people's perception of risk. That is, I understand that I can operate because I don't feel anything waiting later to go to the center, because right now I'm very busy, I have to work, I have to look for money, and I'm also an immigrant [...] I have to look for where I'm going to live.
>
> Another factor, in addition to the personal factor, has to do with the legislature. Not having documentation means that people do not go to health services. If I don't have papers, if I don't have a passport, if I don't have a document, well, most likely, I'm not going to be able to receive the services. So that's also another problem, it's another barrier that very often occurs in people who are migrants. Not having documentation is like giving them problems in accessing services.” (Clinician 123)

2.3.3.3 Non-compliance and non-adherence to care procedures makes it difficult for clinicians and lab personnel to provide the necessary care to patient (Individual Barrier)

The clinicians often mentioned patients not complying or not adhering to care procedures for HIV for multiple reasons. It should be noted that the clinicians often cited the lack of compliance as a symptom of the lack of education patients have about HIV and their difficulties in accessing care. At the hospital, patients must provide written consent to receive a test for HIV, but sometimes, “*el paciente se niega a tomarla. Hay pacientes y cuando [un médico] se sospecha de eso [que el paciente tiene VIH] tiene que firmar su conocimiento para hacerle la prueba. Se niegan*” / “the patient refuses to be tested. There are patients, and when one [a clinician] suspects something [that the patient has HIV], they have to sign their consent to do the test. They refuse” (Médico/Clinician 106). The refusal to be

tested is for various reasons, patients don't want samples taken from them, they don't accept that they may have HIV, or don't think testing is necessary.

When it comes to non-adherence, patients may not retrieve their testing results, may not take their medications, may not disclose if they have HIV and are aware of their diagnosis, or may only come to appointments when they feel bad. Loss to follow up is especially an issue for Haitian immigrants, once again due to their tendency to have a more migratory lifestyle. Médico/Clinician 104 describes what occurs when trying to have a patient follow a treatment plan:

> *"En el programa [de VIH]… hay muchos pacientes ahora, un buen porcentaje de ellos que son un poco… irresponsables en cuanto a venir a las citas, vienen… cuando… se sienten mal, entonces ya como que no se le puede dar un seguimiento óptimo. No siguen el tratamiento porque las citas se le ponen, por ejemplo, a tres meses desde la última vez y los medicamentos se le da para esa cantidad de tiempo. Entonces hay pacientes que se quizás se sienten bien, abandonan, no llegan a la cita. Ese tipo de cosas no ayudan para darle seguimiento al paciente. Quizá también los recursos del paciente. [La falta de recursos] puede que no nos ayudan tampoco, porque […] son los que quizás puede dificultar el seguimiento." (Médico 104)*

> "In the [HIV] program... There are a lot of patients now, a good percentage of them who are a little... Irresponsible about coming to appointments, they come... when... they feel bad, then they can't be followed up optimally. They do not follow the treatment because the appointments are given, for example, three months from the last time and the medications are given for that amount of time. Then there are patients who may feel good, drop out, do not come to the appointment. That kind of thing doesn't help to follow up with the patient. Perhaps also the patient's resources. [The lack of resources] may not help us either […] because that

kind of thing is what can perhaps make it difficult to follow." (Clinician 106)

The lab personnel also discussed non-compliance in that patients do not follow the requirements for blood sample collection (such as fasting) or refuse to comply with getting their sample taken. These factors, greatly affect the lab personnels' ability to perform testing. When asked what has been essential to having a good sample, Personal del Laboratorio/Lab Personnel 210 stated:

> *"A veces que el paciente miente… a veces el paciente también que tiene las vías difíciles, no colabora, no contribuyen, a veces vienen que… tú le dices que tiene que venir en ayuna, no vienen en ayuna o a veces te mienten también. Una toma de muestra va a depender de eso de la honestidad de un paciente y que yo tenga una buena vía para tomarla [la muestra]." (Personal del Laboratorio 210)*

> "Sometimes the patient lies… sometimes the patient also has difficult veins, does not collaborate, does not contribute, sometimes you tell them that they have to come fasted, they don't come fasted or sometimes they lie to you too. A sample is going to depend on the honesty of a patient and that I have a good vein to take it [the sample]." (Lab Personnel 210)

2.3.3.4 Patients following care procedures, especially following up with their results is a facilitator to obtaining HIV diagnoses (Individual Facilitator)

In terms of patient behaviors that facilitate HIV diagnoses, this was not a commonly coded topic, but the participants discussed that it is essential that the patient is "persistent" (Médico/Clinician 110), that they want to do their treatments,

and that good sample collection depends upon honesty. Loss to follow up has improved over the years with Médico/Clinician 110 informing us that:

> *"Antes era más dificultoso porque… el paciente no venía […]. Esas barreras mejoraron. Era muy frecuente usted encontrar muchísimos resultados y nadie venía a buscarlo... Eso [ha mejorado] muchísimo." (Médico 110)*
>
> "Before it was more difficult because... the patient did not come […]. Those barriers improved. It was very common to find many results and nobody came looking for it... That [has improved] a lot." (Clinician 110)

2.3.4 Barriers and Facilitators at the Interpersonal Level

2.3.4.1 Cultural and linguistic differences between patients and clinicians can lead to challenges in providing patients with care (Interpersonal barrier)

The Dominican Republic shares the island of Hispaniola with Haiti and many clinicians informed us that nationals from their "hermano país" or "brother country" (Haiti) are the second largest population at the hospital after Dominicans, and some also noted an increase in Haitians coming to the hospital that have HIV. This presents a language barrier for those from Haiti coming to receive care, as the national languages are Haitian Creole and French, and that of the Dominican Republic is Spanish. When asked how one communicates to Haitians that don't speak Spanish regarding their diagnosis or other healthcare matters, Médico/Clinician 111 stated:

> *"Eso es un problema, pero por lo general uno trata. Por ejemplo, esos pacientes llegan acá con un amigo que es un nacional haitiano también, pero que hable español o que hablan más o menos un poco el español y se entiende. De*

> *lo contrario nosotros tratamos de buscar a alguien que sea aquí en el hospital, algún estudiante o algún médico que sea haitiano o que hable creole para que nos traduzca y así la comunicación entre el paciente y médico sea efectivo." (Médico 111)*
>
> "That's a problem, but usually one tries. For example, those patients come here with a friend who is a Haitian national as well, but who speaks Spanish or who speaks more or less a little Spanish and one understands. Otherwise, we try to find someone who is here in the hospital, some student or someone who is Haitian or who speaks Creole so that they can translate for us so that communication between the patient and doctor is effective." (Clinician 111)

Often Haitian interns or residents will translate for patients that need it, despite not being official translators; family and friends of the patients may also serve as translators. Other immigrants such as Venezuelans and Colombians have the advantage of speaking Spanish thus language is not an issue.

Another aspect that makes communication difficult is the difference is culture and religion between Haitian patients and their Dominican clinicians. Médico/Clinician 110 explains: *"Ellos miran la enfermedad como algo como castigo y contexto mágico*" / "They look at diseases like a punishment and in a magic context". All together these communication differences lead to challenges in providing patients with care.

2.3.5 Sample collection not being done properly leads to challenges for laboratory personnel to analyze a sample (Interpersonal barrier)

Many of the laboratory personnel (eight of the thirteen interviewed) discussed how the sample collection not being done properly can affect their ability to give results and the time to result. Samples are collected in two main ways: by first year medical

residents or by laboratory personnel in the “Toma de Muestras” or “Sample Collection” department. Since medical residents are in training, Personal del Laboratorio/Laboratory Personnel 201 stated that may influence the quality of sample that is received, as, *“que quizás no tienen los conocimientos adecuados de cómo tomar una muestra adecuadamente, cómo tomar una muestra adecuadamente, cómo almacenarla, cómo…homogenizarla, cómo transportarla”* / “they may not have the proper knowledge of how to take a sample properly, how to store it, homogenize it, how to transport it.”

Additionally, sample collection and analysis can be difficult due to a patient having difficult to access veins, leading to a low sample volume for analysis, or even because samples are not sent to the correct department. A major barrier to sample analysis are samples that contain a medicine, drug, or compound that affects their analysis. This could occur due to communication issues with patients or due to the protocol not being followed when the sample is collected. Personal del Laboratorio/Laboratory Personnel 204 describes what occurs if they do not have the information they need when analyzing a sample:

> *“Dificultades… por ejemplo, el tema de tener que repetir muchas veces un mismo paciente porque desconozco, porque quise hacer toma de muestra no le, no le preguntaron lo que… anteriormente lo que está tomando anticoagulantes, está tomando eso… por este proceso no siempre se cumplen el protocolo. Entonces eso hace que tenga yo un inconveniente en el método de procesar porque yo tenga que desarmar el equipo por el trato porque no me está dando, pero que no conozco [el problema].” (Personal del Laboratorio 204)*
>
> “Difficulties… for example, the issue of having to repeat the same [patient’s samples] many because […] they [the

> sample collector] did not ask them [the patient] what… previously what they're taking, blood thinners, [if] they're taking that... this process does not always comply with the protocol. So that makes me have a problem in the processing method because I have to disassemble the equipment because it is not giving me [a result], but I do not know [the problem].” (Laboratory Personnel 204)

2.3.6 Communication between patients with family members, clinicians, and/or lab personnel facilitates patients’ understanding of their HIV diagnosis and treatment options (Interpersonal Facilitator)

To ensure optimal outcomes with patients, it is essential to have good communication. All of the participants discussed various pathways in which communication is important amongst various stakeholders, (patients, clinicians, and lab personnel) which is shown in Figure 2.1. The most notable aspects of communication were what specific topics must be discussed to educate the patient about HIV and who has been essential (like Haitian creole-speaking interns) in facilitating their communication.

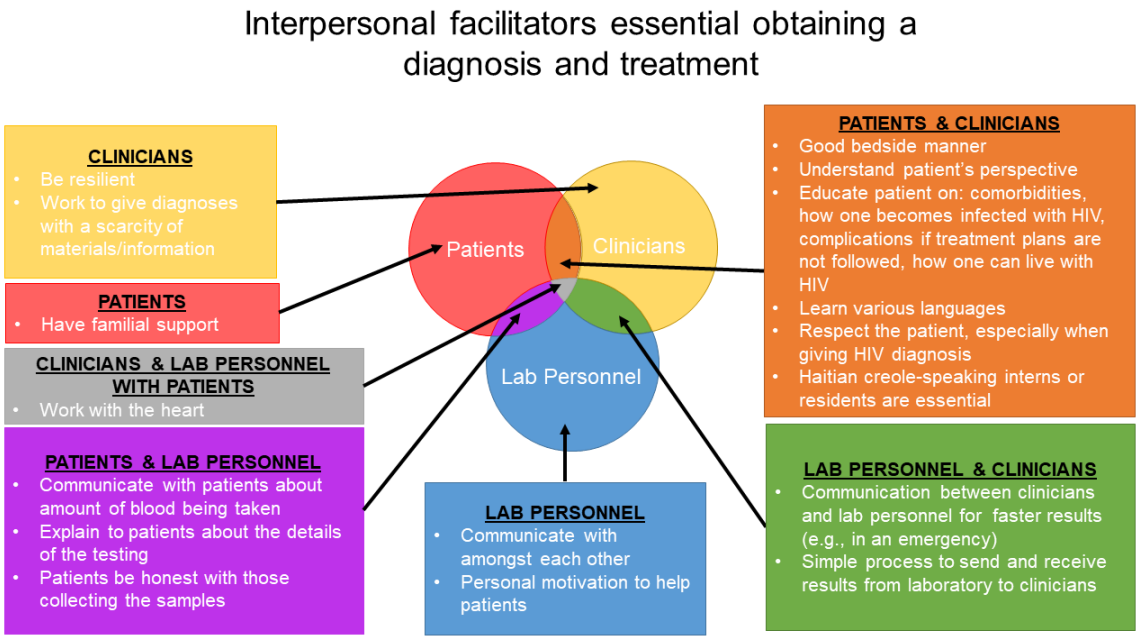


Figure 2.1: Various interpersonal communication facilitators amongst patients, clinicians, and laboratory personnel.

Personal del Laboratorio/Laboratory Personnel 206 explained how important communication is between them, the clinicians, and patients to perform their job well:

> *Tengo un resultado crítico. Tengo que llamar al médico para que un médico empiece a proceder con ese paciente. Y la comunicación con el paciente, porque a partir de los datos que el paciente me dé, yo puedo hacer un mejor diagnóstico. (Personal del Laboratorio 206)*

> "I have a critical result. I have to call the doctor so that a doctor will start proceeding with that patient. And communication with the patient, because from the data that the patient gives me, I can make a better diagnosis. (Laboratory Personnel 206)

For both clinicians and laboratory personnel, some participants emphasized how it is important for them to “tener corazón” / “have heart” (Médico/Clinician 119) when doing this work to lead to better outcomes for the patients.

2.3.7 Barriers and facilitators at the Organizational level

2.3.7.1 Inadequate, insufficient, or inefficient laboratory supplies leads to inconsistent access to diagnostic testing (Organizational Barrier)

The laboratory in the hospital has a lot of capabilities in various departments such as viral testing (HIV, Hepatitis A and B, etc.) or the chemical department (electrolytes). However, the laboratory has inconsistent access to testing materials. At times reagents to perform a certain analysis are not available and “*tú puedes tener un día reactivos, otro día el suplidor no te [los[manda. Entonces, cuando el suplidor no me [los] mandas, pues, no puedo de trabajar, pues no tengo otra plataforma diagnóstica*.” / “You can have reagents one day, another day the supplier doesn’t send them to you. So, when the supplier doesn't them to send me, well, I can't work, because I don't have another diagnostic platform" (*Personal del Laboratorio*/Laboratory Personnel 201). This could be due to disruptions in the supply chain due to COVID-19 or other world events (e.g., wars). Interruptions in supplies can take a couple days to replenish, which may halt testing on some machines and for certain conditions. Patients either have to wait for their results or go to a private lab and pay out of pocket to get results.

The laboratory also does not have all types of tests required; some specialized tests may have to be performed at other labs in Santiago de Los Caballeros, or in the capital city of Santo Domingo. In terms of HIV, CD4 testing was not being done, even at the national level. It is not immediately clear as to why

that was. Generally, though, the hospital laboratory (and other public laboratories) uses rapid tests to diagnose or HIV, which "*que eso [prueba rápida] es uno de los métodos que nos ayuda a avanzar, pero no es un método tan, eh… no es que no es confiable, sino que son unos métodos, eh, que tienen muy amplia."* / "That [rapid test] is one of the methods that helps us move forward, but it's not a method so, uh... It's not that it's not reliable, but it is one of a few methods, uh, that are very broad." (*Personal del Laboratorio*/Laboratory Personnel 203).

2.3.7.2 Inefficient laboratory or hospital workflow and limited lab hours slow down the time to result (Organizational Barrier)

When discussing the laboratory in the hospital the participants discussed several aspects of its workflow that negatively affect their ability to have samples collected and/or analyzed. Firstly, the laboratory has limited hours, and for HIV testing Médico/Clinician 101 explained that, *"después de las seis de la tarde habría que esperar al otro día para poder saber que si el paciente tiene VIH o no tiene VIH, entonces sería una barrera realmente"* / "after six in the afternoon you have to wait until the next day to know if the patient has HIV or not, and therefore that is a barrier." When a clinician expresses that there is an emergency, though, the testing can be done outside of the typical hours that HIV testing is performed.

At the hospital, a psychologist is necessary to give a patient an HIV diagnosis, which many see as a positive (see Section 2.3.7.6), but a few clinicians saw it as an extra, time-consuming, process that slows down the time to give a patient a result. Additionally, to protect the patient's information, HIV results must be retrieved by the clinicians in person, by speaking face-to-face with laboratory personnel. Some clinicians described this as tedious, as for all samples the

residents, who typically collect samples, must take the samples down to the laboratory. The hospital has multiple floors and a few elevators, so some clinicians have described this as time consuming and interrupts time with their patients. The hospital has begun to implement a system in which bioanalysts from the laboratory go floor by floor to collect samples in the mornings, but this process was just starting when we interviewed the participants. One clinician said that they are faster at collecting samples than the laboratory personnel. Lastly, within the hospital, Médico/Clinician 120 described the hospital as a little "*caótico"* / "chaotic", with a lot of noise that leads to a lack of privacy with the patient, which is important when discussing one's results or treatment options.

With the laboratory, several clinicians described how the laboratory is sometimes slow to give a result, which affects when a patient can be discharged from the hospital, and if a clinician will ask a patient to seek results from an external laboratory. Médico/Clinician 104 described what occurs when the results are slow:

> *"Hay veces que uno necesita saber algo, quizás de un tiempo límite, rápido. Y quizá el laboratorio no lo tiene o lo pone difícil para hacerlo. Entonces en esos casos nos adaptamos, o sea, los mandamos a hacer aquí o se manda hacer fuera si lo queremos más rápido y tratamos de que el familiar lo agilice, el proceso, por ejemplo, eh… ¿cómo lo digo? No específicamente analíticas, pero, por ejemplo, tenemos un paciente con una anemia severa que necesitamos que buscar la sangre, quizá que no hay o si hay la ponen difícil o… tiene que ir afuera para buscarlo más rápido. El punto es que uno busca la manera, se adapta a lo que tenemos y lo que el paciente puede conseguir fuera o aquí mismo." (Médico 104)*

> "There are times when you need to know something, maybe in a limited time, fast. And maybe the lab doesn't have it or makes it difficult to do it. So, in those cases we adapt, that is, we send them to be done here or it is sent to do it outside if we want it faster and we try to get the family member to speed it up, the process, for example, eh ... how do I say it? Not specifically analytical, but, for example, we have a patient with severe anemia that we need to look for blood, maybe there is not any or if there is they make it difficult or... They have to go outside to look for it faster. The point is that you look for the way, you adapt to what we have and what the patient can achieve outside or right here." (Clinician 104)

Another aspect of the laboratory's workflow that lead to delays or slowdowns. Firstly, clinicians do not have access to the laboratory's electronic system for tracking results, only laboratory personnel do, which delays the time to result. At times patient samples do not arrive to the correct department, which delays work as well. With the machines that the laboratory uses, some laboratory personnel feel as if the sample analysis process is inefficient because one sample has to go through multiple machines to be able to give a result, as described by Personal del Laboratorio/Laboratory Personnel 204:

> *"Tenemos que trabajar con muchas máquinas. Un un solo paciente tiene que pasar hasta por cuatro máquinas porque está más de la analítica, pero esta no me la hace y también tengo que saltar [a otra máquina]. Debe ser más rápido en todo." (Personal del Laboratorio 204)*

> *"We have to work with a lot of machines. A single patient has to go through up to four machines because it is more than the analytical, but this one does not do it and I also have to jump [to another machine]. It must be faster in everything." (Laboratory Personnel 204)*

2.3.7.3 The hospital needs more laboratory personnel hired (Organizational Barrier)

There is a limited laboratory workforce, which was expressed by all participants, especially laboratory personnel. Médico/Clinician 111 pondered about the supply and demando of the hospital when it came to testing and said, *"la cuestión es que el hospital debe de tener el personal necesario para que supla la demanda, que no lo hay"* / "the question is that the hospital should have the necessary personnel for the demand, which there isn't." This makes it take longer to obtain a result or perform a test, increases the amount of work that one person does a day, and makes it difficult for personnel to take leave and get their area covered. A senior member of the laboratory explained why they were experiencing a low number of personnel at the time of the interview, because *"hace como tres años, casi 50% de nuestro personal se fue porque llegó su tiempo (se jubiló)"* / "three years ago, almost 50% of our personnel left because their time came (they retired)." (*Personal del Laboratorio*/Laboratory Personnel 212)

2.3.7.4 Having a laboratory in the hospital centralizes testing for HRUJMCB (Organizational Facilitator)

As a Tercer Nivel/Third Level hospital, HRUJMCB has a large laboratory inside of it to perform most of the testing needs for the patients. The participants discussed how the laboratory is good to have from a financial perspective, so that patients do not have to get their testing done at a private laboratory and pay out-of-pocket to do so. From a health perspective, having the laboratory in the hospital is helpful because then patients, especially immunosuppressed ones, do

not have to get their testing done elsewhere and risk getting even sicker. Lastly, the hospital laboratory is a convenience for all stakeholders involved, patients, clinicians, and especially family members because then they do not have to leave the hospital to get the sample analyzed. When family members are tasked with collecting a sample this requires them to physically take the collected sample themselves to a private laboratory which is a safety risk and also a risk in the sample not being transported properly. Médico/Clinician 101 discussed many of these topics when asked if the benefits of having the laboratory in the hospital:

> *Sí, yo creo que la forma de poder tener en nuestro laboratorio este tipo de pruebas nos facilita muchísimo a nosotros […] Entonces, entenderá que un paciente que tiene VIH tiene un sistema inmunológico más deprimido, tiene muchísimos más factores de riesgos para poder contagiarse de otra enfermedad. Este tipo de pacientes, cuando rondamos, tratamos de que sea el menor flujo de personas porque, es inmunológico, un paciente que tiene el sistema deprimido, que cualquier cosa puede afectarle. Entonces, el hecho de que tú tengas las pruebas dentro del hospital, y tú sepas que ese paciente es inmunocomprometido, vas a ayudar a ese paciente a que no tenga otras patologías dentro del hospital. (Médico 101)*

> "Yes, I believe that being able to have this type of test in our laboratory makes it much easier for us […] So, one will understand that a patient who has HIV has a more depressed immune system, has many more risk factors to be able to get another disease. This type of patient, when we do rounds, we try to make it the least flow of people because, they are immunologically, a patient who has a depressed system, that anything can affect them. So, the fact that you have the tests inside the hospital, and you know that that patient is immunocompromised, you're going

to help that patient not have other pathologies within the hospital." (Clinician 101)

2.3.7.5 A well-resourced and well-managed laboratory is critical for high quality results in a timely manner (Organizational Facilitator)

The participants overall described several ways in which the laboratory in the hospital has facilitated them getting results, by discussing what they were currently satisfied with, what would be good to implement, and describing aspects of the laboratory that are particularly important to obtaining a high-quality result (Table 2.2).

Table 2.2: Laboratory resources and management aspects that the participants are currently satisfied with, would like to implement, and think are essential to have.

Currently satisfied with	Would be good to implement	Essential to have
• Time to result (depends on laboratory department) • Quality of result • Types of testing done • Lab machine improvements over the years • That lab personnel know how to do testing manually, so they do not have to rely on the machines • Good training of lab personnel • Good management of the laboratory	• CD4 and viral load testing at the hospital (biomolecular testing center) • Laboratory to be open more hours for HIV testing • Further advanced laboratory machines and testing abilities • Easier-to-obtain laboratory materials • Laboratory personnel and mini laboratories on each floor to do sample collection and some analyses	• Well-trained laboratory personnel • Good management and procedures for sample collection and distribution within the laboratory • Sufficient and high-quality reagents and machines • Ability to use manual methods to compare to machine results • Well-organized laboratory • Proper sample type, volume/amount, and

• Procedure of obtaining HIV results personally/face-to-face from lab personnel • When laboratory personnel collect samples from patients on each floor • Manageable workload (depends on the laboratory department)	• More anonymous sample collection procedure when patients have to go to the laboratory for sample collection • Database only clinicians and laboratory personnel can access to see testing results	sample storage for laboratory analyses • Various types of analytical offerings • Well-managed and happy employees in the laboratory • Reasonable time to result to not worry patients

In terms of what participants are currently satisfied with, many agreed that the training of personnel and management of the lab is good. When asked how long it takes for personnel to learn how to use new laboratory devices, Personal del Laboratorio/Laboratory Personnel 202 informed us that:

> *“Algunas [maquinas] se utiliza casi a la semana porque sí el entrenamiento de la máquina es, que es fluida y es fácil, pues, tú lo utilizas de una vez. Si [es] muy complicada, que alguna cosa puede durar dos semanas, pero generalmente cuando traen estos equipos a la semana ya se están utilizando.” (Personal del Laboratorio 202)*
>
> "Some [machines] are used almost that week because if the training of the machine is fluid and easy, well, you use it at once. If it is very complicated, that’s something can last two weeks, but usually when they bring this equipment, that week they are already being used."(Laboratory Personnel 202)

When it comes to what participants would like to see implemented in the laboratory to facilitate sample collection and analysis, several suggestions were made, some of which were actively being addressed. For example, some participants expressed an interest in expanding the capabilities of the laboratory

to be able to analyze molecular samples such as those that rely on nucleic acid testing, like viral load. Additionally, both clinicians and lab personnel discussed wanting sample collection to be done by lab personnel instead of by residents, and perhaps for there be to be smaller versions of the laboratory on each floor of the hospital to better facilitate sample collection and some analyses. When asked what processes they would like to implement to make the sample collection and analysis process more fluid, *Personal del Laboratorio*/Laboratory Personnel 201 *stated:*

> *"Procesos. Bueno, la opción sería que en cada estación del hospital existiera un área de toma de muestras. O sea, que ellos, se genera una petición de una toma de muestras, pues de ahí se toma y se… se traslada [al laboratorio]. O si pudiera existir en cada planta un mini laboratorio. Para que alguien pueda procesar las pruebas que son de urgencias, hacerlas en esa planta, sin necesidad de tener que bajar a laboratorio." (Personal del Laboratorio 201)*

> "Processes. Well, the option would be for each hospital station to have a sampling area. That is, they generate a request for a sampling, and from there it is taken and it is moved [to the laboratory]. Or if there could be a mini laboratory on each floor. So that someone can process the tests that are urgent, do them on that floor, without having to go down to the laboratory." (Laboratory Personnel 201)

Lastly, we discussed with the participants what was essential or important to have to ensure that sample collection and analysis is fluid and efficient. The most coded topic was the quality of the sample collected, as that was essential for the laboratory personnel to give a good, trustworthy result. *Personal del Laboratorio*/Laboratory Personnel 203 discussed the importance of a well-prepared sample:

> *“Que la muestra tiene que estar bien rotulada, organizada, también la muestra tiene que estar alta para lo que es la prueba, en este caso es el HIV. Entonces de ahí pasa muy bien lo que es el proceso, que ya es parte del proceso, lo que es la distribución y la organización de las muestras para poder terminar el proceso lo más claro posible en el tiempo adecuado.” (Personal del Laboratorio 203)*

> "That the sample has to be well labeled, organized, also the sample has to be high for what the test is, in this case it is HIV. So, from there the process proceeds very well, the distribution and organization of the samples to be able to finish the process as clearly as possible in the right time.” (Laboratory Personnel 203)

2.3.7.6 The HIV program (CEDI) and psychologists are essential HIV care so that patients are well-supported (Organizational Facilitator)

When it comes to HIV diagnosis and care at the hospital, there are specific procedures that they follow to provide this service, which are described in Organizational Barriers (2.3.7.2). The hospital has pre-counseling and post-counseling for those suspected of having HIV. That is, if one is tested for HIV, they will receive counseling with a psychologist before they receive their result and after they have their result (whether they have HIV or not). Having a psychologist counsel patients was described by several participants as essential, as there is a lack of education around HIV and HIV is stigmatized in the DR so patients may have a difficult time coping with the social implications of an HIV diagnosis on their lives. Médico/Clinician 120 explained their thoughts on the imperative role of a psychologist in the HIV care cascade:

> *“Entiendo que lo primero es que el paciente se de un seguimiento por salud mental o psicología, ah, porque todavía para los pacientes sigue habiendo ese estigma de que “aye si tengo VIH me voy a morir.” Entonces ese acompañamiento por la parte de salud mental es esencial y también que el paciente entienda las opciones de tratamiento que no se va a morir… que hay bastantes extensas opciones de tratamiento y eso…pero que eso lo fundamental, es que el paciente entienda que hay opciones y que no es el fin del mundo eso.” (Médico 120*)
>
> "I understand that the first thing is that the patient is followed up for mental health or psychology, ah, because there is still that stigma for patients that "aye if I have HIV I'm going to die." So that accompaniment by the mental health part is essential and also that the patient understands the treatment options that they are not going to die ... that there are quite a few extensive treatment options and that... But that the fundamental thing is that the patient understands that there are options and that it is not the end of the world."(Clinician 120)

Similarly, CEDI, the hospital’s HIV-specific disease program (similar programs are found in other public hospitals) was discussed as fundamental to HIV patients’ care. This is a group of specialists that HIV patients are referred to quickly after being diagnosed with HIV or are referred to for long-term care. Participants described CEDI as having a positive impact on patients as they can give holistic care via psychological care, perform viral load and CD4 testing (via external laboratories), provide antiretroviral therapy, provide resources in Haitian creole, and are a team of educated, specialized individuals that can discuss HIV with patients without stigma. *Médico/*Clinician 119 explained how quickly those who receive a positive HIV test typically begin to interact with the program:

> *“En las 24 horas el paciente está con nosotros y el paciente puede iniciar la toma de ARV, antirretroviral. No pasa de*

> *siete días. Y en [CEDI], la única forma de que pase de siete días es si el paciente se pierde, y nosotros tratamos de llamarlo y a veces da número falso. Pero… siempre a los siete días, cinco días, seis días, a veces el mismo día si tiene todo. Se puede iniciar tratamiento para el VIH." (*Médico *119)*
>
> "Within 24 hours the patient is with us and the patient can start taking ARV, antiretroviral. It doesn't go beyond seven days, it doesn't go beyond seven days. And in [CEDI], the only way seven days pass is if the patient is lost, and we try to call them and sometimes they gives a false number. But... Always at seven days, five days, six days, sometimes the same day if you have everything. HIV treatment can be started." (Clinician 119)

2.3.8 Barriers and Facilitators at the Community Level

2.3.8.1 Difficulties sending samples from the hospital to private laboratories in Santiago de Los Caballeros (Community Barrier)

If the laboratory is unable to perform a certain type of testing, the clinicians will either wait for the testing to be available (if the issue is a lack of reagents) or send the sample to another lab in Santiago de Los Caballeros for testing. Sending these samples to, most often, private laboratories can be a barrier to obtaining a diagnosis as the patient has to pay for it out of pocket, it can take even longer to receive a result, or the sample could get lost or contaminated in the transport of the sample by family members to the external laboratories. Médico/Clinician 101 explained the barriers of obtaining a result from another laboratory in the city:

> *"[…] enviar una muestra afuera tiene que ver mucho si el paciente puede costear esa muestra afuera. O sea, aquí no se le va a cobrar por una muestra, ¿entiende? Se le hace gratuitamente. Entonces, enviarlo fuera sería perder más*

> *tiempo para poder diagnosticar. Y segundo, que el paciente cuente con el factor económico para poder costear esa prueba. Entonces, lo más efectivo puede hacerlo aquí mismo. El paciente está aquí, no se le va a cobrar y ya tú tienes el resultado inmediatamente." (Médico 101)*
>
> "[…] sending a sample outside has a lot to do with whether the patient can afford that sample outside. Like, you're not going to be charged for a sample here, understand? It is done for free. So, sending it out would be wasting more time to be able to diagnose. And second, that the patient has the economic factor to be able to pay for that test. So, the most effective is being able to do it right here. The patient is here, they will not be charged, and you have the result immediately." (Clinician 101)

2.3.8.2 Community organizations provide support to non-Dominican patients (Community Facilitator)

The value of community organizations in Santiago de Los Caballeros was discussed by a few of the participants, who help immigrants, especially Haitians, in navigating the healthcare system. *Médico*/Clinician 110 explained how there is *"un grupo de mujeres, eh, de nacionalidad haitiana que trabajan acompañando a [haitianos que no hablan español]"/* "a group of women who are Haitian nationals who work, accompanying [Haitians who do not speak Spanish]", which has provided additional support so they can more easily access care.

2.3.9 Barriers and Facilitators at the Societal Level

2.3.9.1 Stigma around HIV is a significant barrier to HIV care (Societal Barrier)

All of the clinicians interviewed highlighted the stigma around HIV in the DR as whole. The clinicians described how this is shown socially in that people think that those who have HIV have a certain lifestyle (use of drugs, promiscuous), that one can get the disease just by skin-to-skin contact, or their family members may reject them. In general, they often become isolated from society. Thus, this stigma or taboo around HIV leads patients to not want to take medications, get tested for HIV, to be around other HIV patients in the HIV clinic/program, and to believe that they will die if they are diagnosed with HIV. *Médico*/Clinician 103 described the effects of stigma on one's willingness to seek and follow HIV treatment plans:

> *"Si el paciente ya sabe su diagnóstico se le explica cómo tiene que tomar sus medicamentos. Porque los medicamentos afectan la vida social del paciente. […] Y también la parte de la vergüenza, la parte de que todo el mundo lo va a señalar, "eres un H." […] Hay mucho tabú. La gente es muy… […] los aíslan. Entonces por eso es que esos pacientes entran en un estado psico afectivo muy complicado. Entonces por eso aquí hay mucho tabú. Sí, muchos estigmas del VIH. […] Si tú te tomas tus medicamentos, tú sigues una vida saludable, una vida privada normal. Bien, tú puedes vivir una vida completa. […] El VIH uno puede vivir con eso." (Médico 103)*

> "If the patient already knows their diagnosis, it is explained to them how to take their medications. Because medications affect the patient's social life. [...] And also the shame part, the part that everyone is going to point out, "you're an H." [...] There is a lot of taboo. The people are very... [...] They

> isolate them. So that's why these patients enter a very complicated psycho-affective state. […] If you take your medications, you follow a healthy life, a normal private life. Well, you can live a full life. [...] HIV you can live with." (Clinician 103)

Also, though not often discussed, within the healthcare system clinicians and laboratory personnel themselves may contribute to the stigmatization of those living with HIV. One clinician, *Médico*/Clinician 119, described an experience one of their patients had in which they were spoken to "*en una manera brusca*"/"harshly" in the sample collection department, which caused them to "*abandonar tratamiento en el hospital*"/"abandon treatment at the hospital". Luckily, though, they were able to continue to treatment at a different location in the private sector.

2.3.9.2 Inconsistent funding in public health by the government limits laboratory personnel, clinicians, and the HIV program from providing optimal care (Societal Barrier)

When asked if the government supports patients with HIV well, participants had mixed responses. They believed the government was both a barrier and facilitator to HIV patient support. In terms of being a barrier, this was discussed in three aspects. Firstly, in terms of the testing for HIV, specifically CD4 counts and viral load testing, at the time of the interviews, CD4 testing was not being performed in the country for the public sector. It is not immediately clear from the interviews why that is, but the participants pondered that it was due to a lack of reagents or a change in government funding to support other diseases. When viral load testing is performed, it takes about 15 days to receive the results,

as the samples have to be sent and analyzed in Santo Domingo, which is about 3 hours away (driving) from the hospital in Santiago de Los Caballeros. This lack of quick testing or testing at all can lead to patients be lost to follow up, discussed by *Médico*/Clinician 108:

> ***Entrevistadora B:*** *¿Los pacientes tienen que esperar como 15 días [para recibir los resultados de] ((…ininteligible…)) carga viral de Santo Domingo? ¿Sí?*
> ***Médico 108:*** *Mhm.*
> ***Entrevistadora B:*** *Entonces, ¿qué hacen en este tiempo?*
> ***Médico 108:*** *Esperar.*
> ***Entrevistadora B:*** *Ok. ¿Y vienen acá otra vez?*
> ***Médico 108:*** *Sí, regresan, si se interesan mucho. [O] lo abandonan.*

> **Interviewer B:** Do patients have to wait like 15 days [to receive results from] ((... unintelligible...)) Santo Domingo viral load? Yes?
> **Clinician 108:** Mhm.
> **Interviewer B:** So what do they do in this time?
> **Clinician 108:** Wait.
> **Interviewer B:** Ok. And do they come here again?
> **Clinician 108:** Yes, they come back, if they are very interested. [Or] they abandon it.

In addition, it affects how CEDI personnel can provide care to their HIV patients, as they are not able to consistently provide care procedures. Lastly, some participants felt like while the government does support CEDI and other HIV programs, it does not *"incentivar más la gente a pertencer a este tipo de programas*"/ "incentivize people more to belong to these programs" (*Médico*/Clinician 101).

The second aspect that participants discussed in terms of government barriers was when it comes to providing reagents for the hospital itself, some participants discussed how “burocracia”/ ”bureaucracy” was a barrier to reagents arriving in a timely manner. Additionally, a few laboratory personnel discussed how there is a lack of scientific innovation in the country. One participant explained to us that there is a lack of people studying biotechnology. So, they were disappointed in the lack of advanced machines or machines able to do certain types of testing, which requires the hospital’s laboratory to send samples to another laboratory outside of the hospital. On this topic, *Personal del Laboratorio*/Laboratory Personnel 204 stated:

> *“Mira aquí cuando, por ejemplo, llega una prueba que no se realiza [por el laboratorio], obviamente porque no tenemos lo- las, las, los utensilios, pues se envían [las pruebas o muestras] a otro centro. ¡Aquí no inventan! Aquí, simplemente si no tenemos la máquina para hacer la prueba, se envían a otro lugar.” (Personal del Laboratorio 204)*

> "Look here when, for example, a test arrives that is not performed [by the laboratory], obviously because we do not have the utensils, they [the testing or samples] are sent to another center. They don't invent here! Here, simply if we don't have the machine to do the test, they are sent somewhere else." (Laboratory Personnel 204)

The third aspect of government support that the participants felt was not sufficient was in terms the education provided regarding HIV. They felt that the government does not provide sufficient public education for HIV such that people

could be more aware of comorbidities with HIV and understand more about the virus in general. *Médico*/Clinician 102 explained how the HIV programs are helpful, but the lack of public education around HIV is still a barrier:

> *"Según lo que he escuchado de como yo no he trabajado ahí [en CEDI]. Veo que están ayudando. Lo único que faltaría sería un poco más de educación a nivel público, no para el mismo paciente que lo tiene, sino a los que no. Eso sí, siento que faltare un poco, pero ellos están ayudando, los programas" (*Médico *102)*
>
> "According to what I've heard since I haven't worked there [at CEDI]. I see that they are helping. The only thing missing would be a little more education at the public level, not for the same patient who has it, but for those who do not. Of course, I feel that I am missing a little, but they are helping, the programs" (Clinician 102)

2.3.9.3 Government investment in public health is improving certain diseases' care, but outreach is needed to educate the public (Societal Facilitator)

As previously mentioned, the participants expressed that the government is both a barrier and facilitator to HIV care. In terms facilitators for HIV diagnosis and treatment, most of the participants discussed how the HIV programs provide a lot of support, resources, and free medications to those living with HIV. Also, they are easy to access, national programs, that provide testing and care in all the provinces of the DR. *Médico*/Clinician 111 explained how they thought the government's efforts for HIV were very good:

> *"Considero que nuestro gobierno ha hecho los esfuerzos necesarios para tratar esa población de pacientes. Tengo entendido que en todas las provincias hay una institución donde se encarga de dar las consultas, prevención,*

> *diagnóstico, y tratamiento de los pacientes con HIV. O sea que es totalmente gratis. El gobierno las suple. Esas necesidades son suplidas por el gobierno. Los pacientes no tienen que preocuparse por pagar una consulta, ni tampoco tiene que preocuparse por los medicamentos porque estos van a estar suplidos por el estado. Entonces, considero que el esfuerzo que se ha hecho es muy bueno."*
>
> "I think our government has made the necessary efforts to treat that population of patients. I understand that in every province there is an institution where they are responsible for consultation, prevention, diagnosis, and treatment of HIV patients. Like, it's totally free. The government supplies them. These needs are met by the government. Patients don't have to worry about paying for a consultation, nor do they have to worry about medications because they're going to be supplied by the state. So, I think the effort that has been made is very good."

Some participants also mentioned how the government supports other diseases via similar programs like tuberculosis, and also is funding disease or conditions that weren't previously funded like autism and pulmonary fibrosis. *Médico*/Clinician 123 explained how the country has advanced due to the COVID-19 pandemic, in the context of HIV:

> *"Una de las cosas fundamentales que ha hecho que la pandemia, no estamos en las mejores posiciones porque pudiésemos estar mejor. Pero hemos cambiado. O sea, la República Dominicana avanzado mucho en materia de VIH y eso se ha dado fundamentalmente por tres cosas. Una, la voluntad política. O sea, los existen programas de VIH que son manejados, dirigidos, y financiado por el Ministerio de Salud Pública. O sea, el Estado da los medicamentos. Tener los medicamentos era algo que en el pasado no existía. Lo segundo es la movilización social. Las organizaciones de base comunitaria de República*

Dominicana se han involucrado en la respuesta de VIH. Son organizaciones que siempre están activas y que siempre están procurando que el Ministerio haga su trabajo y que tengamos los medicamentos. Y tercero, las organizaciones que trabajan con el paciente, con el usuario. Yo creo que el tener organizado un grupo de personas que ti que viven con VIH ha hecho que pueden hablar en público y que pueden pedir en público a las autoridades que hagan el trabajo. El país tiene autonomía de medicamentos antirretrovirales en la actualidad se los compra, no necesita que nadie lo done. Y las pruebas de VIH, de diagnóstico y de seguimiento son gratuitas en el sector público, los medicamentos, y también el tratamiento." (Médico 123)

"One of the fundamental things that the pandemic has done, we are not in the best position because we could be better, but we have changed. In other words, the Dominican Republic has made a lot of progress in terms of HIV and that has been fundamentally due to three things. One, political will. That is, there are HIV programs that are managed, directed, and funded by the Ministry of Public Health. In other words, the State gives the medicines. Having the drugs was something that didn't exist in the past. The second is social mobilization. Community-based organizations in the Dominican Republic have become involved in the HIV response. They are organizations that are always active and that are always trying to get the Ministry to do their job and that we have the medicines. And third, organizations that work with the patient, with the user. I believe that having organized a group of people living with HIV has made it possible for them to speak in public and that they can publicly ask the authorities to do the work. The country has autonomy of antiretroviral drugs, currently buys them, does not need anyone to donate it. And HIV testing, diagnosis and follow-up are free in the public sector, medicines, and also treatment." (Clinician 123)

The participants also discussed various topics they would like to see the government support more that would facilitate HIV diagnosis and treatment, and the most frequent response was that they would like expanded HIV testing capabilities. That is, decentralizing CD4 and viral load testing such that testing does not only occur in Santo Domingo (which can take as long as 15 days to receive a result). *Médico*/Clinician 119 discussed how decentralization of viral load is lacking at the hospital:

> *"[…] descentralizar la… donde se envía la muestra. Si tuviésemos una, un centro, biomolecular acá en este hospital, que es un hospital que vienen pacientes de diferentes hospitales de referencia. Eso sería malo porque estamos hablando que dos o tres días ya tuviesen una carga viral o CD4 ahí y cuidado si el mismo día para nuestro hospital, eso ayudaría bastante" (Médico 119)*
>
> *"[…] decentralize the... where the sample is sent. If we had one, a center, biomolecular here in this hospital, which is a hospital that patients come to from different reference hospitals. […] Having a viral load or CD4 there and care if the same day for our hospital that would help a lot" (Doctor 119)*

The participants also discussed the need for an increase of personnel in the laboratory as well as for the monitoring of people living with HIV.

In terms of mitigating stigma in the country, all the clinicians suggested campaigns to educate the public about HIV. With country-wide campaigns, the clinicians suggested advertisements via the radio, television, via influencers, and digital campaigns. They also discussed how sexual education should be

emphasized in public schooling and aim to normalize HIV more in society. On this topic, *Médico*/Clinician 111 said:

> *"Yo considero que se debe promocionar más la campaña de prevención y que también las personas hagan conciencia de que las personas con HIV, hecho de que tú tengas un diagnóstico de HIV no significa que tú te vas a morir. Claro está, si tú te descuidas puedes llegar a empeorar la salud y llegar a producir la muerte. […] Entonces, no significa que, que sea una persona que tú tengas que rechazar. Entonces se debe trabajar un poco más en la promoción, bueno de la promoción de la prevención y también para tratar de no discriminación a esas personas…" (Médico 111)*

> "I think that the prevention campaign should be promoted more and that people also become aware that people with HIV, just because you have an HIV diagnosis does not mean that you are going to die. Of course, if you neglect yourself you can worsen your health and lead to death. […] So, it does not mean that they are a person that you have to reject. So, promotion should be worked on a little more, good promotion of prevention and also to try not to discriminate against those people ..." (Clinician 111)

2.3.10 What participants would like in a new device

The final part of the interview discussed characteristics participants would like in a new point of care device, for the home. Participants often would describe how they would like their device to be similar to a glucómetro/glucometer. When asked what characteristics they would give to their ideal device, *Médico*/Clinician 109 said:

> *"De que sea como un glucómetro, un glucómetro, sí. Por ejemplo, si me va a hacer el HIV [prueba] que yo lo ponga con la sangre y me diga de una vez sí es positivo y el conteo de CD4 qué tiene. [ríe]" (Médico 109)*

> "That it's like a glucometer/. For example, if I am going to do HIV [test], I should put it in my blood and tell me at once if it is positive and the CD4 count is there. [laughs]" (Clinician 109)

In terms of characteristics that the participants would like to see in a new device, the most cited were: easy to use (24/36), fast (18/36 participants), reliable (14/36 participants), can analyze various analytes in one device (10/36 participants), small in size/weight (10/36 participants). We have summarized the other responses into Table 2.3.

Table 2.3: Participants descriptions of what they would like in a new device for the point of care.

Characteristic	Responses
Cost	• Low-cost • Cost unimportant if high quality device
Physical characteristics	• Long-lasting • Portable • Easy-to-clean • Wearable • Environmentally friendly • Small size/low weight
Ideal time to result	• "Fast" • Ranged from: instantaneous to 24-48 hours (dependent on type of test)
Workflow	• Minimal steps • Automated • Efficient • Simple directions • Reusable • Easy to use

	• Can show or print results
Analytical capabilities	• Multiple analytes • Multiple patients at once • Low sample volume • Accurate and precise (reliable)

2.4 Discussion

This study examined the barriers and facilitators to obtaining (primarily) a HIV diagnosis and/or treatment, from various lenses, to inform device development, especially for the point of care. We used the social ecological model for theme development and found various barriers and facilitators at each level. Each level was not mutually exclusive, though, as the topics of each level were interrelated. For example, since stigma is present in the DR society, this affects patients' willingness to obtain a diagnosis or adhere to treatment. Castro et al. highlighted a similar finding in their study of barriers and facilitators for HIV treatment adherence in Puerto Rico, that the barriers and facilitators are complex and involve multiple systems [56]. We sought to explore these barriers and facilitators to inform point of care device development for the hospital and for other areas that may have similar barriers and facilitators to obtaining a HIV diagnosis or treatment. Though context-specific results have been reported here, we found similar barriers and facilitators to obtaining a diagnosis and monitoring for HIV as several other studies which include barriers like: stigma, socioeconomic status, lack of information about HIV, and long wait-times for results, and include facilitators such as: offering of HIV exams, education of the patient, clinicians, and communities [57]–[60]. This information is critical for public health interventions, but also informs the context in which point of care devices will be developed, which is rarely studied and reported on. We have summarized our key findings and recommendations for point of care device development based on these findings in Table 2.4.

Table 2.4: Key findings of this study and recommendations for point of care (POC) device development

Key findings	Recommendations for point of care (POC) device development
1. Obtaining a HIV diagnosis has multi-system barriers and facilitators	#1: Multiple stakeholders' voices at different levels of healthcare system should be incorporated in POC device development and implementation
2. Stigma affects how one seeks HIV diagnoses and treatment	#2: POC devices should be developed with non-stigmatizing
3. The participants highly regard psychologist involvement in HIV diagnoses and treatment	#3: Psychological support should be incorporated into HIV point of care delivery
4. Cultural and linguistic barriers between patient and clinician are important to overcome when delivering care	#4: POC devices should be able to deliver care in multiple languages
5. A well-resourced and well-managed laboratory in essential to testing at the hospital	#5a: More low-cost point of care devices should be developed to decentralize hospital testing, especially in public hospital settings #5b: POC device developers should reduce the supply chain reliance of LMICs on high-income countries
6. Consistent government funding of public health measures like educational health campaigns and increased testing capacity would improve patient knowledge and willingness to obtain a diagnosis	#6: POC technology developers should work with public health practitioners and other key stakeholders to develop educational campaigns around their technologies
7. Users have a wide variety of needs regarding POC devices	#7: User needs must be well-studied for POC device development and implementation

2.4.1 Multiple stakeholders' voices at different levels of healthcare system should be incorporated in POC device development and implementation (Key Recommendation #1)

As point of care technology demand continues to increase [61], understanding the barriers and facilitators to sample analysis is essential to ensure technologies meet user needs. The needs for point-of-care technology development are often discussed from a strictly technological or scientific perspective, that is, by discussing the need for the development novel methods of sample analysis with paper, microfluidics, biosensors, and more [43], [62]. While the technology must be sound, for successful implementation inclusion of stakeholder perspectives is essential for any intervention, yet these perspectives are lacking from the pre-implementation phase of point of care technologies. Instead, user perspectives are sought out and reported on after implementation, in which stakeholders state their issues with implementation of the technology [63]. The Point-of-Care Technologies Research Network, a group of centers funded by the National Institutes of Health in the US, made a call for qualitative clinical needs assessments such as the one performed here to ensure social implications, stakeholder needs, economic viability, and technical feasibility were considered when implementing new point-of-care technologies [10]. Seeking the inputs of end users of these point of care technologies and various stakeholders involved in one's care is not only beneficial for successful implementation, but also is essential for ensuring the needs of the users are met [10], [64]. We recommend that user voices from various levels of health systems be incorporated into device development and implementation.

2.4.2 POC devices should be developed with non-stigmatizing language (Key Recommendation #2)

People living with HIV are very stigmatized in many places around the world [65], which there reduces their likelihood of seeking care or following up with results [66]. All (100%) of the physicians stated that HIV stigma was present with patients, families, employers, and more broadly in the country. This led patients to not want to seek care or follow up with results. Thus, when developing point of care tests for HIV, especially those for use at-home, it is critical that non-stigmatizing language is used in the instructions for use and for delivering the result. Self-tests for HIV are becoming of interest (or have already been introduced) to several health systems [67], including that of the DR, as told to us by one of our participants. This type of point of care test has been discussed as a way to strengthen healthcare systems, increase access to testing, reduce the need for lengthy clinic visits, ensure confidentiality, promote patient autonomy, and lower stigma. When discussing how self-testing has the potential to reduce stigma, researchers are often discussing stigmatization from healthcare providers or in a healthcare setting [68]. In the DR context, and likely many other contexts, stigmatization is also extremely important to consider from the greater society. However, the adverse outcomes that would be related to non-clinic related stigma of HIV self-testing are rarely studied [69]. Maestre et al. investigated co-designing technologies that incorporated HIV stigma coping strategies, which generated several ways technology could help to reduce HIV-related stigma [70]. While most of the results of this exercise were “futuristic”, an example of a way technology can incorporate non-stigmatizing language is to connect results with mobile applications to deliver informational and supportive messaging. We recommend that POC device development should incorporate non-stigmatizing language such that adverse outcomes such as mental health crises could partially be mitigated at the time of receipt of the diagnosis.

2.4.3 Psychological support should be incorporated into HIV point of care delivery (Key Recommendation #3)

Similar to the importance of non-stigmatizing language with technology use, the mental health impacts of receiving a diagnosis and if using a POC for disease monitoring, must be considered. The participants in this study, both laboratory personnel and clinicians discussed the importance of a psychologist being involved in the process of diagnosing someone with HIV and in their ongoing care. Whether one receives a positive or negative result, if they are suspected of having HIV they are connected with a psychologist as a part of hospital procedures. The importance of psychologists has also been highlighted in other parts of the DR by Agüera and Cruz [71], who found that 100% of the 38 surveyed patients living with HIV in Dajabón, Dominican Republic stated psychologists were essential to their treatment plan. Other studies outside of the DR have more broadly discussed HIV counselling as integral to holistic treatment for those living with HIV [72]. Thus, this finding is in line with other research.

However, this presents a dilemma as the DR and the world moves toward at-home, POC diagnostic testing. There would be a lack of counseling or mental health support if one was to perform a POC test at home, which is one of the arguments against at-home HIV testing [73]. A potential way to address this would be to utilize telehealth to deliver mental health support as one does their at-home diagnostic. Several studies have examined telehealth for HIV to delivery mental health care [74]–[76] which have shown positive outcomes for and improvements in the HIV care cascade, including in LMICs. When it comes to integrating this with laboratory services, there are complex challenges concerns related to regulations, cost, and logistics. We recommend that POC device development, especially for at-home testing, incorporate telehealth to provide mental

health support to those receiving HIV diagnoses or monitoring their health outside of a clinic.

2.4.4 POC devices should be able to deliver care in multiple languages (Key Recommendation #4)

One population that faces many barriers when seek care at the hospital that was discussed in the interviews was immigrants, who were typically from the neighboring country of Haiti. Many participants noted that they were at a higher risk of being diagnosed with HIV, which has been corroborated in the literature [77], with 48% of new HIV diagnoses among Haitian migrants. Some participants discussed the difficulties Haitian immigrants in particular face compared to other immigrant communities (Venezuelans and Colombians), with one of the largest being the language barrier, as the languages spoken in Haiti are Haitian Creole, Haitian French, and French [48]. Additionally, in 2013 the DR rescinded citizenship to Haitians born to undocumented parents after 1929, thus creating thousands of undocumented individuals [77]. Lack of citizenship affects one's access to healthcare financially, removing one's ability to rely on health insurance to offset health costs. Also, Haitian immigrants who are seasonal migrant workers [77] have frequent address, phone number, and location changes, causing loss of provider follow-up to occur. Loss to no follow-up was discussed by many of the physicians as an issue, as well as the language barrier.

As people move and settle in different areas of the world, language needs to be considered in healthcare systems and in technologies that are developed. Batchelor et al. analyzed HIV/AIDS-related policy of various countries and found that language and multilingualism were not concretely outlined for HIV/AIDS interventions [78]. There are several ways to overcome this challenge with online translators, mobile apps, or video calls [79]. However, in the hospital studied here and many other places, internet and cell

phone service was limited. Thus, when delivering results with a POC device, multilingual resources to understanding the result and their implication that do not rely on the internet must be incorporated.

2.4.5 More low-cost point of care devices be developed to decentralize hospital testing, especially in public hospital settings (Key Recommendation 5a)

Many of the participants expressed how they were satisfied with the capabilities of their laboratory, despite some of its shortcomings. The physicians stated that having a laboratory physically in the hospital was a facilitator for successful patient diagnosis and monitoring as it was close in proximity and low-cost or free for patients. The large regional hospitals in the DR have laboratory facilities with many capabilities, which means that patient samples do not always have to be sent to the capital or a private facility for testing. This increases access to healthcare to those with lower resources such as those who make a low income or tourists. There are a few specialized tests, like viral load analyses, that do have to be sent to other laboratories, though. However, recently instead of viral load analyses being performed only in the capital, Santo Domingo, a facility was recently built in the city of the study site which has improved access to this type of testing. This highlights the importance of decentralization of laboratories and ensuring these critical public laboratories have sufficient supplies to provide laboratory services to the large communities they serve.

Decentralization of laboratories was especially important at the start of the COVID-19 pandemic as countries' health systems quickly had to make testing accessible to various parts of the country [80]. In a study by Bianchi et al. where they qualitatively assessed the acceptability of a point-of-care technology before and after implementation, the study participants discussed the difficulties they faced with only a centralized laboratory, before

implementation of the point-of-care testing, which included difficulties in transportation of samples and communication of result challenges [81]. Similarly, Engel et al. described how the coordination of care was difficult in India, but this was due to a lack of communication between hospitals and clinics in which testing was done. Decentralization is at the heart of why point of care technologies are being developed [1], and this study this study highlighted how having a decentralized laboratory within the hospital facilitated communication and eliminated the need for complex transportation systems. The greatest need for decentralization in this setting, and others, is for nucleic acid-based tests like viral load in the case of HIV. There have been commercially available devices that have been developed, but they can be cost prohibitive and complex [82]. Thus, we recommend that more low-cost point of care devices be developed to decentralize hospital testing, especially in public hospital settings.

2.4.6 POC device developers should reduce the supply chain reliance of LMICs on high-income countries (Key Recommendation 5b)

The participants, despite their frustrations with some of the systems in the laboratory, still expressed that the laboratory was an asset to faster and more streamlined sample analysis. This is important to overcome to reduce some of the barriers to testing, which include long wait time for results [66]. Bianchi et al. performed a study similar to that done here by qualitatively assessing perceptions of a point-of-care technology before and after implementation as a part of an intervention's evaluation in eight African countries [81]. Bianchi et al. interviewed laboratory personnel and healthcare workers as well and found that laboratory personnel mentioned one of their most prominent barriers were disruption of healthcare services due to inconsistent supply of laboratory supplies and issues with transportation of samples (the location in which their study took place had a centralized laboratory in which samples had to be transported to). In this study, laboratory

personnel and healthcare workers also discussed disruption in performing their duties, but from two different perspectives. Clinicians described how the limited laboratory hours interfered with their ability to get results for their patients, while the laboratory personnel cited insufficient equipment, inconsistent access to reagents, and inadequate instruments as to why they are unable to sometimes quickly give results to clinicians and/or patients.

As discussed by Engel et al. who performed a qualitative study across various settings in India regarding barriers to point-of-care testing, diagnostic testing does not occur in isolation [83]. That is, there are several players that are required for successful implementation of diagnostic testing that are providers, the government, scientists, and more. Since the laboratory studied here relies on imports from other countries for reagents and devices, if there are global supply chain interruptions or other issues, this causes delays to the receipt of reagents, new instruments, and more as the laboratory personnel described. The challenges of the global biotechnology landscape are complex, but is well illustrated when exploring the challenges the COVAX initiative faced when attempting to have equitable distribution of COVID-19 vaccines [84]. High income countries, specifically the United States and some countries in Europe, tend to have the most innovation, that is new product development, around biotechnology [85]. Other countries will purchase or rent equipment, reagents, etc. from these countries and the intellectual property is within the higher income country, limiting other countries' abilities to manufacture, distribute and develop similar products from within their own boarders. Thus, this leads to a reliance of LMICs on high income countries when it comes to resources for diagnostic testing. Several countries in Latin America, though, have scaled up their investment in biotechnology [85], likely leading to less of a reliance on other continents for supplying their biotechnology sector. We recommend that point of care device developers explore ways to reduce the supply chain reliance of LMICs on high-income countries.

2.4.7 POC technology developers should work with public health practitioners and other key stakeholders to develop educational campaigns around their technologies (Key Recommendation #6)

Though we did not discuss specific populations that were stigmatized, most of the physicians stated that education of the public about HIV via flyers, radio advertisements, and more public health campaigns would help to reduce HIV stigma. The suggested educational campaigns have been shown in a few settings to reduce negative attitudes, but with the caveat of needing to be well-designed and to include people living with HIV in the study design [86]. Additionally, evidence of success of these interventions are missing in many regions such as Central and South America [86]. Budhwani et al. is addressing this gap in the DR, by launching a pilot of an intervention to reduce stigma with sexual and gender minority people living with HIV [87]. This pilot study is ongoing, but demonstrates the desire of physicians to reduce stigma related to HIV in the DR. We recommend that POC technology developers continue to work with public health practitioners and other key stakeholders to develop educational campaigns around their technologies.

2.4.8 User needs must be well-studied for POC device development and implementation

In terms of what participants would like in a new device, they stated similar characteristics that point of care device developers aim to have. POC devices try to be easy to use, portable, fast, affordable, and reliable [1], [43], [62], thus these results confirm that the goals of the technology match those of user needs. Qualitative methods for point of care devices typically involve assessing their implementation and/or evaluating attitudes towards them [88], [89]. However, these studies, as well as the one performed here to understand user needs are rarely, if ever, done. The study performed here showed that there are a variety of user needs, which are context dependent. For example, not everyone

stated that they would like a device that gives an instantaneous result; in other words, “fast” is relative to the person’s perspective. However, many people wanted the device to be reliable, similar to other studies’ results about attitudes toward blood-based POC diagnostics [88]. This therefore gives space for POC device developers to prioritize sensitivity and specificity of the results even if it does take more time. It is essential to incorporate qualitative methods with point of care device design not only to understand user needs, but to understand the variety of ways that they may impact one’s personal, professional, and social life.

2.5 Conclusions

Overall, this study provided insight into the landscape of biological sample analysis in the DR for HIV diagnosis and monitoring. The participants important insight into the challenges and benefits of working at a public hospital in the DR, with laboratory personnel highlighting the specific processes of the laboratory and clinicians largely discussing patient and societal perspectives. The barriers and facilitators present in obtaining a diagnosis and/or treatment are in multiple levels of society, and would require collaboration across several areas to address, including with those who design point of care technologies. Reducing stigmatization, mitigating language barriers, emphasizing mental health care, and ensuring efficient laboratory workflows are among the most important considerations to maximize one’s likelihood to obtain a diagnosis or treatment, especially for HIV.

For point of care technology developers, this study showed firstly the importance of qualitative assessments before implementation of technologies to understand the current landscape and to identify gaps that can be addressed. It can be concluded that providing machines that can be self-maintained, that is without a lot of reliance on outside suppliers, within the setting in which they are implemented is essential to a smooth workflow for

laboratory personnel and physicians. Minimizing disruptions to the workflow of laboratory personnel and physicians is the best manner to maximize positive outcomes for patients, which is the goal of point of care technologies.

This work could be expanded upon by evaluating public health campaigns such as flyers about HIV and via other media to educate the public and reduce HIV stigma. Based on these results, the hospital system has some areas they could focus their efforts on like emphasizing the development of more disease-specific programs like that for HIV or exploring ways to make sample collection from patients more efficient. In terms of POC devices, evaluation of implementation currently used devices or devices that could be used could be explored. Future work in this space should incorporate user perspectives before design and implementation of a POC device with a wider focus than just the technology to strengthen health systems holistically.

2.6 Strengths and Limitations

There were several strengths and limitations to this study. A strength of this study was that questions of the interview guide covered several topics to give a broad view of the political, social, economic, technological, and medical landscape of the DR from which several in-depth studies can expand upon. Also, the use of template style thematic analysis allowed for more extensive theme development for this rich dataset. In terms of the study population, a strength of this study was that they had experience in several different sectors of healthcare, primary care, specialty clinics, private hospitals, and more, so they were able to speak to those areas, enhancing our understanding of the wider DR context. A limitation of this study is that it was performed at a single type of hospital (public and regional), which may the applicability of this study to other hospitals in the DR and in similar countries. The medical residents, who made up most of the clinicians interviewed

were mostly a month into their residency programs, slightly limiting their perspective on some aspects of the study, but they were the closest to the process of sample collection. This study also was limited in the fact that a specific point-of-care technology was not inquired about, so future studies would need to be more specific to their interventions. Lastly, an important strength of this study was the multidisciplinary team that spanned the fields of biomedical engineering, public health, global health, qualitative research, and medicine. This is integral to our shared goal of ensuring biomedical technologies are equitable for sustained use for multiple types of users.

2.7 Acknowledgements

We would like to thank the participants for entrusting us with their stories and their words to be able to do this study. KL and DA would like to thank the School of Public Health and the Emerging Infectious Disease Scholarship program for funding this work. KL and DA would also like to thank Dra. Morell, Karla, Dra. Rodriguez, and Deisy for their help in organizing interviews once at the hospital.

CHAPTER 3: A CAPILLARY FLOW-DRIVEN MICROFLUIDIC DEVICE FOR POINT OF CARE BLOOD PLASMA SEPARATION

Chapter 3 has been published as an original research article.

Brakewood, W.*, **Lee, K.***, Schneider, L., Lawandy, N., and Tripathi, A. “A Capillary Flow-driven Microfluidic Device for Point of Care Blood Plasma Separation”. *Front. Lab on a Chip*, 1 (November):1-12. 2022.
*both authors contributed equally to this work.

3.1 Abstract

Plasma has significant utility as an input for diagnostics and screening for conditions such as viral infections, cancer, and more. However, plasma is difficult to obtain at the point-of-care, as separation from whole blood is typically carried out via centrifugation. We have designed and optimized a low-cost, simple-to-operate microfluidic device which carries out the separation of plasma from whole blood. The device utilizes depth filtration as its separation mechanism and collects plasma via capillary action, allowing for operation without components that drive flow externally. We first optimized device dimensions and operating parameters and demonstrated consistent separation efficiencies for the samples with hematocrits ranging from 25-65%. The impact of input sample hematocrit percentage on flow rate through the device was also examined, with samples with hematocrits greater than 45% decreasing plasma flow rate. Lastly, we evaluated the ability of this device to produce plasma with a high protein concentration and found no significant difference between protein levels in samples from the device compared to samples produced via centrifugation. This system produced plasma with a maximum separation efficiency of 88.5% and achieved a maximum plasma volume of ~14 μL from a 50 μL whole blood input. The low cost, simplicity of operation, and high plasma quality associated with this device give it many advantages in a point-of-care setting. This device could be integrated into plasma-based diagnostic workflows to increase access to various types of disease testing and monitoring.

3.2 Introduction

Blood contains a wealth of information regarding the health status of an individual. Thus, blood-based diagnostic tools have become critically important in medicine, with current and potential applications ranging from human immunodeficiency virus (HIV) diagnosis to the detection of traumatic brain injuries [90], [91]. For many blood-based

diagnostic tests, plasma is used instead of whole blood to eliminate the impact of red blood cells on the assay. The need for plasma isolation presents a challenge in efforts to develop diagnostics for the point-of-care. The current gold standard for plasma separation is centrifugation, which is typically done with large, complex, and expensive equipment not well suited to point-of-care applications [92].

One method for identifying whether a diagnostic method will be useful in point-of-care settings is the ASSURED criteria developed by the World Health Organization, which emphasizes that useful point-of-care diagnostics should be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and delivered [93]. There are significant challenges in developing blood plasma separation strategies which meet these criteria due to the limitations inherent to the standard separation approach of centrifugation.

Since centrifugation techniques are incongruous with point-of-care diagnostics, there have been efforts to develop microfluidic devices that can carry out plasma separation at a small-scale. Microfluidic devices are systems which process fluids in channels with dimensions sized in the micron scale [94]. Microfluidic devices have significant potential for use in point-of-care diagnostics because they allow for diagnostic results to be obtained from small volume inputs, often a few microliters of biological samples. Furthermore, microfluidic devices themselves are physically small objects, with lengths and widths which are often only a few centimeters, making these devices extremely suitable for the distribution and localized use necessary for point-of-care diagnostics [94].

To separate plasma, microfluidic devices have taken several approaches, including leveraging the Zweifach-Fung effect through microchannel geometry, which involves chaining together many branching channels of varying sizes, causing branches to have different flow rates when blood is pumped through the device [95], [96]. Cells have greater mass than the molecules contained within plasma, therefore, their greater momentum at

constant velocity causes them to disproportionately flow down higher flow rate channels, allowing for separation of plasma from red blood cells when sufficient branching paths are placed in series. A similar effect for plasma separation has also been demonstrated by developing microfluidic devices with perpendicular channels that have different flow rates driven by electric fields [97], [98]. Further devices have been developed which carry out blood plasma separation via small-scale centrifugation [99], or by integrating membrane separation into a digital microfluidic [100]. These approaches have been shown to be capable of producing high plasma quality, volumetric yield, and throughput. However, one commonality in many of these microfluidics methods is that they require a force actively driving fluid flow in the device, for example, a pressure difference or an electric field. Because of this requirement, these microfluidic devices often require external components like syringe pumps or electrodes which can significantly increase the overall size, cost, and complexity of a diagnostic tool, limiting their potential for point-of-care diagnostics.

Several plasma separation microfluidic devices have been created which avoid the need for external flow driving components through the leveraging of capillary action. Since capillary forces can be generated without external components, a capillary flow-driven microfluidic has the potential to function as a standalone device, with many of these devices being compiled in a review by *Wang et al* [101]. One capillary flow-driven microfluidic was previously demonstrated by *Maria et al.*, which utilized a wettability gradient to separate plasma from whole blood, obtaining 99.9% pure plasma and >20% volume yield from whole blood samples <10 µL in 15 minutes [102]. However, maintaining the wetting properties of channels within the device requires storage in deionized water under a vacuum, which may impose limitations on the device's usage in some point of care settings. Devices utilizing dead-end filtration have also been demonstrated by *Hauser et al.* and *Son et al.*, which both were capable of producing high-quality plasma from small

whole blood samples with minimal external components required to drive flow; although the device outlined by *Son et al.* does require the use of a desiccator to drive flow via degassing prior to use [103], [104]. A further capillary flow driven device was outlined by *Madadi et al.,* which utilized a microfluidic filtration based system to produce very small quantities of highly pure plasma with no external components required to drive flow in this device, and demonstrated this systems potential for certain point of care applications with low volume requirements by validating the system with the detection of thyroid stimulating hormone [105]. Finally, there have been microfluidic devices carrying out blood plasma separation, which are entirely paper based, including the device developed by *Songjaroen et al.* [106]. While this system is extremely affordable and simple to operate, the plasma produced by the system is confined to the paper of the device, which limits the potential diagnostic applications of the system to those which can currently be carried out on paper. This is a challenge found with lateral flow assays, in which the physical properties must be controlled by physical and chemical modifications to the paper, which may affect the biological molecule or sample of interest [107]. A more thorough list of blood plasma separation microfluidic devices currently existing in the literature can be found in **Table *S*3.1**Supplemental Table 1.

In this chapter, we present a system for the separation of plasma from whole blood which uses a simple microfluidic device. This device consists of a plasma separation depth filter placed over a microchannel cut into a double-sided adhesive. Whole blood is placed onto the filter, and it is pulled from the filter into the microchannel via capillary action where it can be collected via pipette using an outlet well. We first optimized the design of the device in terms of separation efficiency and volume yield, then characterized the capillary flow, and finally compared the concentration of protein in the plasma collected by this device to that of plasma obtained by centrifugation. Our method does not require external

flow or complex equipment, nor does it require material surface modifications for optimization that may affect the integrity of the sample. The microfluidic device developed here uses a small whole blood input volume of 50-100 µL and can produce high-quality plasma in under two minutes. This device is ideal for point of care settings because it is extremely simple to operate, only requiring a pipette to input and collect samples, and is simply and inexpensively manufactured from a glass sheet, a coverslip, double sided adhesive, and a small filter.

3.3 Materials and Methods

3.3.1 Microfluidic device components

The microfluidic device developed here has five components: one 4''x 3'' glass slide base obtained from Ted Pella Inc. (Redding, CA), a 40 x 22 mm Fisherbrand Premium Cover Glass obtained from Thermo Fisher Scientific (Waltham, MA, USA), two acrylic adhesive patches (ARcare 90445Q, Adhesives Research, Glen Rock, PA, USA) and a separation filter made from CF-D23-X1 Whole Blood Separation Media, Cross Flow, Nominal Hct Range, obtained from I.W. Tremont (Hawthorne, NJ, USA). Manufacturing this device also required the use of a 3 mm diameter and 6 mm diameter Accu-Punch disposable skin biopsy punch obtained from Electron Microscopy Sciences (Hatfield, PA).

3.3.2 Human whole blood

Human whole blood was obtained from Research Blood Components, LLC (Watertown, MA, USA) and used sodium heparin as an anticoagulant.

3.3.3 Hematocrit Variation

To vary hematocrit in experiments, whole blood was centrifuged at 3400 RPM for 10 minutes. Plasma was then separated from red blood cells in the centrifuged sample via pipette and transferred to a separate tube. Plasma and red blood cells were then recombined in various volume ratios to create samples at the desired hematocrit.

3.3.4 Plasma Volume Yield Measures

To establish device volume yield, plasma was collected using a pipette set to 1 µL by aspirating around the collection well fifteen times and placing the collected plasma into a tube. The total volume collected was measured by aspirating the final sample into a 20 µL pipette and slowly dispensing air from the tip until plasma began to exit, with the volume setting on the pipette at this point being recorded as the final volume collected. Plasma volume yield was then calculated by dividing this value by 55% of the input volume, which corresponds to the amount of plasma in whole blood for the 55% hematocrit whole blood samples used in volume collection experiments. A further trial was conducted to determine volume yield with no limit on the number of aspirations, which was used to determine the maximum amount of plasma that could be extracted from this device.

3.3.5 Plasma Quality Measures

Plasma quality was measured by comparing the concentration of hemoglobin in plasma samples prepared by this device to the concentration of hemoglobin in both whole blood samples and plasma samples prepared via centrifugation. To obtain hemoglobin measurements, we first diluted samples 1:1 in nuclease-free water and incubated them for 10 minutes at room temperature to lyse any red blood cells present. Any cell debris in the sample was removed to produce a homogenous solution by centrifuging them at 3,400 RPM for 10 minutes and collecting the supernatant via pipette. Hemoglobin concentration was then found by measuring the sample's absorbance at 414 nm via the UV-Vis setting on the NanoDrop™ 1000 Spectrophotometer from Thermo Fisher Scientific (Waltham, MA, USA). This absorbance value was then converted to a separation value by comparing absorbance values of the device samples to those of lysed whole blood and plasma controls. The absorbance of plasma produced by the device could then be converted to a separation efficiency value according to the following formula (Equation 3.1):

$$Separation\ Efficiency = \left(1 - \frac{Abs\ Sample - Abs\ Plasma}{Abs\ Blood}\right) * 100\% \quad \text{(Equation 3.1)}$$

This formula places the 414 nm absorbance of the whole blood sample at 0% separation efficiency and the 414 nm absorbance of centrifuged plasma at 100% separation efficiency. All NanoDrop™ 1000 readings were carried out three times per sample and averaged to ensure the stability and accuracy of measurements. If absorbance values were unstable due to high absorbance values, the sample was diluted until it was within the acceptable operating range of the NanoDrop™ 1000, and the resulting absorbance value was multiplied by a dilution factor.

3.3.6 Protein Quantification

Protein passage through the device filter was measured by comparing the concentration of protein in plasma obtained from this device to that of plasma obtained by centrifugation. To remove any residual impurities which may impact concentration readings, plasma samples produced by the device were centrifuged for 10 minutes at 3,400 RPM and the absorbance of the supernatant at 280 nm was measured using the NanoDrop™ 1000 to determine protein concentration. All absorbance readings were taken three times per sample to ensure consistency between readings. This concentration was then compared to the concentration of protein in plasma produced via centrifugation at 3,400 RPM for 10 minutes.

3.3.7 Statistical Analyses

Statistical analyses were performed in GraphPad Prism 8 (San Diego, CA, USA) or Microsoft Excel (Redmond, WA, USA) with alpha = 0.05. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$. $\alpha = 0.05$ for all analyses. Statistically significant differences are shown in the figures.

3.4 Results

3.4.1 Microfluidic Separation Device Manufacturing

The microfluidic device designed here separates plasma from whole blood and consists of a plasma separation filter placed over a microchannel cut into a double-sided adhesive. To manufacture the plasma separation device a 60 mm x 60 mm patch of double-sided adhesive was first cut. An inlet well, outlet well, and 1 mm x 40 mm rectangular channel connecting both wells were cut into the adhesive using a 3 mm diameter holepunch, 6 mm diameter holepunch, and straight razor, respectively (Figure 3.1A). The bottom side of the cut adhesive was then attached to a glass slide, forming the microfluidic design. A glass coverslip was placed on top of the adhesive to form the main channel such that it was adjacent to, but not covering, the inlet well. The cover of the top of the adhesive was not immediately attached to the coverslip to ensure proper alignment of the outlet well's collection area. To form the collection area of the outlet well a second 20 mm x 20 mm adhesive patch was placed over the outlet well such that its edge was in contact with the coverslip. The position of the outlet well was marked on the second adhesive, and a 3 mm hole was placed in the marked position via holepunch. The top cover for the first adhesive was then removed, and the coverslip and second adhesive were attached to the device in the previous configuration to form the main channel and the collection area (Figure 3.1B). Finally, a 10 mm x 10 mm piece of the filter was placed over the inlet well (Figure 3.1C).

A schematic for this microfluidic device is shown in Figure 3.1. Using a pipette, whole blood is placed on the filter and then plasma passes through it. This filter uses an agglutinating chemistry and retention of 4 μm to trap red blood cells which have been bound into masses much larger than the filter retention by this chemistry, while allowing plasma to be filtered out via gravity [108]. After passing through the filter, the plasma is

drawn via capillary action from the inlet well, through the main channel, into the ring between the top and bottom outlet well (i.e., the collection area) (Figure 3.1D). The plasma can then be collected, with the separation time from the input of whole blood to the start of collection being approximately two minutes.

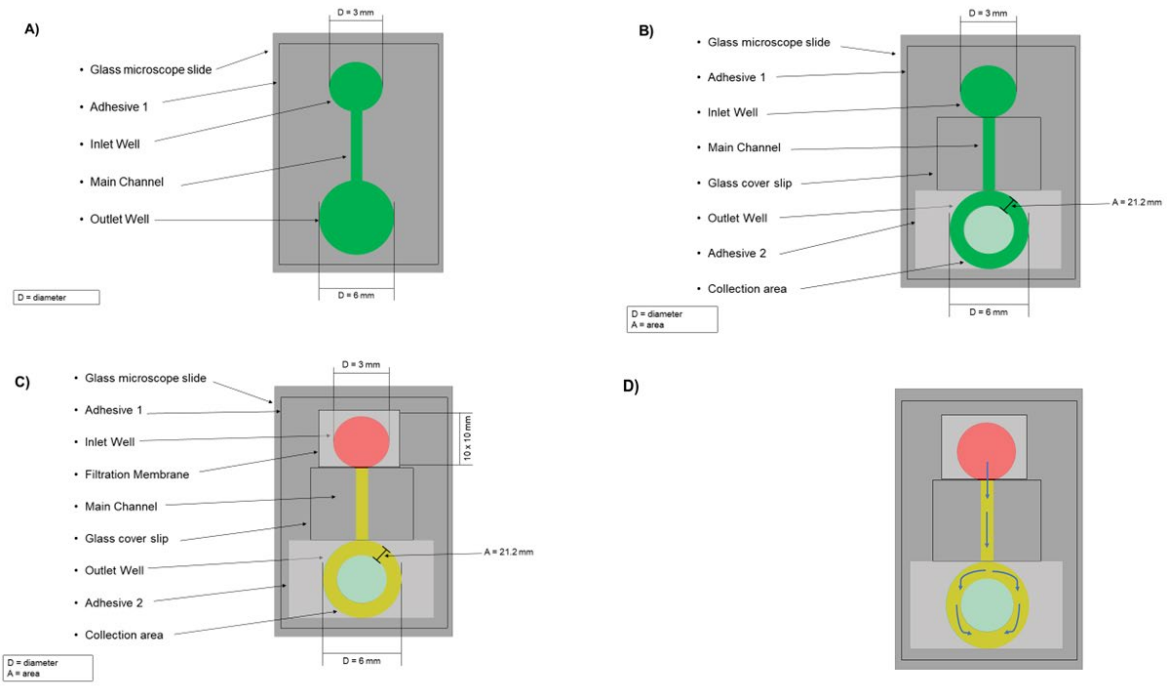


Figure 3.1: **Schematic for the microfluidic blood plasma separation system.** ***(A)*** *A 60 mm x 60 mm patch of double-sided adhesive (Adhesive 1) was first cut, and on this adhesive, an Inlet Well, Outlet Well, and 1 mm x 40 mm rectangular channel (Main Channel) connecting both wells were cut into the adhesive using a 3 mm diameter holepunch, 6 mm diameter holepunch, and straight razor, respectively. The bottom side of the cut adhesive was then attached to a glass microscope slide, forming the microfluidic design.* ***(B)*** *A glass coverslip was placed on top of the adhesive to form the main channel such that it was adjacent to, but not covering, the inlet well. To form the collection area of the outlet well a second 20 mm x 20 mm adhesive patch (Adhesive 2) was placed over the outlet well such that its edge was in contact with the coverslip. The position of the outlet well was marked on Adhesive 2, and a 3 mm hole was placed in the marked position via holepunch to form the collection area (area = 21.2mm).* ***(C)*** *A 10 mm x 10 mm piece of the filtration membrane was placed over the inlet well. Whole blood (red) is pipetted on the top of the filter, trapping the red blood cells. The plasma (yellow) flows through the filter into the main channel.* ***(D)*** *The arrows indicate plasma's flow path through the microfluidic design.*

3.4.2 Optimization of outlet well diameter, channel width, and input volume

To determine the optimal design for this device, we focused on three variables: outlet well diameter, channel width, and input volume. First, we varied the diameter of the bottom outlet well by testing 3-mm, 3.5-mm, 4-mm, and 6-mm-diameters to maximize the volume of plasma collected, using 50 µL of whole blood as the input volume (Figure 3.2A). This experiment deviated from the previously described device manufacturing protocol in that it used a 7.5 mm x 7.5 mm filtration membrane patch. This study was limited to a collection of 1-µL aspirations fifteen times to eliminate the impact of collection time on results, yields were consistently lower than the theoretical maximum this device could achieve. To estimate this maximum, the 6-mm-diameter width study was repeated with no limit on collection time, yielding an average volume of 13.8 µL from a 50 µL whole blood sample containing 27.5 µL of plasma (i.e., the maximum plasma volume yield was 50.3%).

Next, the width of the main channel was varied to determine how this impacted the quality of plasma obtained from the device, with channel widths of 0.5 mm, 1 mm, 1.5 mm, and 2 mm being tested (Figure 3.2B). Finally, the input volume of blood was varied to determine the impact on plasma quality, with 25 µL, 50 µL, 75 µL, 100 µL, 125 µL, and 150 µL inputs being tested (Figure 3.2C).

It was found that volumetric yield for this device consistently increased as the bottom outlet diameter increased from 3-mm to 6 mm. One possible explanation for this is that increasing bottom outlet diameter led to a greater area in the collection mechanism, increasing the volume of plasma produced by the device. Further, it was found that plasma quality was the highest and least variable at a channel width of 1 mm, with channels narrower and wider than 1 mm producing lower separation efficiency. However, this change in quality was not statistically significant, which suggests that filter performance is not significantly related to the main channel of the device. Finally, it was found that there

was a minimum volume of 75 µL needed for a 10 mm x 10 mm filter to be saturated with whole blood and allow for volume to be collected, with no samples being produced for volumes less than that at that filter size. However, as volume increased above 75 µL plasma quality decreased, though not with any statistical significance. This indicates that the filter requires a minimum volume to operate effectively, but that beyond this filter performance is only marginally impacted by input volume variations in the range tested. Based on these results, we determined the optimal device parameters to be the following: the outlet well to have a 6mm outer diameter, the main channel to be 1 mm wide, and the input volume of whole blood for a 10 mm x 10 mm filter to be 75 µL.

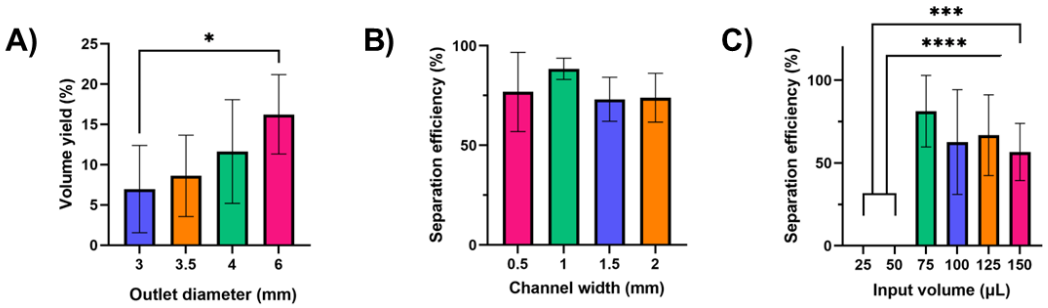


Figure 3.2 Optimization of outlet diameter, channel width, and input volume. *Six samples were tested for each condition. One-way ANOVA with Tukey's multiple comparisons test was performed.* ***(A)*** *Outlet diameter and volume yield.* ***(B)*** *Channel width and separation efficiency. No statistically significant difference was found in the average separation efficiencies.* ***(C)*** *Input volume and separation efficiency.*

3.4.3 The impact of hematocrit on separation efficiency

Following the initial experiments optimizing device features, we tested the separation efficiency of this device over a series of input hematocrit values ranging from 25%-65%. Human blood typically has hematocrit values ranging from 36%-54%, so a slightly

extended range of 25%-65% was selected to determine if samples with abnormally high or low volume percentages of red blood cells might impact device performance [109]. Although there was some variation in separation efficiency, the results shown in Figure 3.3 show no statistically significant difference or clear trend in terms of separation efficiency as hematocrit was varied. For 25%, 35%, 45%, 55%, and 65% hematocrit the average separation efficiencies were 73.2%, 88.2%, 69.2%, 75.3%, and 73.3%, respectively.

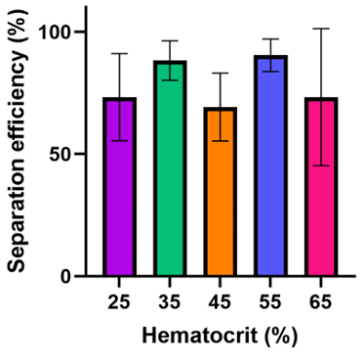


Figure 3.3: Hematocrit impact on device performance. *The separation efficiency and flow time for this device was determined over a hematocrit range of 25-65%. Six samples were tested for each condition. One-way ANOVA with Tukey's multiple comparisons test was performed. Separation efficiency. No statistically significant difference in separation efficiency was found between any hematocrit values.*

3.4.4 Impact of hematocrit on plasma flow rate and flow time

We sought to study how filtration of plasma from samples of varying hematocrit percentages affect flow rate through the device's main channel. To investigate this, the impact of varying input hematocrit from 25-65% (Figure 3.4) on flow rate and flow time in the device was assessed. The flow rate was calculated by measuring the time required to

fill the device's main channel and dividing the volume of the channel by this time. Hematocrit impacts the capillary forces and filter resistance which drive flow in this device due to its impact on the viscosity of fluid passing through the filter and flowing through the main channel. The results of the study are summarized in Figure 3.4A, which shows that increasing hematocrit led to a significant decrease in flow rate in the device. Flow time in the device increased significantly as hematocrit increased (Figure 3.4B).

It should be noted that the calculation for separation efficiency considered the absorbance of whole blood. As the hematocrit value for whole blood varied in this experiment, this absorbance value also changed. While prior results indicate a consistent separation efficiency over a range of hematocrits, this demonstrates the filter performing consistently and not plasma in the main channel having consistent purity regardless of input hematocrit. The variation of input hematocrit would lead to variation in plasma quality in the channel, with input hematocrit being roughly in proportion to outlet hematocrit.

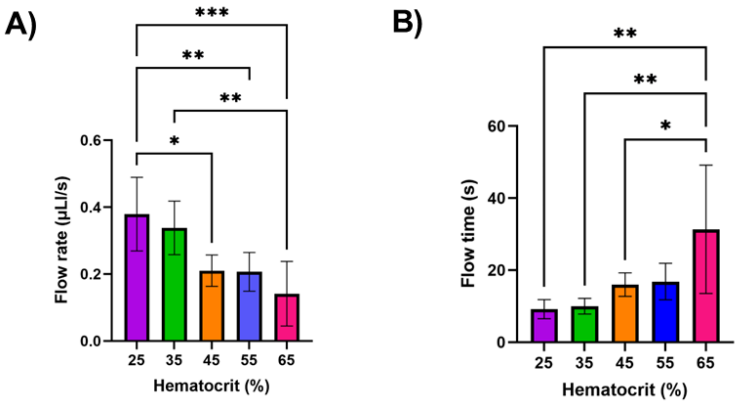


Figure 3.4: Impact of input hematocrit on device flow rate and flow time. *One-way ANOVA with Tukey's multiple comparisons test was performed. Six samples were tested for each condition.* ***(A)*** *Device flow rate assessed at varying hematocrit levels.* ***(B)*** *Flow time.*

3.4.5 Protein Concentration in Plasma from Device

To determine the potential diagnostic value of plasma produced by this device, it was necessary to demonstrate that biomarkers with diagnostic relevance are present in samples from the device at a concentration comparable to other plasma separation strategies. Given the important role of proteins in diagnostics [110], we compared the concentration of protein in plasma obtained from this device to the concentration of protein in plasma produced by centrifugation. No statistically significant difference in protein concentration (Figure 3.5) was found between these two methods, indicating that this device would be effective at producing plasma samples containing protein-based diagnostic targets.

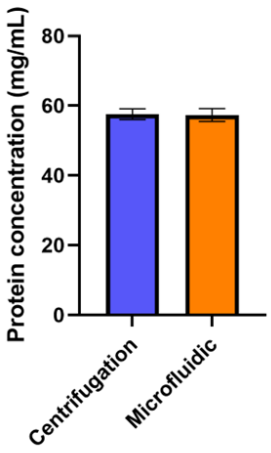


Figure 3.5: Impact of Device on Plasma Protein Concentration. *Protein concentration in plasma samples produced via the microfluidic device was compared to the protein concentration of plasma produced via centrifugation. An unpaired t-test was performed. No statistically significant difference was found between the average protein concentration yields (n=6).*

3.5 Discussion

3.5.1 Collection Area Development

The collection mechanism of the microfluidic device was developed to create a zone at the end of the capillary channel which is both appropriately sized to generate strong capillary forces driving flow into it, while also being flexible enough to enable liquid to be extracted from it. This is necessary because capillary pressure differences decrease as channel depth increases, thus, there is no pressure difference driving plasma to exit the device when the shallow microchannel is exposed to atmosphere. As such, in the first iteration of our device, plasma did not naturally exit the primary microchannel. To resolve this issue, we designed a device with a ring-shaped collection well which a pipette tip could be inserted into, enabling it to collect plasma. The collection mechanism was designed to partially cover the channel with a soft adhesive rather than a glass coverslip, which creates a zone where capillary pressure differences are maintained during device operation. Because this zone is covered by a more flexible adhesive rather than a glass coverslip, it is possible to collect plasma within it either by fitting a pipette tip under the adhesive, or by peeling back this adhesive to expose the plasma. This device functions optimally when the smaller top outlet well is centered around the larger bottom outlet well, as this placement ensures plasma is evenly distributed around the collection mechanism. As seen by our results, increasing the bottom outlet well diameter increases the area of the collection area, giving it a greater volume capacity, leading to greater volume yields. Additionally, increasing the bottom outlet well diameter mitigates manual error of the placement of the second adhesive, because there is more room to place the top outlet well within a larger bottom outlet well such that the wells are centered. It should be noted that the collection well does serve as a limit on volume collection, so the yields determined here for a 50 µL input volume would likely be somewhat lower when the same sized device is used with larger input volumes.

Our maximum volume yield of 50.3% from a 50 µL whole blood sample is comparable to that achieved by *Hauser et al.* with a similar device design of 65% volume yield also from a 50 µL sample [103]. The device developed by *Hauser et al*. carried out separation using a depth filter connected to two capillary channels. It should be noted that the calculation for volume yield used by *Hauser et al* measured the plasma volume collected within the capillary channel, while volume yield in this chapter considers plasma volume which could be removed from the device via the collection mechanism. While this distinction is not highly relevant for many point of care applications, as plasma generally can be used directly in the reservoir for a diagnostic system, it does lead to volume yields being lower in the system outlined in this chapter than they would be if they were measured via the methods used by *Hauser et al*. Beyond device design, one potential reason the volume yields differed between the two devices could have been due to the hematocrit of samples used, which was 55% in our sample compared to 41% in *Hauser et al.* Additionally, *Hauser et al.* did not describe the separation efficiency of their samples, which not only impacts the volume yield but the sample's integrity for downstream processing. A study considering separation efficiency for a plasma separation microfluidic was conducted by *Tripathi et al.* on a system which carried out plasma separation leveraging the Zweifach-Fung effect, with curved channels being used to generate different flow rates via centrifugal force [111]. This study measured separation efficiency by finding red blood cell densities in both plasma produced by the device and whole blood using a hemocytometer. This study found that separation efficiency varied from nearly 100% to just under 85% depending on inlet hematocrit and flow rate. Therefore, we explored the role of hematocrit, channel width, and input volume on the separation efficiency of plasma using our device.

3.5.2 Channel Width does not affect separation efficiency

It was necessary to assess the quality of plasma isolated using this device, as interference from red blood cells and other molecules can negatively affect analyses which may be relevant downstream of the system such as PCR for determining viral load [96]. Plasma quality was not significantly impacted by channel width in experiments. One explanation for this behavior lies in the Young-Laplace equation, which describes the relationship between capillary pressure, the geometry of a microchannel, and the fluid contained within the channel. For a channel with a rectangular cross section, the equation is as follows (Equation 3.2):

$$P = -\gamma\left[\frac{cos\theta_t + cos\theta_b}{h} + \frac{cos\theta_l + cos\theta_r}{w}\right] \qquad \textit{(Equation 3.2)}$$

Where P is capillary pressure, h is channel height, w is channel width, γ is the surface tension of the liquid in the channel, and θt, θb, θl, θr are the top, bottom, left, and right contact angles of liquid associated with each channel well. Given a channel width of 1 mm, a channel depth of 81 μm, and a plasma surface tension of .054 N/m [112], this equation can be tested at different possible contact angles. Assuming full wetting of the surface, meaning the contact angle is 0°, a pressure difference of 1440 Pa would exist across the channel, whereas if the surface is only partially wetted, and contact angle were 30°, the pressure difference would be roughly 1250 Pa.

Examining Equation 3.2:, capillary pressure decreases as both the height and width of the channel increases. Since this device contains a channel with a much greater width than height (500-2,000 μm vs. 81 μm), the terms in the Young-Laplace equation which depend on channel width approach zero compared to the terms which depend on channel height. As such, pressure across this channel is not dramatically impacted by varying

channel width. Given that this device is composed of a dead-end filter connected to a microchannel with both components exposed to the atmosphere, the difference in capillary pressure in the microchannel should equal the difference in pressure across the membrane. As such, changing channel width does not significantly impact the pressure drop across the filter. Pressure drop across the filter is a factor very likely to be driving imperfect separation in this device, as it can damage the filter, cause hemolysis, and potentially cause red blood cells to deform sufficiently to pass through the filter, so changing channel width should not be expected to impact separation efficiency.

3.5.3 Input Volume has Minimum Threshold for Device Functionality

Separation efficiency was consistently zero for input volumes below 75 µL, and nonzero for any input volumes at or above 75 µL. No statistically significant differences were found between the input volumes greater than 75 µL. The separation efficiency values of zero resulted from no volume exiting the membrane filter. It should be noted that the minimum input volume for separation to occur in earlier experiments optimizing the collection mechanism volumetric yield was only 50 µL. This is likely because a 7.5 mm x 7.5 mm patch of filter was used in these experiments rather than the 10 mm x 10 mm filter patch used in all other experiments. This led to the filter area in these experiments being 56.25% of its size in all other experiments, which enabled the filter to function with smaller volume inputs. As such, 50 µL was above the minimum threshold for plasma separation in volume collection experiments.

One possible explanation for the lack of plasma exiting the membrane at low input volumes is that the filter can retain a certain amount of fluid, termed a hold-up volume [113]. For very low input volumes, the volume of whole blood placed on the filter may be less than the hold-up volume, meaning the entire sample is retained in the filter and never enters the capillary channel. This retention would be a result of capillary forces, as the

average pore diameter in this device is 3-5 μm, producing strong capillary forces which must be overcome for plasma to enter the 81 μm x 1000 μm main channel in the device. With sufficiently high volumes, the pressure from whole blood that has not yet entered the membrane filter can overcome this capillary force and allow for flow through the device to occur. It also should be noted that the minimum input volume for separation to occur in earlier experiments optimizing the collection mechanism volumetric yield was only 50 μL. This is likely because a 7.5 mm x 7.5 mm patch of filter was used in these experiments rather than the 10 mm x 10 mm filter patch used in all other experiments. This led to the filter area in these experiments being 56.25% of its size in all other experiments, which would decrease the hold-up volume of the filter accordingly. As such, 50 μL was above the minimum threshold for plasma separation in those experiments. It should also be noted that the hold-up volume would impact the volume yield of the system, with a lower hold-up volume allowing for higher possible volume yields as less plasma is retained by the filter. Given this, it may be desirable to minimize the hold-up volume, which may be accomplished by lowering filter area or changing filter type to allow for larger pore sizes which retain plasma less strongly.

3.5.4 Hematocrit against Separation Efficiency

Hematocrit is the volume of red blood cells compared to the total blood volume and is often expressed in a percentage. The average range for humans is 35-45% in the absence of conditions that affect hematocrit. The hematocrit is a primary determinant of blood viscosity which is essential to human body functions. Hematocrit is not only important for blood flow throughout the human body but also indicates the presence of various pathologies, if higher or lower than average, such as cardiovascular, metabolic, or inflammatory disorders [114]. Therefore, it is essential to examine the impact of

hematocrit on the functionality of the microfluidic device to verify if its utility would be maintained with blood from a variety of pathologies.

Hematocrit did not have a statistically significant impact on separation efficiency. One explanation for this lies in the equation for capillary pressure across a cylindrical capillary. Because the filter in this device can be modeled as many cylindrical capillaries placed in parallel, this equation can be used to estimate pressure across the filter. The equation is as follows (Equation 3.3) [115]:

$$P_C = \frac{2\sigma \cos\theta}{r} \qquad \textit{(Equation 3.3)}$$

where σ is the surface tension of the liquid in the capillary, and θ is the contact angle between the capillary and the liquid in the capillary. which describes how pressure in the filter varies with liquid characteristics. This value for capillary pressure can also be used in the Lucas-Washburn equation to approximate the flow rate through the filter by modeling it as a series of parallel cylindrical capillaries. The Lucas-Washburn equation for velocity within a single capillary is as follows (Equation 3.4) [115]:

$$\frac{dh}{dt} = \frac{(P_A + P_h + P_C)(r^2 + 4\varepsilon r)}{8\mu h} \qquad \textit{(Equation 3.4)}$$

where P_A is unbalanced atmospheric pressure, P_h is hydrostatic pressure across the channel, P_c is capillary pressure, r is capillary radius, ε is coefficient of slip, μ is the viscosity of fluid in the capillary, and h is capillary height. In this device channel radius is 2 μm, the viscosity of plasma is roughly .0012 Pa s [116] and channel depth is .5 mm. In a scenario with complete wetting, one assumes a contact angle θ of 0°, and a slip coefficient of 0, which yields a final volumetric flow rate per pore of .000565 μL/s. To attain flow rates in alignment with those seen experimentally in this device, that would require a pore density of roughly 5 pores per square mm. Given how low this value is, the limiting factor for capillary flow in this device is not transport through the filter, but rather is

transport through the attached microchannel. As a result, the theoretically determined flow rate for this system with a filter connected to a microchannel should be roughly equal to the theoretically determined flow rate through the microchannel alone.

Varying hematocrit does lead to variations in liquid surface tension and contact angle but given that plasma quality was relatively consistent across trials, and the fact that Equation 2 is not very sensitive to variation in these quantities, this was not a substantial enough change to damage the filter through increased pressure. As such, our device is capable of constant separation efficiencies over the hematocrits tested. Compared to other devices that have examined hematocrit and separation efficiency, our device yielded comparable results. Many other devices tested at hematocrit of ~45% and found separation efficiencies ranging from 60% - 99% [109]. Here, we were able to test a range of 25% - 65% hematocrit and showed that the separation efficiency was greater than 65% for all hematocrits tested.

3.5.5 Increasing Hematocrit Decreases Flow Rate

While this device was designed for the separation of plasma from whole blood, we also explored the effect of hematocrit on flow rate and flow time to investigate their potential applicability to a hematocrit-based screening device. That is, one could not only separate plasma using our device, but also measure the flow rate and/or flow time of the plasma in the capillary channel of the device to determine if a person's hematocrit is out of the average range. This approach can be compared to a microfluidic device that has been developed which uses image processing to determine the hematocrit of whole blood samples, with the device being integrated into a smartphone app for easy operation [117]. While colorimetric data may be one effective way of determining if a whole blood sample is within typical hematocrit ranges, a potential alternative method may be to measure the flow rate of a sample in a given capillary tube. This approach may have some potential at

identifying samples with hematocrit well out of the ordinary range, as we observed that increasing input hematocrit resulted in a statistically significant decrease in device flow rate.

The trend of lower hematocrit inputs leading to greater flow rates can be approximately modeled using a simplified solution to the Navier-Stokes equations for a flat and very wide rectangular microchannel, which is as follows (Equation 3.5) [108]:

$$Q = \frac{h^3 w \Delta P}{12 \eta L(t)} \left[1 - 0.630 \frac{h}{w}\right] \qquad \textit{(Equation 3.5)}$$

where ΔP is the pressure difference in the channel as could be found in Equation 3.2, L(t) is the length of the channel containing liquid, h is channel depth, w is channel width, and η is viscosity of the fluid in the channel. Given that lower hematocrit inputs lead to lower viscosity fluid moving through the capillary channel in this device, this should lead to greater flow rates in the channel. Knowing that this device has a channel depth of 81 μm, a width of 1 mm, a length of 4 cm, and that plasma has a viscosity of .0012 Pa s, pressure values associated with different contact angles from Equation 3.2,can be tested for this system. Assuming some limited wetting and a contact angle of 30°, the flow rate in this channel would be 1.10 μL/s. These values are somewhat higher than those seen experimentally, indicating that this channel surface is not fully wetting, and that impurities in experimental plasma samples might be impacting viscosity in the channel.

Our data indicates that it may be possible to identify abnormal hematocrits using this device design. Several improvements could be made to this device to further enable this potential application. First, while removing the filter from this system would prevent it from being used for its primary purpose of plasma separation, it may allow for more precise identification of hematocrit by removing the impact of the transport through the filter on

flow rates in the device. Further, by integrating in a timing mechanism or flow rate sensor into the device, this process of approximating hematocrit could be automated.

3.5.6 Protein Passage in Device

There was no statistically significant difference between protein levels in plasma obtained by this device and plasma obtained via centrifugation. This result is expected, as the average diameter of pores in this filter is 3-5 μm while the size of proteins would typically be measured on the nanoscale [118]. As such, while pores in this filter are small enough to block red blood cells, they are orders of magnitude too large to impede the movement of proteins. This experiment supports the notion that this system could be integrated into blood-based point-of-care diagnostic systems, as it indicates that protein biomarkers with diagnostic potential can pass through this device into plasma samples without issue. This pore size should also allow for other biomarkers of interest to be detected in plasma produced by this device, including DNA, RNA, or metabolites.

3.6 Conclusions

In this work a microfluidic device capable of separating plasma from whole blood utilizing a passive borosilicate glass microfiber filter and capillary collection mechanism was demonstrated, optimized, and tested. The device was shown to be capable of collecting multiple microliter samples of plasma with separation efficiencies of up to 88.5 ± 5.4%, and device features including the collection mechanism, channel width and input volume were optimized for both output volume yield and plasma quality. The device was found to function over input hematocrit values ranging from 25%-65%. The impact of input hematocrit on flow in the device was assessed, with lower hematocrit samples producing higher flow rates at. The possibility of using capillary flow rate as a proxy for hematocrit in diagnostics was also discussed. Finally, the device was tested to ensure that it produces plasma containing protein biomarkers.

This platform is low cost, can be assembled manually in under 15 minutes, separates plasma in under 2 minutes, and can be used either to collect plasma samples with the collection mechanism, or can be easily integrated into existing microfluidic systems that require plasma as an input. However, some potential limitations of this device include that it produces plasma samples with variable red blood cell contamination, and that it requires a minimum sample size on the order of tens of microliters, with both factors limiting the potential diagnostic applications of the system. Future work should examine the elution of molecules such as DNA and RNA to further expand its impact for the point of care. Further testing could also be done to determine if reducing filter size and main channel size could enable this system to function with even smaller input volumes. The simplicity and low operating requirements of this device render it a strong candidate for integrating into point-of-care diagnostic platforms.

3.7 Data Availability

The data that support the findings of this study are available at this link: https://doi.org/10.7910/DVN/GHE3BD

3.8 Supplemental Information

Table SI 3.1: Review of Existing Blood Plasma Separation Microfluidics

Citation	Separation Strategy	Maximum Separation efficiency	Volume Yield	Throughput (Input volume per batch and batch time, or input flow rate)	External Equipment
[95]	Bifurcation Law using flow rates set by device geometry, PDMS chip	100%	15-25%	10 µL/hr	Syringe Pump
[102]	Capillary flow against gravity in a channel with hydrophobic and hydrophilic sections, PDMS device	99.9%	Not given in paper, but >36% assuming 55% hematocrit	10 µL blood per use, 15 minutes per use	Must be stored in DI water under vacuum
[96]	Bifurcation law using flow rates set by device geometry PMMA	60.8%	40%	2 mL/hr	Syringe Pump
[109]	Cross flow filtration in PDMS based microfluidic	Not Quantified as a percentage	8%	.65 µL/min	Compressed air to drive flow
[99]	On chip centrifugation	>99%	Not specified	2 mL samples processed in 2.5-10 minutes	Motor

[114]	Inducing RBC agglutination on chromatography paper with pores larger than RBC aggregates	99.5% (.5% hematocrit in purified plasma)	Not Applicable to paper-based system	7 μL per device	None
[119]	PDMS chip, separation via microchannel geometry	97.05%	3.47%	200 μL/min	External pump
[100]	Digital microfluidic, membrane separation	100%	Not quantified	50 μL	External components used for digital microfluidics
[97]	PDMS chip using perpendicular electrical fields to generate bifurcation effect	Not quantified as a percentage	8-26%	.5 μL per use, 5 minutes per use	Electrical components for generating potentials
[120]	PDMS chip with filtration	99.55%	Not quantified	.5 uL/min	External pump
[103]	Passive Membrane Filtration	Not quantified as a percentage	65%	50 μL sample per use, 10 minutes per use	None
[104]	Gravity assisted cell sedimentation with filtration	Not quantified as a percentage	20%	Produced up to 4 μL plasma over 20 minutes	Degassing of system required before use

[105]	Capillary driven filtration	>98%	>3.5%	5 μL per sample, 3-5 minutes per sample	None

CHAPTER 4: PARALLEL DNA EXTRACTION FROM WHOLE BLOOD FOR RAPID SAMPLE GENERATION IN GENETIC EPIDEMIOLOGICAL STUDIES

Chapter 4 has been published as an original research article.

Lee, K. and Tripathi, A., "Parallel DNA extraction from whole blood for rapid sample generation in genetic epidemiological studies," *Front. Genet.,* vol. 11, no. 374, pp. 1–16, Apr. 2020.

4.1 Abstract

Large-scale genetic epidemiological studies require high-quality analysis of samples such as blood or saliva from multiple patients, which is challenging at the point of care. To expand these studies' impact, minimal sample storage time and less complex extraction of a substantial quantity and good purity of DNA or RNA for downstream applications are necessary.

Here, a simple microfluidics-based system that performs genomic DNA (gDNA) extraction from whole blood was developed. In this system, a mixture of blood lysate, paramagnetic beads, and binding buffer are first placed into the input well. Then, the gDNA-bound paramagnetic beads are pulled using a magnet through a central channel containing a wash buffer to the output well, which contains elution buffer. The gDNA is eluted at 55^{o}C off the chip. The 40-minute microfluidic protocol extracts gDNA from six samples simultaneously and requires an input of 4 μL of diluted blood and a total reagent volume of 75 μL per reaction. Techniques including quantitative PCR (qPCR) and spectrofluorimetry were used to test the purity and quantity of gDNA eluted from the chip following extraction.

Bead transport and molecular diffusional analysis showed that an input of less than 4 ng of gDNA (~667 white blood cells) is optimal for on-chip extraction. There was no observable transport of inhibitors into the eluate that would greatly affect qPCR, and a sample was successfully prepared for Next-Generation Sequencing (NGS). The microfluidics-based extraction of DNA from whole blood described here is paramount for future work in DNA-based point-of-care diagnostics and NGS library workflows.

4.2 Introduction

Genetic testing from various starting samples such as blood, urine, and saliva is becoming more popular due to the rise in next-generation sequencing (NGS). Genetic epidemiological studies allow for greater possibilities in personalized medicine, but to be more accessible, sample collection should be adapted to the point of care [121]. Point-of-care (POC) devices are devices that allow biological tests to be performed on-site, not requiring expensive equipment and/or mobility of the samples. For a genetic epidemiological study, this would signify the preparation of the sample for NGS on-site, reducing the need for shipment and storage of biological samples. However, isolation and preparation of DNA samples for sequencing can be time-consuming.

DNA extraction has evolved from using harsh chemicals such as chloroform to a method called solid-phase extraction (SPE) [27]. Solid-phase extraction is based on liquid and solid phases, with DNA (or RNA) being adsorbed onto the solid phase depending on the pH and salt concentrations of the buffers used. After the DNA is adsorbed onto the solid phase, it must be washed and eluted off. Various solids for adsorption have been used in SPE and typically involve centrifugation, vacuum filtration, or column separation during the wash and elution steps [28]. These processes during the wash and elution steps increase experimental time and require additional equipment. Advances in SPE methods have allowed for the use of magnetic beads as the solid phase. DNA binds to the magnetic beads, and they are washed with wash buffers by pipetting, which removes the need for centrifugation, vacuum filtration, or column separation. To elute the DNA off the beads, the DNA-bound beads are simply placed into an elution solution, leaving the isolated DNA in the supernatant.

Whole blood is a common biological starting sample for DNA extraction. Compared to other minimally invasive sources of genomic DNA (gDNA), such as saliva or buccal cells, gDNA yield is higher and less fragmented [122]. Whole blood contains red blood cells (RBCs), white blood cells (WBCs), platelets, and plasma, with gDNA found in the nuclei of WBCs. gDNA found in blood is of high quality and is used in forensics, cancer diagnoses, and various other biological tests. Previously, there a few POC devices have been created that use magnetic beads to extract gDNA from whole blood [123]–[125]. These POC devices each use small, handheld tools that handle microliter-scale volumes, known as microfluidic devices. They also utilize magnetic beads for purification and highlight gDNA extraction efficiencies. The yield is sufficient for preparation for an Illumina® Sequencing platform, which requires as little as 1 ng. In each of these studies, however, besides the performance of PCR amplification, there is a lack of an extensive description of the extracted gDNA solution's purity, which would indicate its suitability for downstream use in NGS. More quantitative methods, such as quantitative PCR (qPCR) can be used to observe inhibition, or contamination, in amplified samples. . To obtain accurate NGS data, the DNA samples must be as free from contamination as possible [126]. Contamination can arise from a variety of sources such as bacteria and inhibitors of polymerase chain reaction (PCR), which is an integral part of DNA sequencing. In blood, hemoglobin, heme, and anticoagulants such as EDTA and Heparin may inhibit PCR efficiency, as may the reagents used in DNA extraction protocols [127].

This chapter proposes a simple, automated microfluidics-based system that performs gDNA extraction from human whole blood. A solution of blood lysate, binding buffer, and DNA-bound paramagnetic beads are added to a microfluidic chip, where the beads are moved using a magnet through a wash buffer and end up in an elution buffer. The microfluidic chip reduces the number of wash steps needed compared to a manual protocol. This system has several advantages compared to those previously developed. Firstly, the polyvinyl alcohol magnetic particles (M-PVA Magnetic Beads) being used have low, unspecific protein binding properties, high functionalization potential, and high magnetite content. These factors contribute to maximizing the purity of the DNA eluate and the speed of extraction. Additionally, these magnetic beads do not require the use of chaotropic salts for nucleic acid binding [30]. Chaotropic salts, which are typically present in lysing and binding buffers during SPE, are PCR inhibitors so the reduction of their presence increases the likelihood of successful PCR [31]. The system is simple to use, utilizes small sample and reagent volumes, does not require continuous flow, and can perform multiple extractions in parallel. The microfluidic protocol is based on diffusion principles and can be automated but can easily be performed manually, signifying that the DNA extractions can be done in the absence of electrical equipment. The lack of a need for electricity increases the accessibility of the device to a variety of settings, such as at the point of care. Overall, the microfluidic chip described here extracts gDNA of high quality and purity from whole blood, which has the potential to significantly reduce the time and cost of sample generation for genetic epidemiological studies.

4.3 Materials and Methods

4.3.1 Human whole blood

Human male whole blood was purchased from Golden West Biosolutions (Temecula, CA, USA). The blood samples were stored in 5-mL tubes, with either Sodium Heparin or Sodium EDTA anticoagulants. The samples were tested for common infectious diseases by Gold West Biosolutions and were received one day after the bleed date. All samples were stored at 4^{o}C.

4.3.2 Off-chip workflow

The chemagic^{TM} DNA Blood250 Kit (PerkinElmer, Waltham, MA, USA) is a manual gDNA extraction kit that was converted for on-chip use. Unless otherwise stated, all reagents used are from that kit. The workflow for this kit is depicted in Figure 4.1, which will be

referred to as “off-chip.” First, 250 µL of whole blood is mixed with 350 µL of Lysis Buffer 1 and incubated for 5 minutes at room temperature. Then, 50 µL of magnetic beads and 950 µL of Binding Buffer 2 are added to the lysed blood cells, and these are mixed and incubated for 5 minutes at room temperature. The tube containing lysed blood cells and the DNA-bound magnetic beads is placed onto a magnetic rack to separate the DNA-bound magnetic beads from the lysate for 2 minutes at room temperature. The supernatant is discarded, and 800 µL of Wash Buffer 3 is added to the tube; the beads are resuspended, and the tube is placed back onto the magnetic rack for 1 minute. The supernatant is discarded, and the washing procedure is repeated using Wash Buffer 4 and Wash Buffer 5. After Wash Buffer 5 is removed, the tube is left in the magnetic rack, and 1.5 mL of Wash Buffer 6 is added without disrupting the pellet. This is left for 90 seconds, and the supernatant is discarded. Then, 200 µL of Elution Buffer 7 is mixed with the DNA-bound magnetic beads and incubated for 10 minutes at 55°C for DNA elution. The tube is then placed onto the magnetic rack, and the beads are separated from the eluate. The eluate contains purified DNA. The expected yield is 5–10 µg of genomic DNA from normal healthy whole blood.

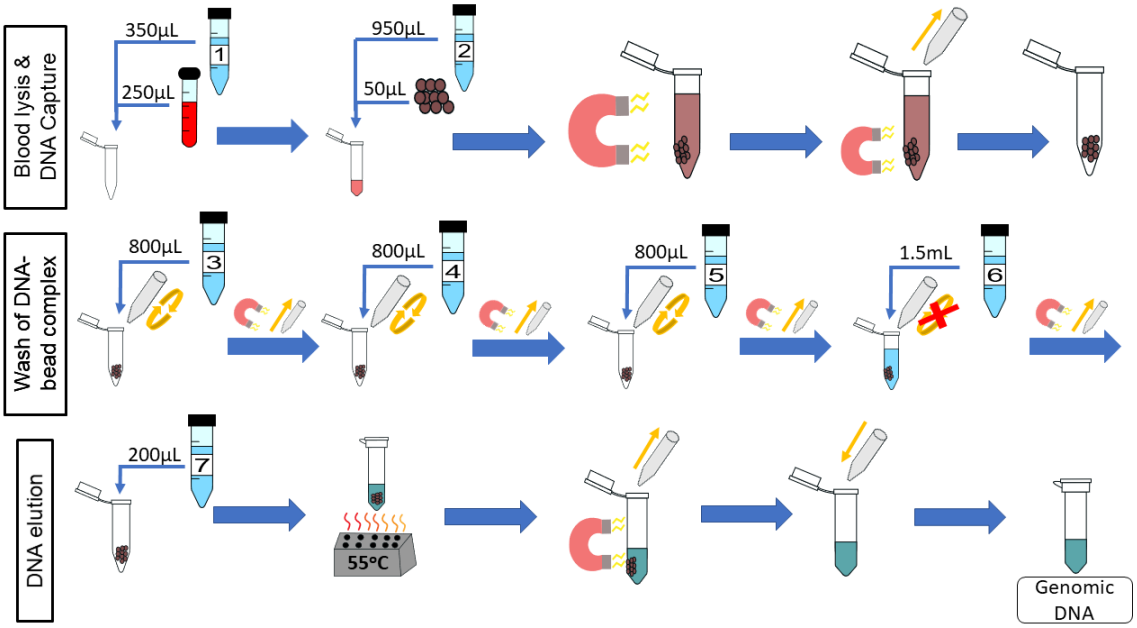


Figure 4.1: Off-chip Workflow, a graphic of the chemagic™ DNA Blood250 Kit protocol. *A 250-µL sample of blood is lysed, and then magnetic beads and a binding buffer are added to the solution to bind the gDNA. The gDNA-bound magnetic beads are moved to the side of the tube using a magnet, and the supernatant is removed. The gDNA-magnetic bead complex is washed four times, and an elution buffer is added. The gDNA elutes off of the beads at 55°C, and the supernatant contains the gDNA of interest.*

4.3.3 Determination of DNA yield

Spectrofluorometry was performed based on guidelines from ThermoFisher Scientific Inc. (Waltham, MA, USA) using the Quant-iT™ PicoGreen™ dsDNA Assay Kit. Signal measurements of all samples were performed using the EnVision 2105 Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). Subsequent linear regression analysis was performed in Microsoft Excel (Microsoft, Redmond, WA, USA). For all experiments, the standard curve used for linear regression had $R^2 > 0.99$.

4.3.4 Microfluidic chip fabrication

The PDMS-glass microfluidic chip has two rows of six microfluidic separators per chip that are spaced 9 mm apart, such that DNA extraction can be performed from 6 individual blood samples simultaneously with a multichannel pipette.

The wells of the device were formed by curing the polydimethylsiloxane (PDMS) in a sandwich mold composed of an SU-8 master mold to form the microfluidic features and a machined aluminum mold to form the wells of the device (Figure SI 4.1). The two molds are aligned and held together with spring clamps. Uncured PDMS is then poured into the top opening into the gap between both molds. Once cured at 70°C, the film of PDMS formed in the gap is released from both molds. The wells have been formed by the aluminum mold, but a thin layer of PDMS occludes them, which is hole-punched out. The PDMS film is then bonded to a 75 x 50 mm glass slide. This method allows for more precise well formation when compared to conventional PDMS hole-punching methods.

4.3.5 Magnet movement

To remove variability in the magnet's movement when performing experiments, an in-house device was used to standardize magnet motion. The device consists of an x-stage for left-right motion. The stage is a linear screw-drive stage (igus® plastics for longer life®, Providence, RI, USA) with a stepper motor used to control the stage motion (Applied Motion Products, Inc., Watsonville, CA, USA) based on an in-house script. The magnet is a 2" x 1/2" x 1/4" thick neodymium bar magnet (Grade N42, Item #BY084), with a surface field of 3424 Gauss, purchased from K & J Magnetics, Inc. (Pipersville, PA, USA).

4.3.6 Magnetic Force Modeling

Modeling was performed with COMSOL Multiphysics (Burlington, MA, USA).

4.3.7 Primers for real-time PCR

Real-time PCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). TaqMan™ Gene Expression Assay ID# Hs00243216_s1, a primer for human sex-determining region Y (SRY) gene, was used (ThermoFisher Scientific Inc., Waltham, MA, USA).

The Taqman™ Gene Expression Master Mix (ThermoFisher Scientific Inc., Waltham, MA, USA) was used for the assay.

4.3.8 Pure genomic DNA

Human genomic DNA (male) was obtained from Promega Corporation (Madison, WI, USA).

4.3.9 Next-Generation Sequencing preparation

Extracted DNA samples were prepared for Illumina® Sequencing using the NEXTFLEX® Rapid DNA-Seq Kit (PerkinElmer, Waltham, MA, USA). For analysis, the Agilent DNA 1000 Kit was used on the Agilent 2100 Bioanalyzer device (Agilent Technologies, Santa Clara, CA, USA).

4.3.10 Statistical analysis

Statistical analyses were performed in GraphPad Prism 8 (San Diego, CA, USA). *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$.

4.4 Results & Discussion

4.4.1 Reduced blood volume for translation to the chip

One of the goals of the microfluidic chip was to reduce the number of wash steps needed in the gDNA extraction protocol. To identify one wash buffer, or combination of wash buffers, the off-chip protocol was performed only using one wash step with one wash buffer per experiment. Wash Buffer 3 was found to have comparable DNA yield and purity to the original protocol (data not shown).

The volumes of the remaining chemagic™ protocol reagents needed to be scaled down significantly, as the wells of the microfluidic chip can hold a maximum volume of ~70 µL.

Various linear scale-downs of the off-chip protocol were tested, meaning each reagent was scaled down by the same factor. The best results were found via scaling the starting volume of blood from 250 µL to 4 µL, and therefore all reagents were scaled linearly by a factor of 62.5. Thus, Lysis Buffer 1 was scaled to 5.6 µL, Binding Buffer 2 to 15.2 µL, and the magnetic beads to 0.8 µL. Together, this volume of 25 µL constitutes the input to the microfluidic chip. The output is the eluate containing Elution Buffer 7, and the scaling factor made the required volume 3.2 µL. However, this volume would be too small to be pipetted from the microfluidic chip for elution, and since the microfluidic chip is based on diffusion, this stark difference in volume between the input and output would cause the input to diffuse into the output well. To (1) maintain similar volumes between the input and output and (2) not overdilute the gDNA eluted such that the concentration would be difficult to quantify, an elution volume of 16 µL was used, which makes the solution 5 times more dilute than that of the full protocol.

The full protocol starting with 250 µL of blood and the 4 µL reduced-blood volume protocol were each performed off-chip, and the results are compared in Figure 4.2 to indicate whether off-chip gDNA yield was similar between the two protocols. The full protocol was performed two days after the bleed date of the donor, and the reduced protocol was performed four days after the full protocol. As mentioned previously, since the elution volume for the reduced-volume protocol is 5 times more dilute than that of the full, off-chip protocol, the concentration of DNA eluted using the reduced protocol was multiplied by 5 for comparison purposes. Following the original protocol, EDTA-anticoagulated blood yielded 8.46ng/µL, and Heparin-anticoagulated blood yielded 8.35ng/µL. Using the reduced protocol, EDTA-anticoagulated blood yielded 8.15ng/µL, and Heparin-anticoagulated blood yielded 6.20 ng/µL. There was no statistically significant difference between the yields of the original protocol and the reduced protocol for either anticoagulant type. Thus, it was concluded that the reduced protocol, using a starting blood volume of 4 µL, was appropriate for translation onto the microfluidic chip.

The expected yield for the chemagic™ protocol is 25–50 ng/µL, but the yield here with both protocols when quantified with spectrofluorometry was approximately 8 ng/µL. Using spectrometry via Nanodrop™ (ThermoFisher Scientific Inc., Waltham, MA, USA), the extracted gDNA samples were measured to be at the expected concentration, but due to the presence of residual reagents, the Nanodrop™ concentration values are easily variable. Thus, only spectrofluorometry was used as the quantification technique for gDNA

concentration here. Other studies have compared the use of these two quantification techniques and also reported lower concentration measurements using spectrofluorometry [128].

Additionally, DNA yield from blood can vary dramatically, even from samples with a similar white blood cell count. The wide error bars are likely a result of this, which contributes to the lack of a statistically significant difference between the Heparin samples' protocols. In addition, the delay between gDNA extraction of the samples plays a role in the lower recovery of the reduced protocol samples [129]. However, EDTA acts as a DNA stabilizer, and therefore it is expected that DNA concentration will remain similar despite delays in extraction and blood collection. The anticoagulant type was not significant for this study but was explored in order to investigate the applicability to human blood of Kotikalapudi and Patel's (2015) work, which compared the two anticoagulants' effects on long-term cattle DNA extraction and storage [130]. Similarly, they concluded that the anticoagulant type was not significant for DNA extraction and storage.

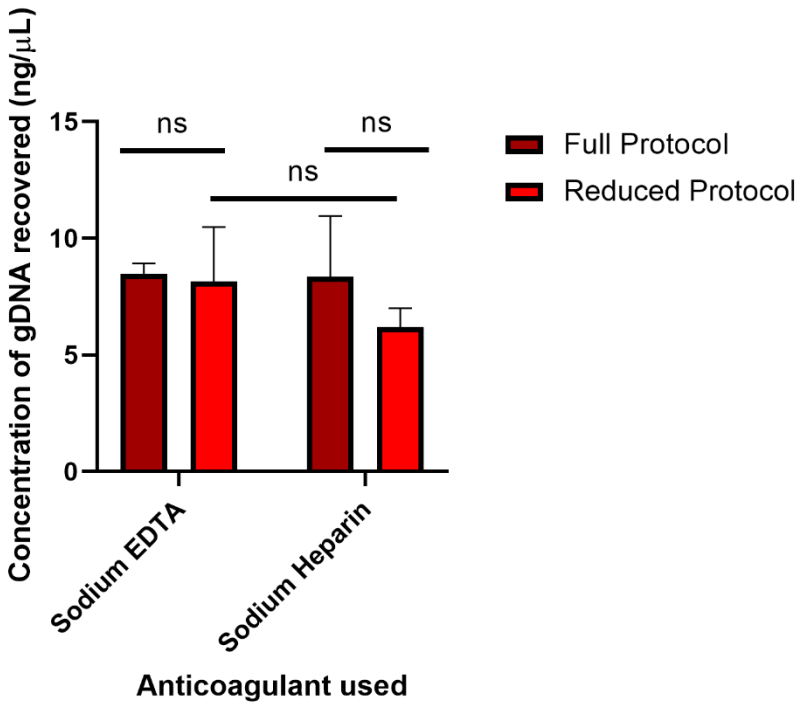


Figure 4.2 Concentration of gDNA from starting whole blood volumes of 250 µL and 4 µL off-chip. *The concentrations of gDNA extracted under the full protocol and reduced protocol were compared in two different anti-coagulated whole blood samples. The full protocol starting blood volume is 250 µL, while the reduced protocol starting blood volume is 4 µL. To compare the two anticoagulants, multiple unpaired t-tests were performed. The mean difference in recovery between*

the two protocols (Sodium EDTA difference = 0.31, SE = 1.16, and Sodium Heparin difference = 2.14, SE = 1.37) was not significantly greater than 0 (Sodium EDTA: t(5) = 0.27, two-tail adjusted p = 0.80; Sodium Heparin: t(6) = 1.57, two-tail adjusted p = 0.31), providing evidence that either anticoagulant type can be used in the gDNA extraction protocol. To compare the mean gDNA recovery of the two protocols, as well as the anticoagulant types, two-way ANOVA was performed (α = 0.05). The mean difference between Sodium EDTA and Sodium Heparin was not significantly greater than 0 (p = 0.28). The mean difference between the full protocol and the reduced protocol was not significantly greater than 0 (p = 0.21). Lastly, using Sidak's multiple comparisons test, there was no statistically significant difference between Sodium EDTA and Sodium Heparin using the reduced protocol (p = 0.32). Together, these results provide evidence that neither the anticoagulant type nor the starting blood volume has a significant effect on the gDNA yield.

4.4.2 The microfluidic chip

One of the microfluidic separators and its dimensions, well, and channel names are shown in Figure 4.3A. The purpose of a separator is to simplify the number of wash steps needed for magnetic bead-based DNA extraction from blood. To reduce magnetic bead aggregation in the corners of each segment as the beads move through the chip, the well is angled inward. The wash channel has an hourglass shape to reduce hydrodynamic flow. This also slows the magnetic beads, allowing them to be more effectively washed.

Figure 4.3B depicts the procedure for liquid loading and magnet movement for the microfluidic chip-based gDNA extraction. In Figure 4.3B, black represents empty wells and channels before loading, and they are filled as follows. First, 20 µL of Wash Buffer 3 (green) is loaded into the Primary Wash Buffer Loading Well (Figure 4.3Ba) and moves via capillary action through the center Wash Channel to the Secondary Wash Buffer Loading Well (Figure 4.3Bb). The wash buffer does not seep into the Sample Well or the Elution Well due to the capillary flow-controlled Wash Channel. Then, the input solution (red), which is the 25 µL volume obtained after lysing the blood and binding the gDNA to the magnetic beads (using the Reduced Protocol from Figure 4.2), and 16µL of Elution Buffer 7 (blue) are simultaneously loaded into the Sample Well and the Elution Well, respectively (Figure 4.3Bc). The two solutions stay separated due to the length of the wash channel, which contributes to the slow diffusion time between the two wells. A magnet is used to move the magnetic beads from the Sample Well, through the Wash Channel, and into the elution well (Figure 4.3Bd-e).

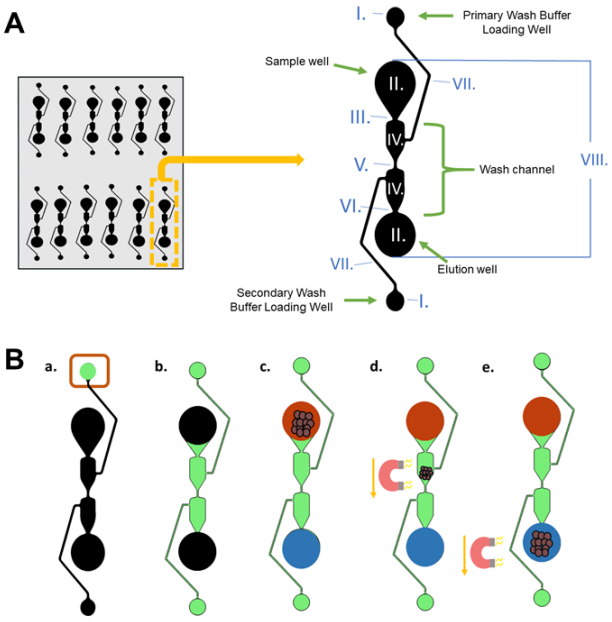


Figure 4.3 DNA extraction from whole blood procedure. ***(A)*** *A single microfluidic separator with labeled well and channel names, along with the dimensions of the chip. The entire length of one separator is 15.5 mm, and they are spaced 9 mm apart horizontally. The channels have a depth of 150 µm, and the sample well, elution well, and wash buffer loading wells are 7.65 mm in depth. I. Wash Buffer Loading Wells, radii = 0.75 mm; II. Sample well and elution well: radii = 1.75 mm; III. Width = 0.212 mm; IV. Width = 1.517 mm; V. Width = 0.134 mm; VI. Width = 0.148 mm; VII. Width = 0.125 mm; VIII. Length = 12 mm.* ***(B)*** *(a-b) Wash Buffer 3 is loaded into the Primary Wash Buffer Loading Well and diffuses through the center Wash Channel to the Secondary Wash Buffer Loading Well. (c) The input solution of lysed blood and DNA-bound magnetic beads and output solution, Elution Buffer 7, are added simultaneously to the sample well and elution well, respectively. (d-e) A magnet moves the DNA-bound magnetic beads from the sample well through the wash buffer into the elution well.*

4.4.2.1 Magnet movement for pulling beads through the chip

Once the appropriate volumes for the microfluidic chip were established, the reduced protocol was transferred to the microfluidic chip. The movement of the magnet necessary for on-chip extraction was first investigated manually via moving a magnet beneath the microfluidic chip by hand. To standardize, this movement was automated using the x-stage of an in-house device. The microfluidic chip is placed onto the x-stage and moved over a magnet, which is depicted in Figure 4.4. The movement was first optimized for use with magnetic beads that were not bound to gDNA and then modified for those that were.

The movement of the x-stage begins with the magnet beneath the sample well to first concentrate the magnetic beads to the bottom of the sample well (Figure 4.4A). The variable x_{mag} refers to the location on the microfluidic separator in which the edge of the

bar magnet is located, with the top of the sample well (shown in red) being x_{mag} = 0 mm and the bottom of the elution well (blue) being x_{mag} = 15.5 mm. t_{mag} refers to the length of time that the x-stage is moving or holding at a position (HOLD). The x-stage then moves the microfluidic chip such that the magnet is beneath the center of the microfluidic chip (Figure 4.4A-B). Here, the x-stage holds for 5 seconds to allow the magnetic beads to move towards the magnet (Figure 4.4B). Next, the x-stage moves for 1 second down the length of the separator, holding at the start of the elution well for 8 seconds (Figure 4.4C). Then, the x-stage moves past the elution well for 1 second and holds to concentrate the magnetic beads into the elution well for 10 seconds (Figure 4.4D). The magnetic beads were found to aggregate in two major points in the wash channel, indicated by the stars in Figure 4.4D. Thus, the magnetic field needed to be concentrated at these two points but not intensify aggregation. The magnet was therefore moved repeatedly between the two aggregation points for 20 cycles, as shown in Figure 4.4E. One cycle consists of (1) brief (less than 0.5 seconds) movement away from the elution well, and (2) brief movement toward the elution well followed by a 0.5-second pause. This movement allows the magnetic beads that are trapped in the upper part of the microfluidic chip to be attracted to the magnet while ensuring that the magnetic beads are spending more time being pulled toward the elution well. Lastly, to gather the magnetic beads fully into the elution well, the magnet is held at the bottom edge ($x_{mag,8}$ = 15.5 mm) of the elution well for 30 seconds to ensure complete movement of the magnetic beads to the elution well (Figure 4.4F). The reduction of aggregation cycles and the 30-second hold at the elution well were then repeated 4 times, a number that was determined by observation to maximize the number of beads moving through the chip.

Once the magnet motions are complete, the contents of the elution well are removed from the microfluidic chip and placed into a separate tube and incubated for 10 minutes at 55^{o}C. The tube is then placed onto a magnetic rack, and the beads separated from the eluate. The eluate contains purified gDNA.

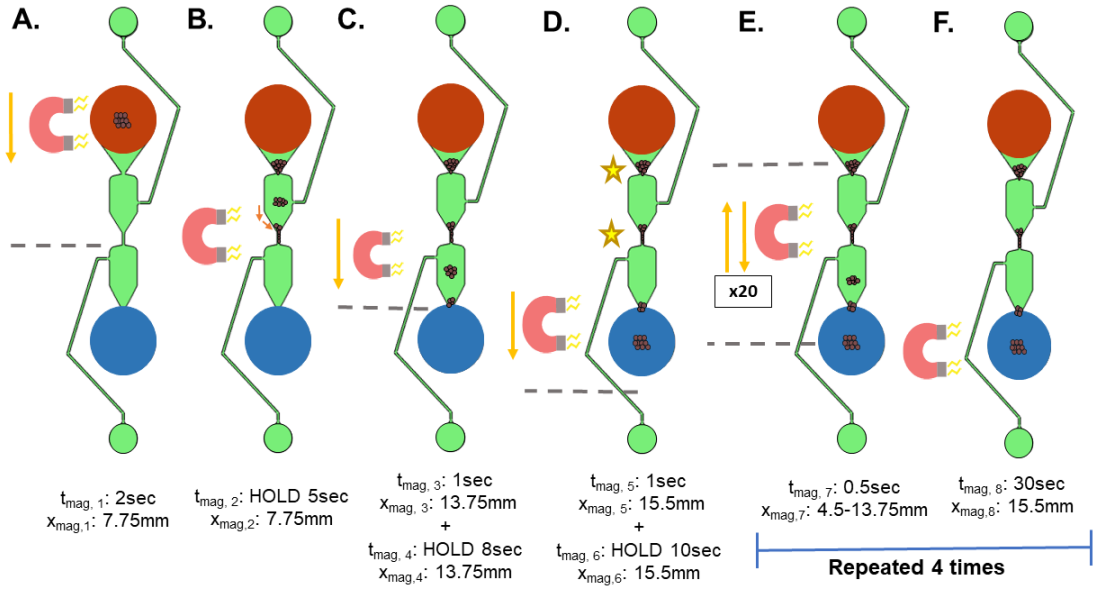


Figure 4.4: Standardized magnet movement. *The movement of the x-stage holding the microfluidic chip over a magnet was based on an in-house script. The variable x_{mag} refers to the location on the microfluidic separator in which the edge of the bar magnet is located, with the top of the sample well (shown in red) being x_{mag} = 0 mm. t_{mag} refers to the length of time that the x-stage is moving.* ***(A-B)*** *The x-stage then moves the microfluidic chip 7.75 mm ($x_{mag,1}$) over 2 seconds ($t_{mag,1}$) such that the magnet is beneath the center of the microfluidic chip.* ***(B)*** *The x-stage pauses ($x_{mag,2}$) for 5 seconds ($t_{mag,2}$) to allow the magnetic beads to move towards the magnet.* ***(C)*** *The x-stage moves for 1 second ($t_{mag,3}$) to 13.75 mm, the length of the separator ($x_{mag,3}$), pausing at the start of the elution well for 8 seconds ($x_{mag,4}$ & $t_{mag,4}$).* ***(D)*** *The x-stage moves for 1 second ($t_{mag,5}$) past the elution well to 15.5 mm, the length of the microfluidic separator ($x_{mag,5}$), and pauses to concentrate the magnetic beads into the elution well for 10 seconds ($x_{mag,6}$ & $t_{mag,6}$). The x-stage must move the microfluidic chip repeatedly such that the magnet is beneath two key aggregation points (indicated by the stars), at the narrowest parts of the microfluidic separator.* ***(E-F)*** *To encourage movement of the magnetic beads at these locations towards the elution well, the x-stage cycles between the dotted lines, which are $x_{mag,7}$ = 4.5 mm and $x_{mag,8}$ = 13.75 mm. One cycle consists of (1) movement away from the elution well, and (2) movement toward the elution well, followed by a 0.5-second pause. Each cycle was repeated 20 times, followed by a 30-second pause ($t_{mag,8}$). These two steps were repeated 4 times.*

4.4.3 gDNA reduction for successful movement through the microfluidic chip

Initial results showed scarce amounts of gDNA recovered from the microfluidic chip due to there being little to no movement of the magnetic beads from the aggregation points to the elution well. Various experiments were done to isolate the cause of this, including magnetic force calculations on the glass slide using varying magnet strengths, varying the volume of magnetic beads, and performing extraction with a pure gDNA sample to remove

the influence of blood's many components. It was found that the magnetic beads' movement was significantly altered by the presence of pure gDNA. Without gDNA present, the magnetic beads moved quickly through the chip, but when gDNA was bound to the beads, they aggregated at the aggregation points (Figure 4.4D) and there was remarkably less movement through these points. It was concluded that the starting amount of gDNA in the starting sample well heavily influenced the ability of the magnetic beads to move to the elution well. The use of a stronger magnet was considered, and modeling showed that the magnetic force would increase by 10N (Figure SI 4.2) if the supplier's strongest magnet of similar dimensions was used (the current magnet being used is the second strongest). However, this slight increase of magnetic force would cause the magnetic beads to more quickly and forcefully aggregate, increasing the difficulty of overcoming the DNA-DNA interactions. The amount of starting gDNA needed to be reduced.

Anticoagulated whole blood samples were diluted in either nuclease-free water or 1X PBS to a final volume of 4 µL, and gDNA was extracted using the reduced protocol. It was found that there was no significant difference between diluting blood in 1X PBS and in nuclease-free water in terms of the percent recovery of gDNA on- and off-chip (data not shown). Nuclease-free water may not be available to all users of this device, so 1X PBS was used for dilution of the blood.

4.4.3.1 Dilution of blood to various concentrations to characterize the starting dilution needed to maximize gDNA recovery

To better understand the restraints of the microfluidic chip with respect to the amount of gDNA in the starting sample, various dilutions of whole blood were tested. The dilutions were done off-chip and on-chip and compared. To reduce sample variability between the off- and on-chip protocols, both were started from the same starting sample. Stock solutions of whole blood were diluted 1:10 and 1:20 in 60µL of 1X PBS, and 1:40 in 80µL of 1X PBS. From there, the initial starting sample was made in one tube at two times the on-chip protocol volume, with 8 µL of the diluted blood. All other reagents were scaled accordingly. Six separate replicates of each stock dilution were placed into separate tubes. For each, the solution made after the binding step was divided equally into two for on-chip protocol and off-chip protocol washing (Figure 4.5); the starting amount of gDNA for each protocol was from a 4-µL sample and eluted in 16µL of Elution Buffer 7. The yields from both protocols at the various dilutions were compared. Figure 4.6 shows the amount of

gDNA recovered from various dilutions of whole blood. It should be noted that the blood samples leading to the results in Figure 4.2 and Figure 4.6 are not from the same donor. Additionally, the amount recovered shown in the graph does not take into account the dilution factor of the elution volume, unlike Figure 4.2. For these experiments, Sodium Heparin anticoagulated blood was used, and the extractions occurred 9 days after the donor's bleed date.

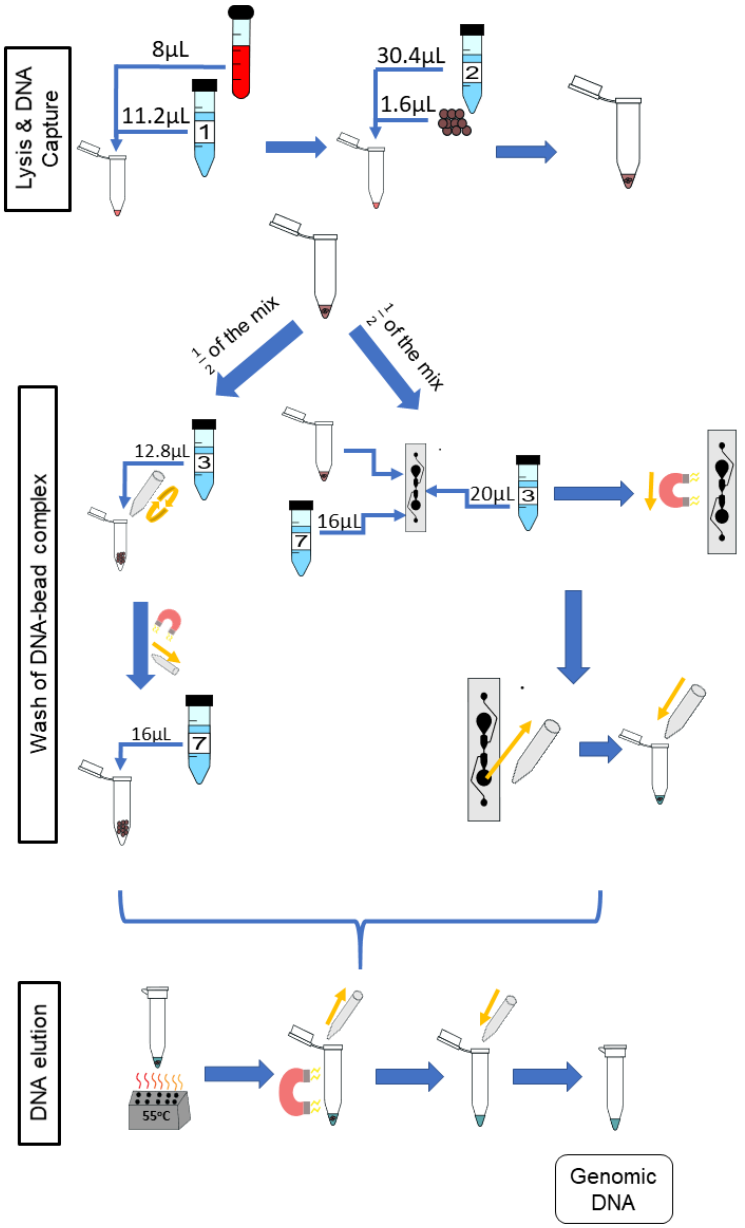


Figure 4.5: Paired protocol to compare gDNA extraction off- and on-chip. *The starting volume of the sample was doubled to 8 µL from 4 µL so as to have a starting sample that would be split equally between the two protocols for comparison purposes. The reagents were scaled accordingly. Half of the starting sample (diluted blood, lysing buffer, binding buffer, and magnetic beads) was used with the off-chip protocol (left) and the other half with the on-chip protocol (right).*

Using the Picogreen™ assay, the average gDNA yield in the undiluted blood sample was measured to be ~14 ng off-chip, and the average amount of DNA recovered on-chip was 0.15 ng. The dilutions should theoretically be starting amounts of 1.4 ng, 0.7 ng, or 0.35 ng, and on-chip, they recovered an average of 1.2 ng, 2.38 ng, and 2.85 ng, respectively. As the blood sample becomes more diluted, i.e., a lower starting amount of gDNA, the on-chip protocol and off-chip protocol recover similar amounts of gDNA, with the on-chip protocol surpassing that of the off-chip at the highest dilution factor. The samples have similarly sized error bars, which is most likely due to the uneven distribution of white blood cells in the samples, which will cause increased variability in the gDNA present in the starting sample between replicates.

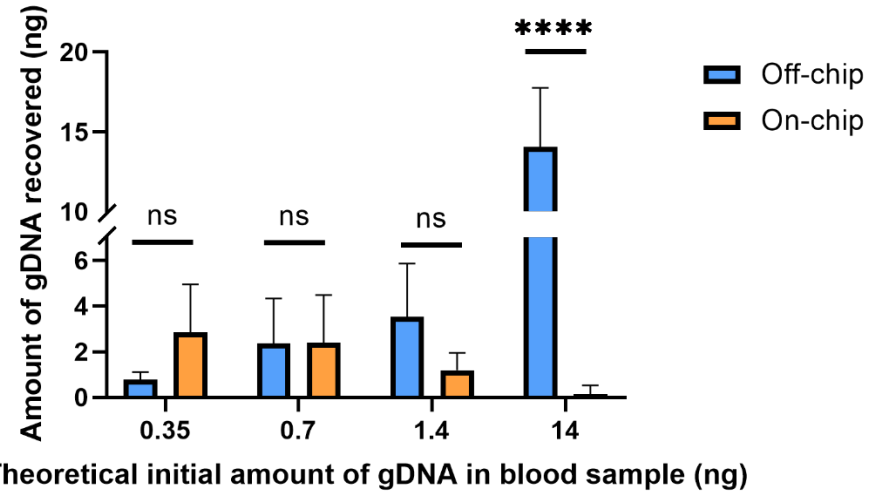


Figure 4.6: Amount of gDNA extracted on- and off-chip with varying dilutions of blood. *Whole blood was diluted to various starting amounts to compare the recovery of gDNA on-chip to that off-chip. The undiluted starting amount (14ng) of gDNA in whole blood was found using the off-chip 4-µL protocol. The subsequent dilutions were simply divided by their dilution factors to yield the values in the x-axis. Two-way ANOVA (α = 0.05) was performed to compare off-chip versus on-chip and n=6. One sample from the positive control, 14 ng off-chip, was excluded as an outlier due to significantly low yield. Dunnett's multiple comparisons test was used for deeper analysis, and 14 ng, $p < 0.0001$ (SE of difference =1.183), and all others $p > 0.2$.*

The theoretical initial amounts on the x-axis of Figure 4.6 were calculated by dividing the undiluted off-chip sample's yield by the solutions' respective dilution factors; these may not be their true starting amounts. To better compare the off-chip and on-chip yields at the various dilutions, a percent recovery (Equation 4.1) was calculated by comparing the on-chip protocol to the off-chip protocol at that specific sample's starting amount (Table 4.1). This was done as an alternative to comparing all values to the undiluted starting amount, which originated from a different stock solution to the others. The percent recovery indicates that there is a point below 0.7 ng at which the on-chip protocol captures more gDNA than the off-chip protocol. A similar trend was seen with another data set [data not shown]. The trend in gDNA recovery on-chip is such that lower amounts of starting gDNA led to improved recovery of the gDNA. To further investigate this, along with the significant increase in percent recovery for both protocols at lower amounts of starting gDNA, mathematical modeling was performed,

$$\%\, recovery(i) = \frac{onchip\ extracted\ amount(i)}{offchip\ extracted\ amount(i)} * 100\% \qquad \textit{(Equation 4.1)}$$

where i = specified sample's concentration.

Table 4.1: Percent recovery off-chip and on-chip of various diluted blood samples.

Sample Starting Amount (ng)	% Recovery
14	1.06%
1.4	40%
0.7	101%
0.35	366%

4.4.4 Mathematical and mechanistic understanding of magnetic bead transport

For this gDNA extraction technique to be successful in general, the magnetic beads must (1) bind the gDNA and (2) be transported in the presence of a magnetic field. The reagent protocol for binding the gDNA off-chip and on-chip was unchanged and was thus not considered for modeling purposes. For bead transport, the original protocol uses tubes, so bead movement is largely unrestricted. Here, and in other microfluidic devices,

the magnetic beads must be transported through more restrictive and complex geometries.

The amount of gDNA present in the starting sample was shown to have a significant effect on bead transport. gDNA is very long, can interact with itself, and exists in a variety of topologies [131]. Once bound, magnetic beads are introduced into the multiple gDNA strand complex, and then, together, they must be transported under the influence of a magnet. The gDNA-magnetic bead complex can be thought of as a deformable cluster of complicated geometrical shapes. The deformability of the cluster depends on the geometry through which it is moving but, more importantly, also depends on the magnetic force felt by the paramagnetic beads and the number and strength of the interactions of DNA molecules. Understanding of the physics behind interactions between DNA molecules is complex and largely theoretical; however, it is known that such interactions are present when the molecules are in close proximity in an aqueous solvent [132]. More gDNA present in this system means that there is a higher density of the gDNA networks and more opportunities for intermolecular forces between gDNA molecules (Figure 4.7). The higher density causes the magnetic beads to be entangled in the gDNA network, with the intermolecular forces between molecules overpowering the magnetic force, negatively affecting the beads' transport through the intricate geometry. With less gDNA present, the opposite appears to be true; the magnetic force is greater than that of the intermolecular forces between gDNA molecules, causing movement of the gDNA-magnetic bead complex through the microfluidic chip.

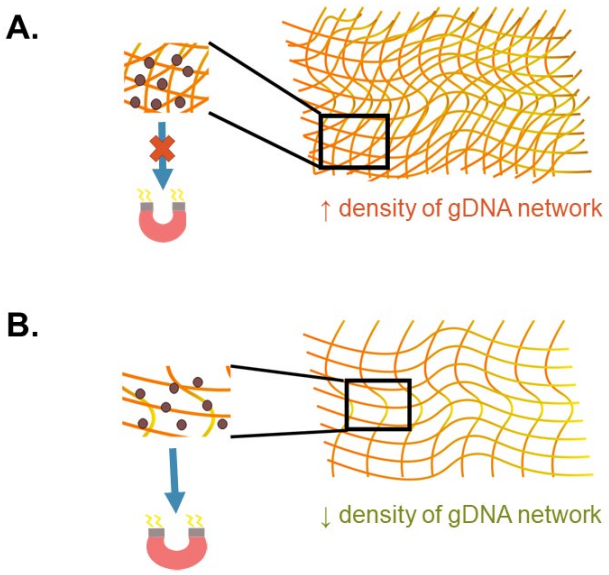


Figure 4.7: Mechanistic understanding of bead transport. ***(A)*** *A higher density of gDNA means there are more opportunities for interaction between DNA molecules, increasing the presence of intermolecular forces. These forces are likely greater than that of the magnetic force, which causes inhibition of the gDNA-magnetic bead complex's movement through the microfluidic chip.* ***(B)*** *A lower density of gDNA signifies fewer opportunities for interaction between gDNA molecules. The magnetic force is likely greater than the intermolecular forces between the gDNA-gDNA networks, allowing the gDNA-magnetic bead complex to move more freely through the microfluidic chip.*

To look at the relationship between gDNA density and the magnetic beads more simply, the relationship between the available surface area of the spherical beads and the surface area of gDNA was investigated. Using a hemocytometer, the concentration of the magnetic beads (C_{bead}) was measured to be approximately $23x10^6$ beads/µL, and their diameters are 1.5µm. The total available surface area (Equation 4.2) for a given volume ($V_{bead}(x)$) of magnetic beads can therefore be calculated.

$$Total\ available\ magnetic\ bead\ surface\ area = SA_{bead} * C_{bead} * V_{bead}(x) \quad \textit{(Equation 4.2)}$$

where x is dependent upon the initial sample volume. Here, the bead volume was kept constant for all dilutions; therefore, the total available magnetic bead surface area was found to be 1.31 cm^2.

Theoretical values were used to find the total available surface area for gDNA in the system. The concentration of leukocytes in fresh, whole blood was used to find the number of cells in a given volume, N_{cells}, of which, on average, there are 5,500 leukocytes/µL [17]. There are 6.4 billion base pairs in the human genome [133], and the size of extracted gDNA fragments once purified is 100–200kbp. [134]. Here, it will be

assumed that the gDNA present in the system is already in the ~150kbp fragments. Using these parameters, the number of fragments present in one white blood cell can be calculated, F_{gDNA}.

We then calculated the total available gDNA surface area (Equation 4.3). For modeling purposes, the gDNA fragments were assumed to be spherical, using the calculated radius of gyration of ~0.9 μm [135], [136] to calculate the surface area of one gDNA fragment (SA_{gDNA}).

$$Total\ available\ gDNA\ SA = N_{cells}(y,z) * F_{gDNA} * SA_{gDNA} \qquad \textit{(Equation 4.3)}$$

where $F_{gDNA} = \frac{human\ genome\ length}{size\ of\ extracted\ fragments}$ and the number of cells, N_{cells}, is dependent upon y, the volume of blood used, and z, the dilution factor of the blood.

From Equation 4.2 and Equation 4.3, the ratio of DNA surface area to bead surface area can be calculated (Equation 4.4):

$$gDNA{:}\,bead\ ratio = \beta = \frac{Total\ available\ gDNA\ SA}{Total\ available\ magnetic\ bead\ SA} \qquad \textit{(Equation 4.4)}$$

Variables x, y, and z were varied to estimate the gDNA:bead ratio (β) at the various dilution factors for which results are shown in Figure 4.6 (Table 4.2).

Table 4.2: gDNA:bead ratio (β) calculations for varying dilutions of whole blood.

Dilution factor	Undiluted blood volume [μL]	N_{cells}	Total gDNA surface area available [cm^2]	β
None	4	22,000	100	76
1:10	0.4	2,200	10	7.6
1:20	0.2	1,100	5	3.8
1:40	0.1	550	2.5	1.9

β is significant for successful movement of the gDNA-magnetic bead complex through the microfluidic chip. For the undiluted sample, β = 76, and once diluted, β decreases by a factor of 10. This indicates that there is a critical ratio of β<10 at which the transport of magnetic beads in a gDNA complex improves, because this correlates with the findings shown in Figure 4.6 that gDNA extraction is improved with blood dilution. The number of beads in a given area increases with the dilution factor. A larger number of beads in an

area allows a greater force to be applied to the DNA networks to separate them for transport through the microfluidic chip. This estimation of β is not precise, as gDNA topology varies heavily in cells. However, the trend is what is significant for this study, which indicates the importance of the gDNA:bead ratio in the successful movement of the gDNA-bead complex through the microfluidic chip.

4.4.5 Quantitative PCR of gDNA extracted from diluted blood samples

A downstream preparation step for NGS is PCR. To investigate the purity and quality of the diluted gDNA samples, quantitative PCR (qPCR) was performed using Taqman probes for the human SRY gene. The average C_t value for three selected samples from each of the starting amounts shown in Figure 4.6 is presented in Figure 4.8. Replicates 2, 3, and 5 from each experimental group were selected for qPCR analysis at random. In Figure 4.8, the eluates of on-chip and off-chip extractions from the undiluted sample and the diluted samples were not significantly different when compared. However, replicates that did not amplify within the 40-cycle PCR were excluded from the graph; these were two on-chip samples from 14 ng, two on-chip samples from 0.35 ng, and one on-chip sample from 0.7 ng. For the replicates that did not amplify, their extracted gDNA amounts were cross-checked with data from Figure 4.6. One of the excluded 14 ng replicates did not have gDNA recovered on-chip, matching its C_t value. For all the other excluded values, however, the replicates did have sufficient gDNA recovered yet did not amplify. This indicated the presence of inhibition due to a component in the eluate on-chip. This was further investigated.

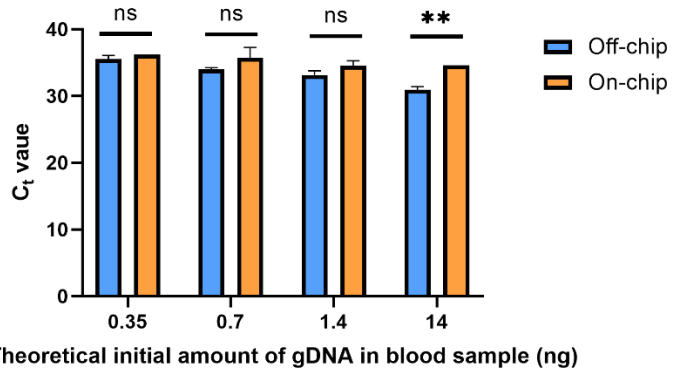


Figure 4.8: C_t values of gDNA extracted from diluted blood samples. *qPCR using Taqman chemistry was performed on the gDNA samples from Figure 6. Samples that did not have a C_t value in the 40-cycle PCR were omitted; these were two on-chip samples from 14 ng, two on-chip samples from 0.35 ng, and one on-chip sample from 0.7 ng. Data were analyzed using two-way ANOVA (α=0.05) and Sidak's multiple comparisons test for further analysis, n=3. For 14 ng, p = 0.004 and all others, p > 0.091. Standard curve using 5-fold dilutions: R^2 = 0.9993, slope = -3.32.*

4.4.6 PCR inhibition from Wash Buffer 3

PCR inhibition can arise from a variety of sources in this system. Anticoagulated blood has PCR inhibitors such as heme and Heparin [137]. The protocol does not involve a Proteinase K digestion; therefore, it is unlikely that heme is contributing to the inhibition [138]. Heparin inhibits ribonucleases, which is also not relevant here [139]. Carryover of blood lysate components like proteins is possible but, is unlikely due to the negative charge of the M-PVA beads [30]. Though the contents of the reagents used are proprietary, general knowledge about their contents provided the possibility of them containing PCR inhibitors.

The volume of Elution Buffer 7 put into the elution well is 16µL, and the sample volume input onto the chip is 25 µL. Since this microfluidic chip is based on diffusion, the difference in volume between the two wells could cause hydrodynamic flow, causing Wash Buffer 3 to flow into the elution well. Additionally, when the eluate is removed from the microfluidic chip, it is pipetted up and down to gather all the magnetic beads. There is a sudden change in volume, which could cause the sample well contents to quickly flow into the wash channel, causing the wash buffer to flow into the elution well. Wash buffers typically contain ethanol, which has been shown to inhibit PCR [140].

To see the effects of Wash Buffer 3 on qPCR, varying percentages of Wash Buffer 3 were added to pure gDNA solutions. Pure human male gDNA was used to eliminate the possibility of inhibition from blood components. First, 10-µL solutions of 1.6, 8, and 40 ng/µL of pure gDNA were made, containing either 0%, 20%, 40%, or 80% Wash Buffer 3 (Figure 4.9). Then, 1 µL of this solution was used in the qPCR reaction, using the same Taqman probes as in Figure 4.8. For all solutions, 20% of Wash Buffer 3 did not significantly inhibit the reaction. However, above 20% of Wash Buffer 3, none of the solutions amplified within the 40-cycle PCR. It was concluded that small amounts of Wash Buffer 3 ingress into the eluate greatly inhibit qPCR.

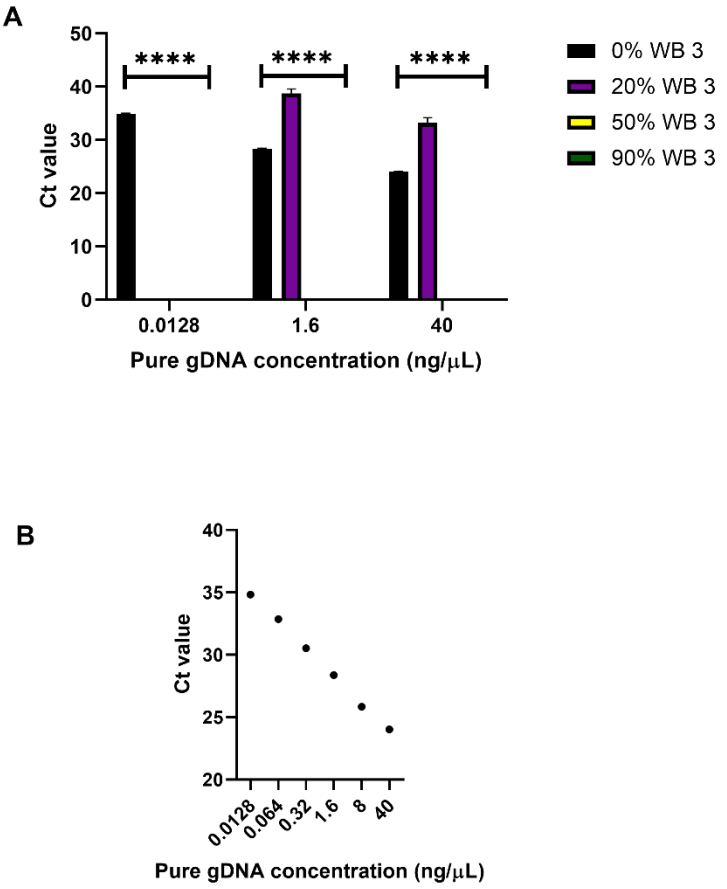


Figure 4.9: Wash Buffer 3 inhibition. ***(A)*** *C_t values from qPCR of 0%, 20%, 40%, and 80% solutions of Wash Buffer 3 with pure genomic DNA solutions and n=1 with 3 replicates. Statistical significance was found by two-way ANOVA (α= 0.05), using Dunnett's multiple comparisons test for deeper analysis. Within each concentration, C_t values were compared to 0% Wash Buffer or a pure solution of gDNA. P-values were <0.0001 for all comparisons.* ***(B)*** *Standard curve for (A) using 5-fold dilutions. R^2 = 0.9973, slope = -3.543.*

4.4.6.1 Reduction of Wash Buffer Ingress into the Elution Well

Wash Buffer 3 fills the wash channel via capillary action and does not enter into the sample or elution wells as it fills. Therefore, the source of the contamination had to be due to wash buffer ingress into the elution well via a different phenomenon. Analysis with colored liquids showed that Wash Buffer 3 ingresses into the elution well when the eluate is removed for elution, as hypothesized. To reduce this effect, two changes were made to the on-chip protocol. Firstly, the elution buffer volume was increased to 32 µL. This was done to reverse the direction of diffusion such that some of the elution buffer will diffuse into the wash channel instead of vice versa. Secondly, to remove the eluate from the

elution well for the off-chip heated elution, volumes in the sample well and elution well were removed simultaneously (just as they were added simultaneously) such that equal volumes remained after removal. Therefore, when the eluate is pipetted up and down, there is no volume difference to cause the sample well contents to flow into the elution well. With these changes, the sample well has 23 µL of solution, and the elution well has 32 µL of solution. A volume of 20 µL is removed from the sample well, and a volume of 28 µL is removed from the elution well, respectively. The sample volume removed is discarded, and the eluate is placed into a separate tube for the heated elution.

This new protocol was used, and the samples were analyzed with qPCR in Figure 4.10. Each sample was from the same stock solution of Heparin anticoagulated blood diluted to a concentration of 0.5 ng/µL. The C_t values off-chip and on-chip are closer in value compared to those in Figure 4.8. In Figure 4.8, as mentioned previously, some samples were omitted due to lack of amplification. All samples were included in Figure 4.10, which further shows the repeatability of this updated protocol.

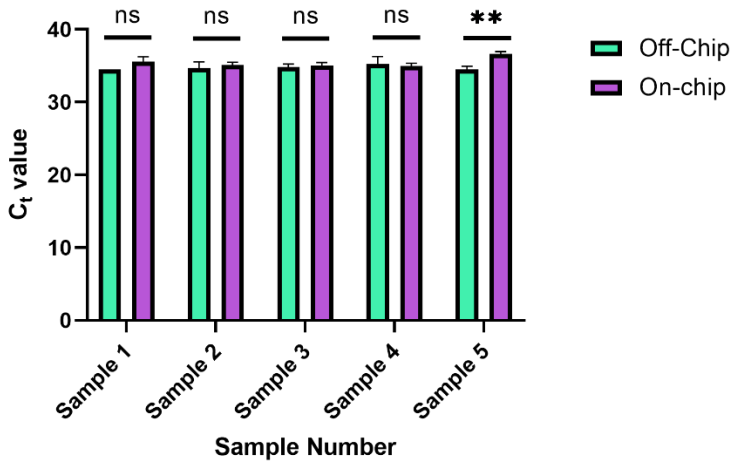


Figure 4.10: Reduced ingress of Wash Buffer 3 C_t values. *C_t values obtained for five separate diluted blood samples, with the on-chip extraction utilizing a new method of eluate removal from the chip. Statistical significance was found by multiple unpaired t-tests (α = 0.05). Sample 5, p = 0.0029, and all others p > 0.222.*

4.4.7 Final on-chip protocol

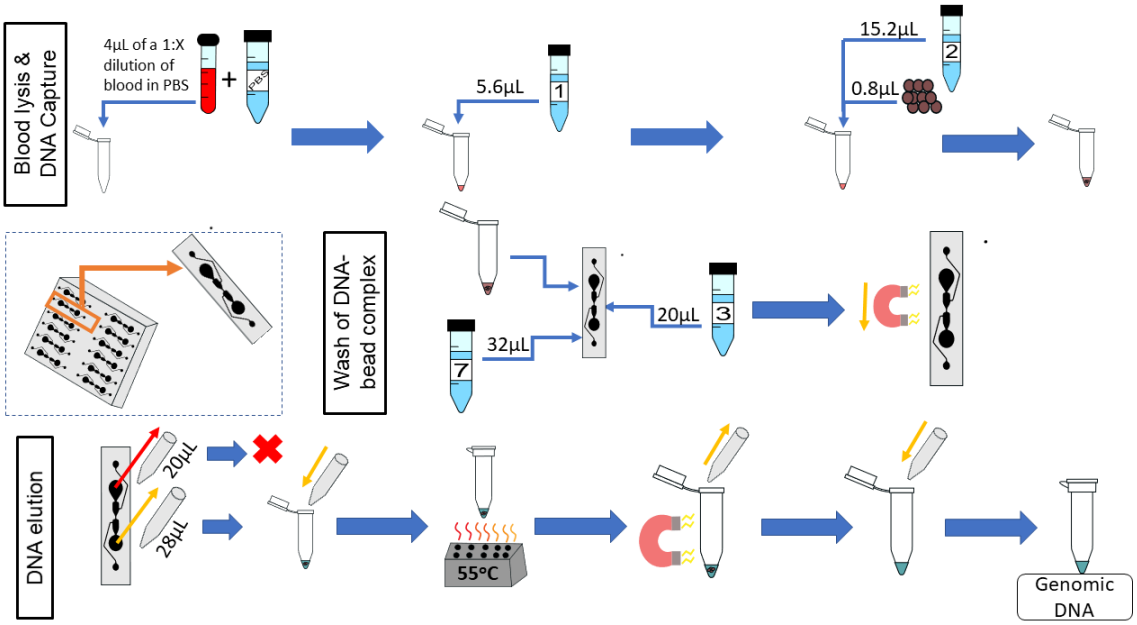


Figure 4.11: On-chip workflow. *The microfluidic chip has 12 microfluidic separators; the protocol described is for one of these microfluidic separators. Whole blood is diluted 1:X with PBS, where X is dependent upon the age of the blood sample; 4µL of this dilution is used for the on-chip protocol. Next, 5.6 µL of Lysis Buffer 1 is added to the diluted blood, mixed, and incubated for 5 minutes at room temperature. Subsequently, 0.8 µL of magnetic beads and 15.2 µL of Binding Buffer 2 are added to the lysed blood cells, and the combination is mixed and incubated for 5 minutes at room temperature. During the second incubation, 20 µL of Wash Buffer 3 is added to the Primary Wash Buffer Loading well. Then, 23 µL of the sample (lysed blood, binding buffer, and magnetic beads) is added to the sample well simultaneously with 32 µL of Elution Buffer 7. A magnet moves the magnetic beads from the sample well to the elution well. To elute the DNA, 28 µL of the eluate is removed from the elution well simultaneously with 20 µL of the sample well contents to reduce the effects of diffusion. The 28-µL sample is placed in a tube, which is placed onto a heat block at 55°C for 10 minutes. The tube is then placed onto a magnetic rack, and the supernatant is reserved.*

The complete on-chip protocol is shown in Figure 4.11 . Whole blood is diluted 1:X, in PBS, where X is dependent upon the length of time the blood sample has been stored. The concentration of gDNA in blood decreases with increasing storage time, so a sample that has been stored for longer would need to be diluted less [129]. It is recommended that the sample be diluted to at least 1:10, but as shown here, fresher samples should be diluted by a higher dilution factor. The volumes of the protocol can vary if key physical restraints are met (Table 4.3).

Table 4.3: Restraints of the microfluidic chip

Parameter	Restraint
Input DNA amount	Below 4 ng, or 667 white blood cells

Reagent volumes	Scaled linearly with sample volume
Sample Well volume	Volume must be lower than that of the elution well
Elution Well volume	Volume must be higher than that of the sample well
Sample loading	Sample well and elution well contents must be loaded simultaneously
Eluate removal	Elution well and sample well contents must be removed simultaneously, leaving equal residual volumes in both wells

4.4.8 Next-Generation Sequencing preparation of gDNA extracted on-chip

The goals of this device were to reduce the cost and time of gDNA extraction from whole blood as well as yielding gDNA that is of a quality suitable for NGS. To show the quality of the gDNA extracted, an on-chip washed sample that contained approximately 0.24 ng of gDNA was prepared for NGS using the 1-ng preparation protocol of the NEXTFLEX® Rapid DNA-Seq Kit. This kit produces libraries for Illumina® sequencing. The electropherogram and corresponding gel of the DNA library are shown in Figure 4.12. The prepared sample's electropherogram appears as it should do according to the manufacturer's expected results. The peaks at 15bp and 1500bp are the upper and lower markers, which are specific DNA sequences that are a part of the Agilent DNA 1000 kit, used to guide the sizing of the input sample. The peak at 120bp is an adapter dimer, which was expected, as excess adapter was added to exaggerate the results. The prepared library is the "bump" seen in the 200-1500bp range, which contains PCR-amplified fragmented gDNA sequences with the necessary modification for Illumina® platforms.

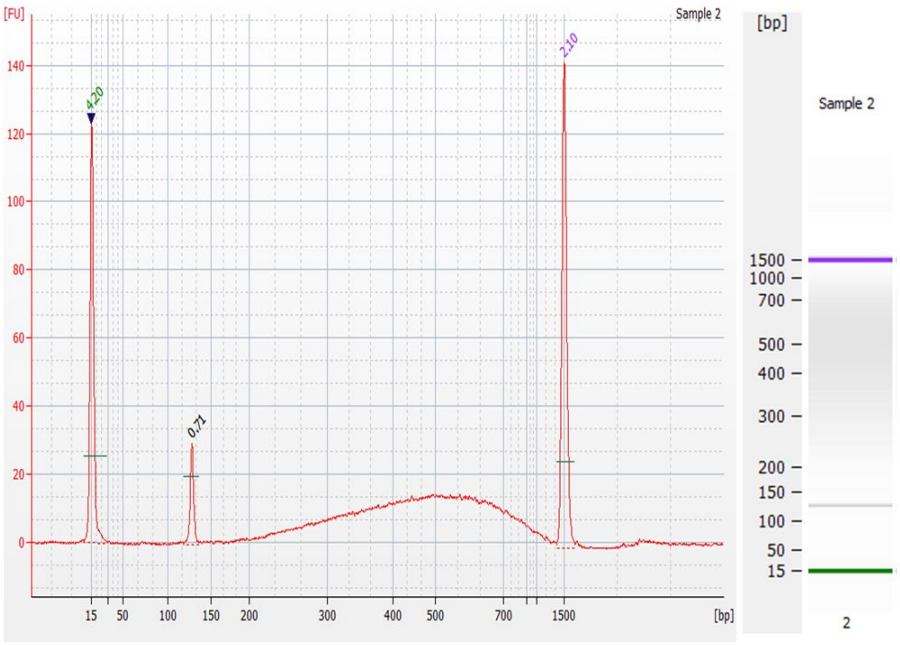


Figure 4.12: Preparation of gDNA extracted from the microfluidic chip for Next-Generation Sequencing. *Electropherogram (left) and corresponding gel (right) of the DNA library prepared from an on-chip washed sample of gDNA using the NEXTFLEX® Rapid DNA-Seq Kit. The peaks at 15bp and 1500bp are the upper and lower markers, the peak at 120bp is an adapter dimer, and the "bump" seen in the 200–1500bp range is the prepared library.*

4.5 Conclusion

Genetic epidemiological studies aim to understand the role of genetics in disease etiology. These studies require analysis of multiple patient samples so as to obtain more conclusive measures of association. High-quality and pure DNA samples are needed, which involves complex sample preparation protocols. These protocols are successful for gDNA extraction using macroscale volumes and multiple washes, but there is still limited knowledge on the quantitative extraction efficiency in microscale geometries. Here, a microfluidic chip was designed that reduces the number of wash steps necessary for genomic DNA extraction from whole blood. Six extractions can be performed simultaneously, increasing the throughput of sample collection. The volume of blood needed is less than 1 µL (undiluted), the total volume of reagents needed is 75 µL, and the entire protocol takes approximately 40 minutes to extract gDNA from six samples.

The microfluidic chip designed here has the potential to reduce sample processing time and shipping costs associated with genetic epidemiological studies. When compared to similar technologies [123]–[125], though the blood is diluted, the gDNA yield is similar. Additionally, the yield of ~3 ng is sufficient for NGS preparation, which can have an input of ~1 ng. The device does not use electricity or complicated equipment, which increases its accessibility for a variety of settings. Even though we are still at an early stage of technology development, we believe that this fundamental understanding of molecular diffusional analysis and bead transport phenomena will provide insightful guidance for point-of-care biological diagnostic platforms that use small volumes of whole blood, such as from finger pricks. There are opportunities for optimization of the reaction components, such as increasing magnetic bead volumes and adjusting buffer ratios. Future directions of this device include the design of more specific assays targeting genes of interest and integration of an on-chip heated elution step to further reduce manual steps. Additionally, an advantage of using magnetic beads is that it allows for further automation of the protocol. Overall, this microfluidic chip is simple to use while resulting in high-quality DNA samples, which can increase the throughput of genetic epidemiological studies.

4.6 Acknowledgments

Authors acknowledge Dr. Lothar A. Brassard's guidance on the use of chemagic™ reagents. We thank KL's funding sources and Dr. Francis Cui for his guidance in the early stages of this work.

4.7 Data Availability

The data for this work can be found at the following link: https://doi.org/10.7910/DVN/KKYWSB

4.8 Supplemental Information

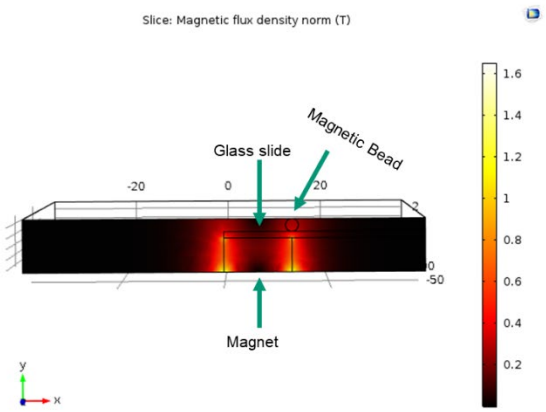


Figure SI 4.1: COMSOL model of the microfluidic chip with the magnetic field. *To model the magnetic force felt by the glass slide, and hence the paramagnetic beads, COMSOL Multiphysics was used. With an air box, the magnet is on the bottom, above it is the glass slide that the microfluidic chip would be mounted upon, and above the glass slide is a mock magnetic bead (for visual purposes). The input for the magnet was the remnant flux density, given by the manufacturer for the N42 magnet as 13,200 Gauss and for their strongest magnet (N52) this value is 14,800 Gauss. The image depicts the magnetic flux density in Tesla. Force calculations revealed that the glass slide feels a force of 37.1N with a N42 magnet and 46.7N with a N52 magnet.*

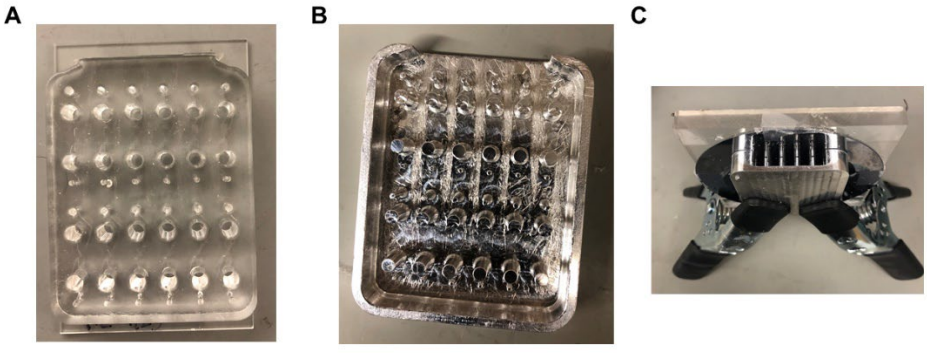


Figure SI 4.2: Microfluidic chip formation. ***(A)*** *The PDMS-glass microfluidic chip.* ***(B)*** *The aluminum mold.* ***(C)*** *The sandwich mold composed of the SU-8 master mold, uncured PDMS, and the aluminum mold.*

CHAPTER 5: AN INVESTIGATION INTO IMPROVING RNA INTEGRITY FOR TRANSCRIPTOMICS WITH AN ELECTROKINETICALLY-DRIVEN MICROFLUIDIC PROTOCOL

Chapter 5 has been published as an original research article.

Lee, K. and Tripathi, A., "An investigation into improving RNA integrity for transcriptomics with an electrokinetically-driven microfluidic protocol". *Biomicrofluidics*, vol 16, no. 044107, pp. 1-13, 2022.

5.1 Abstract

Current methods for total RNA extraction are time-consuming and require several hands-on steps and specialized equipment. Microfluidic devices can offer the opportunity to reduce the number of hands-on steps, decrease the volumes of reagents required for purification, and make extraction high throughput. Here, we investigated the translation of a high volume magnetic-bead-based total RNA extraction method (from human whole blood) onto a low input volume microfluidic device. Our results first show that RNA integrity is maintained when the reagent volumes are scaled down by a factor of 22 and the wash buffers are combined 1:1. With our microfluidic method, the number of wash steps can be reduced from four to one. Thus, the time to complete RNA extraction can be reduced from two hours to forty minutes. These manipulations to the conventional protocol yielded RNA amplifiable within 40 cycles of RT-qPCR when using the microfluidic device to simplify the wash steps. To improve the purification mechanism of the RNA during the bead transport through the microchannel, we also investigated the effect of a synergetic application of the electrokinetic flow. Our results show that DNase I and other contaminants surrounding the beads get washed away more effectively via electrophoretic transport. Most notably, RNA adsorption on the beads is strong enough to counter electrophoretically-driven desorption. In all, our work opens new ways to extract high-quality total RNA rapidly and simply from a small quantity of blood, making the process of RNA extraction more accessible.

5.2 Introduction

DNA and RNA can be isolated from viruses, circulating tumor cells, and more to assess a patient for various pathologies. The isolation and purification of nucleic acids from a biofluid such as blood or saliva requires several steps and complex equipment. When analyzing multiple peoples' biofluids for research studies or clinical diagnoses, the

large number of preparation steps, the need for cold storage, and the lack of low-cost automation [141] limits the performance of nucleic acid preparations to well-resourced laboratories. Automated equipment, large numbers of personnel, sufficient quantities of reagents, equipment for cold storage, and more are required for high-quality nucleic acid extraction from multiple samples within a reasonable timeframe. RNA isolation and purification, though, has further challenges as RNA molecules are less stable than DNA, and are degraded by ribonucleases (RNases) which are present on surfaces and in the air [26]. Here, we focus on total RNA extraction from white blood cells, which requires isolation of white blood cells from whole blood, lysis of white blood cells, DNase treatment to degrade the DNA, and lastly, purification of the RNA. To reduce the volume of solutions used, increase throughput, and decrease the amount of time to extract total RNA from cells, this paper shows the significance of using a microfluidic device in combination with magnetic bead-based solid phase extraction for the miniaturization and simplification of RNA purification.

Microfluidic devices allow for miniaturization of processes which often reduces the volume of reagents and samples required. Additionally, they are portable, can utilize fluid dynamic phenomena for actions like mixing and separation, and are automation-friendly [142]. All these characteristics make microfluidic devices an excellent candidate for simplifying nucleic acid extraction for low-resource settings. However, due to the ubiquitous presence of RNases in the environment, the creation of microfluidic chips for RNA extraction is difficult, which likely has limited their development [143]. In addition, whole blood is a complex biofluid that contains inhibitors of downstream nucleic acid analysis techniques, like PCR [138]. Thus, there are few studies have developed microfluidic workflows starting from whole blood to simplify RNA extraction.

Of the devices that have extracted total RNA from human whole blood on a microfluidic chip, most use a capture matrix [142]–[149]. These methods involve drying of the membrane with a vacuum and/or a pump for constant fluid flow, which increases the volume of reagents and samples necessary for extraction. Magnetic beads are more automation-friendly and require only a magnet to manipulate them (compared to a centrifuge for non-microfluidic capture matrices). One of the few studies that use magnetic beads to extract total RNA from whole blood lysates using microfluidics was done by Jebrail et al., who designed a digital microfluidic device [150]. Digital microfluidic devices are automation-friendly and can integrate further analyses methods like reverse transcription and PCR, which helps to reduce the need for cold storage of the RNA. With this device, researchers were able to complete the RNA extraction process in one device, but their protocol, which does not include DNase digestion, was tested by our group and the RNA eluate contained significant DNA contamination, lowering the quality of RNA eluted (data not shown). Additionally, digital microfluidics are complex in their design and their method required multiple external pieces of equipment.

Du et al. used magnetic beads for purification in a microfluidic device developed to isolate and fluorescently identify Ebola virus RNA from a spiked whole blood sample [151]. This device is a great for the point-of-care, but since they focused on hybridizing the RNA of interest to the beads for fluorescence detection, their method did not have to consider the washing away of contaminants in blood lysate to yield RT-PCR-amplifiable RNA. Wash steps will follow the lysing of the cells or viruses, to wash away contaminants from the cells and viruses as well as wash away other reagents used in the purification process. Lysis buffers often contain guanidium salts, wash buffers contain ethanol or isopropanol, and DNase solutions contain DNases which all inhibit PCR [152], [153]. Therefore,

insufficient removal of buffers used in nucleic acid extraction can affect the integrity of diagnoses and sequencing.

To maximize impact for low-resourced laboratories, minimal laboratory equipment is essential, but many devices, such as those developed for the point of care (POC), still use methods that require specialized equipment [13]. Here, a method to simplify the washing of RNA extracted from whole blood was developed, without the need for equipment beyond the microfluidic device, pipettes, a magnet, and the reagents involved. We also investigated the use of electrophoretic transport for improved purification. Previous work in our group has used electroosmosis and magnetic beads on microfluidic chips for DNA purification [154], [155]. In Schneider et al., adapter-dimer DNA fragments were being purified out of a solution for downstream sequencing using magnetic beads [154]. Deraney et al. purified PCR inhibitors for DNA extraction from plasma [155]. Their work showed strong evidence of the utility of electroosmosis in those applications for getting rid of the contaminants of interest, which is why its applicability was investigated in this work. Here, the contaminants differ in terms of electrical charge, type, and amount. Overall, this novel work developed a simple microfluidic-based protocol for RNA purification and used the combination of electrokinetics and magnetic bead-based purification to increase the integrity of RNA following RNA extraction from whole blood.

5.3 Materials and Methods

5.3.1 Human whole blood

Human male whole blood was purchased from Golden West Biosolutions (Temecula, CA, USA). The blood samples contained either sodium heparin or disodium EDTA anticoagulants. The samples were tested for common infectious diseases by Gold West Biosolutions. All samples were stored at 4^{o}C.

5.3.2 Conventional RNA extraction reagents

Reagents from the chemagic™ RNA Blood Kit Special H96 (PerkinElmer, Waltham, MA, USA) were used for RNA extraction from whole blood. Millipore Sigma's Red Blood Cell Lysis Buffer (RLB) (Burlington, MA, USA) was used for cell lysis and isolation for the white blood cells. Instead of a 2:1 ratio of RLB to whole blood, a 3:1 ratio of RLB to whole blood was used for the first red blood cell lysis step to lyse the red blood cells more efficiently. Adjusting the ratio of RLB to whole blood was suggested by the manufacturer's protocol if one did not see sufficient lysis, i.e., the resulting solution was not semi-transparent. We found that with a 3:1 ratio, the lysis was more efficient. β-mercaptoethanol (Millipore Sigma, Burlington, MA, USA) was used to stabilize the RNA.

5.3.3 2.3 Determination of RNA yield & integrity

RNA yield and integrity were found using the Agilent Bioanalyzer 2100 and either the Agilent RNA 6000 Pico Kit or the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, California, USA). RNA Integrity Numbers (RINs) were calculated by the Agilent Technologies 2100 Bioanalyzer 2100 Expert software, version B.02.11.SI811.

5.3.4 Microfluidic chip fabrication

The microfluidic chip was fabricated using polydimethylsiloxane (PDMS) soft lithography. The microfluidic channels were designed using Fusion360 (version 2.0.11415, Salt Lake City, Utah, USA) and the SU-8 mold was made by FlowJem (Toronto, Ontario, Canada). Following the PDMS curing directions for Sylgard 184, a 10:1 mixture of Sylgard 184 elastomer base and a curing agent from Krayden, Inc. (Denver, Colorado, USA) was made and placed into a vacuum chamber for 30 minutes to remove air bubbles.

The channels wells of the microfluidic chip were formed by pouring the PDMS into a sandwich mold composed of an SU-8 master mold to form the microfluidic features (150μm in depth) and a machined aluminum mold to form the wells of the device [153].

The two molds are aligned and held together with spring clamps. Uncured PDMS is then poured into the top opening into the gap between both molds, which is 13.4mm in width. Once cured at 70^{o}C, the film of PDMS formed in the gap is released from both molds. The wells have been formed by the aluminum mold, but a ~3mm length of PDMS occludes them, which is hole-punched out using 3.5 or 1.5mm diameter hole punchers, according to the respective well size. The PDMS film is then bonded to a 75 x 50 mm glass slide. This method allows for more precise well formation when compared to conventional PDMS hole-punching methods. After plasma bonding, the microfluidic chip stayed at 70^{o}C overnight and then was brought to room temperature for at least 30 minutes. Depending on the ionic strength of the solutions used in a microfluidic chip, the temperature may play a more significant role in the zeta potential uniformity [156].

5.3.5 Magnet movement

To remove variability in the magnet's movement when performing experiments, an in-house device was used to standardize magnet motion. The device consists of an x-stage for left-right motion. The stage is a linear screw-drive stage (igus® plastics for longer life®, Providence, Rhode Island, USA) with a stepper motor used to control the stage motion (Applied Motion Products, Inc., Watsonville, California, USA) based on an in-house script. The magnet is a 2" x 1/2" x 1/4" thick neodymium bar magnet (Grade N42, Item #BY084), with a surface field of 3424 Gauss, purchased from K & J Magnetics, Inc. (Pipersville, Pennsylvania, USA). To move any mangetic beads that did not move with the (non-optimized) in-house device movement, a bar magnet was used manually.

5.3.6 Primers for reverse transcription qPCR (RT-qPCR)

Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA) and was performed the day of RNA extraction, and the resulting cDNA was stored at -20^{O}C.

Real-time PCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA). TaqMan™ Gene Expression Assay ID# Hs00168719_m1, a primer for the peptidylpropyl isomerase B (PPIB) gene was used, which is a good reference gene for human peripheral blood (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) [157]. The Taqman™ Gene Expression Master Mix (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) was used for the assay.

5.3.7 Human Peripheral Blood Leukocytes Total RNA – RT-qPCR standard curve

Human Peripheral Blood Leukocytes Total RNA (ThermoFisher Scientific Inc., Waltham, Massachusetts, USA) was used to make a standard curve RT-qPCR.

5.3.8 2.8 Autodesk® Fusion360™ and COMSOL Multiphysics® - Theoretical Modelling

Theoretical modelling was performed using COMSOL Multiphysics® Software (Stockholm, Sweden), version 5.6. Two-dimensional CAD models were designed in Fusion360 (Draper, Utah, USA) and imported into COMSOL as the geometry for the simulations.

5.3.9 Statistical analyses

Statistical analyses were performed in GraphPad Prism 8 (San Diego, California, USA) or Microsoft Excel (Redmond, Washington, USA) with alpha = 0.05. Statistical analyses were performed in GraphPad Prism 8 (San Diego, CA, USA). *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$. Further information regarding statistical comparisons can be found in the Supplementary Information.

5.4 Results and Discussion:

5.4.1 Conventional protocol

The extraction of RNA from whole blood requires several biochemical steps, including lysis of cells, separation of DNA and RNA, isolation of RNA, and buffer exchanges. Since the protocols are lengthy and often complex to understand, we designed a visual way to describe the protocol used here in Figure 5.1, where all steps and conditions are described in three parts, I, II, and III. First, the red blood cells are lysed, and the white blood cells are isolated following the protocol for Millipore Sigma's Red Blood Cell Lysis Buffer (RLB). About 1.1 x 10^7 white blood cells (~ 5,500 per μL) were theoretically expected in this step [17]. Once the white blood cells were isolated, reagents from the RNA extraction kit are used to lyse the white blood cells, and β-mercaptoethanol is used to stabilize the RNA. The RNA and DNA in the white blood cell lysate are bound to magnetic beads using a binding buffer, and the solution is constantly mixed for five minutes via pipetting up and down. The supernatant is removed, and similar mix steps are done for the wash steps. The solution is then degraded by a DNase I solution for 20 minutes at room temperature. This step removes any DNA contamination, which is a prerequisite for any RNA-based expression profiling. From there, the RNA is re-bound to the magnetic beads using a binding buffer, and the RNA-magnetic bead complex is washed. The RNA is eluted using an elution buffer. About 3.2-16μg of RNA was expected from 2mL of blood [158], which is a concentration of 16-80ng/uL in 200uL of elution buffer. This protocol takes approximately 3 hours to complete for one sample.

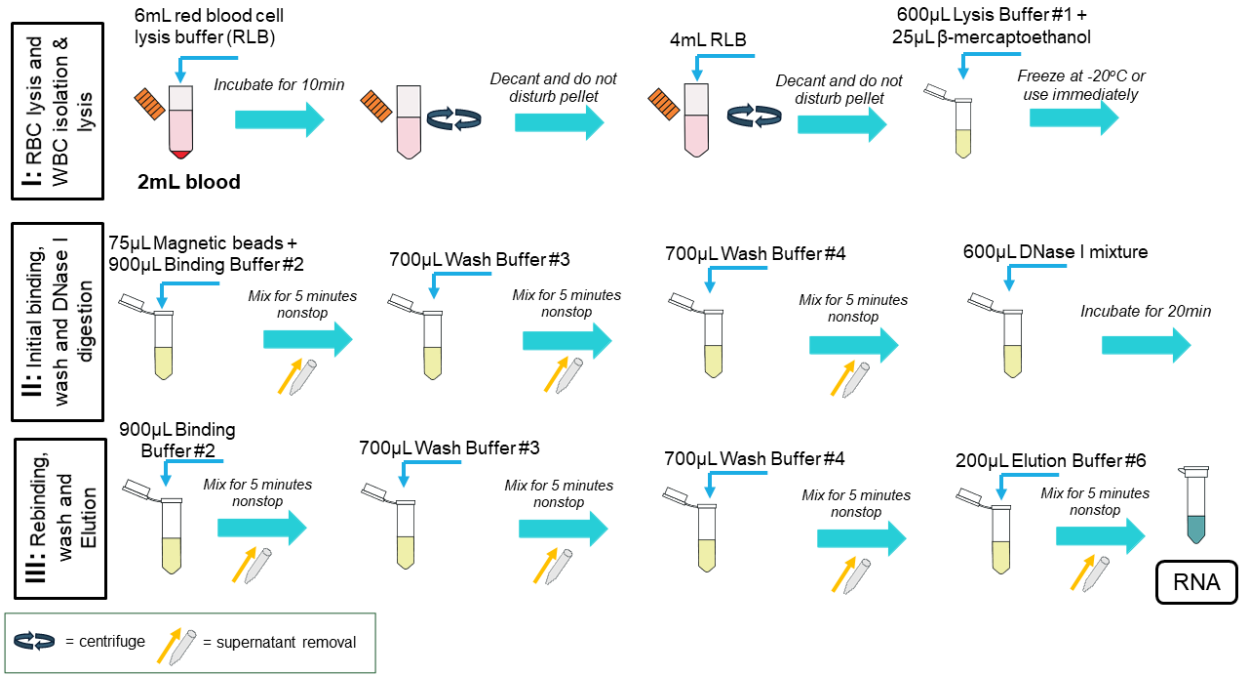


Figure 5.1: RNA extraction from whole blood protocol. *The protocol for red blood cell (RBC) lysis and white blood cell (WBC) isolation used Millipore Sigma's Red Blood Cell Lysis Buffer (RLB) and RNA extraction used the Chemagic™ RNA Blood Kit Special H96. All steps were performed at room temperature.*

Following the method in Figure 5.1, three human whole blood samples were tested, using disodium EDTA or sodium heparin as anticoagulants. These samples were stored for a variety of times after the blood was drawn: 2 days, 5 days, and 8 days, contributing to the standard deviation of the yield measurements. Disodium EDTA and sodium heparin anticoagulated blood yielded average RNA concentrations of 25ng/µL and 20ng/µL, respectively (Table 1). The average RNA integrity numbers (RIN) and average C_q values were 4.3 and 30.06, respectively, for disodium EDTA anticoagulated blood. The average RNA integrity numbers (RIN) and average C_q values were 6.7 and 29.01, respectively, for sodium Heparin anticoagulated blood. There was no statistically significant difference between the values measured for disodium EDTA versus sodium heparin. In comparison to other methods, RNA integrity for the conventional protocol is similar; the concentration of the samples tested here is 2-3x fold higher than other methods [159].

5.4.2 Scale down of Part I of the conventional protocol

Reducing the starting volume of the blood or the white blood cell lysate and scaling down the subsequent reagents linearly was essential for future microfluidic protocol development. Part I of the conventional protocol (Figure 5.1) involves centrifuging 2mL of blood and lysing the red blood cells to isolate the white blood cells. Thus, two methods of reducing the volume of the reagents in these steps were investigated. The first was reducing the starting volume of blood (from 2mL to 500µL, 250µL, 100µL, or 50µL) and the second was reducing the starting volume of the isolated white blood cells, lysis buffer, and β-mercaptoethanol (from 625µL to 156µL, 78µL, 39µL, or 28.4µL). The rest of the reagents were scaled down linearly with the starting sample. At the two highest volume reductions, reducing the starting volume of blood resulted in a lower concentration and RIN (measured using the Agilent Bioanalyzer 2100) than by reducing the volume of the white blood cell mixture. However, at the two lowest volume reductions the concentration and RIN were similar between the two methods (data not shown).

We decided to move forward with the second method to scale down of Part I to reduce the sample preparation time for future experiments. Using this method, the starting volume of blood is always 2mL and the white blood cells are isolated from the blood using red blood cell lysis buffer. From there multiple small aliquots (28.4µL) of the white blood cell lysate can be used for multiple experiments and analyses. When using the first method many individual replicates of the white blood cell lysate would have needed to be prepared, while the second requires one large solution of white blood cell lysate that can be sampled from multiple times. Either method would be sufficient for reducing the volume of the conventional protocol. However, when isolating RNA from smaller cell counts, the second method using lower volumes of blood may not have sufficient yield of less concentrated RNA.

5.4.3 Scale down of Part II & III of the conventional protocol and combining wash buffers

The lowest volume tested used 1/22 of the 625µL white blood cell lysate mixture, which is a starting volume of 28.4µL after performing Part I of the conventional protocol. Therefore, the volumes of the reagents in Parts II and III of the conventional protocol were scaled down by a factor of 22. The scaled-down elution volume of 9µL was multiplied by 2.2 for a final 20µL elution volume to ensure sufficient volume for pipetting and downstream analyses. These experiments were done with 5-day old blood. Additionally, in a separate experiment, to investigate the possibility of reducing the number of wash steps, wash buffers #3 and #4 were mixed 1:1. These experiments were done with 2-day old blood, with two common anticoagulants: disodium EDTA and sodium heparin. We used two types of anticoagulated blood for investigational purposes, not for comparison. The results for average concentration (in elution buffer), average RIN, and average C_q are shown in Table 5.1. The results for the altered protocols were compared to the conventional protocol, and no statistically significant difference was found between them. The actual yield per µL of blood for the scale downs is lower than the expected yield which could be due to the age of the blood and lower volumes being more sensitive to measurements since the scaled down concentration was found using the RNA Pico Kit. However, since the C_q and RIN values were comparable to the conventional protocol we concluded that reducing the volume of reagents and combining the wash buffers into one wash buffer is acceptable to translate onto the microfluidic chip.

Table 5.1: Average concentration, RIN, and C_q value of various protocols to simplify RNA extraction. *The conventional protocol was performed on three disodium EDTA and sodium heparin anticoagulated human whole blood. Using a t-test assuming unequal variance, the results of average concentration, average RIN, and average C_q value for three samples were compared between disodium EDTA and sodium heparin anticoagulants. There was no significant difference ($p > 0.05$) between any of the aforementioned values between the two anticoagulants. The volume of the reagents in the conventional protocol were scaled down by a factor of 22, and a separate experiment mixed the two wash buffers 1:1 to reduce the number of wash steps. Using the conventional protocol as a reference group, all measured values of the adjusted protocols were*

compared to that of the reference group and were not statistically significant. "Aver." – Average; "Std. Dev." – Standard Deviation.

Anticoagulant	Protocol	Aver. RNA yield (ng/µL elution buffer) ± std. dev.	Aver. actual yield (ng/µL blood)	Expected yield (ng/µL blood)	Aver. RIN ± std. dev.	Aver. C_q value ± std. dev.
Disodium EDTA	*Conventional protocol*	25 ± 7.21	2.5	1.6-8	4.3 ± 3.0	30.06 ± 4.43
	1/22 scale down	54.05 ± 24.42	0.54		3.4 ± 0.5	29.60 ± 0.85
	1:1 Wash buffer mix	26.22 ± 4.00	2.6		4.7 ± 1.9	26.49 ± 1.16
Sodium heparin	*Conventional protocol*	20 ± 5.67	2		6.7 ± 1.6	29.01 ± 2.97
	1/22 scale down	36.66 ± 15.02	0.37		3.6 ± 0.7	30.25 ± 1.54
	1:1 Wash buffer mix	31.83 ± 14.90	3.2		4.6 ± 0.6	26.78 ± 0.95

Note: for the "1/22 scale down" concentration values in the elution buffer - this is not the actual yield of the 1/22 scale down protocol, but for comparison purposes, the measured value was multiplied by 22.

5.4.4 The microfluidic separator

Previous work in our group led to the development of a microfluidic device that integrates magnetic separation and electrokinetic purification (MSEP) of DNA [153], [154]. These microfluidic devices were developed to simplify the washing and purification steps of the DNA extraction process and are made of a PDMS mold bonded to a glass slide. The device operates by first placing a wash solution into the Primary Wash Buffer Loading Well, which flows throughout the Wash Channel to the Secondary Wash Buffer Loading Well (green,Figure 5.2A-B). Then, the sample, which contains nucleic acids, is pipetted into the Sample Well simultaneously with the Elution Well solution (a wash buffer or an elution buffer) (yellow, Figure 5.2C). Our prior work used a purified DNA sample [154] or diluted whole blood as the starting sample [153]. A magnet is then placed beneath the separator to transport the magnetic beads through the wash solution in the Wash Channel,

washing the beads. This movement is repeated until all beads have been transported to the Elution Well, and the solution from the Elution Well is removed from the microfluidic separator (Figure 5.2D-E). This also must be done simultaneously with the Sample Well's contents such that they do not flow into the Elution Well, but the Sample Well's contents are discarded. Multiple separators are designed to be on one microfluidic chip, with six in parallel and in two rows (for a total of 12 separators), with each separator being 9mm apart such that multiple purifications can be performed simultaneously with a multi-channel pipettor and a long magnetic.

For RNA purification, which was investigated here, several changes were made to the microfluidic separator design used in Lee et al. Firstly, the number of beads used in the RNA extraction protocol compared to the DNA extraction protocol used in Lee et al. is 4.25x higher, so the width of the narrowest section of the wash channel and the width of the entrance point of the sample and elution wells were increased to be 4.25x larger. This helped to reduce the possibility of the magnetic beads getting stuck and unable to move through the separator and to keep the time to move the magnetic beads through the entire separator similar to that of the DNA extraction protocol. Next, the width of the entrance to the elution well was made to be the same as that of the sample well (instead of smaller) to ensure the magnetic beads could efficiently enter the elution well. The Secondary Wash Buffer Loading Channel was made to be twice the width of the Primary one because with a previous iteration of this RNA microfluidic separator, the wash solution would not flow from the Primary Wash Buffer Loading Well to the Secondary Wash Buffer Loading Well but would stop once the Wash Channel was filled. To reduce the hydrodynamic resistance in the Secondary Wash Buffer Loading Well, the width of the channel was doubled which did cause the Wash Channel and wells to be filled as hoped. Lastly, the height of the Sample and Elution Wells (and subsequently the Wash Buffer Loading Wells) was

increased via increasing the depth of the aluminum mold used to make the microfluidic chip, such that 100µL of solution could fit into the Sample and Elution Wells.

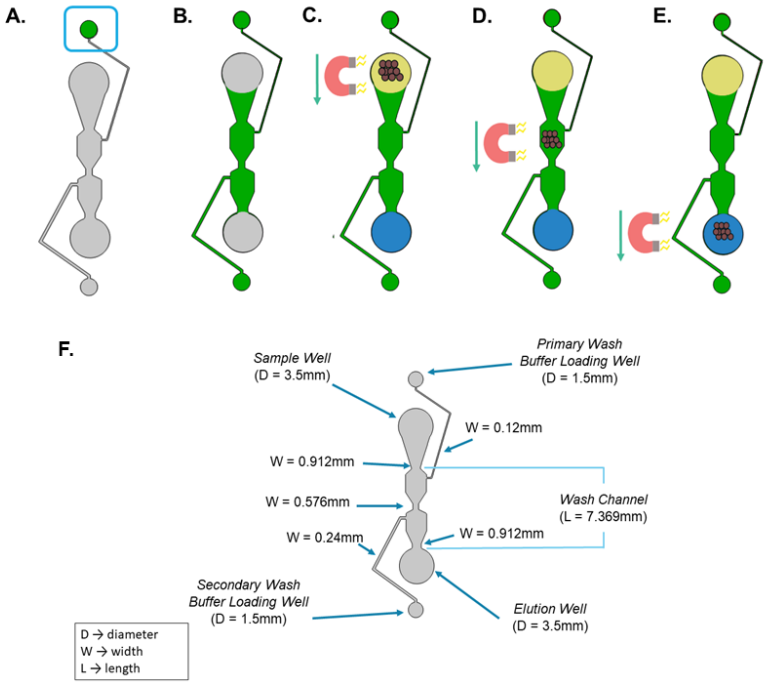


Figure 5.2: Solution loading and dimensions of the microfluidic separator. (*A-B)* *A wash buffer loaded into the Primary Wash Buffer Loading Well which flows throughout the Wash Channel into the Secondary Wash Buffer Loading Well. This takes approximately 20 seconds.* ***(C)*** *A solution with nucleic acids is pipetted into the Sample Well (top, yellow) at the same time as either a wash buffer or elution buffer into the Elution Well (bottom, blue).* ***(D-E)*** *Then, using a magnet placed beneath the microfluidic separator, the magnetic beads are transported from the Sample Well to the Elution Well, through the wash buffer in the Wash Channel. D-E are repeated until all magnetic beads have moved to the Elution Well, which takes 2-3 minutes.* ***(F)*** *The dimensions of a microfluidic separator.*

5.4.5 Investigation of a reduced number of wash steps and hands-on time from the conventional protocol

Parts II and III of the conventional protocol have ~25 minutes in hands-on washing steps each. The microfluidic separator was designed to simplify washing of magnetic beads, therefore, we investigated how the number of steps could be reduced for microfluidic purification. The blood samples here were from two different 2mL aliquots from

the same donor and was 2 days old. The controls (following the 1/22 scale down protocol) had an average C_q value of 26.88 for disodium EDTA anticoagulated blood and 28.08 for sodium heparin anticoagulated blood. There was no statistically significant difference between the two average C_q values of each anticoagulant.

To first test a reduction of the number of wash steps, all the wash steps were removed from the 1/22-scaled down protocol (termed “No washes”), and the resulting products’ C_q values were measured. Without the wash steps, the average C_q value for disodium EDTA anticoagulated blood was 34.05, and that of sodium heparin anticoagulated blood was 34.90 (Figure 5.3) which were both higher than their respective control protocols. Additionally, out of six disodium EDTA replicates, only three amplified; for sodium heparin, two out of six of amplified. The inconsistency in amplification via RT-qPCR indicates that removing all the wash steps is not repeatable and that some of the unwashed reagents inhibit PCR.

We hypothesized that DNase I was essential to being washed off the magnetic beads for successful qPCR. To test this, the 1/22-scaled down protocol was performed but without the wash steps after the DNase digestion (termed “No wash 2”). This trial was performed with the 1:1 mixture of wash buffers in place of two separate wash steps. The disodium EDTA “samples had an average C_q value of 33.86, and the sodium heparin anticoagulated samples had an average C_q value of 37.61, which were significantly higher than their respective controls. Four of the six disodium EDTA samples amplified, and one of the six sodium heparin samples amplified, leading to a similar conclusion to that of the “No washes” samples, which skipping the wash steps after the DNase I digestion are not repeatable.

Lastly, to further reduce the number of hands-on steps for the microfluidic protocol, the need for the five-minute constant mixing after every reagent addition was investigated.

For this experiment, the 1/22-scaled down protocol was performed without any mixing where the conventional protocol indicates to do so. Instead, the sample was incubated for five minutes at room temperature during the indicated mixing times. For the disodium EDTA anticoagulated blood the average C_q value was 33.14, and that of sodium heparin was 29.54. There was no statistically significant difference between no mixing with sodium heparin anticoagulated blood and the positive control; there was a statistically significant difference between no mixing with disodium EDTA anticoagulated blood and the positive control. Sodium heparin and disodium EDTA have both been shown to affect PCR [160]. Disodium EDTA chelates Mg^{2+} ions, while sodium heparin is thought to directly interact with nucleic acids [127]. This could explain the more significant difference between the sodium heparin protocol variations and their corresponding control since RNA amplification may be more affected by heparin presence in the absence of washes.

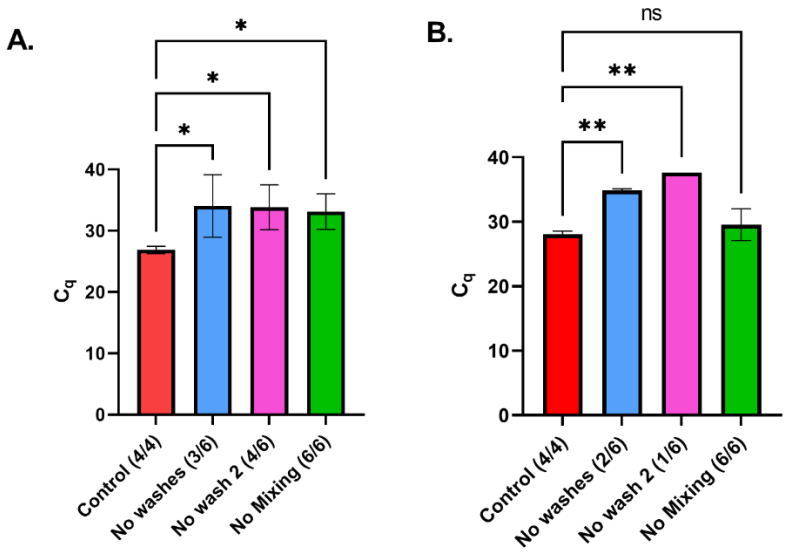


Figure 5.3: C_q values of trials with the removal of wash steps at different stages in the 1/22-scaled down protocol using disodium EDTA and sodium heparin anticoagulated blood. *Four samples were tested for the control protocol and six samples were tested for the experimental protocols. The values in parentheses are the number of samples that amplified during RT-qPCR*

(numerator) over the total number of samples analyzed (denominator). All samples were generated following the 1/22-scaled down volumes. "Control" refers to trials following the 1/22-scaled down protocol. "No washes" refers to trials in which none of the wash steps were performed. "No wash 2" followed the 1/22-scaled down protocol, and also used a 1:1 mixture of the wash buffers for the wash steps in Part I, and only skips the wash steps in Part III, after DNase I treatment. Lastly, "No mixing" refers to all mixing steps being replaced with five-minute incubations. To compare the mean C_q value of the trials to the positive controls, one-way ANOVA and Dunnett's multiple comparisons test were performed. ***(A)*** *Disodium EDTA anticoagulated blood.* ***(B)*** *Sodium heparin anticoagulated blood.*

To summarize, the protocol variations tested showed that: 1) the scaled-down volume of 1/22 and combining the wash steps into one 1:1 mixture had a comparable C_q values and RINs to the conventional protocol, 2) washing the RNA-magnetic bead complex after the DNase digestion was an essential step for repeatable results, and 3) the manual mixing steps could be replaced with room temperature incubations. DNase I is a PCR inhibitor since it degrades DNA, and this shows that DNase I must be sufficiently washed away for the resulting RNA sample to be RT-qPCR amplifiable. Taken together, these results allowed us to reduce the starting volume of the white blood cell lysate, reduce the volumes of other reagents used, combine the wash buffers, and reduce the amount of hands-on time needed by the researcher to perform RNA extraction. These are significant steps towards simplifying the RNA extraction process and reducing the volume of reagents implies a reduction in cost to do RNA extraction. A detailed analysis into conventional protocol reduction has rarely been described in the literature, which is important for not only microfluidic translation but in understanding essential steps in RNA extraction and purification.

5.4.6 The microfluidic separator – solution loading procedure for RNA purification

We first tested the microfluidic separation by using the solution in Part II before the wash steps, which contained white blood cell lysate, DNA-RNA-bound magnetic beads and binding buffer, as the sample for the Sample Well and the 1:1 mix of the wash buffers in the Elution Well. This showed that due to the high nucleic acid content of the sample,

the magnetic beads were too difficult to transport from the Sample Well to the Elution Well. This is because both DNA and RNA are on the beads, and they have strong bonds between them, that cannot be broken up by the magnetic pull on the magnetic beads [153]. Thus, our focus shifted to using the microfluidic separator for the wash steps in Part III, after the DNAse I digestion and re-binding of the RNA to the magnetic beads.

Using the insight from the conventional protocol's variations, the finalized microfluidic protocol is shown in Figure 5.4. Part I remains the same between the conventional protocol and the microfluidic protocol. 28.4μL of the solution from Part I was mixed with 3.4μL of magnetic beads and 41μL Binding Buffer #2 and incubated for 5 minutes at room temperature. The supernatant was removed and 27.4μL of DNase I solution is added to the magnetic beads. Then, 41μL of Binding Buffer #2 was added to the DNase I/beads solution and incubated for 5 minutes at room temperature. ~50μL of a 1:1 mixture of wash buffers 3 and 4 are added to the Wash Channel. Then, the DNase I/beads/Binding Buffer #2 solution (72μL) was added to the Sample Well of a microfluidic separator simultaneously with a 1:1 mixture of the two wash buffers added to the Elution Well. Then the microfluidic separator was used to clean the RNA-magnetic bead complex. The magnetic beads were moved from the Sample Well to the Elution Well until all magnetic beads are transported to the Elution Well, which is verified by visual inspection. Then the solution from the Elution Well is placed into a tube, and the supernatant is removed. 20μL of the elution buffer was added to the RNA-magnetic beads complex and incubated for 5 minutes. The supernatant contains the isolated total RNA. The 1:1 wash buffer mix was prepared fresh daily due to the uncertainty around the stability of that solution long-term. The volume of 85μL was removed from the Sample Well (a larger volume than was input) to ensure all the contents of that well are removed from the separator such that none flows into the Elution Well. If the Sample Well contents flow into

the Elution Well, the RNA did not amplify in RT-qPCR, due to the presence of DNase I (data not shown).

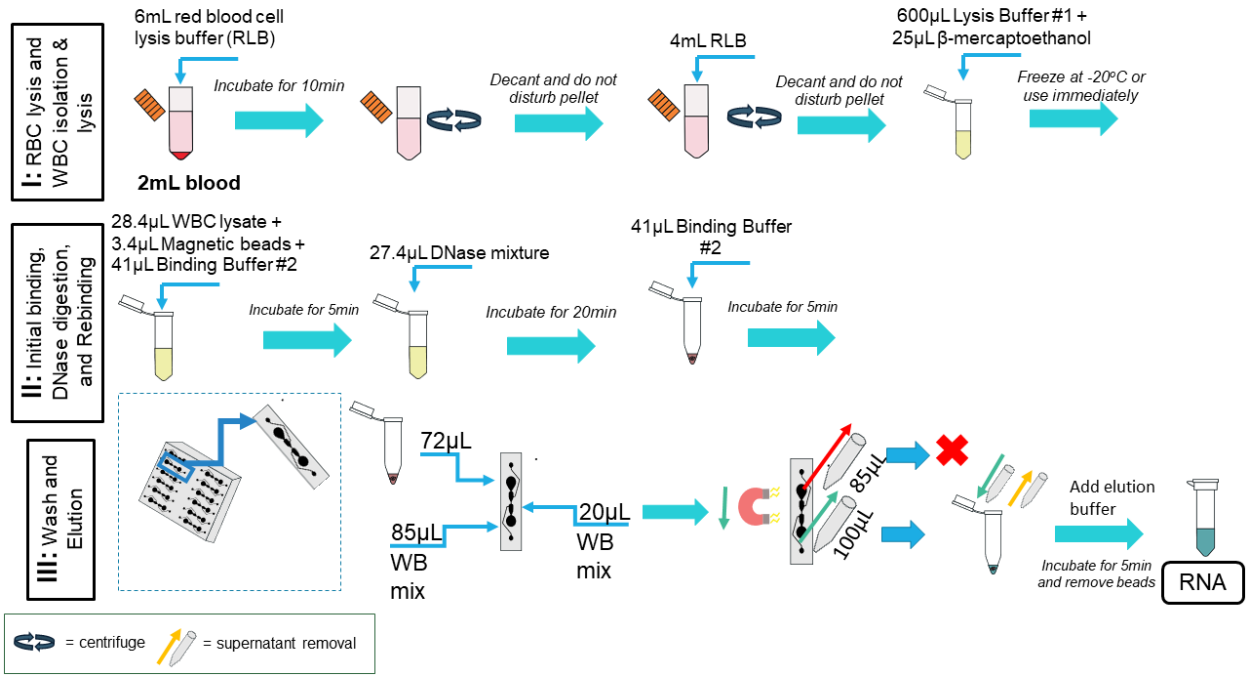


Figure 5.4: Procedure for solution loading for microfluidic washing of total RNA after DNase I digestion. *Part I remains the same as the conventional protocol. In Part II, the wash steps are skipped compared to the conventional protocol, and the solutions are incubated where indicated instead of mixed. In Part III the microfluidic chip is used to perform the wash steps, and the elution is performed off-chip.*

5.4.7 Can the microfluidic separator replace the post-DNase I treatment wash steps?

Following the steps in Figure 5.4, we tested if the microfluidic separator was sufficient to perform the wash after the DNase I digestion and re-binding of the RNA to the beads (Figure 5.5). The average C_q value for disodium EDTA anticoagulated samples was 31.82 and that of sodium heparin anticoagulated samples was 33.57. In this experiment, all six samples tested amplified, the indicating repeatability and that the microfluidic separator was sufficient to wash away any PCR inhibitors, including DNase I. Additionally,

there was no significant difference between using the microfluidic separator for washing compared to the conventional protocol or the 1/22-scaled down protocol. “On-chip wash” does have more variability in C_q value than the control, which is likely due to the slight differences between separators and their formation. “On-chip wash” was performed on an earlier iteration of the microfluidic chip in which the Elution Well was a smaller diameter than the Sample Well (instead of being the same diameter), which made removing solutions from it via pipetting a bit challenging. This issue was solved by making the Elution Well’s diameter larger and the variability between microfluidic separators was reduced as seen in subsequent results.

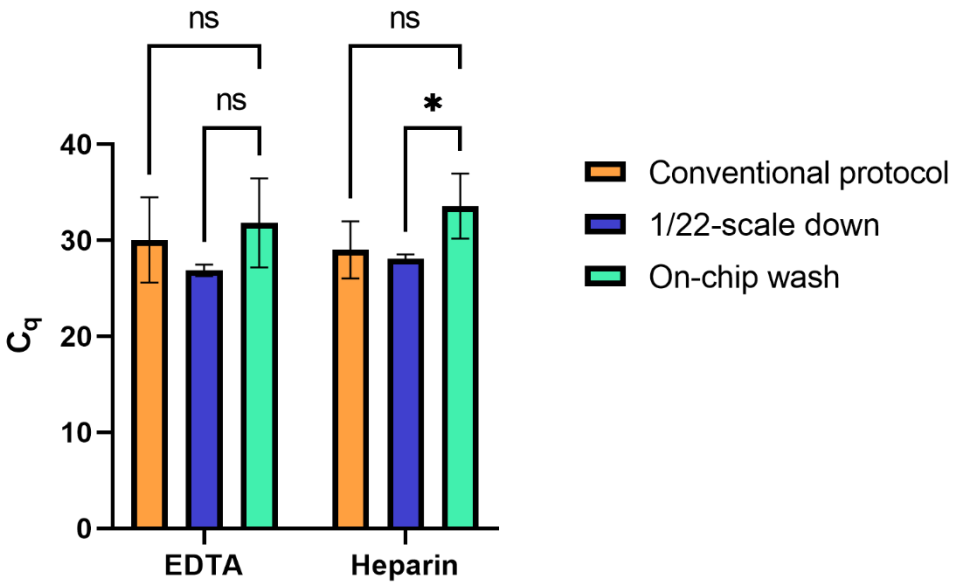


Figure 5.5: On-chip washing versus the conventional protocol and the 1/22-scale down. *“On-chip wash” uses the volumes of the 1/22-scale down, skips the wash step in Part II, and the washes for Part III are performed on the microfluidic chip. To compare the mean C_q value of the trials to that of the 1/22-scale down and the conventional protocol, two-way ANOVA was performed, with Tukey’s multiple comparisons test.*

5.4.8 Electrophoretic applications to the microfluidic separator for increased purification

Building upon previous work in our group, we sought to investigate if the use of electrokinetics to improve purification of the RNA samples. Here, when referring to electrokinetics, we are referring to both electroosmotic flow and electrophoretic transport. The total velocity of the solute would be the sum of the electroosmotic and the electrophoretic velocity (Equation 5.1).

$$v_{total} = v_{eof} + v_{ep} \qquad \textit{(Equation 5.1)}$$

Electroosmotic flow is the bulk flow of fluid in the presence of an electric field. For this study, the glass slide to which the PDMS microfluidic chip is bonded is negatively charged, causing the positive charges of the fluid to be attracted to the slide creating an electric double layer [161]. Thus, along the surface of the glass slide, in the presence of an electric field, the bulk flow of the fluid is towards the negative electrode due to the positive charges of the fluid. Electrophoresis is the movement of a solute in response to an electric field, depending upon the charge of the solute. For example, cations will move towards a negative electrode, and anions will move towards a positive electrode [162]. Schneider et al. and Deraney [154], [155] found that electroosmotic flow improved purity of the magnetic bead-bound DNA samples they were purifying because contaminants in the bulk solution would flow out of the Wash Channel into the Wash Buffer Loading Well that contained the negative electrode.

There are multiple contaminants in the solution that likely surround the beads, which are positively and negatively charged and will reduce RNA purity. The solutes are likely the DNase I enzyme, binding buffer salts, and fragmented genomic DNA. DNase I requires positive ions such as Mg^{2+} and Ca^{2+} to degrade DNA [163]. The magnetic beads were dragged through microchannel with applied electric field, and the fluid drag on beads

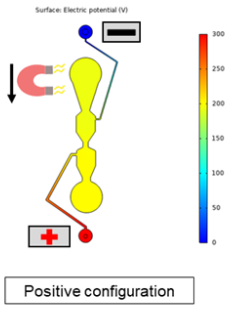
$6\pi\eta R v_b$ separates any unbound DNase, salts or fragmented genomic DNA. Fluid viscosity, bead aggregate radius and bead velocity are denoted by η, *R*, and v_b, respectively. However, the no-slip condition on the bead aggregate is less likely to separate the unbound species close to bead surfaces. Hence, electric field driven electrokinetic flows were enacted to effectively separate unbound species next to beads. More specifically, we were interested in exploiting electrophoretic transport as this would theoretically remove both positively and negatively charged contaminants from Wash Channel.

5.4.9 Experimental investigation of electrophoresis for improving RNA purification on the microfluidic separator

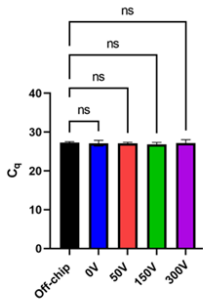
Similar to our prior work, we applied an electric field onto the microfluidic separator via placing electrodes into the Primary and Secondary Wash Buffer Loading Wells to induce electrophoretic and electroosmotic velocities. To obtain uniform zeta potential between different microfluidic chips, it was essential for the microfluidic chip to not be used until it sat overnight following plasma bonding between the PDMS and glass slide. A negative electrode was placed into the Primary Wash Buffer Loading Well, and a positive electrode was placed into the Secondary one, causing the electroosmotic velocity to be in the opposite direction as magnetic bead motion and electrophoretic transport to occur according to the respective electrode (i.e. negatively charged contaminants flow towards the positive electrode and vice versa) (Figure 5.6A). Electroosmotic flow in the opposite direction of the bead motion would mean that the bulk flow of the fluid and contaminants would not be towards the Elution Well to aid in purifying the sample. Electrophoretic transport towards both electrodes would mean that both positively and negatively charged contaminants are flowing out of the Wash Channel to the Wash Buffer Loading Wells.

We compared the use of the electric field to an off-chip sample which followed the microfluidic protocol (Figure 5.4) but instead of using the microfluidic chip in Part III, the wash was performed in a tube. We tested off-chip (average C_q = 27.28) and compared them to 0V (average C_q = 27.12), 50V (average C_q = 26.31), 150V (average C_q = 27.35) and 300V (average C_q = 25.62). There was no significant difference between the average C_q values of any of the tested conditions versus off chip (Figure 5.6B).

A.



B.



C.

Sample Name	Concentration (pg/µL)	RIN
Off-chip	4,100	2.8 ± 0.6
0V	1,049	4.2 ± 0.7
50V	1,111	4.1 ± 0.2
150V	1,506	4.8 ± 1.3 (*)
300V	924	5.8 ± 0.1 (**)

Figure 5.6: C_q values, concentrations, and RINs of when voltages were applied the microfluidic separator in the positive configuration using sodium heparin anticoagulated blood. ***(A)*** *The positive configuration of the electric potential, shown with 300V.* ***(B)*** *Six replicates were tested for off-chip. Four replicates were tested for 0V, 50V, and 300V. Five replicates were tested for 150V. To compare the mean C_q value of the trials to that of the off-chip protocol, one-way ANOVA was performed, with Dunnett's multiple comparisons test.* ***(C)*** *To compare the mean concentration of off-chip to the mean concentrations of 0V, 50V, 150V, and 300V one-way ANOVA was performed, with Dunnett's multiple comparisons test. One replicate did not yield a value for RIN for both 150V and 300V, therefore the concentration values for those samples were excluded.*

C_q values are a function of both RNA concentration and RNA sample quality, i.e., presence of inhibitors or degradation of RNA. Therefore, to further characterize the RNA extracted, capillary gel electrophoresis was performed using the Agilent Bioanalyzer (Figure 5.6C). In this analysis, each sample's replicate in was measured three times with the Bioanalyzer, and those values were averaged. Then each replicate's measurements were averaged. The Bioanalyzer measures concentration and an RNA Integrity Number

(RIN). The concentration values were highly variable with the Bioanalyzer measurements, but the yields of 0V, 50V, 150V, and 300V were not statistically significantly different than that of off-chip. The lack of statistical difference is likely due to the high variability in the off-chip concentration measurements. The off-chip concentration measurements ranged from 871pg/μL (RIN of 4) to 14,001pg/μL (RIN of 2.1), and the median was 1,351pg/μL (Table SI 5.1). With RIN, there was a statistically significant difference between off-chip and 150V, and off-chip and 300V, with the RIN of 150V and 300V being higher than that of off-chip.

Since C_q values depend on a combination of purity and concentration, the combination of the RNA concentration and purity values in Figure 5.6 explain why C_q does not show an increase in purity. The average off-chip concentration is higher than that of any of the on-chip samples, and the average RIN for the on-chip samples are higher than those of the off-chip samples. Thus, this results in similar C_q values for all samples. A study by White et al. [164] showed some correlation between RIN and C_q value, with C_q values being lower with higher RIN. However, it has been shown that amplification of transcripts can happen despite RNA degradation [165].

We tested a reversal of the electrodes, termed the negative configuration, to ensure electrophoretic transport was dominating over electroosmotic flow. If electroosmotic velocity was dominating, we would see a reduction of purity of the RNA samples because the contaminants would flow in the direction of the Elution Well [155], which we did not see (Figure SI 5.1). We also tested disodium EDTA anticoagulated blood in the positive configuration and had similar results for the C_q values comparisons (Figure SI 5.2). For the Bioanalyzer results, however, the RIN values could not be measured reliably, likely due to the concentration of EDTA being higher than the recommended limits for the RNA Pico 6000 Kit. These results are in Table SI 5.2. The electropherogram traces

for disodium EDTA samples were similar to that of the sodium heparin ones, thus the threshold for the RIN measurements could be adjusted to estimate RIN values with less confidence.

Overall, these results show that electrophoretic transport increased purity of the RNA extracted using the microfluidic separator. The higher the voltage the better the purity, or the higher the RIN, of the sample improves. Even in the absence of electrokinetics, 0V appeared to improve purity of the RNA (though not statistically significant) indicating that the microfluidic separator alone, may clean the sample better than the off-chip protocol. This could be due to the differing geometries between an Eppendorf tube and the microfluidic wash channel, where the channel is more spread out allowing for the magnetic beads to experience advection more strongly. On the other hand, all samples tested on the microfluidic separator had a lower concentration than when using a tube off-chip. This demonstrates the importance of treating the microfluidic device with a solution to eliminate RNases (which was not done here), since the concentration of RNA purified on the microfluidic device was lower than that of off-chip. The lower concentration could also be due to adsorption of RNA to the walls of the PDMS or glass of the microfluidic chip [166]. Lastly, since the concentration was not significantly reduced between the voltages tested, this shows that the RNA remained bound to the magnetic beads despite the effects of electrophoresis present in the microfluidic separator.

5.4.10 Mechanistic understanding of flow of unbound species in the microfluidic channel

To validate our experimental results, we mathematically modeled the electric field on the microfluidic separator using COMSOL Multiphysics to observe the effect of electrokinetic velocities. For the Wash Channel, the solution chosen for modeling was ethanol, which is likely a component of the proprietary wash buffer used experimentally.

The solute was chosen to be DNA fragments of 150bp is size with a large negative charge, but the solutes are much more complex than that. Three modules were used in COMSOL: Electric Currents, Laminar Flow, and Transport of Diluted Species, to solve for the electric field, electroosmotic flow, and the convection, diffusion, and electrophoresis of charged species, respectively. For further details regarding the model, see the Supplementary Information. Figure 5.7 shows the concentration of the solute at 3 minutes of the simulation time for 150V and 300V with z_i = -9000, where z_i is the charge number of the ionic species (dimensionless). The electroosmotic velocity, electrophoretic flux, and electric field for 150V and 300V are shown in Figure SI 5.3 and Figure SI 5.4. This shows that electrophoresis is what dominates the velocity of the solutes since the solutions (in red) are moving towards the positive electrode instead of the negative one. If the solution has a positive charge of the same magnitude (z_i = 9000) the same profile is seen but towards the negative electrode (Figure SI 5.5). Electroosmotic velocity is also occurring (Figure SI 5.3), though it is not greater than electrophoretic velocity as evidenced by the concentration profile and increases with increasing voltage.

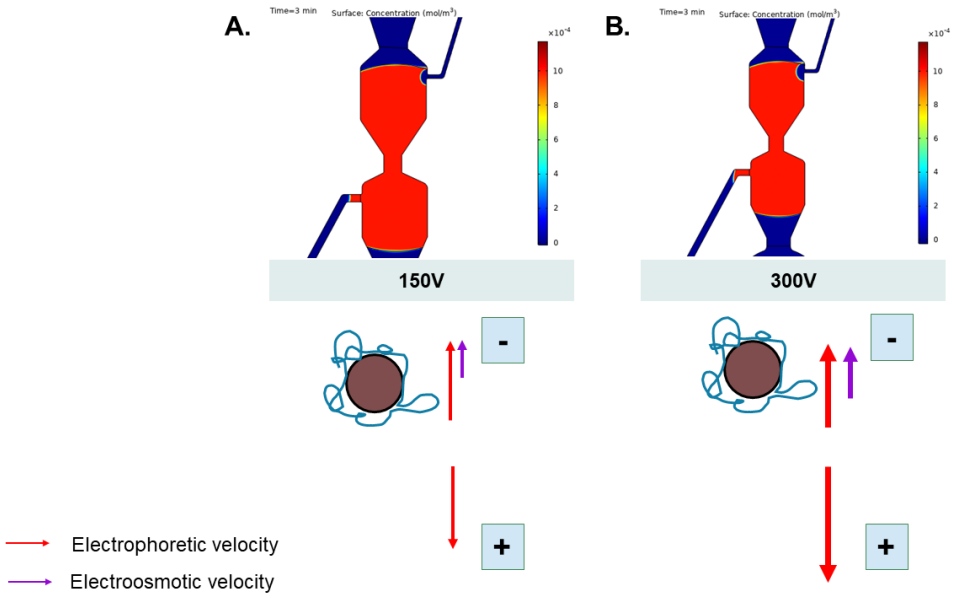


Figure 5.7: Movement of negatively charged solutes' concentration boundary when 150V and 300V are applied for 3 minutes and a depiction of electroosmotic and electrophoretic velocity. *Top: the red color indicates the solute concentration (set as 0.001mol/m^3) and the boundary can be seen moving toward the Secondary Wash Buffer Loading Well due to electrophoresis. Bottom: Electroosmotic velocity and electrophoretic velocity in this microfluidic separator are in opposition for negatively charged molecules but not for positively charged molecules. The magnitudes of the velocities are depicted by the arrow thickness and length, based on the experimental results.* ***(A)*** *150V is shown.* ***(B)*** *300V is shown.*

Modeling in COMSOL aided in understanding the movement of the unbound species in the Wash Channel of the microfluidic separator. This showed that in the positive configuration, negatively charged ions move towards the bottom of the chip due to electrophoresis, while the positive ions travel upwards due to electroosmosis and electrophoresis.

5.5 Conclusions

High-quality total RNA extraction is essential for various downstream applications like transcriptomic or viral load analyses. Our aim was to investigate ways to shorten the total RNA extraction process while yielding high-quality RNA. Compared to the conventional protocol, the microfluidic protocol reduced the starting blood volume from 2mL to 91μL, reduced the number of wash steps from 4 to 1, reduced the total volume of reagents from ~5,500μL to ~400μL, and eliminated the manual mixing steps. This reduction allows for a lower volume of both reagents and blood sample, which is beneficial to both the patient and researchers. A lower volume of reagents lowers the cost and reduces the physical space needed for the storage of these reagents. Additionally, all the reagents are stored at room temperature except for DNase I (which can be lyophilized and rehydrated), expanding access to performing RNA extraction in a variety of environments. The use of a microfluidic design and magnetic beads for RNA extraction allows for automation of this process and for multiple RNA extractions to be performed in parallel. For example, performing the extractions in parallel was possible with this microfluidic design and was

done in the 0V experiments which made the extraction process significantly faster. Compared to other magnetic bead-based microfluidic devices developed for RNA extraction and purification, our method uses minimal equipment and does not require the use of electricity or fluid pumping, which greatly simplifies this process.

COMSOL modeling showed that electrophoresis dominates in the microfluidic Wash Channel, which means that this microfluidic design introduces a novel way to minimize electroosmotic velocity in the absence of additives like coatings. Electrophoretic velocity further purified positively and negatively charged contaminants while maintaining the eluted RNA concentration at higher voltages. Both negatively charged genomic DNA fragments and positively charged salts needed to be washed away to maximize the purity of eluted RNA, and this microfluidic separator was able to wash away both types of charged contaminants, with and without an applied voltage. Thus, we showed that a microfluidic device is essential for the success of lower volume protocols, since the modifications made to the conventional protocol were best performed on the microfluidic chip.

A limitation of this study is that the gene used for qPCR amplification was one that was abundant in white blood cells. Future studies should investigate the purification of a less abundant gene, perhaps with a virus such as human immunodeficiency virus (HIV) RNA in plasma. We did not alter the concentrations of buffers relative to each other, which would be a good study to investigate more ways to increase RNA yield. Future work should incorporate RNA amplification and detection platforms to the microfluidic protocol for the POC to reduce the need for RNA storage. To improve the precision and accuracy of the PDMS's formation, alterations to the SU-8 can be made to align the aluminum mold more precisely with it, improving the accuracy of the intended location of the well formations. The parameters of the microfluidic design as well as surface modifications can be

investigated to further explore the role of electrokinetic forces on the purification of RNA. Overall, this study provided an in-depth insight and understanding of the essential steps of RNA purification protocols. The RNA purification process was greatly simplified which will aid in expanding access to RNA purification to lower resourced laboratories, and eventually to the point of care.

5.6 Acknowledgements

Thank you to Tim Weissbach for his insight in the early stages of this project.

5.7 Data Availability

The raw data that support the findings of this study are available at this link: https://doi.org/10.7910/DVN/DFBFEV

5.8 Supplementary Information

5.8.1 Further detail on statistical comparisons

Figure 5.2: The microfluidic separator and its dimensions. *The microfluidic separator is 20.986mm in length. The length from the Sample Well (3.5mm diameter) to the Elution Well (3.5mm diameter) is 10.957mm, with the Wash Channel being 7.369mm in length. The width of the channel leaving the Sample Well is 0.912mm, the width of the channel between the Sample and Elution Wells is 0.576mm, and the width of the channel entering the Elution Well is 0.912mm. The Primary and Secondary Wash Buffer Loading Wells are 1.5mm in diameter. The Primary Wash Buffer Loading Well is 0.12mm in width and the Secondary Wash Buffer Loading Well is 0.24mm in width.*

Figure 5.3*: For disodium EDTA anticoagulated blood, the mean differences between the average C_q value of the positive control and the average C_q value of "No washes", "No wash 2", and "No mixing" were statistically greater than 0, where p = 0.032, p = 0.024, p = 0.026 respectively. For sodium heparin anticoagulated blood, the mean differences between the average C_q value of the positive control and the average C_q value of "No washes", "No wash 2", and were statistically greater than 0, where p = 0.006 and p = 0.004 respectively. The mean difference between the average C_q value of the positive control and the average C_q value of "No mixing" was not statistically significant.*

Figure 5.5*: For EDTA anti-coagulated blood, the mean difference between the average C_q value of the positive controls and the experimental values was not significant. For Heparin anti-coagulated blood, the mean difference between the average C_q value of the 1/22-scale down and the experimental C_q values was statistically greater than zero, where p = 0.048.*

Figure 5.6B: *To compare the mean C_q value of the trials to that of the off-chip protocol, one-way ANOVA was performed (α = 0.05), with Dunnett's multiple comparisons test (α =*

0.05). The mean difference between the average C_q value of the off-chip and 0V, 50V, 150V, and 300V were not statistically greater than zero.

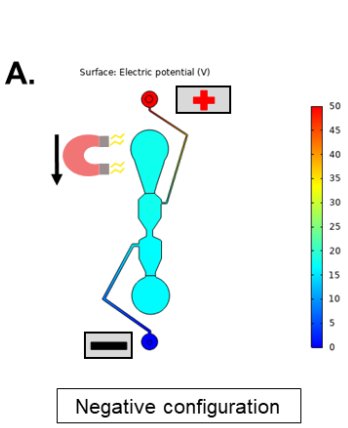
Figure 5.6C*: To compare the mean concentration of off-chip to the mean concentrations of 0V, 50V, 150V, and 300V one-way ANOVA was performed (α = 0.05), with Dunnett's multiple comparisons test (α = 0.05). The mean difference between the average C_q value of the off-chip and 0V, 50V, 150V, and 300V were not statistically greater than zero. To compare the mean RIN of the off-chip to the mean RINs of 0V, 50V, 150V, and 300V one-way ANOVA was performed (α = 0.05), with Dunnett's multiple comparisons test (α = 0.05). The mean difference between the average RIN between off-chip and 150V and 300V were statistically greater than 0, with p = 0.04 and p = 0.003 respectively. One replicate did not yield a value for RIN for both 150V and 300V, therefore the concentration values for those samples were excluded.*

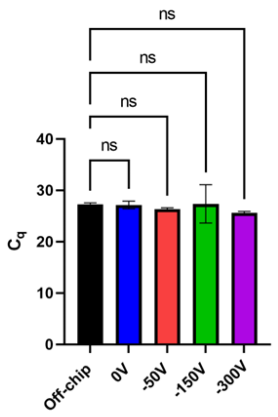
5.8.2 Further experiments with electrophoresis in microfluidic separator

Table SI 5.1: Concentration and RIN Values for voltages tested with sodium Heparin anticoagulated blood.

Sample Name	Concentration (pg/µL) ± standard deviation	RIN ± standard deviation
Off-chip	4,100 ± 5,252	2.8 ± 2.8
0V	1,049 ± 673	4.2 ± 0.6
50V	1,111 ± 294	4.1 ± 0.4
150V	1,506 ± 1,514	4.8 ± 1.3
300V	924 ± 316	5.8 ± 0.1

-50V had a statistically significantly higher average RIN than off-chip (Figure SI 5.1). For each of the voltages tested in the negative configuration, one replicate did not have a RIN. This indicates a slight reduction in repeatability for the negative voltages, though not significant. Lastly, the variability in the RIN values is greater for the negative configuration than the positive configuration.





C.

Sample Name	Concentration (pg/µL)	RIN
Off-chip	4,100 ± 5,252	2.8 ± 0.6
0V	1,049 ± 673	4.2 ± 0.7
-50V	632 ± 196	4.2 ± 0.2 (**)
-150V	1,770 ± 1,788	3.7 ± 2.3
-300V	1,025 ± 406	5.0 ± 1.3

Figure SI 5.1: The negative configuration; C_q values and RIN measurements for the negative configuration (Sodium Heparin anticoagulated blood). ***(A)*** *The negative configuration of the electric potential, shown with -50V.* **(*B*)** *To compare the mean C_q value of the trials to that of the off-chip protocol, one-way ANOVA was performed (α = 0.05), with Dunnett's multiple comparisons test (α = 0.05). The mean difference between the average C_q value of the off-chip and 0V, -50V, -150V, and -300V were not statistically greater than zero.* **(*C*)** *To compare the mean concentration of off-chip to the mean concentrations of 0V, -50V, -150V, and -300V one-way ANOVA was performed, with Dunnett's multiple comparisons test (α = 0.05). One replicate did not yield a value for RIN for -50V, -150V, and -300V, therefore the concentration values for those samples were excluded. To compare the mean concentration of off-chip to the mean concentrations of 0V, -50V, -150V, and -300V one-way ANOVA was performed (α = 0.05), with Dunnett's multiple comparisons test (α = 0.05). The mean difference between the average C_q value of the off-chip and 0V, -50V, -150V, and -300V were not statistically greater than zero. To compare the mean RIN of the off-chip to the mean RINs of 0V, -50V, -150V, and -300V one-way ANOVA was performed (α = 0.05), with Dunnett's multiple comparisons test (α = 0.05). The mean difference between the average RIN between off-chip and -50V was statistically greater than 0, with p = 0.002.*

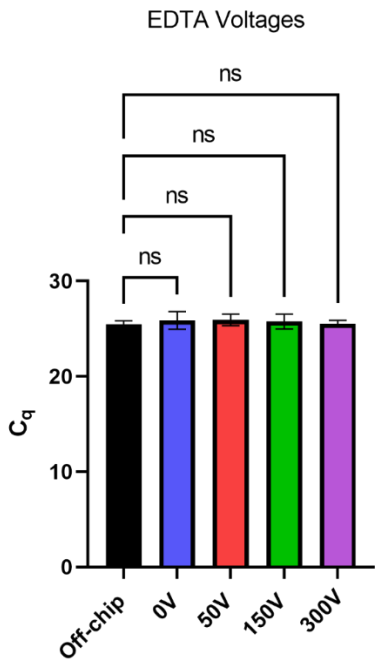


Figure SI 5.2: C_q values for EDTA voltages tested in the "positive configuration". *To compare the mean C_q value of the trials to that of the off-chip protocol, one-way ANOVA was performed (α = 0.05), with Dunnett's multiple comparisons test (α = 0.05). The mean difference between the average C_q value of the off-chip and 0V, 50V, 150V, and 300V were not statistically greater than zero.*

Table SI 5.2: Concentration and RIN Values for voltages tested with sodium EDTA anticoagulated blood. *For the unadjusted RIN values, only those with a RIN value were included. For off-chip this was 2 of 6 samples, for 0V - 2 of 4 samples, for 50V - 3 of 4 samples, for 150V – 1 of 4 samples, and for 300V – 0 of 4 samples. To compare the mean concentration values and the RIN values of the trials to that of the off-chip protocol, one-way ANOVA was performed (α = 0.05), with Dunnett's multiple comparisons test (α = 0.05). The mean difference between the average concentration values and RIN values of the off-chip and 0V, 50V, 150V, and 300V were not statistically greater than zero.*

Sample Name	Concentration (pg/μL) ± standard deviation	RIN ± standard deviation
Off-chip	1,346 ± 1,157	4.3 ± 2.1
0V	7,983 ± 0.42	2.7 ± 0.4
50V	1,255 ± 0.2	2.7 ± 0.4
150V	907	N/A

300V	N/A	N/A

5.8.3 Further details on the COMSOL model inputs and model results

For the Electric Currents module, the model inputs where the electrical conductivity (σ = 0.554μS/cm) [167] and relative permittivity (ε_r = 24.3) of ethanol [168]. The out-of-plane thickness was set to 150μm, which is the depth of the microchannels. The channel walls were electrically insulated. The electrical potentials were applied to the top and bottom wells and adjusted to match experimental values.

The electroosmotic velocity (v_{eof}) is defined as the electroosmotic mobility (μ_{eof}) multiped by the magnetic of the applied electric field (E):

$$v_{eof} = \mu_{eof}\, E \qquad \textit{(Equation SI 5.1)}$$

$$where\ \mu_{eof} = \frac{\varepsilon\, \zeta}{4\pi\eta} \qquad \textit{(Equation SI 5.2)}$$

ζ is the zeta potential of the capillary wall, η is the buffer's viscosity, and ε is the buffer's dielectric constant [162].

In the Laminar Flow module, the density and dynamic viscosity were built-in the COMSOL software from ethanol set as the material used for the microchannel. The boundary condition was electroosmotic velocity, which required the input of ζ_{glass} = -66.2mV [169] and ethanol's relative permittivity. The wall was assumed to be a sliding wall with the velocity of the moving wall being equal to the electroosmotic velocity along the PDMS wall the microchannel, using $\varepsilon = \varepsilon_r * \varepsilon_0$ as the permitivity using the zeta potential of the PDMS, ζ_{PDMS} = -67.6mV [170] and the viscosity of ethanol η = 1.095cP [171]. The top of the sample well was set to have an inlet velocity of 0m/s and the bottom of the elution well was set to have an outlet pressure of 0Pa.

For the electrophoretic mobility, the equations used are derived from Fick's law in the Transport of Diluted Species module, which include convection and migration in an electric field:

$$\frac{\partial c_i}{\partial t} + \nabla \cdot \boldsymbol{J_i} + \boldsymbol{u} \cdot \nabla c_i = R_i \qquad \textit{(Equation SI 5.3)}$$

$$where\ \boldsymbol{J_i} = -D_i \nabla c_i - z_i u_{m,i} F c_i \nabla V \qquad \textit{(Equation SI 5.4)}$$

where $\mathbf{J_i}$ is the diffusive flux vector, c_i is the concentration of species i (mol/m^3), D_i is the diffusion coefficient of species i (m^2/s), **u** is the fluid velocity (m/s), F is Faraday's constant (A*s/mol), V is the electric potential (V), z_i is the charge number of the ionic species (dimensionless), and $u_{m,i}$ is its ionic mobility (mol*s/kg) [172].

The concentration (c_i) was set to 0.001mol/m^3 in the Wash Channel. The diffusion coefficient (D_i) was calculated using the equation by Lukacs et al. [173]: $D_i = 4.9 \times 10^{-6} \times$ [bp size]$^{-0.72}$ for 150bp fragments, assuming the fragments are very small due to DNase I digestion [174]. Convection was applied via **u,** which was found from the Laminar Flow module. The electric potential (V) was found from the Electric Currents module. The charge number (z_i) was set to -9000. The ionic mobility was found from the Nernst-Einstein relation:

$$u_{m,i} = \frac{D_i}{RT} \qquad \textit{(Equation SI 5.5)}$$

where R is the molar gas constant (J/mol*K) and T is the temperature (K).

The temperature was set to room temperature, 293.15K. The simulation was run in two steps, first getting the Electric Currents and Laminar Flow modules to steady-state and then running the Transport of Diluted Species module for 5 minutes with sampling done every one minute, to represent the maximum amount of time the beads are in the microchannel under these conditions.

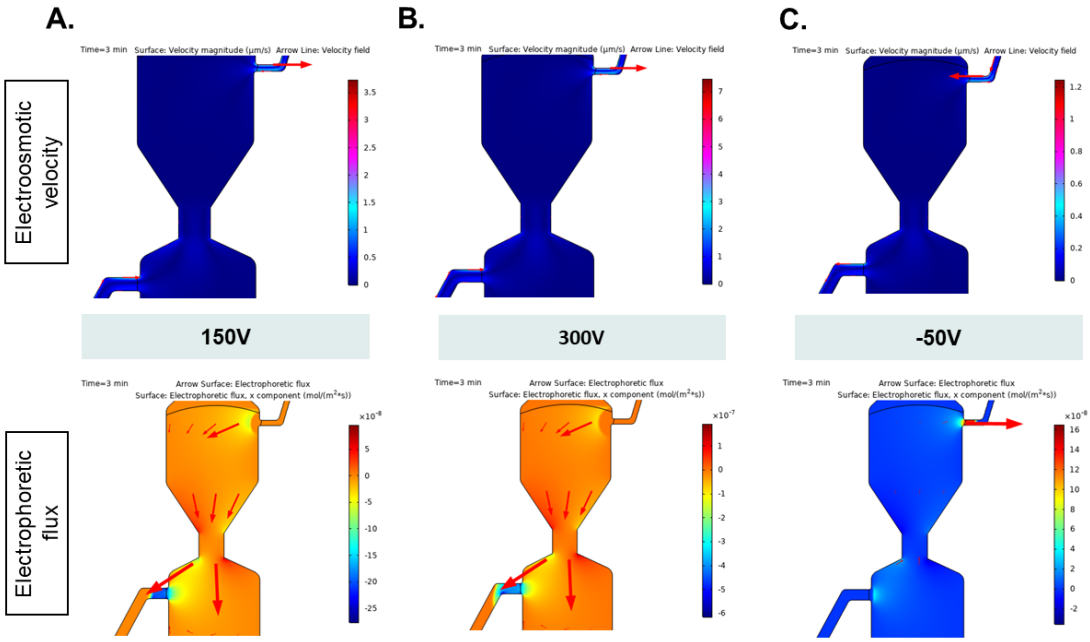


Figure SI 5.3: Electroosmotic velocity and electrophoretic flux for 150V, 300V, and -50V.

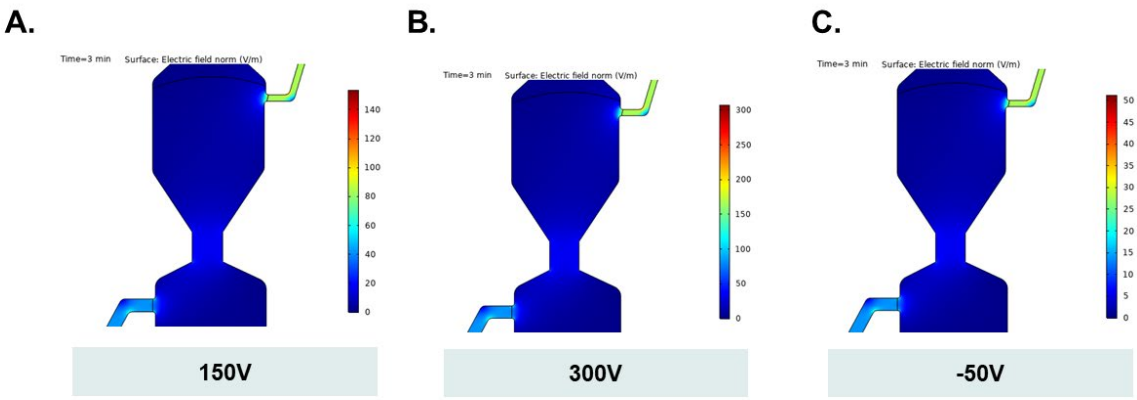


Figure SI 5.4: Electric field for 150V, 300V, and -50V.

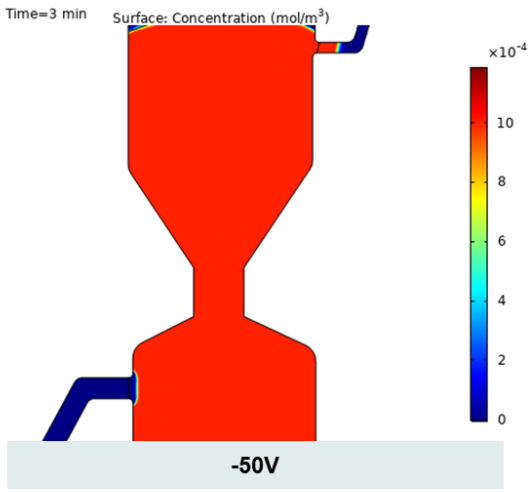


Figure SI 5.5: Concentration boundary showing electrophoresis when -50V are applied.

For -50V it appears that the concentration of the RNA was lower, and since the beads spend more time in the top half of the Wash Channel, the RNA was removed from the beads due to electrophoresis. This conclusion is further strengthened by looking at -300V, where the electroosmotic velocity is stronger than with -50V which likely restoring the loss of the RNA while maintaining the purity. Interestingly for 150V and -150V, the standard deviation is the highest amongst their respective configurations. This implies that there are forces that are opposing each other, causing competition among which force will dominate.

CHAPTER 6: INSIGHT INTO INCREASED RECOVERY AND SIMPLIFICATION OF GENOMIC DNA EXTRACTION METHODS FROM DRIED BLOOD SPOTS

Chapter 6 is an original research article that is accepted for publication at Biopreservation and Biobanking.

Lee, K. and Tripathi, A., "Insight into increased recovery and simplification of genomic DNA extraction methods from dried blood spots" *Biopreservation and Biobanking (accepted)*

6.1 Abstract

There is no consensus on how to perform the manual extraction of nucleic acids from dried blood spots (DBSs). Current methods typically involve agitation of the DBSs in a solution for varying amounts of time with or without heat, then purification of the eluted nucleic acids with a purification protocol. We explored several characteristics of genomic DNA (gDNA) DBS extraction such as extraction efficiency, the role of red blood cells in extraction and critical kinetic factors to understand if these protocols can be simplified while maintaining sufficient gDNA recovery. We found that agitation in a red blood cell lysis buffer before performing a DBS gDNA extraction protocol increases yield 1.5 to 5-fold. The use of an alkaline lysing agent along with heat and agitation was sufficient to elute PCR amplifiable gDNA in 5 minutes. This work adds insight into the extraction of gDNA from DBSs with the intention of informing a simple, standardized manual protocol for extraction.

6.2 Introduction

The use of dried blood spots (DBSs) began as a method for more than 50 years for neonatal metabolic disease screening. Their use has expanded beyond infant disease screening to a method of sample collection for pharmacologic testing, genetic analyses, and monitoring of viral infections [175]. In more remote areas and for self-sampling, DBSs are particularly beneficial compared to venipuncture because they do not require specialized skills for sample collection and their transport and storage is less reliant on the cold chain process [176]. DBSs use a few drops of capillary blood from a finger prick that are placed on a cellulose-based, light-weight paper card and dried for four to twenty-four hours. Viral RNA and DNA, proteins, antibodies, and metabolites are stable on these cards when they are placed in a bag with a desiccant and maintain stability for longer at ambient temperatures when compared to wet blood [177]. Paper is porous, which is why it is a

good storage method for molecules [178], and is easily affected by humidity or liquids [179], which is why DBSs must be stored with a desiccant [180]. Nucleic acid extraction from DBSs is important for analyses such as viral load or other nucleic acid-based diagnostics [175], [181], [182].

DBS nucleic acid extraction can be performed manually and there is a lack of consensus on the best method for manual nucleic acid extraction from DBSs [182]. DBS has been automated by the development of various benchtop devices [36], [181], [183]–[185]. While the automation of DBS extraction is significant and leads to the standardization of DBS nucleic acid extraction, not all laboratories have access to such devices [184], [186]. Generally, manual DBS nucleic acid extraction protocols have similar processes, which are to use buffers like phosphate-buffered saline (PBS), strong bases, strong acids, or salt solutions to re-hydrate and/or lyse the blood contents on the DBS. For gDNA, a method involving phenol had been used for a number of years[187], which then required a large concentration of Proteinase K to be used, which increased cost of extraction. Extraction methods then shifted to using either just a nucleic acid extraction and purification kit with or without agitation in an extraction solution, often made in-house, beforehand [188]–[192]. Many protocols have been developed and are used, but the literature in general lacks explanations for why the specified reagents or chosen kinetic factors (agitation, heat, etc.) were chosen.

In addition to the lack of consensus on method of nucleic acid elution, critical information regarding DBS elution barriers and facilitators are not well studied. A few studies have examined inhibitory components to elution of any molecule with DBSs which include the heparin anticoagulant [24], [193], [194]. Others have seen that red blood cells lyse upon drying, so using a red blood cell lysis buffer did not improve their yield [193]. In addition, it is also not clear if using multiple or larger punches will increase yield.

Researchers use varying quantities of 1 – 6 mm punches. For nucleic acids though, to our knowledge, there are no studies that have explicitly examined important constraints of DBSs such as the percent elution yield of nucleic acids or the impact of the aforementioned factors on yield. Thus, in this manuscript we sought to explicitly examine barriers and facilitators to extraction of nucleic acids, specifically genomic DNA (gDNA) from DBSs. gDNA elution from DBSs is essential for viral diagnoses and whole genome sequencing from this storage matrix [195]–[197]. We also sought to understand which kinetic factors were essential for gDNA DBS extraction. This work provides insight into DBS elution of nucleic acids to aid researchers in optimizing a simpler, standardized manual elution protocol where access to automated devices is limited.

6.3 Materials and Methods

6.3.1 Human whole blood

Human male whole blood was purchased from Golden West Biosolutions (Temecula, California, USA) or Research Blood Components, LLC (Watertown, Massachusetts, USA). The blood samples contained either sodium heparin or disodium EDTA anticoagulants. We refer to sodium heparin anticoagulated blood as “heparin” and disodium EDTA anticoagulated blood as “EDTA”. The samples were tested for common infectious diseases and were stored at 4 ^{o}C.

6.3.2 Preparation of Dried Blood Spots

Dried blood spots were prepared by pipetting 75 μL of whole blood onto each circle onto Whatman™ 903 protein saver cards (Millipore Sigma, St. Louis, Missouri, USA). Then, they were left to dry overnight at room temperature. After drying, each card was placed into a bag with a desiccant and stored at -20 ^{o}C. To reduce the impact of storage time on the results, the DBSs used in this manuscript were not stored for longer than 1 month before use.

6.3.3 Reagents, Instruments, and Protocols for DBS gDNA extraction (Protocols 1-6)

For nucleic acid extraction from DBSs, protocols typically require punched-out spots or the entire circle to be shaken for various amounts of time in a solution. Heat or other kinetic factors may also be applied to increase the extraction yield. Following the shaking, a nucleic acid extraction protocol is typically performed to purify the eluted gDNA, which are necessary not only to increase gDNA yield, but as we briefly investigated, to also isolate the gDNA in a solution that is PCR-compatible (Table SI 6.1).

Table 6.1: Six protocols for gDNA extraction from dried blood spots. *A summary of the reagents, shake times, and shake temperatures for six protocols from across the literature for DNA extraction from dried blood spots.*

Protocol number and source	***Solution***	***Agitation Time***	***Agitation temperature***
1 [198], [199]	1mL 1X PBS/0.05% Tween-20/0.08% sodium azide	Overnight	RT
2 [200]	1 mL 1X PBS	30 min	RT
3 [201]	150 µL 1X PBS/600 µL Lysis Buffer	2 h	RT
4 [202], [203]	2 mL Lysis Buffer	1 h	RT

5 [204]	2 mL Lysis Buffer	30 min	37 °C
6 [205]	130 µL KOH/Trizma®	40 min	25-70 °C

For Protocols 1-5 used in this manuscript (Table 6.1), they each followed a similar procedure in which a dried spot punch was placed into a solution and agitated on a plate shaker for a given time, which are specified in Table 6.1. Protocol 1 required Tween® 20 and sodium azide which were obtained from Millipore Sigma (St. Louis, Missouri, USA). Protocols 1, 2, and 3 [198]–[201] required 1X Gibco™ Phosphate buffered saline (PBS), pH 7.4 was obtained from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA). Protocols 3, 4, and 5 [201]–[204] required a Lysis Buffer and the NucliSENS® Lysis Buffer (Biomérieux, Marcy-lÉtoile, France) was used. After shaking, all these protocols required DNA purification, which was done using the NucliSENS® Magnetic Extraction Reagents (Biomérieux, Marcy-lÉtoile, France). The elution volume of the gDNA was 80 µL for all samples. As originally described, Protocol 1 was developed to obtain DBS eluates for the analysis antigens and antibodies of infectious diseases [198], [199] and thus did not include a nucleic acid purification step, but we used one here. When testing Protocols 1-5, either 2 x 6mm punches or the entire circle were used for each biological replicate (Figure 6.1). For example, for 2 x 6 mm punches, the two punches would be placed into a singular tube, which was one biological replicate.

Protocol 6 was developed by PerkinElmer (Waltham, Massachusetts, USA) for congenital cytomegalovirus screening for newborns. The KOH/Trizma® buffer used in Protocol 6 utilizes KOH for alkaline lysis [206], [207] of the blood cells for DNA release as the KOH/Trizma® solution is very basic with a pH of ~12, and Trizma® base for DNA

stabilization. The protocol uses a 3 mm DBS punch that is placed into 80 µL of the KOH/Trizma® solution on a 96-well plate. The plate is sealed then shaken for 3 minutes at 1000rpm at 25°C. The buffer is discarded then the plate is incubated in a plate shaker, here the Trinest™ incubator shaker, for 10 min while the shaker heats up to 70 °C. Then, 50 µL of the KOH/Trizma® solution is added to the well for elution and the plate is shaken for 20 minutes at 70 °C at 1000 rpm. Here, we used one 6 mm DBS punch or two per well for each biological replicate and adjusted the volumes accordingly. Additionally, the incubation time was increased to 15 minutes because that is how long it took the Trinest™ incubator shaker to heat up to 70 °C. Since Protocol 6 used a 96-well plate a whole circle was unable to fit into a well, therefore, either 1 x 6mm punches or 2 x 6mm punches were used (Figure 6.1).

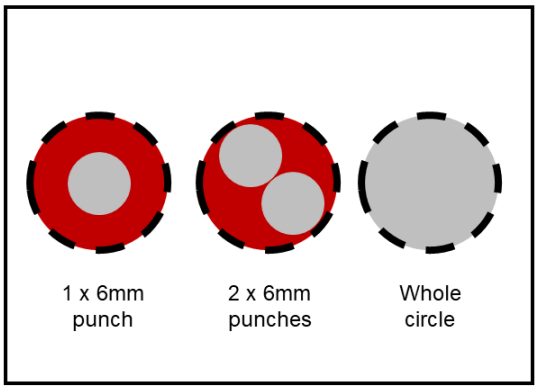


Figure 6.1: Depiction of the types of DBS punches used as inputs for the protocols tested. *In grey is a depiction of 1 x 6mm punches, 2 x 6mm punches, or the whole circle taken from the dried blood spots to be inputs into the protocols tested. Protocols 1-5 used 2 x 6mm punches or the whole circle; Protocol 6 used 1 x 6mm punches or 2 x 6mm punches.*

6.3.4 Development of mixtures of parts of whole blood for assessment of DNA DBS extraction yield

We made mixtures of white blood cells (WBCs) by themselves, WBCs mixed with plasma, or WBCs mixed with red blood cells (RBCs) and spotted them onto the DBS paper. The detailed protocols of how the solutions were made are included in the Supplementary Information. We pipetted 75 µL of each mixture onto each circle of DBS paper. The percent yield of gDNA eluted from 1 x 6 mm punch of each mixture was calculated by standardizing the yields of each sample to that of the sample that yielded the largest concentration for "whole blood" (Equation 6.1); we assumed that this sample would be our theoretical maximum yield. 5 punches were tested for each condition, all samples were from the same donor.

$$\frac{measured\ concentration\ of\ each\ sample}{sample\ with\ highest\ concentration\ for\ "whole\ blood"} x\ 100\% \qquad \text{(Equation 6.1)}$$

6.3.5 Red blood cell lysis buffer

To isolate the white blood cells for the experiments described in Section 3.4, and to test the impact of red blood cell lysis on gDNA extraction yield, Millipore Sigma's Red Blood Cell Lysis Buffer (RLB) (Burlington, Massachusetts, USA) Protocol was used.

6.3.6 Real-time (or quantitative) PCR (qPCR)

Real-time PCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA). TaqMan™ Gene Expression Assay ID# Hs00243216_s1, a primer for human sex-determining region Y (SRY) gene, was used (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The Taqman™ Gene Expression Master Mix (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) was used for the assay. 3 technical replicates were averaged for

each for biological replicate analyzed.1:5 or 1:10 dilutions of purified human genomic DNA were used as a standard for all qPCR reaction plates.

6.3.7 Determination of DNA concentration

Spectrophotometry was performed using the NanoDrop™ 1000 based on guidelines from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA). Spectrofluorometry was performed based on guidelines from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA) using the Quant-iT™ PicoGreen™ dsDNA Assay Kit. Signal measurements of all samples were performed using the EnVision 2105 Multimode Plate Reader (PerkinElmer, Waltham, Massachusetts, USA). Subsequent linear regression analysis was performed in Microsoft Excel (Microsoft, Redmond, Washington, USA). For all experiments, the standard curve used for linear regression had $R^2 > 0.99$. 3 technical replicates were averaged for each for biological replicate analyzed.

6.3.8 Statistical analyses

Statistical analyses were performed in GraphPad Prism 8 (San Diego, California, USA) with alpha = 0.05. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$. Further information regarding statistical comparisons can be found in the Supplementary Information.

6.4 Results

6.4.1 Investigation of five DBS elution protocols from across the literature

There are several dried blood spot extraction protocols in the literature for various molecules, and vary based on time for elution, temperatures used, if agitation is required, buffers used, and more. We selected several DBS nucleic acid extraction protocols to compare to each other (Table 6.1) for gDNA extraction, primarily based on simplicity of buffers for extraction. We also investigated the need for chemical lysis by comparing protocols that used a lysis buffer and those that did not. The cycle threshold (C_q) values

measured for all the protocols' eluates are shown in Figure 6.2. We chose to measure C_q values to compare the ability of the samples to amplify during PCR, which has implications in the sample quality, starting gDNA yield, and the sample's potential of success for sequencing [208]. There was no significant difference in the C_q values between EDTA and heparin DBSs for Protocols 1-5, so their results were combined (Figure 6.2).

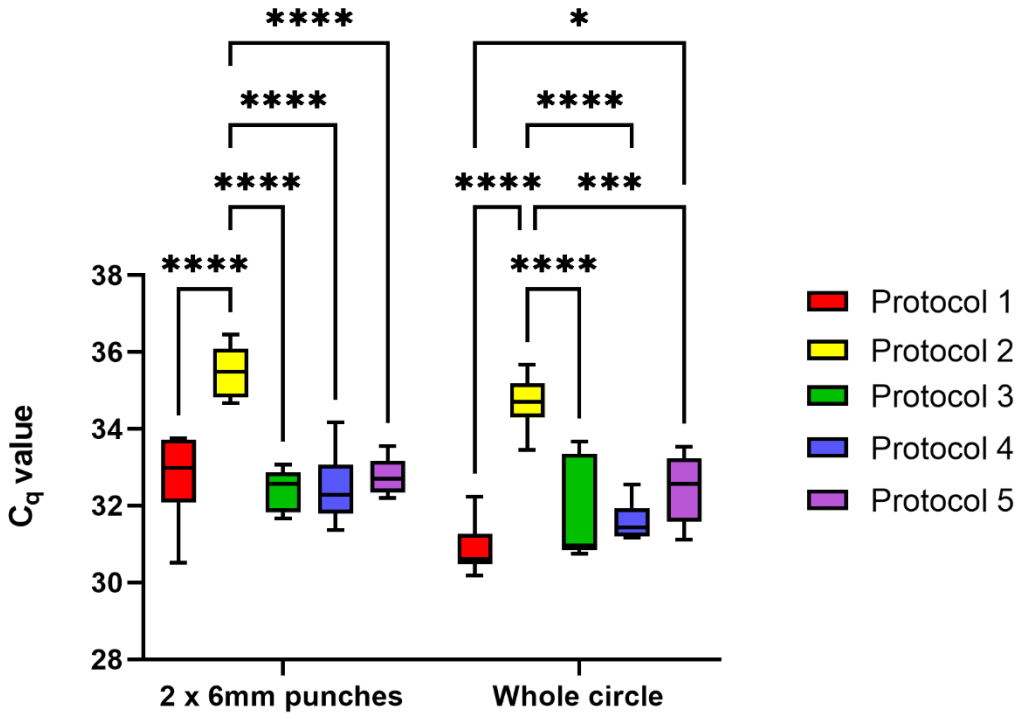


Figure 6.2: Comparison of C_q values for Protocols 1-5. *6 replicates were tested for each protocol and DBS sample input type. All samples were from the same donor. Comparison of C_q values for Protocols 1-5 for 2 x 6 mm punches and whole circles. CV (%) for 2 x 6 mm punches: Protocol 1: 1.5%; Protocol 2: 1.9% Protocol 3: 1.7%; Protocol 4: 2.9%; Protocol 5: 2.2%. CV (%) for whole circle: Protocol 1: 2.3%; Protocol 2: 2.1%; Protocol 3: 4.2%; Protocol 4: 1.6%; Protocol 5: 2.9%.*

After comparing Protocols 1-5, we also wanted to investigate a protocol that firstly, did not require nucleic acid purification steps, which reduces the number of reagents one requires for DBS gDNA extraction and secondly, required multiple kinetic factors which

we could investigate. Thus, we next explored the use of Protocol 6 [205], which does not require nucleic acid purification steps. There was no significant difference in the average C_q values between the anticoagulants nor the number of punches for Protocol 6 (Figure 6.3). Protocol 6 had a comparable C_q value to the other protocols (for 2 x 6 mm punches). For future experiments, we sought to use a buffer in which gDNA would be stable to store, a protocol with minimal hands-on time and a method that was high throughput. Thus, for subsequent experiments, we used Protocol 6 to better understand DBS gDNA yield, explore methods to improve yield, and to investigate the kinetic factors of DBS DNA extraction.

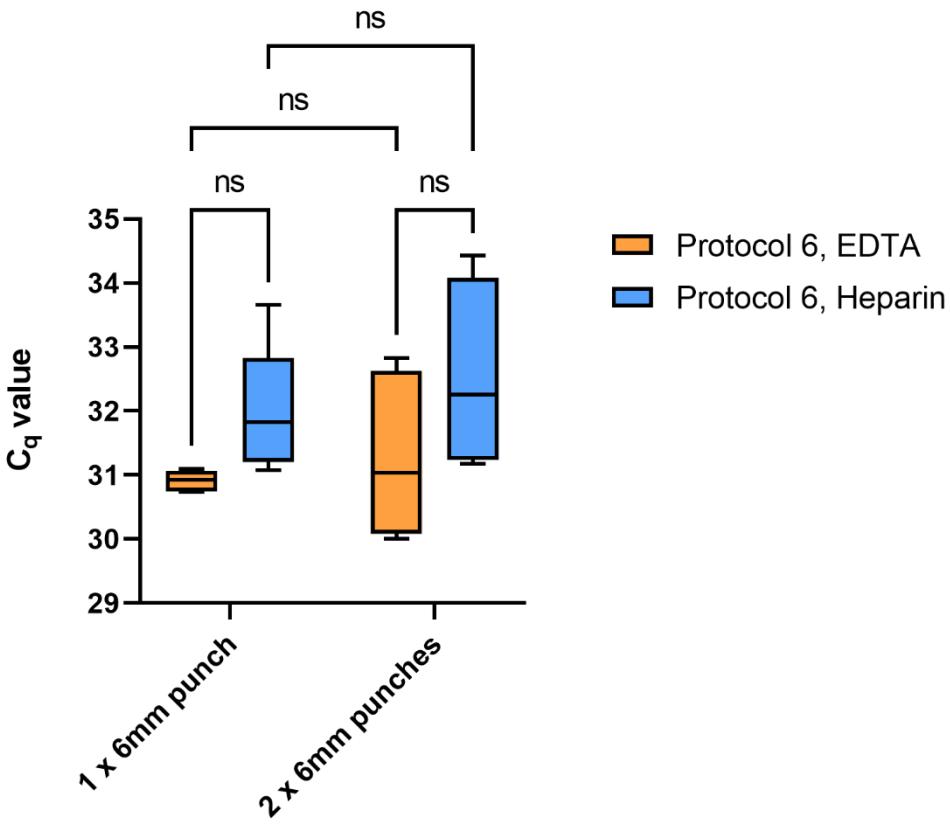


Figure 6.3: Comparison of 1 x 6 mm punch and 2 x 6 mm punches for Protocol 6. *6 replicates were tested for each protocol and DBS type. All samples were from the same donor. Comparison of C_q values for Protocol 6 for 1 x 6 mm punch and 2 x 6mm punches. CV (%) for 1 x 6 mm punches: EDTA 0.5%; Heparin 3.0%. CV (%) for 2 x 6 mm punches: EDTA: 3.8%; Heparin: 4.2%.*

6.4.2 Exploration of individual blood components' impact on gDNA extraction yield from DBSs

To explore the impact of individual blood components on gDNA extraction from DBSs, we created dried spots with mixtures of whole blood, white blood cells (WBCs) only, WBCs and plasma, and WBCs and red blood cells (RBCs). Figure 6.4A shows the gDNA yield of the mixtures of EDTA anticoagulated blood; whole blood, WBCs only, WBCs and plasma, and WBCs and RBCs had normalized yields of 94.1%, 26.3%, 42.4%, and 68.28%, respectively. For heparin anticoagulated blood (Figure 6.4B), the normalized percent yields for whole blood, WBCs only, WBCs and plasma, and WBCs and RBCs were 92.98%, 67.5%, 83.9%, and 49.07%, respectively.

We then tested the impact of the use of red blood cell lysis buffer on gDNA extraction yield from the WBCs + RBCs dried spots (Figure SI 6.1). The protocol for this experiment is described in the Supplementary Information. Briefly we agitated the punches in the red blood cell lysis buffer before performing Protocol 6. As seen in Figure 6.4C, there was an increase in the gDNA yield for both anticoagulants for both whole blood and WBCs + RBCs after RBC lysis, but this increase was more significant for the heparin anticoagulated blood.

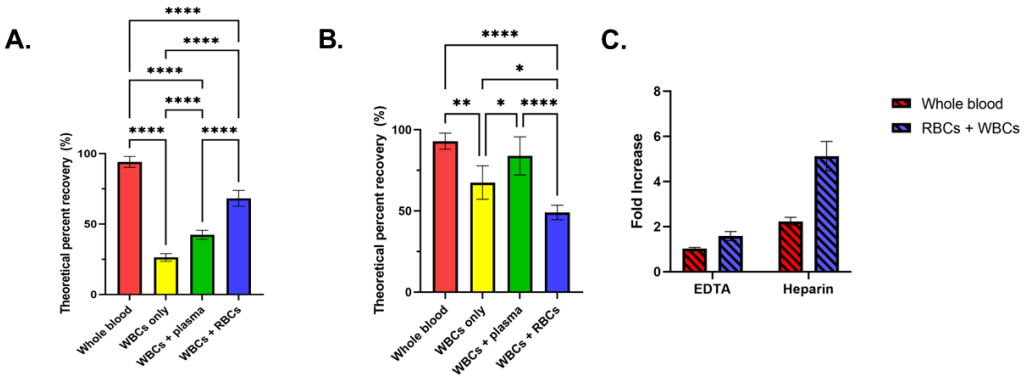


Figure 6.4: gDNA percent recovery from various mixtures of white blood cells, plasma, and red blood cells; fold increase of gDNA yield with an additional RBC lysis step to DBS elution. *5 replicates were tested for all conditions. All samples were from the same donor.* ***(A-B)*** *Comparison of percent gDNA recovery from DBSs for disodium EDTA (A) or sodium heparin (B) anticoagulated blood using whole blood, white blood cells (WBCs) only, WBCs + plasma, and WBCs + red blood cells (RBCs) with fresh 1X PBS. CV (%) for EDTA: whole blood: 4.1%; WBCs only: 10.3%; plasma + WBCs: 7.5%; RBCs + WBCs: 8.2%. CV (%) for Heparin: whole blood, 5.3%; WBCs only, 15.3%; plasma + WBCs: 13.9%; RBCs + WBCs: 9.1%.* ***(C)*** *Using whole blood and WBCs + RBCs DBSs from A-B, an RBC lysis step was done before performing Protocol 6. CV (%) for EDTA fold increase: whole blood: 5.4% and RBCs + WBCs: 12.8%. CV (%) heparin fold increase: whole blood: 8.0%; RBCs + WBCs: 12.8%.*

6.4.3 Understanding essential kinetic factors of gDNA extraction from DBSs using Protocol 6

After investigating DBS gDNA release, we sought to examine which steps of Protocol 6 were essential for gDNA extraction by altering one parameter of this protocol at a time (Figure 6.5). Knowing this would help in understanding what kinetic factors were most important for future work to establish a more simplified protocol for DBS gDNA extraction. For clarification, we did not incorporate the RBC lysis step into the protocol, these experiments were based on the manufacturer's instructions for Protocol 6. We chose to test two buffers, KOH/Trizma® and 1X PBS, to further investigate the importance of chemical lysis in DBS extraction. We first compared the use of 1X PBS instead of the KOH/Trizma® buffer to perform Protocol 6 (Figure 6.5A). The results showed that when

using KOH/Trizma® the gDNA extracted a significantly lower C_q value. We also found a similar result when keeping the anticoagulant constant for both buffers (Figure SI 6.2).

Next, using the two buffers, a reduction of the standard 20-minute 1000rpm shake at 70 ^oC for DNA elution was investigated by reducing the shaking time to 5, 10, or 15 min (Figure 6.5B). For the KOH/Trizma®-extracted gDNA there was no statistically significant difference between the C_q values for any of the varying shake times. When using 1X PBS, the C_q value decreased with increasing shake time. We then performed the elution step at 25 ^oC instead of 70 ^oC (Figure 6.5C). The change in temperature had a large effect on the PBS-eluted samples, but no significant effect on the KOH/Trizma® samples. Next, we tested if the two shaking steps, one to wash the DBS and one to elute the gDNA, at 1000rpm were necessary by not shaking the DBSs throughout the protocol (Figure 6.5D). The average C_q value for gDNA from 1X PBS-eluted samples without shaking was slightly higher than PBS with shaking ($p = 0.04$) but was significantly higher than the Protocol 6 control with KOH/Trizma®. Lastly, we looked at skipping the discard step of the supernatant after the wash step, which is normally discarded after 3 minutes of shaking at 1000rpm at room temperature. There was no significant difference between the two buffers' C_q values but compared to the Protocol 6 control, PBS did have a higher C_q value (Figure 6.5E).

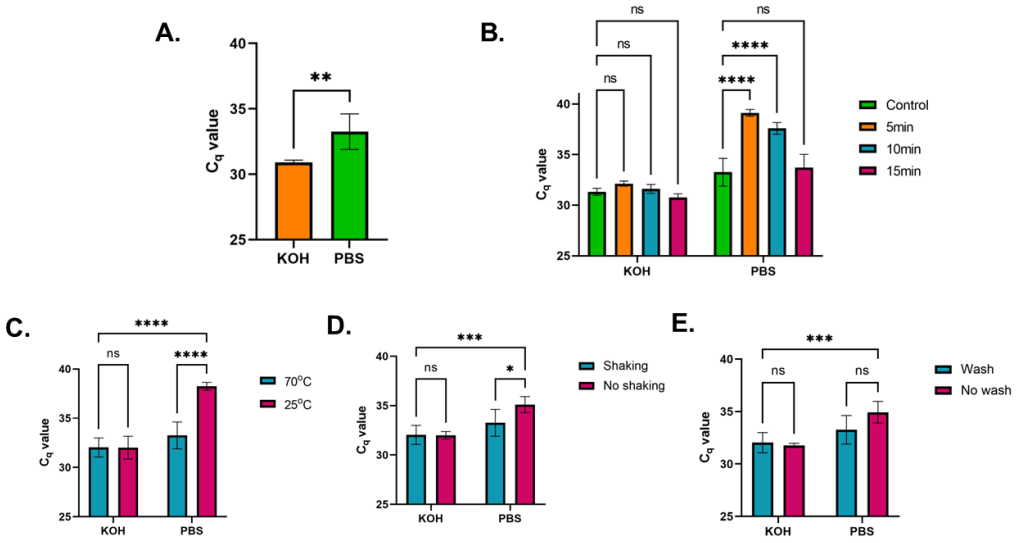


Figure 6.5: Investigation of alterations to Protocol 6 using KOH/Trizma® and 1X PBS. *5 replicates were tested for each condition. For KOH/Trizma®, EDTA anticoagulated DBS samples were used and for 1X PBS heparin anticoagulated DBS samples were used.* ***(A)*** *Comparison of the two buffers. To compare the mean C_q values of the buffers used to each other, an unpaired t-test was performed. CV (%): KOH/Trizma®: 0.5%; PBS: 4.1%.* ***(B)*** *Reduced shake time. CV (%) for KOH/Trizma®: 5 minutes: 0.8%; 10 minutes: 1.4%; 15 minutes: 1.2%. CV (%) for PBS: 5 minutes: 0.9%; 10 minutes: 1.5%; 15 minutes: 3.9%.* ***(C)*** *Performance of the elution step at 70 °C versus 25 °C. CV (%) for 25°C: KOH/Trizma®: 3.6%; PBS: 1.1%.* ***(D)*** *Performance or no performance of the two 1000rpm shaking steps. CV (%) for no shake steps: KOH/Trizma®: 1.2%; PBS: 2.3%.* ***(E)*** *Performance or no performance of the wash step. CV (%) for no wash step: KOH/Trizma®: 0.6%; PBS: 2.9% All samples from A and C-E were from the same donor; samples in B were from a different donor.*

Since the changes made to Protocol 6 when using KOH/Trizma®, as tested in Figure 6.5, seemed to not make a significant difference in gDNA yield, we decided to test not including heat, shaking, and the wash together. This essentially means that the samples were incubated at room temperature for ~30 minutes in the buffer. Here we saw a significant change in the C_q value for KOH/Trizma®-eluted gDNA, with the C_q value being higher than the control for both buffers (Table 6.2).

Table 6.2: C_q values of 30-minute incubation of DBSs in KOH/Trizma® and PBS at room temperature. *5 replicates were tested for each buffer.*

Buffer	C_q value, 30min at room temperature, no shaking	C_q value, control
KOH/Trizma®	38.8	31.33
1X PBS	N/A	33.26

6.5 Discussion

6.5.1 The buffer type, but not the type of sample punch input significantly affects gDNA extraction yield from DBSs

This study explored various parameters to inform the development of a standardized protocol for DNA extraction from DBSs. We first tested a variety of buffers, agitation times, and agitation temperatures with a variety of DBS punch sizes. We tested several punch sizes as researchers will vary punch sizes depending on physical tube constraints or to maximize the yield of the sample [201], [209]. There was no significant difference between C_q values of eluates from different sample inputs, 1 x 6 mm punches, 2 x 6 mm punches, or the whole circle, for any of the protocols except for Protocol 1. For Protocol 1 the whole circle had a lower C_q value than that of the 2 x 6mm punches, implying that the yield was higher for the whole circle. As discussed by Carpentieri et al., the use of a whole circle would reduce the effects of diffusion on the extraction from DBSs [180], but as we showed here smaller punches appear to be acceptable as well. Compared to all the other protocols Protocol 2, which used 1X PBS for agitation, resulted in eluates with C_q values, on average, that were significantly higher for both types of sample inputs. This is likely because there is no lysing agent to lyse the cells for gDNA release, preservative, or surfactant to help the gDNA release, compared to the other protocols. Panda et al. compared various DNA isolation protocols from DBSs for malaria determination and found similar results, that the protocols they studied had similar specificities [186].

To summarize, the gDNA eluted from the multiple protocols did not significantly differ from each in terms of C_q value other except when using Protocol 2. Additionally, the amount of sample input (i.e. 2 x 6 mm punches vs. the whole circle) made a difference only for Protocol 1. There are limited studies that explore the differences in the number of punches used and to our knowledge none that explore various input types for nucleic acid extraction. When examining protein yield, Björksten et al. that showed that using two versus one punch did not lead to a significant difference [177].

6.5.2 We hypothesize that red blood cells are a facilitator and barrier to gDNA extraction from DBSs

We tested the gDNA yield from various mixtures of blood components spotted onto the DBS paper to see the impact of the components on gDNA extraction yield. Overall, the yield was lower for heparin than for EDTA anticoagulated blood (Supplementary Information), which was expected as other studies have shown how heparin (especially with DBSs that are stored for a long time) is harmful to nucleic acid DBS extraction yield [24], [193], [194]. Based on prior work analyzing gDNA yield from human whole blood obtained via venipuncture [153], the expected yield for one 6mm punch was ~0.9 – 1.2 ng/µL. Here, the yield was ~33% recovery for EDTA and ~13% recovery for heparin whole blood DBSs.

Our study is the first (to our knowledge) to examine the impact of individual blood components on nucleic acid elution from dried blood spots. We hypothesize that these results show how RBCs are both a facilitator and barrier to gDNA elution. When comparing the anticoagulants, the mixture had the lowest percent yield varied, which was WBCs only for EDTA and WBCs + RBCs for heparin. This shows that the RBCs seem to reduce the yield of gDNA extracted from DBSs when using heparin as an anticoagulant. By lysing the RBCs and removing the lysate, the gDNA yield improved drastically improved. Therefore,

we hypothesize that RBCs facilitate gDNA extraction because the viscous nature of RBCs aid in proper spread of a solution onto the DBS cards, as seen by the lower yield of WBCs only. On the other hand, they could be a barrier to gDNA elution due to the RBC components that are typically inhibitors of DNA measurement techniques [210]. Belkacem et al. did not see a difference in recovery when they used a red blood cell lysis buffer versus 1X PBS for DBS extraction [193]. They were examining WBC recovery, though, with flow cytometry, which is a different application than the one presented in this work.

Lastly, we also tested, with similar mixtures, the elution of purified gDNA spotted on the DBS paper, which had a significantly lower yield than using WBCs as the gDNA source (Supplemental Information). Additionally, the RBC-containing mixtures had a lower recovery than the plasma ones. This further adds to our hypothesis of how RBCs can negatively impact gDNA yield and offers insight into another source of gDNA's extraction from DBSs.

6.5.3 A lysis buffer used with either heat or shaking is sufficient for gDNA DBS extraction

From the results in Figure 5, it appears that when using KOH/Trizma® for gDNA extraction from DBSs with Protocol 6 the agitation in the elution step can be performed for 5 minutes, the elution temperature can be lowered to room temperature, agitation is not necessary for any step, and the wash step can be skipped to yield PCR-amplifiable gDNA comparable to the manual protocol. One may think why the manufacturer of Protocol 6 would include these steps. It is important to note that Protocol 6 was not written for gDNA extraction but for a less abundant gene. Thus, to maximize DNA elution, these steps are likely necessary. For 1X PBS, however, all the steps of the protocol are necessary; this again highlights the importance of using a buffer that lyses cells to maximize yield. Lastly, it is also important to note that there was a donor-dependent response in terms of using

PBS as an elution buffer since the Cq values of the eluate from one donor's DBSs using PBS for extraction were comparable to those of KOH/Trizma® and for another donor they were not.

We can conclude that in addition to a lysing agent, at least one other element is important for higher gDNA elution from DBSs which is heat or shaking, based on our results. In the absence of a lysing agent, more than one kinetic element is important for elution. Heat is important not only helpful for relaxing the bonds molecules make, but also hemolyzes the RBCs [211] and likely partially degrades or destabilizes proteins like hemoglobin [212]. Agitation also increases the speed at which molecules move, increasing the likelihood that bonds will be broken between the paper and the molecules bound to it.

6.6 Conclusions

DBSs have great potential to expand access to multiple types of diagnostic tests but are not well-studied mechanistically. We first showed the DNA yields from multiple protocols from across the literature, which showed the vast difference between DBS nucleic acid extraction protocols and the lack of consensus of what is necessary for DBS nucleic acid extraction. All protocols had similar yields despite having different elution buffers and sample inputs, though. We then added further insight into DBS nucleic acid extraction by showing how red blood cells both facilitate and inhibit gDNA extraction from DBSs. They facilitate gDNA extraction due to their viscosity ensuring proper spreading on the paper and inhibit gDNA yield due to their contents. An addition of an RBC lysis step improve yield 2- to 5-fold, depending upon the anticoagulant used. Our results led to similar conclusions as previous studies about heparin and DBSs, that this anticoagulant reduces gDNA yield. Lastly, we experimentally analyzed the significance of heat, agitation, wash steps, and length of elution time to find that for a buffer that lyses cells only heat or

agitation is significant in extracting gDNA from DBSs. For a non-lysing buffer, elution time, heat, and agitation were important for elution of gDNA from DBSs.

Overall, this work demonstrated what is essential for gDNA extraction from DBSs. This is important for those developing DBS extractions protocols, especially those who may be limited by access to certain equipment. The complexity of the buffers and steps required for gDNA extraction can be a barrier to using DBSs in a wide array of settings. A limitation of this work is that these experiments were analyzing gDNA which is abundant in white blood cells and a relatively stable molecule. Therefore, future studies should examine elution of less abundant or stable molecules such as RNA and/or viral nucleic acids such as HIV RNA. This work added to the literature on gDNA DBS extraction, which could be used for downstream processes like whole genome sequencing. These results were a step in creating a standardized manual protocol to maximize gDNA elution from DBSs for a variety of applications.

6.7 Acknowledgements

We would like to thank the donors for their blood samples.

6.8 Data Availability

The data in relation this study is available through this link: https://doi.org/10.7910/DVN/NG1ITP

6.9 Supplementary Information

6.9.1 Detailed statistical analyses for each figure

Figure 6.2: Comparison of C_q values for Protocols 1-5 for 2 x 6mm punches and whole circles.

- *CV (%) for 2 x 6 mm punches: Protocol 1: 1.5%; Protocol 2: 1.9% Protocol 3: 1.7%; Protocol 4: 2.9%; Protocol 5: 2.2%*
- *CV (%) for whole circle: Protocol 1: 2.3%; Protocol 2: 2.1%; Protocol 3: 4.2%; Protocol 4: 1.6%; Protocol 5: 2.9%*

- *To compare the mean C_q value of the protocols to each other within their input sample type, two-way ANOVA and Tukey's multiple comparisons test were performed (α = 0.05). With 2 x 6mm punches, the mean differences between Protocol 2 ($\overline{C_q}$ = 35.48) and Protocol 1 ($\overline{C_q}$ = 32.76), Protocol 3 ($\overline{C_q}$ = 32.43), and Protocol 4 ($\overline{C_q}$ = 32.45), and Protocol 5 ($\overline{C_q}$ = 32.74) were statistically greater than 0, with p < 0.0001. With whole circles, the mean difference between Protocol 2 ($\overline{C_q}$ = 34.69) and Protocols 1 ($\overline{C_q}$ = 30.86), Protocol 3 ($\overline{C_q}$ = 31.74), Protocol 4 ($\overline{C_q}$ = 31.59), and Protocol 5 ($\overline{C_q}$ = 32.44) were statistically greater than 0, with p < 0.0001 for Protocols 1 and 2 and p = 0.0007 for Protocol 5. The mean difference between Protocol 1 and Protocol 5 was statistically greater than 0, with p = 0.0375. To compare mean C_q value of the protocols between input sample type, two-way ANOVA and Šídák's multiple comparisons test was used. The differences in mean of 2 x 6mm punches and the respective whole circle was not statistically greater than 0, except for Protocol 1, in which p = 0.0018.*

Figure 6.3: Comparison of C_q values for Protocol 6 for a 1 x 6mm punch and 2 x 6mm punches.

- *CV (%) for 1 x 6 mm punches: EDTA 0.5%; Heparin 3.0%*
- *CV (%) for 2 x 6 mm punches: EDTA: 3.8%; Heparin: 4.2%*
- *To compare the mean differences between average C_q values for the number of punches as well as the anticoagulant type (for 1 punch: $\overline{C_q}$ = 30.91 for disodium EDTA and $\overline{C_q}$ = 32.03 for sodium heparin; for 2 punches: $\overline{C_q}$ = 31.26 for disodium EDTA and $\overline{C_q}$ = 32.56 for sodium heparin), two-way ANOVA and Tukey's multiple comparisons tests were performed. There was no statistically significant difference between the average C_q value for the anticoagulant type or number of spots used. Protocol 6 was compared to Protocols 1-5 for 2 x 6mm punches using two-way ANOVA and Tukey's multiple comparisons test (α = 0.05) the mean difference between Protocol 2 ($\overline{C_q}$ = 35.48) and 6's ($\overline{C_q}$ = 31.91) average C_q values were statistically greater than 0, with p < 0.0001.*

Figure 6.4A*: gDNA percent recovery from various mixtures of white blood cells, plasma, and red blood cells, disodium EDTA.*

- *CV (%) for EDTA: whole blood: 4.1%; WBCs only: 10.3%; plasma + WBCs: 7.5%; RBCs + WBCs: 8.2%*
- *To compare the average percent recovery for the different conditions tested, one-way ANOVA and Tukey's multiple comparisons tests were performed. The mean differences between whole blood (94.1%) and WBCs only (26.3%), whole blood and WBCs + plasma (42.4%), whole blood and WBCs + RBCs (68.28%), WBCs only and WBCs + plasma, WBCs only and WBCs + RBCs, and WBCs + plasma and WBCs + RBCs were statistically greater than 0 with p < 0.0001 for all comparisons.*

Figure 6.4B*: gDNA percent recovery from various mixtures of white blood cells, plasma, and red blood cells, sodium heparin.*

- *CV (%) for Heparin: whole blood, 5.3%; WBCs only, 15.3%; plasma + WBCs: 13.9%; RBCs + WBCs: 9.1%*
- *To compare the average percent recovery for the different conditions tested, one-way ANOVA and Tukey's multiple comparisons tests were performed. The mean differences between whole blood (92.98%) and WBCs only (67.5%), whole blood and WBCs + RBCs (49.07%), WBCs only and WBCs + plasma (83.9%), WBCs only and WBCs + RBCs, and WBCs + plasma and WBCs + RBCs were statistically greater than 0 with p = 0.001, p < 0.0001, p = 0.03, p = 0.02, and p < 0.0001, respectively.*

Figure 6.4C: RBC lysis buffer effect on yield. CV (%) for EDTA fold increase: whole blood: 5.4% and RBCs + WBCs: 12.8%. CV (%) heparin fold increase: whole blood: 8.0%; RBCs + WBCs: 12.8%

Figure 6.5A: Comparison of KOH/Trizma® and PBS buffers using Protocol 6. To compare the mean C_q values of the buffers used to each other, an unpaired t-test was performed. The mean difference was statistically significantly greater than 0 with p = 0.0003. KOH/Trimza®: $\overline{C_q}$ = 30.91. PBS: $\overline{C_q}$ = 33.26. CV (%): KOH/Trizma®: 0.5%; PBS: 4.1%

Figure 6.5B: Reduced shake time for both KOH/Trizma® and PBS buffers. To compare the mean C_q values of the time reductions to the control, two-way ANOVA and Šídák's multiple comparisons test were performed. For KOH/Trizma®, the mean differences between the control ($\overline{C_q}$ = 31.33) and 5 minutes ($\overline{C_q}$ = 32.13), 10 minutes ($\overline{C_q}$ = 31.61), and 15 minutes ($\overline{C_q}$ = 30.75), were not statistically greater than 0. For PBS, the mean differences between the control ($\overline{C_q}$ = 33.26) and 5 minutes ($\overline{C_q}$ = 39.12), and 10 minutes ($\overline{C_q}$ = 37.59) were statistically greater than 0, with p < 0.0001 and p < 0.0001. The mean difference between the control and 15 minutes ($\overline{C_q}$ = 33.72) was not statistically greater than 0. CV (%) for KOH/Trizma®: 5 minutes: 0.8%; 10 minutes: 1.4%; 15 minutes: 1.2%. CV (%) for PBS: 5 minutes: 0.9%; 10 minutes: 1.5%; 15 minutes: 3.9%

Figure 6.5C: Performance of the elution step at 70°C versus 25°C for KOH/Trizma® and PBS buffers. To compare the mean C_q values of the time reductions to the control, two-way ANOVA and Tukey's multiple comparisons test were performed. For KOH/Trizma®, the mean difference between 70°C ($\overline{C_q}$ = 32.03) and 25°C ($\overline{C_q}$ = 32.01) was not statistically greater than 0. For PBS, the mean difference between 70°C ($\overline{C_q}$ = 33.26) and 25°C ($\overline{C_q}$ = 38.24) was statistically greater than 0, with p < 0.0001. Comparing 25°C PBS to Protocol 6, the mean difference was statistically greater than 0, with p < 0.0001. CV (%) for 25°C: KOH/Trizma®: 3.6%; PBS: 1.1%

Figure 6.5D: Performance or no performance of the two 1000rpm shaking steps for KOH/Trizma® and PBS buffers. *To compare the mean C_q values of the time reductions to the control, two-way ANOVA and Šídák's multiple comparisons test were performed. For KOH/Trizma®, the mean difference between shaking ($\overline{C_q}$ = 32.03), and no shaking ($\overline{C_q}$ = 31.99) was not statistically greater than 0. For PBS, the mean difference between shaking ($\overline{C_q}$ = 33.26), and no shaking ($\overline{C_q}$ = 35.11) was statistically greater than 0 with p = 0.04. Comparing no shaking PBS to Protocol 6, the mean difference between was statistically greater than 0, with p = 0.0003. CV (%) for no performance of shake steps: KOH/Trizma®: 1.2%; PBS: 2.3%*

Figure 5E: Performance or no performance of the wash step for KOH/Trizma® and PBS buffers. To compare the mean C_q values of the time reductions to the control, two-way ANOVA and Tukey's multiple comparisons test were performed. For KOH/Trizma®, the mean difference between washing ($\overline{C_q}$ = 32.03) and no washing ($\overline{C_q}$ = 31.77) was not statistically greater than 0. For PBS, the mean difference between washing ($\overline{C_q}$ = 33.26) and no washing ($\overline{C_q}$ = 34.93) was not statistically greater than 0. Comparing no washing PBS to Protocol 6, the mean difference between was statistically greater than 0, with p = 0.0008. CV (%) for no wash step: KOH/Trizma®: 0.6%; PBS: 2.9%

Table 6.2: CV (%): KOH/Trizma®: 1.6%

6.9.2 Protocols for individual blood components experiments (Figure 6.4)

Figure 6.4, solution development: 2 mL of whole blood were centrifuged and 150µL of plasma or 135 µL of RBCs were placed into separate tubes. These volumes were chosen to keep the ratio of RBCs and plasma to 0.9 (45% vs. 55% in whole blood) when the experiments were compared. The red blood cells lysis buffer protocol was performed with the remaining blood solution to isolate the WBCs. The pelleted WBCs were resuspended in 1,400 µL of 1X PBS, and 350 µL of this solution was mixed with the plasma and 365 µL was mixed with RBCs. For the WBCs only solutions 2,000 µL of 1X PBS was used to resuspend the WBCs. 1X PBS was used to prevent RBC lysis and all samples, including whole blood, theoretically contained the same concentrations of gDNA. Then, 75 µL of each mixture (WBCs only, WBCs and plasma, or WBCs and RBCs) were spotted onto each circle of the DBSs. Background signal was not subtracted from these samples, thus the gDNA yields may be higher than the true values for the WBCs only, WBCs and plasma, and WBCs and RBCs.

Figure 6.4A: Whole blood, WBCs only, WBCs and plasma, and WBCs and RBCs had a gDNA yield of 0.39 ng/µL, 0.11 ng/µL, 0.17 ng/µL, and 0.28 ng/µL respectively.

Figure 6.4B: The gDNA yield for whole blood, WBCs only, WBCs and plasma, and WBCs and RBCs were 0.16 ng/µL, 0.12 ng/µL, 0.14 ng/µL, and 0.08 ng/µL, respectively.

Figure 6.4C: To do this, we shook DBSs in 200 µL of Red Blood Cell lysis buffer at 1000 rpm for 10 minutes at room temperature, using the same plate shaker used for Protocol 6. We discarded the supernatant and then performed Protocol 6.

6.9.3 Table S1: Compatibility of the solutions used in the six protocols with qPCR

We tested a mixture of 90% of each of the solutions used for the six protocols with 10% purified gDNA to see if the gDNA would be amplifiable each solution. The gDNA amplified for all solutions except those that contained the lysis buffer (**Table SI 6.1**).

Table SI 6.1: Compatibility of solutions used in Protocols 1-5 with qPCR.

Solution (Protocol Number)	Compatible with qPCR?
1X PBS, Tween-20, Sodium Azide	Yes
NucliSENS® Lysis Buffer, 1X PBS (1)	No
1X PBS (2)	Yes
NucliSENS® Lysis Buffer (3, 4)	No
KOH/Tris (6)	Yes

6.9.4 Solution development for elution efficiency insight into DBS gDNA elution from purified gDNA (Figure S1)

Human genomic DNA (male) was obtained from Promega Corporation (Madison, Wisconsin, USA).

Figure S1A: 5 solutions of purified gDNA diluted in 1X PBS were prepared to ~40ng/μL, ~20ng/μL, ~10ng/μL, ~5ng/μL, and ~2.5ng/μL from a stock solution of purified gDNA at ~242ng/μL. 1 x 6mm punch and 2 x 6mm punches were used with Protocol 6, at half the standard protocol volumes. The DBSs were pipetted on one day and analyzed 24 hours later.

Figure S1B: To make solutions of gDNA and plasma or gDNA and RBCs, 66.2μL of a ~242ng/μL stock solution of pure gDNA was mixed into 150μL of plasma or 135μL of RBCs and the entire solution was brought up to 500μL with 1X PBS. The final concentration of purified gDNA therefore was ~29ng/μL. The yield for plasma and gDNA for EDTA was 0.91ng/μL and for RBCs and gDNA was 0.50ng/μL. The yield for plasma and gDNA for heparin was 0.83ng/μL and for RBCs and gDNA was 0.46ng/μL.

Figure S1C: To do this, we centrifuged 3mL of sodium heparin whole blood and pipetted out 150μL of plasma and 135μL of RBCs and mixed them together in a separate tube. The volumes of plasma and RBCs were chosen to keep the ratio of plasma and RBCs the same as in whole blood, while ensuring there was sufficient volume of white blood cells or gDNA to detect via spectrofluorometry using the Picogreen assay. 215μL of a 40 or 20ng/μL solution of purified gDNA diluted in 1X PBS was then added to the mixture of RBCs and plasma. A blank solution of 1X PBS was also mixed with the RBCs/plasma to be used as a measure of background or WBC gDNA that got into the solution since pipetting RBCs from the centrifuged blood requires the pipette penetrating the WBC layer. 75μL of these mixtures were then pipetted on to the DBS circles; the final concentration for 40ng/μL solution was measured and calculated to be 13.8ng/μL and for the 20ng/μL

solution was 8.4ng/μL. The yield for “14ng/μL” was 0.16ng/μL and for “8ng/μL” was 0.07ng/μL.

6.9.5 Investigation into DBS gDNA elution efficiency with purified gDNA

To understand the elution efficiency of DBSs, we first examined the elution of purified gDNA from DBS paper (Figure S1). Protocol 6 was used at half the standard protocol volumes to ensure detection with spectrofluorimetry (the Picogreen™ assay). We pipetted 75 μL of purified gDNA only at various concentrations onto the dried blood spot circles and found the normalized percent recovery based on the concentration of the gDNA solution put onto the paper. That is, the normalized yield was calculated for each sample, X, using this equation:

$$\frac{measured\ concentration\ of\ sample\ X}{input\ concentration\ for\ sample\ X}\ x\ 100\% \qquad \text{(Equation SI 6.1)}$$

and then averaged the percent yield of the samples for each mixture.

The normalized recovery for ~40ng/μL, ~20ng/μL, ~10ng/μL, ~5ng/μL, and ~2.5ng/μL were around 1% for 1 x 6mm punch and around 2.5% for 2 x 6mm punches. There was no statistically significant difference between the 1 x 6mm punch recoveries, but for 2 x 6mm punches, the recovery for 2.5ng/μL was significantly lower than the other concentrations. Blood is more viscous than the solution we diluted the gDNA in (1X PBS). We visually observed that the viscosity aids blood to not spread beyond the dotted circle on the DBS paper. Therefore, we next spiked gDNA into plasma and red blood cells (RBCs) separately.

To isolate plasma and RBCs we centrifuged whole blood and pipetted portions of the red blood cell (RBC) and plasma layers out and mixed them with the same amount of gDNA. This was performed with both EDTA and heparin anticoagulated DBSs. We tested these two anticoagulants to see if there was a difference between them in terms of yield since heparin has been shown to be detrimental for DBS stability [24]. The normalized recovery of gDNA in plasma or RBCs for EDTA was 3.1% and 1.7%, respectively. The normalized recovery of gDNA in plasma or RBCs for heparin was 2.8% and 1.5%, respectively. The samples containing RBCs had a statistically significantly lower concentration of gDNA yield for both anticoagulants. Also, the large error bar with the heparin anticoagulated sample for the gDNA and plasma mixture highlights how heparin interferes with the fluorescence of the Picogreen dye. The plasma contains more concentrated heparin (compared to whole blood), thus the effect of heparin on this quantification method is more evident[213]. Lastly, we made mixtures of purified gDNA with whole blood whose white blood cells were removed (Figure 3C). The normalized recovery for “14ng/μL” was 1.1% and for “8ng/μL” was 0.84% (with the background signal of residual WBCs subtracted). There was no statistically significant difference between their percent recoveries.

For all samples, the normalized percent recovery was low, ranging from ~0.7% - 3%. We believe this result is due to the conformational differences in gDNA extracted from cells, which is supercoiled, compared to purified gDNA which is likely more relaxed and elongated [214]. Since the purified gDNA is more elongated, it has more chances to make bonds with the DBS paper, which would cause it to be more tightly bound than the supercoiled gDNA. This is the first work to examine the elution efficiency of gDNA elution

from Whatman™ 903 cards. Though not as clinically relevant, this work shows that if one wanted to store gDNA on DBS paper, to not rely on cold storage the recovery may not be that high, especially for a gene that is less abundant. The lower sample viscosity and use of purified gDNA may be barriers to the recovery of gDNA from DBSs, therefore we performed a similar analysis with WBCs as the gDNA source.

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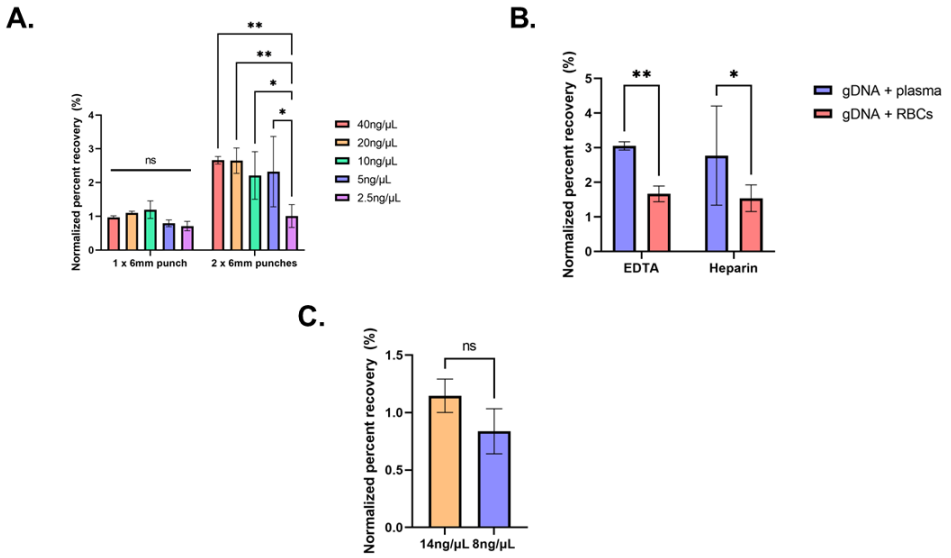

Figure SI 6.1: Elution efficiency of purified gDNA from various concentrations and mixtures of WBC-free blood, plasma, and RBCs. *gDNA was extracted using Protocol 5 and measured using spectrofluorometry.* ***(A)*** *Comparison of purified gDNA percent recovery from purified gDNA dilutions (diluted in 1X PBS). A 1 x 6mm punch was used with 3 replicates for each condition.* ***(B)*** *Comparison of purified gDNA yield from DBSs for EDTA and heparin anticoagulated blood using mixtures of gDNA + plasma and gDNA + RBCs. A 1 x 6mm punch was used with 6 replicates for each condition.* ***(C)*** *Comparison of purified gDNA from WBC-free blood at concentrations of 14ng/µL and 8ng/µL. 5 samples (1 x 6mm punch) were tested for all; all samples were from the same donor. All samples were and gDNA was extracted using Protocol 6 and measured using spectrofluorometry.*

Figure SI 6.1A*: Comparison of purified gDNA percent recovery from purified gDNA dilutions (diluted in 1X PBS).* To compare the mean concentration of the mixtures to each other, an unpaired t-test was performed. The mean difference was not statistically significantly greater than 0.

Figure SI 6.1B: Comparison of purified gDNA yield from DBSs for EDTA and heparin anticoagulated blood using mixtures of gDNA + plasma and gDNA + RBCs. To

compare the mean concentration of the mixtures to each other, two-way ANOVA and Šídák's multiple comparisons test were performed. The mean difference between gDNA + plasma and gDNA + RBCs for disodium EDTA as well was between gDNA + plasma and gDNA + RBCs for sodium heparin was statistically greater than 0, with p = 0.0092 and p = 0.02, respectively.

Figure SI 6.1C: Comparison of purified gDNA normalized percent recovery from WBC-free blood at concentrations of 14ng/µL and 8ng/µL. To compare the mean concentration of the mixtures to each other, two-way ANOVA and Tukey's multiple comparisons test were performed. The mean difference between the 1 x 6mm punch dilutions was not statistically greater than 0. For 2 x 6mm punches, the mean differences between 2.5ng/µL and 40ng/µL, 20ng/µL, 10ng/µL, and 5ng/µL were statistically greater than 0, with p = 0.0015, p = 0.0017, p = 0.025, and p = 0.013, respectively.

6.9.6 Disodium EDTA anticoagulated blood (Donor 1) and sodium heparin anticoagulated blood (Donor 2), KOH/Trizma® vs PBS extraction with Protocol 6

Donor 1: Tested KOH and PBS for both anticoagulants

Donotr 2: Tested KOH and PBS for both anticoagulants

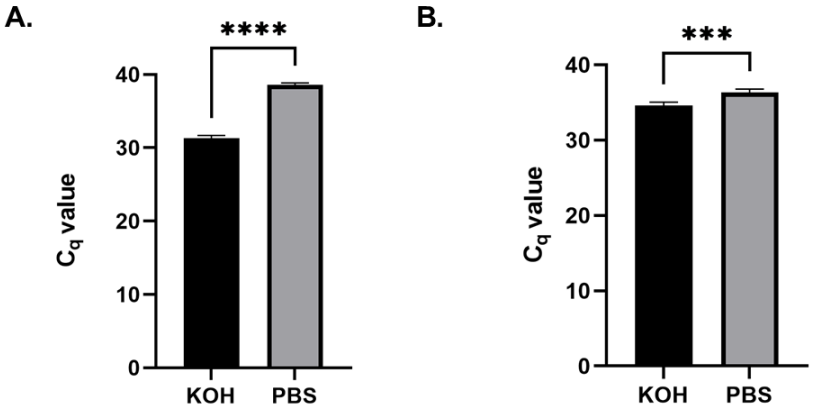

Figure SI 6.2: Disodium EDTA anticoagulated blood (Donor 1) and sodium heparin anticoagulated blood (Donor 2), KOH/Trizma® vs PBS extraction with Protocol 5. (A) *EDTA, Donor 1 To compare the mean Cq value of KOH/Trizma® and PBS to each other, an unpaired t-test was performed. The mean difference was statistically greater than 0, with p < 0.0001. CV (%) for KOH/Trizma®: 1.1%; for PBS: 0.8%* ***(B)*** *Heparin, Donor 2. To compare the mean Cq value of KOH/Trizma® and PBS to each other, an unpaired t-test was performed. The mean difference was statistically greater than 0, with p = 0.0003. CV (%) for KOH/Trizma®: 1.2%; for PBS: 1.2%.*

CHAPTER 7: ELECTRO-DBS: A SIMPLE METHOD TO RAPIDLY EXTRACT GENOMIC DNA FROM DRIED BLOOD SPOTS

Chapter 7 has been published as an original research article.

Lee, K., Murphy, J., and Tripathi, A., "Electro-DBS: A Simple Method to Rapidly Extract Genomic DNA from Dried Blood Spots". *Anal. Chem*. 2022.

7.1 Abstract

Dried blood spots (DBSs) have been used for more than 50 years and are used as a method of sample collection for pharmacological testing, genetic analyses, monitoring of viral infections and more. Protocols for nucleic acid extraction from DBSs involve several steps that require specialized equipment and can be lengthy. Thus, we sought to explore ways to reduce the analytical burden of DBSs. We developed a DBS extraction method that uses synergistic action of electrophoretic and diffusive transport mechanisms to extract gDNA through DBS matrix. This method (which we termed “Electro-DBS”) reduces the time of extraction from 40 minutes to 5 minutes and removes the need for heat, shaking, and DNA purification while maintaining gDNA quality and yield. We found that the electrophoretic transport speeds up gDNA elution, allowing diffusive transport of PCR inhibitors to be minimized due to a reduced elution time. Overall, this work added mechanistic insight into the extraction of DNA from DBSs and developed a method that was ideal for the point of care and automation.

7.2 Introduction

Dried blood spots (DBSs) have been used for neonatal screening for a multitude of diseases for more than 50 years. In that time, their use has expanded beyond metabolic disease screening in infants to a method of sample collection for pharmacologic testing, genetic analyses, and monitoring of viral infections [175]. In more remote areas and for self-sampling, DBSs are particularly beneficial because they do not require specialized skills for obtaining the blood (unlike venipuncture) and their transport and storage is less reliant on the cold chain process [176]. DBSs use a few drops of capillary blood from a finger prick that are placed on a cellulose-based paper card and dried. Viral RNA and DNA, antibodies, and metabolites are stable on these light-weight cards when they are placed in a bag with a desiccant and maintain stability for longer at ambient temperatures

when compared to wet blood [19]. The stability of these molecules, particularly RNA and DNA, is essential for sequencing and viral load determination [201]. However, there is a large analytical burden for processing DBSs.

Generally, DBS nucleic acid extraction protocols have similar processes, which are to use buffers like Tris-EDTA (TE) buffer, phosphate-buffered saline (PBS), strong bases, strong acids to re-hydrate and/or lyse the blood contents on the DBS and then purifying the DNA from the supernatant using a DNA extraction kit [215]–[217]. The extraction of DNA (or RNA) from DBSs ranges from 45 minutes to over 24 hours of processing times (Table 1) and within this time, heating steps, agitation, and/or centrifugation are often required. Not only is this time-consuming, but it requires specific equipment that may not be available in remote settings or low-resourced laboratories. To make nucleic acid analysis from DBSs feasible in low-resource settings, extraction of nucleic acid from DBSs should require minimal equipment. DBS extraction has been automated by the use of commercially-available devices and assays, but these devices require maintenance, are bulky, and their applicability is limited due to previously specified assay targets [36], [218].

Here, we used Whatman™ 903 cards and human whole blood to explore a series of questions. Firstly, we wanted to investigate how the yields of various protocols in the literature compare to each other. Next, we wanted to understand what inhibits DNA yield from DBSs. Then, we explored the essential kinetic factors in DBS extraction. Our overall goal was to design a customizable sample preparation system for DNA extraction from DBS. Expanding upon previous work in our group [205], we applied an electric field to a DBS to extract high-quality nucleic acids for downstream processes like quantitative PCR. We aimed to eliminate the need for heat, shaking, and additional DNA purification steps and developed a novel device which extracts DNA from DBSs in 5 minutes, with a yield comparable to conventional methods.

7.3 Materials and Methods

7.3.1 Human whole blood

Human male whole blood was purchased from Golden West Biosolutions (Temecula, California, USA) or Research Blood Components, LLC (Watertown, Massachusetts, USA). The blood samples contained either sodium heparin or disodium EDTA anticoagulants. We refer to sodium heparin anticoagulated blood as “heparin” and disodium EDTA anticoagulated blood as “EDTA”. The samples were tested for common infectious diseases and were stored at 4 ^{o}C.

7.3.2 Preparation of Dried Blood Spots

Dried blood spots were prepared by pipetting 75 μL of whole blood onto each circle onto Whatman™ 903 protein saver cards (Millipore Sigma, St. Louis, Missouri, USA). Then, they were left to dry overnight at room temperature. After drying, each card was placed into a bag with a desiccant and stored at -20 ^{o}C.

7.3.3 Real-time (or quantitative) PCR (qPCR)

Real-time PCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA). TaqMan™ Gene Expression Assay ID# Hs00243216_s1, a primer for human sex-determining region Y (SRY) gene, was used (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The Taqman™ Gene Expression Master Mix (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) was used for the assay. 3 technical replicates were averaged for each for biological replicate analyzed.1:5 or 1:10 dilutions of purified human genomic DNA (Promega Corporation, Madison, Wisconsin, USA) were used as a standard for all qPCR reaction plates.

7.3.4 Determination of DNA concentration

Spectrophotometry was performed using the NanoDrop 1000 based on guidelines from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA). Spectrofluorometry was performed based on guidelines from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA) using the Quant-iT™ PicoGreen™ dsDNA Assay Kit. Signal measurements of all samples were performed using the EnVision 2105 Multimode Plate Reader (PerkinElmer, Waltham, Massachusetts, USA). Subsequent linear regression analysis was performed in Microsoft Excel (Microsoft, Redmond, Washington, USA). For all experiments, the standard curve used for linear regression had $R^2 > 0.99$. 3 technical replicates were averaged for each for biological replicate analyzed.

7.3.5 Reagents for DBS DNA elution

1X Gibco™ Phosphate buffered saline (PBS), pH 7.4 was obtained from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA). The 20 mM KOH and 30 mM Trizma® solution was obtained from PerkinElmer (Waltham, Massachusetts, USA).

7.3.6 Protocol for DNA extraction from DBS using KOH/Trizma® solution

This protocol was created by PerkinElmer (Waltham, Massachusetts, USA) for congenital cytomegalovirus screening for newborns. The protocol uses a 3 mm DBS punch that is placed into 80 µL of the KOH/Trizma® solution on a 96-well plate. The plate is sealed then shaken for 3 minutes at 1000rpm at 25^{o}C. The buffer is discarded then the plate is incubated in a plate shaker, here the Trinest™ incubator shaker (Waltham, Massachusetts, USA), for 10 min while the shaker heats up to 70 ^{o}C. Then, 50 µL of the KOH/Trizma® solution is added to the well and the plate is shaken for 20 minutes at 70 ^{o}C at 1000 rpm. Here, we used one 6 mm DBS punch or two, and adjusted the volumes accordingly. Additionally, the incubation time was increased to 15 minutes because that is how long it took the Trinest™ incubator shaker to heat up to 70 ^{o}C.

7.3.7 Electro-DBS

The in-house electrical device was fabricated from two Platinum (99.9%) Plate Electrodes coated with PTFE with a gold-plated copper rod from Newvision1981 and purchased from Amazon.com (Seattle, Washington, USA). The two electrodes were taped together, the plates were cut to fit into a well of a 96-well plate with electrical tape and connected to an external power supply. The electrodes were connected to the DP700 Series Programmable Linear DC Power Supply (RIGOL, Beijing, China) for applying the electric field.

7.3.8 Conductivity measurements

Conductivity of 1X PBS and the KOH/Trizma® buffer were measured on the Oakton PC2700 Benchtop Meter (Cole-Parmer, Vernon Hills, Illinois, USA).

7.3.9 COMSOL Multiphysics® - Theoretical Modelling

Theoretical modelling was performed using COMSOL Multiphysics® Software (Stockholm, Sweden), version 5.6.

7.3.10 Statistical analyses

Statistical analyses were performed in GraphPad Prism 8 (San Diego, California, USA) with alpha (α) = 0.05. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$. Further information regarding statistical comparisons can be found in the Supplementary Material.

7.4 Results and Discussion

7.4.1 gDNA extraction from EDTA and heparin anticoagulated DBSs using a KOH/Trizma®-based method

There are several dried blood spot extraction protocols that are used across the literature [198]–[204], which typically require punched-out spots to be shaken for various amounts of time, ranging from 30 minutes to overnight, in an elution solution. From there

the nucleic acids must be purified for downstream processing using a nucleic acid purification kit. We chose to focus our work with a 40-minute KOH/Trizma® solution-based extraction [205] which is used by the manufacturer for cytomegalovirus DNA extraction from human whole blood for newborn screening. The chemical-based lysis of the viral particles and blood cells for DNA release relies on KOH for alkaline lysis [206], [207], as the KOH/Trizma® solution is very basic with a pH of ~12, and Trizma® base for DNA stabilization. This protocol was ideal for our work because most nucleic acid extraction protocols require a nucleic acid cleanup step [200]–[204] for downstream qPCR and sequencing, while the KOH/Trizma® protocol did not. Additionally, this method is high throughput since the extraction is performed on a 96-well plate, allowing for multiple extractions to be performed at once.

We performed DNA extraction using the KOH/Trizma® method with EDTA and heparin anticoagulated DBSs. We tested these two anticoagulants to see if there was a difference between them in terms of yield since heparin has been shown to be detrimental for DBS stability [24]. Researchers will use one 6 mm punch, two 6 mm punches, or the whole circle depending on physical tube constraints or to maximize the yield of the sample. In other words, a whole circle would be unable to fit into the well of a 96-well plate and thus 6 mm punches would need to be used, as is the case for this protocol which requires one 6 mm punch. On the other hand, to increase yield, one may use the entire circle instead of a portion of the sample (as is done with 6 mm punches). The elution volume used was 100 µL for 1 x 6mm punch.

The C_q values and concentration of gDNA released from one 6 mm punch, with each anticoagulant graphed separately (Figure 7.1). The average C_q values for EDTA were statistically lower than those of heparin anticoagulated blood and had less variability

than those of heparin. The concentration of gDNA was statistically significantly higher ($p = 0.048$) for EDTA anticoagulated blood compared to heparin.

Based on prior work analyzing gDNA yield from human whole blood obtained via venipuncture[153], the expected yield for one 6mm punch was ~0.9 – 1.2 ng/µL, Here, the yield was ~0.1 ng/µL for 1 punch, which is approximately 8-11% recovery of gDNA from DBSs. To our knowledge our study is the first to examine the percent recovery of gDNA from healthy donor DBSs. This relatively low recovery indicates a challenge with DBS extraction that should be further investigated. As previously shown, heparin anticoagulated DBSs did perform less favorably compared to those anticoagulated with EDTA, though not significantly. Heparin was not detrimental to the gDNA eluted from the DBSs in these experiments, though the DBSs used had only been stored for a few days. Thus, we experimented with both EDTA and heparin DBSs for future experiments.

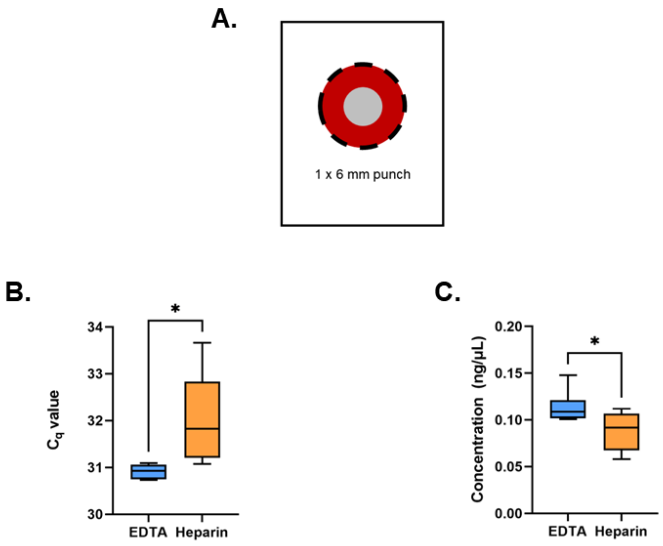

Figure 7.1: C_q values and concentration measured for 1 x 6 mm DBS punches using the KOH/Trizma® method. *6 replicates of 1 x 6 mm punches were tested for EDTA or heparin anticoagulated DBSs.* ***(A)*** *In gray is a depiction of 1 x 6 mm punch that would be taken from the dried blood spots for extraction.* ***(B)*** *Comparison of C_q values.* C) *Comparison of concentration (using the Picogreen™ assay).*

It should be noted that when it comes to the lack of agreement between the C_q values and Picogreen™ assay concentration the measurements generate different information about the sample. The Picogreen™ dye labels all double-stranded DNA while C_q values measure the amount of amplification of a specific gene which is based on the concentration of the starting sample, purity of the starting sample, and the reaction's efficiency[219]. Thus, C_q values could be higher for samples that have a higher starting concentration of gDNA due to the presence of PCR inhibitors since the presence of inhibitors would slow amplification during qPCR. Since this protocol does not require a DNA purification step, all samples generated have qPCR inhibitors present from the blood lysate [210]. qPCR is a gold standard for DNA measurement, typically for quantification of the amount of DNA present in a sample. Here, we use another gold standard, Picogreen™ to quantify the amount of gDNA in our samples and use C_q values to investigate and quantify the samples' ability to amplify in a 40-cycle PCR as PCR is often necessary for further nucleic acid-based analyses such as diagnosis or sequencing.

7.4.2 Comparison of 1X PBS to KOH/Trizma® for DBS DNA elution

We chose 1X PBS as a non-lysing buffer control to test if chemical lysis is necessary for our Electro-DBS method. First, we examined the substitution of 1X PBS into the KOH/Trizma® protocol, using one 6 mm punch. We made EDTA DBSs from one donor (Donor 1) and heparin DBSs from a second donor (Donor 2). These donors differ from the donor in Figure 7.1. The average C_q values and concentrations of the eluted gDNA are shown in Figure 7.2. We did not compare between donors as they may differ biologically in terms of white blood cell count, hematocrit, etc. which would affect gDNA elution.

As expected, samples that were eluted with 1X PBS had a higher C_q value than those eluted with KOH/Trizma®. Interestingly the concentrations of gDNA eluted were

higher for that of 1X PBS even though these samples had higher C_q values. We theorized that this mismatch with 1X PBS was due to inhibition of the qPCR reaction due to higher amounts of contaminants present when eluting gDNA from DBSs, which was explicitly examined in subsequent sections. 1X PBS preserves red blood cells (RBCs) and their contents while the KOH/Trizma® lyses and degrades them. RBCs contain various PCR inhibitors[210], thus it was theorized that the non-lysing buffer (1X PBS) would lead to an increased C_q value. Overall, 1X PBS can be used for DBS gDNA elution, though this buffer does not have additives to stabilize DNA thus we do not recommend its use for long-term storage of eluted gDNA.

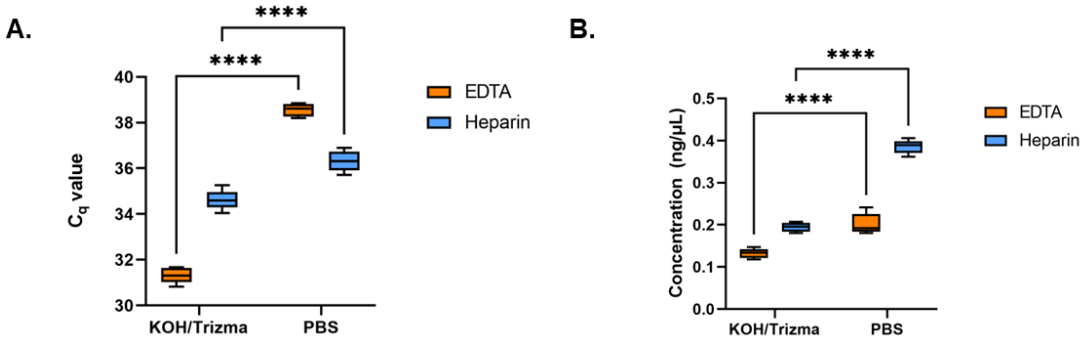

Figure 7.2: Disodium EDTA anticoagulated blood (Donor 1) and sodium heparin anticoagulated blood (Donor 2) with KOH/Trizma® vs PBS extraction. *5 replicates were tested for each number of punches and each anticoagulant.* ***(A)*** *Comparison of average C_q values for EDTA or heparin DBSs eluted with KOH/Trizma® or 1X PBS.* ***(B)*** *Comparison of average concentration of gDNA from EDTA or heparin DBSs eluted with KOH/Trizma® or 1X PBS.*

7.4.3 Electro-DBS

Building upon previous work from our group by Machado et al. [205], we decided to investigate the effect of an applied electric field on the DBSs to see if it sped up DNA extraction. To do so, we developed the device that is shown in Figure 7.3A. To use this device, the user places a 6 mm dried blood spot punch between the two platinum electrode plates, and then places the device into a solution in one well of a 96-well plate. The electric field is applied via an external direct current (DC) power supply. After the electric field is applied the Electro-DBS is removed from the well and placed into another well full of ethanol to clean it.

We developed a model using COMSOL Multiphysics® of the electrodes in a well of a 96-well plate (Figure 7.3B-C). The well was modeled to have a diameter of 5 mm and a height of 10 mm. The model used the software's built-in materials instead of the true materials of the device and the solutions; we set the electrodes as copper, and the solution as water. We left a gap of 0.1 mm between the electrode plates since they do not touch. In the model the electrode plates are perfect semi-circles, but in practice they have a less uniform shape since we had to trim the originally square plates to fit them into the well and not have the electrodes touch. The electric field is shown as a surface model and with arrows. Together, these show that the electric field is non-uniform and in the center the voltage is about ½ of the voltage applied.

To better understand the transport phenomena of gDNA within the DBS punches, we estimated diffusive flux and electrical flux using a scalar analysis. When paper is wetted the pore size is altered due to changes in bonds, fiber volume, and the influence of capillary forces [220]. In the absence of an electric field (or heat or shaking) diffusive flux (J_D) is what causes the gDNA to be eluted into the elution solution (Equation 7.1).

$$J_D \sim D_f \frac{c}{l} \qquad \textit{(Equation 7.1)}$$

where l is the thickness of the membrane, c is the concentration of gDNA, and D_f is the fractal diffusion of gDNA through the pores (Equation 7.2):

$$D_f = \frac{kT}{\zeta} \qquad \textit{(Equation 7.2)}$$

where ζ is the friction of coefficient through the pores, k is the Boltzmann constant and T is temperature of the membrane.

When an electric field is applied, electric flux is introduced (Equation 7.3):

$$J_E = \mu * E * c \qquad \textit{(Equation 7.3)}$$

where μ is the electrophoretic mobility of gDNA (Equation 7.4) and E is the electric field:

$$\mu = \frac{q_{net}}{\zeta} \qquad \textit{(Equation 7.4)}$$

Thus, the total flux when an electric field is applied is J_E + J_D. To estimate the magnitude of J_E to J_D we divided J_E by J_D (Equation 7.5). The ratio of convective flux, driven by the electric field, and diffusive flux can be described by the modified Peclet's number Pe_E:

$$Pe_E = \frac{J_E}{J_D} = \frac{-q_{net} * E * l}{kT} = \frac{\mu E l}{D_f} \sim 10^8 \qquad \textit{(Equation 7.5)}$$

We approximated Pe_E to be ~10^8, based on μ ~ 10^{-4} m^2/Vs [221], E* l = 6 V, and D_f ~10^{-12} m/s [214]. This shows that the migration of DNA is dominated by the electric flux. It is important to note that diffusion of DNA through interconnected pores are essential before DNA get swept out of the matrix via electric forces, which therefore should lead to an increased extraction of gDNA in when an electric field is applied to a DBS punch (Figure 7.3D). This equation does not consider the complexity of blood and its lysate or the bonds between gDNA molecules and the paper. In the manual protocol heat (70°C) is applied, which hemolyzes the RBCs [211] and partially degrades or destabilizes proteins like hemoglobin [212]. RBC and protein destabilization both occur in the presence of an

electric field as well [222], [223]. Thus, we experimentally evaluated the effect of applying an electric field to the DBSs with two different elution buffers, KOH/Trizma® and 1X PBS, to see if this method can simplify and speed up gDNA elution from DBSs while yielding high-quality gDNA.

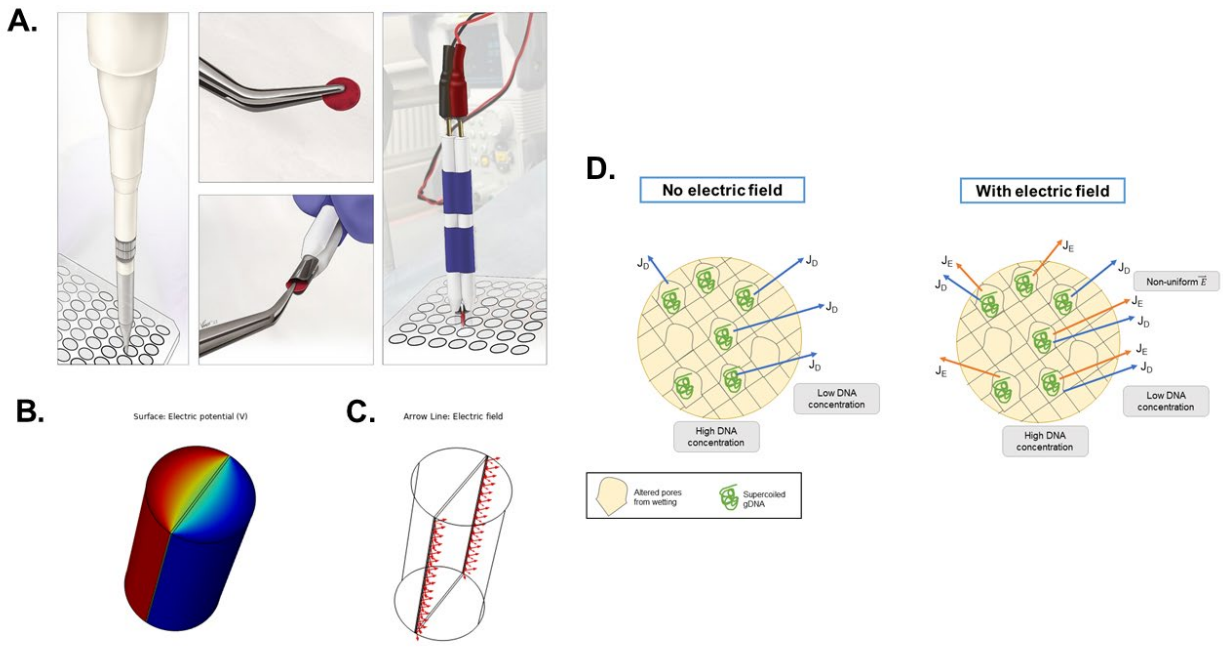

Figure 7.3: Electro-DBS, COMSOL Multiphysics® model of the electric field, and depiction of the fluxes acting on the punch. (*A*) *The Electro-DBS method setup. A 1 x 6 mm DBS punch is placed between two platinum electrodes and placed into a well of a 96-well plate.* ***(B-C)*** *Electric potential model, surface and lines.* ***(D)*** *A depiction of the fluxes acting on the punch.*

7.4.4 Electro-DBS with KOH/Trizma® and 1X PBS

To first establish an appropriate volume of solution to use in the device, we simply used a volume that would not overflow when the electrodes were placed into the well, yet also covered the dried blood spot entirely when the device was placed into the well. This volume was 200 μL for the 96-well plate we used. Next, taking into account the results

from our group's prior work[205], we faced the front of the DBS (the side in which the blood is placed onto), which is typically darker in color than the back, towards the positive electrode or the negative electrode. Machado et al. found that facing the electrode towards the positive electrode had a significantly better yield than facing it towards the negative electrode. This was due to the electrophoretic effects of the electric field on the DBS in a uniform electric field. Since the electric field in the circular well used here was not a uniform electric field (Figure 7.3B-C), we did not expect to see as stark of a difference in DNA yield between DBS orientations as Machado et al. did. We set a time of 5 min as the length of time to apply the electric field. To establish the appropriate voltage to use, we used a DBS punch to test various voltages in 1 V increments and picked the highest one that did not create too many bubbles (which would evaporate the eluate) within 5 min of applying the voltage.

After establishing the operational parameters, we tested KOH/Trizma® and 1X PBS buffers with the electrical device. All experiments were performed at room temperature in a temperature-controlled environment, so at 20-25 ^{o}C. For KOH/Trizma® we found the optimal voltage to be 6 V and for 1X PBS to be 3 V. This difference in voltage was due to the ion content difference between the two buffers, as 1X PBS was measured to have a conductivity of 17.17mS/cm and KOH/Trizma® had a conductivity 2.72mS/cm. We also tested changing the direction of the DBS punch such that the top was facing the negative electrode instead of the positive one; this is indicated by a negative sign in our results. We tested the opposite direction to see if we would see a drastic difference in yield as the work of Machado et al. showed. As shown in Figure 4, we measured performed qPCR and the Picogreen™ assay to quantify gDNA yield directly from the eluate.

In Figure 7.4A we tested KOH/Trizma® as the buffer and used DBSs from Donor 2, which was heparin anticoagulated. We tested 0 V (no electric field applied, just

incubated in the solution for 5 minutes) and 6 V and compared them to the manual protocol. There was not a statistically significant difference between the test conditions in terms of average C_q value and average concentration. In Figure 7.4B we once again tested KOH/Trizma® as the buffer, but used DBSs from Donor 1, which was EDTA anticoagulated. We tested 0 V, 6 V, and -6 V and found that in increasing order 6 V had the lowest C_q value, followed by -6 V, manual, and 0 V with the highest. There was not a statistically significant difference in the average C_q values of the manual protocol and 6 V. -6 V had a statistically significantly higher average C^q value compared to the manual protocol. For the Picogreen™ assay, there was only a statistically significant difference in yield between 6 V and 0 V, with 6 V having a higher yield. Taken together, for KOH/Trizma® Electro-DBS yielded lower C_q values and similar or higher concentration yields of DNA extracted from DBSs compare to the manual protocol. We also tested a longer application of 6 V for Donor 2, for 10 min in KOH/Trizma®, which led to a higher C_q value and lower concentration of gDNA eluted (Figure SI 7.1) than 0 V. We theorize this is due to the electric field negatively affecting the stability of the gDNA [222], [223].

Figure 7.4C and Figure 7.4D show the results of using Electro-DBS with 1X PBS, applying 0 V, 3 V, or -3 V. Figure 7.4C used DBSs made with heparin anticoagulated blood, and found that 3 V yielded the lowest C_q value, followed by the manual protocol, and the 0 V condition did not amplify sufficiently within 40 cycles. For concentration, however, this result is reversed in which 3 V had the lowest gDNA yield, followed by 0 V, and the manual protocol had the highest yield. For Donor 1 (Figure 7.4D), 3 V and -3 V had the lowest C_q value, followed by the manual protocol, and the 0V condition did not amplify sufficiently within 40 cycles. Like Donor 2, the concentration results were reversed, where 3 V and -3 V had the lowest concentration, then 0 V, and the manual protocol had

the highest yield. Taken together, for 1X PBS, Electro-DBS yields lower C_q values than the manual protocol but not a higher concentration.

Overall, Electro-DBS yielded lower C_q values than the manual protocol. For KOH/Trizma®, the reversed direction of the DBS punch led to a higher C_q value, but similar concentration of gDNA eluted compared to the positive electrode-facing direction. As previously mentioned, the electric field here is not uniform, so the electrophoretic effects are not uniformly occurring for DNA elution toward a singular electrode. Therefore, for DBS gDNA extraction using Electro-DBS, it can generally be stated that the positive electrode facing direction is best, but the opposite direction is also acceptable. Also, for KOH/Trizma® the concentrations measured with the Picogreen™ assay generally aligned with the C_q values, i.e., the replicates with higher concentrations had lower C_q values. However, for EDTA the statistical difference in C_q values between experimental groups was greater than the measured concentrations.

When using 1X PBS for extraction, the C_q values and concentration measurements, they seemingly opposed each other, i.e., samples with higher concentrations had higher C_q values. Since 0V had a high concentration, but did not amplify within 40 PCR cycles, the stabilization of the RBCs and their contents was detrimental. The application of the electric field destabilizes the RBC contents[222], [223], allowing for a more successful qPCR reaction. Additionally, the 1X PBS results show an advantage of the Electro-DBS-eluted since the manual protocol performed worse than the electrical conditions despite having a significantly higher gDNA concentration yield. Due to the additional electric flux via the applied electric field, the gDNA elutes out of the paper faster and the non-charged contaminants elute more slowly. In other words, using Electro-DBS, less contaminants elute into the sample due to the shortened elution time, improving the quality of gDNA eluate despite a lower concentration of gDNA being eluted.

Considering the larger difference between the C_q values for the KOH/Trimza®-eluted EDTA DBSs and the results for 1X PBS-eluted DBSs, we evaluated the presence of inhibitory molecules in our experimental groups.

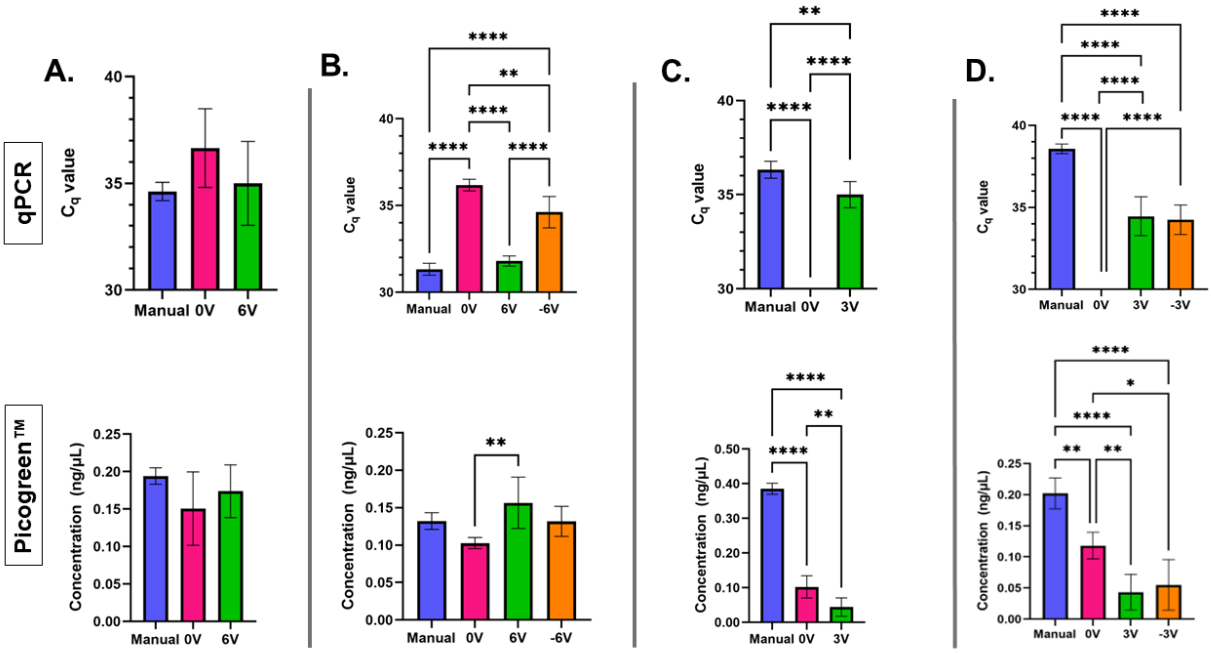

Figure 7.4: qPCR and Picogreen™ measurements from gDNA eluates of 5-min application of an electric field to 1 x 6mm DBS punches in either KOH/Trizma® or 1X PBS, from Donor 1 or Donor 2. *qPCR results are shown in the top row and Picogreen™ assay results are in the bottom row. 5 samples were tested for each condition. The manual protocol is protocol 6. The negative sign indicates a change in which electrode the top of the DBS punch faces, which is toward the negative electrode.* ***(A)*** *KOH/Trizma® for sodium heparin (Donor 2).* ***(B)*** *KOH/Trizma® for disodium EDTA (Donor 1).* ***(C)*** *1X PBS for sodium heparin (Donor 2).* ***(D)*** *1X PBS for disodium EDTA (Donor 2).*

7.4.5 Purity of Electro-DBS-eluted gDNA using Nanodrop™ 260/280 and 260/230 ratios

We sought to analyze the purity of the gDNA obtain using the Electro-DBS by first comparing using the gold standard, Nanodrop™ 260/280 and 260/230 ratios of the

samples in Figure 7.4 to each other. 260/280 and 260/230 ratios are ratios of absorbance at 260nm and 280nm or 260nm and 230nm and are found when analyzing a nucleic acid sample with spectrophotometry. DNA absorbs at 260 nm, while proteins, phenol, EDTA, guanidinium salts, carbohydrates, or other contaminants absorb around 280 nm or 230 nm. For the most “pure” DNA samples, a ratio of ~1.8 is expected for 260/280, and a ratio of ~2 is expected for 260/230 [224].

Examining at the results in Figure 7.5, the 260/280 ratios for KOH/Trizma® for both donors were higher than 1.8, but high 260/280 ratios are not necessarily indicative of an issue with the sample [224]. However, the 260/230 ratios for both buffers were generally significantly lower than 2. This implies that with both buffers, similar contaminants absorbing at 230 nm are present. Looking more closely at heparin DBSs eluted using KOH/Trizma® (Figure 7.5A), the manual protocol had the more ideal 260/280 ratio while the 6 V protocol had the more ideal 260/230 ratio. These results help to explain Figure 4A, as the 0 V had the worst 260/280 values and 260/230 values, and lowest concentration to have the highest C_q (though not statistically significant). Additionally, 6 V and manual were similar in terms of C_q because the 6 V had a better 260/230 but worse 260/230 than that of the manual protocol, so they “equaled out” with similar overall contamination from different sources. For EDTA DBSs eluted with KOH/Trizma® (Figure 7.5B), 0 V had the least ideal 260/280 and 260/230 ratio of the groups tested, explaining why its C_q value was more significantly lower than the other groups, despite the concentration not being significantly lower (Figure 7.4B). -6 V had a more statistically significant difference from the manual protocol in terms of 260/280 ratio, which explains why these samples differed more in terms of C_q than concentration in Figure 7.4B.

When using 1X PBS to elute the gDNA from DBSs, for both donors the 260/230 ratios were not significantly different across the conditions tested (Figure 7.4C-D). The

260/280 ratios, however, help to explain the results in Figure 7.4C-D. 3 V (and -3 V for disodium EDTA DBSs) had the most ideal 260/280 ratio, followed by manual and 0 V. Thus, despite the higher concentration of gDNA eluted from the manual protocol, since the purity of the sample was lower, the gDNA did not amplify as efficiently due to inhibition of the reaction.

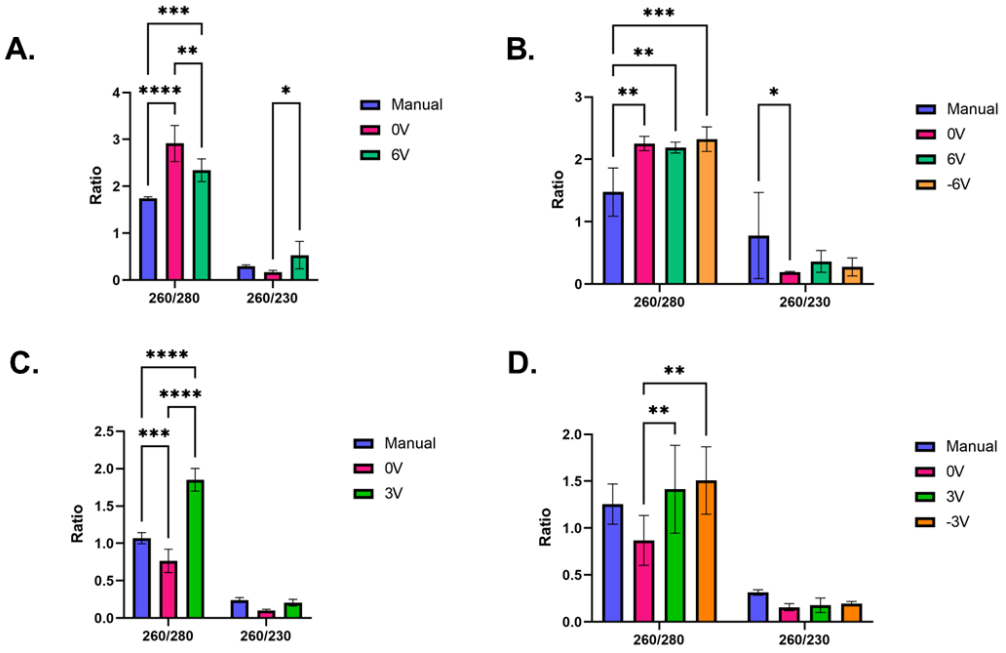

Figure 7.5: Comparison of 260/280 and 260/230 values for each non-electric and Electro-DBS condition. *2 technical replicates for each of the 5 samples were averaged.* ***(A)*** *KOH/Trizma® for sodium heparin (Donor 2).* ***(B)*** *KOH/Trizma® for disodium EDTA (Donor 1).* ***(C)*** *1X PBS for sodium heparin (Donor 2).* ***(D)*** *1X PBS for disodium EDTA (Donor 2).*

These results show that similar impurities are present when using either buffer. Due to the complexity of blood and blood lysate, the specific contaminants are difficult to identify and would need further analysis. Additionally, similar conclusions are drawn about the direction of the punch, that towards the positive is better for gDNA purity, but towards

the negative is also acceptable. These results also aid in explaining the comparison between the C_q values and concentration measurements of the samples in Figure 7.4, specifically when using 1X PBS. The ratios are less favorable for the manual protocol compared to the electrical conditions, leading to increased inhibitor presence which affects the qPCR reaction efficiency. We sought to further investigate and characterize the effect of the eluted samples on the qPCR reaction efficiency in the following section.

7.4.6 Analysis of qPCR inhibition with diluted Electro-DBS-eluted gDNA

qPCR is a well-studied technique that has been used for decades. Assuming perfecting doubling of the number DNA template molecules (N_0), the equation for the number of target molecules after c cycles (N_c) is (Equation 7. 6):

$$N_c = N_0 2^c \qquad \textit{(Equation 7.6)}$$

The perfect doubling in every cycle of qPCR is not likely, thus the PCR efficiency is also considered in (Equation 7.7):$N_c = N_0(1+E)^{(c-1)}$ *(Equation 7.7)*

The efficiency is calculated by (Equation 7.8), in which the slope is found from the slope of a line where y = C_q values of the dilutions and x = $\log_{10}$(diluted concentration).

$$E = 10^{(-1/slope)} - 1 \qquad \textit{(Equation 7.8)}$$

Therefore, when performing a 10-fold dilution series the slope, or difference in C_q values between each dilution, assuming E = 100% is -3.3. The efficiency (E) depends on several factors such as the primers and the purity of the starting sample [219]. Thus, we investigated the inhibition of the qPCR efficiency based on this concept.

To evaluate the effect of the Electro-DBS on qPCR efficiency we compared the difference between a non-diluted and a 1:10 diluted sample for each replicate of the experimental groups:

$$\Delta diluted\ C_q = (diluted\ C_q\ value) - (nondiluted\ C_q\ value) \qquad \textit{(Equation 7.9)}$$

The efficiency of the reaction was evaluated with each qPCR reaction plate using purified gDNA to make a standard curve, and all reactions were E = ~110%. In other words, the efficiency was not decreased due to the primer design. The result of this analysis is shown in Figure 7.6. It should be noted that except for all the manual samples and all samples in Figure 7.6B, the samples tested were from the same donor, but from DBSs stored for a longer time. Thus, the age of the samples may influence the variability in the elutions, especially for the heparin DBSs.

Overall, the results support that of Figure 7.4 and Figure 7.5,with similar trends regarding the amount of inhibition for each experimental group. All the heparin DBSs eluted with KOH/Trizma® had similar levels of inhibition. Interestingly, the gDNA of disodium EDTA DBSs eluted with KOH/Trizma® manually had the most inhibition, though there was a high amount of variability within this group of eluates. For both anticoagulants when using 1X PBS to elute gDNA, the manual protocol had the most inhibition, which once again explains the mismatch in the high concentration, yet lower C_q value trends in Figure 7.4C-D.

Generally, the differences in C_q between non-diluted and diluted samples were not ~3.3 cycles, which was expected considering both the non-diluted and diluted samples do not contain purified gDNA. However, this data strengthened our theory of increased inhibition of qPCR with the manual protocol when using 1X PBS compared to with Electro-DBS, due to increased diffusive flux of contaminants over a longer elution time. Overall, Electro-DBS was shown to be a method to reduce the time of gDNA elution, reduce the amount of PCR inhibitors in gDNA eluted from DBSs, and to simplify gDNA elution. This work is a significant step to reducing the burden of DBS nucleic acid extraction, while maintaining the quality of nucleic acids obtained.

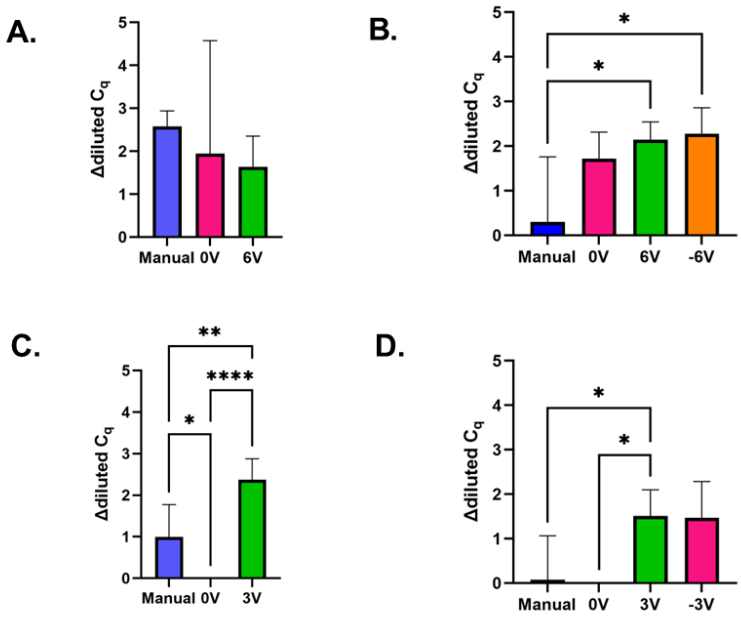

Figure 7.6: Evaluation of difference in C_q values of 1:10 diluted to non-diluted samples for each non-electric and Electro-DBS condition. ***(A)*** *KOH/Trizma® for sodium heparin (Donor 2).* ***(B)*** *KOH/Trizma® for disodium EDTA (Donor 1).* ***(C)*** *1X PBS for sodium heparin (Donor 2).* ***(D)*** *1X PBS for disodium EDTA (Donor 2).*

7.5 Conclusions

This work developed a device that uses an electric field to extract DNA from DBSs, that we termed Electro-DBS, to reduce the burden of DBS nucleic acid extraction. Using our novel device, we were able to reduce the time to extraction from 40-minute multi-step protocol to 5 minutes. With our device we also eliminated the need for complex equipment like a plate shaker and device to heat the DBS punches. The voltage that is applied is also low, which indicates that we could power our tool with a battery, which is great for the point of care. The device is also re-usable, as one can clean the electrode plates off with ethanol or bleach and then use them in another well of a 96-well plate. In terms of a buffer for gDNA extraction, 1X PBS or KOH/Trizma® could be used, with the latter better for DNA

samples that need to be stored long term. Our method could easily adaptable to different voltages and buffers as well. The gDNA eluted with Electro-DBS from DBSs of healthy human whole blood had comparable or better purity and C_q values to a manual protocol. Our method does not require a buffer that lyses cells for successful gDNA elution, which means that this method is more accessible to a variety of users since a specialized buffer is not needed. Since the gDNA eluted in this work was not purified before downstream processing, the contents of the buffers and blood lysate do interfere with all the DNA quantification techniques used. Despite this, the Electro-DBS system still performed as well or better than the manual protocol that was performed under comparable conditions.

There are several future directions for Electro-DBS to evaluate its impact for point of care applications. We used healthy human blood and looked at gDNA only, so its application to less abundant and differently sized DNA should be investigated. Similarly, the elution of RNA from DBSs should also be investigated. Characterizing the extraction with different hematocrit ranges also is of interest to better define the device protocol developed here and DBS extraction more generally. Electro-DBS should also be investigation for the elution of other molecules such as proteins and pharmacological compounds. Using an electric application means that there are multiple parameters that can be tuned like current, different waveforms, and more to improve yield. Overall, our work helps to increase the understanding of DBSs mechanistically and is a step towards reducing the burden of DBS nucleic acid extraction.

7.6 Acknowledgements

We would like to thank Vinald Francis for his work in Figure 7.3.

7.7 Data Availability

The raw data that support the findings of this study are available from the following link:

https://doi.org/10.7910/DVN/JGJQTT .

7.8 Supporting Information

7.8.1 Detailed statistical analyses for each figure

Figure 7.1B: Comparison of C_q values for a 1 x 6mm punch for EDTA and heparin anticoagulated blood. *To compare the mean differences between average C_q values for the number of punches as well as the anticoagulant type ($\overline{C_q}$ = 30.91 for disodium EDTA and $\overline{C_q}$ = 32.03 for sodium heparin) an unpaired t-test was performed. There was a statistically significant difference between the two conditions with p = 0.02.*

Figure 7.1C: Comparison of concentration for a 1 x 6mm punch for EDTA and heparin anticoagulated blood. *To compare the mean differences between average concentrations for the number of punches as well as anticoagulant type (avg. conc. = 0.11 ng/µL for disodium EDTA and 0.09 ng/µL for sodium heparin), an unpaired t-test was performed. There was a statistically significant difference between the two conditions with p = 0.048.*

Figure 7.2A: Comparison of C_q value for KOH/Trizma® and PBS with EDTA and Heparin DBSs. *To compare the mean differences between average C_q values for the buffer as well as the anticoagulant type (for KOH/Trizma®: $\overline{C_q}$ = 31.33 for disodium EDTA and $\overline{C_q}$ = 34.62 for sodium heparin; for 1X PBS: $\overline{C_q}$ = 38.57 for disodium EDTA and $\overline{C_q}$ = 36.32 for sodium heparin), two-way ANOVA and Tukey's multiple comparisons tests were performed. The mean differences between all comparisons were p < 0.0001.*

Figure 7.2B: Comparison of concentration for KOH/Trizma® and PBS with EDTA and Heparin DBSs. *To compare the mean differences between average concentrations for the buffer as well as anticoagulant type (for KOH/Trizma®: avg. conc. = 0.13 ng/µL for disodium EDTA and 0.19 ng/µL for sodium heparin; for 1X PBS: avg. conc. = 0.2 ng/µL for disodium EDTA and 0.39 ng/µL for sodium heparin), two-way ANOVA and Tukey's multiple comparisons test were performed. There was no statistically significant difference between the average concentrations of KOH/Trizma® heparin and PBS EDTA DBSs. The mean differences between all comparisons were p ≤ 0.0001.*

Figure 7.4A: KOH/Trizma® for sodium heparin (Donor 2). *To statistically compare the mean values of the manual ($\overline{C_q}$ = 34.62; avg. conc. = 0.19ng/µL), 0V ($\overline{C_q}$ =36.65; avg. conc. = 0.15ng/µL), or 6V ($\overline{C_q}$ = 35; avg. conc. = 0.17ng/µL), conditions to each other, one-way ANOVA with Tukey's multiple comparisons test was performed. The mean difference for all comparisons was not statistically greater than 0, for both C_q values and concentrations.*

Figure 7.4*B:* KOH/Trizma® for disodium EDTA (Donor 1). *To statistically compare the mean values of the manual, 0V, 6V and -6V conditions to each other, one-way ANOVA with Tukey's multiple comparisons test was performed. For C_q values: the mean difference between manual ($\overline{C_q}$ = 31.33) and 0V ($\overline{C_q}$ = 36.17), manual and -6V ($\overline{C_q}$ = 34.62), 0V and 6V ($\overline{C_q}$ = 31.81), 0V and -6V, and 6V and -6V were statistically greater than 0, where p <*

0.0001, p < 0.0001, p < 0.0001, p = 0.001, and p < 0.0001 respectively. For average concentration measurements: the mean difference between 0V (avg. conc. = 0.10ng/μL) and 6V (avg. conc. = 0.16ng/μL) was statistically greater than 0 with p = 0.0043. The mean difference between all other comparisons were not statistically significant. -6V (avg. conc. = 0.13ng/μL) and manual (avg. conc. = 0.13ng/μL).

Figure 7.4*C:* 1X PBS for sodium Heparin (Donor 2). T*o statistically compare the mean values of the manual, 0V, or 3V conditions to each other, one-way ANOVA with Tukey's multiple comparisons test was performed. For C_q values: the mean difference between manual ($\overline{C_q}$ = 36.32) and 0V ($\overline{C_q}$ = N/A), manual and 3V ($\overline{C_q}$ = 35) and 0V and 3V, were statistically greater than 0, where p < 0.0001, p = 0.0026, and p < 0.0001, respectively. For average concentration measurements: the mean difference between manual (avg. conc. = 0.39ng/μL) and 0V (avg. conc. = 0.1ng/μL), manual and 3V (avg. conc. = 0.04ng/μL), and 0V and 3V were statistically greater than 0 with p < 0.0001, p < 0.0001, and p = 0.01, respectively.*

Figure 7.4*D:* 1X PBS for disodium EDTA (Donor 1). T*o statistically compare the mean values of the manual, 0V, or 3V conditions to each other, one-way ANOVA with Tukey's multiple comparisons test was performed. For C_q values: the mean difference between manual ($\overline{C_q}$ = 38.57) and 0V ($\overline{C_q}$ = N/A), manual and 3V ($\overline{C_q}$ = 35), manual and -3V ($\overline{C_q}$ = 34.25), 0V and 3V, and 0V and -3V were statistically greater than 0, where p < 0.0001 for all. For average concentration measurements: the mean difference between manual (avg. conc. = 0.20ng/μL) and 0V (avg. conc. = 0.12ng/μL), manual and 3V (avg. conc. = 0.04ng/μL), manual and -3V (avg. conc. = 0.06ng/μL), 0V and 3V, and 0V and were statistically greater than 0 with p = 0.002, p < 0.0001, p < 0.0001, p = 0.006, and p = 0.02, respectively.*

Figure 7.5A: Purity of KOH/Trizma® for sodium heparin (Donor 2*).* *To statistically compare the mean ratios of the manual, 0V, and 6V conditions to each other, two-way ANOVA with Tukey's multiple comparisons test was performed. For 260/280: the mean difference between manual (1.74) and 0V (2.91), manual and 6V (2.34), and 0V and 6V were statistically greater than 0, where p < 0.0001, p = 0.0007 and p = 0.0012, respectively. For 260/230: the mean difference between 0V (0.17) and 6V (0.53) was statistically greater than 0 with p = 0.04. For the manual protocol the 260/230 ratio was 0.3.*

Figure 7.5B: Purity of KOH/Trizma® for sodium EDTA (Donor 1*).* *To statistically compare the mean ratios of the manual, 0V, 6V, and -6V conditions to each other, two-way ANOVA with Tukey's multiple comparisons test was performed. For 260/280: the mean difference between manual (1.48) and 0V (2.25), manual and 6V (2.19), and manual and -6V (2.32) were statistically greater than 0, where p = 0.0013, p = 0.0022 and p = 0.0005, respectively. For 260/230: the mean difference between manual (0.78) and 0V (0.19) was statistically greater than 0 with p = 0.02. For 6V and -6V, the average 260/230 ratio was 0.36 and 0.28, respectively.*

Figure 7.5C: Purity of PBS for sodium heparin (Donor 2). *To statistically compare the mean ratios of the manual, 0V, and 3V conditions to each other, two-way ANOVA with Tukey's multiple comparisons test was performed. For 260/280: the mean differences between manual (1.07) and 0V (0.77), manual and 3V (1.85), and 0V and 3V were statistically greater than 0, where p = 0.0001, p < 0.0001 and p < 0.0001, respectively. For 260/230: the mean differences between manual (0.24), 0V (0.1) and 3V (0.21) were not statistically greater than 0.*

Figure 7.5D: Purity of PBS for disodium EDTA (Donor 1). *To statistically compare the mean ratios of the manual, 0V, 3V, -3V conditions to each other, two-way ANOVA with Tukey's multiple comparisons test was performed. For 260/280: the mean differences between 0V (0.87) and 3V (1.41) and 3V and -3V (1.51) were statistically greater than 0, where p = 0.0065, and p = 0.0012, respectively. For the manual protocol the average 260/280 ratio was 1.26. For 260/230: the mean differences between manual (0.31), 0V (0.16), 3V (0.18) and -3V (0.2) were not statistically greater than 0.*

Figure 7.6A: Δ diluted C_q of KOH/Trizma® for sodium heparin DBSs (Donor 2). *To statistically compare the mean ratios of the manual, 0 V, and 6 V conditions to each other, two-way ANOVA with Tukey's multiple comparisons test was performed. Manual: Δ = 2.58; 0 V: Δ = 1.94; 6 V: Δ = 1.53. The mean difference between all comparisons were not statistically greater than 0.*

Figure 7.6B: Δ diluted C_q of KOH/Trizma® for disodium EDTA DBSs (Donor 2). *To statistically compare the mean ratios of the manual, 0 V, 6 V, and -6 V conditions to each other, two-way ANOVA with Tukey's multiple comparisons test was performed. Manual: Δ = 0.31; 0 V: Δ = 1.73; 6 V: Δ = 2.15; -6 V: Δ =2.28. The mean difference between the following were not statistically greater than 0: manual and 0 V; 0 V and 6 V; 0 V and -6 V; 6 V and -6 V. The mean differences between the following were statistically greater than 0: manual and 6 V (p = 0.02) and manual and -6 V (p = 0.01).*

Figure 7.6C: Δ diluted C_q of 1X PBS for disodium EDTA (Donor 2). *To statistically compare the mean ratios of the manual, 0 V, and 3 V conditions to each other, two-way ANOVA with Tukey's multiple comparisons test was performed. Manual: Δ =0.99; 0 V: Δ = N/A; 3 V: Δ = 2.37. The mean differences between all comparisons were statistically greater than 0: manual and 0 V (p = 0.03); manual and 3 V (p = 0.004); and 0 V and 3 V (p < 0.0001).*

Figure 7.6D: Δ diluted C_q of 1X PBS for disodium EDTA DBSs (Donor 2). *To statistically compare the mean ratios of the manual, 0 V, 3 V and -3 V conditions to each other, two-way ANOVA with Tukey's multiple comparisons test was performed. Manual: Δ =0.08; 0 V: Δ = N/A; 3 V: Δ = 1.51; -3 V: Δ =1.47. The mean difference between the following were not statistically greater than 0: manual and 0 V; manual and -3 V; 0 V and -3 V; 3 V and -3 V. The mean differences between the following were statistically greater than 0: manual and 3 V (p = 0.04) and 0 V and 3 V (p = 0.03)*

7.8.2 Variations of time of electric field application with the Electro-DBS device

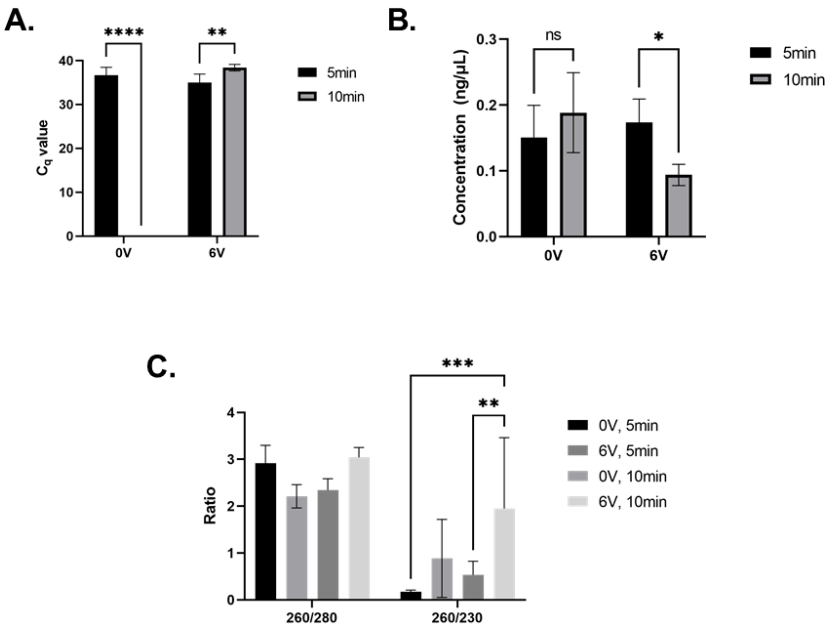

Figure SI 7.1: 5min and 10min of electric field application using sodium heparin anticoagulated blood (Donor 2) in KOH/Trizma®. *5 samples were tested for each condition.* **(A)** C_q values. *To compare the mean C_q value of the mixtures to each other, two-way ANOVA and Šídák's multiple comparisons test were performed. The mean difference between 6V for 5min versus 10min was statistically greater than 0, with p = 0.0025. The mean difference between 0V for 5min versus 10min was statistically greater than 0, with p < 0.0001.* ***(B)*** *Picogreen™ concentration measurement. To compare the mean concentration of the mixtures to each other, two-way ANOVA and Šídák's multiple comparisons test were performed. The mean difference between 6V for 5min versus 10min was statistically greater than 0, with p = 0.02. The mean difference between 0V for 5min versus 10min was not statistically greater than 0.* ***(C)*** *260/280 and 260/230 purity measurements. To compare the mean difference between the test conditions for 260/280 and 260/230 to each other, two-way ANOVA and Tukey's multiple comparisons test were performed. For 260/280, the mean difference between the test conditions was not statistically greater than 0. For 260/230, the mean difference between 0V for 5min and 6V for 10min and 0V for 10min vs 6V for 10min was statistically greater than 0, with p = 0.0008 and p = 0.008, respectively.*

CHAPTER 8: INVESTIGATION OF THE ELECTRO-DBS METHOD FOR TOTAL RNA EXTRACTION FROM DRIED BLOOD SPOTS

8.1 Introduction

Dried blood spots (DBSs) are a method for storage of blood for longer periods of time in general and at room temperature. They are also used as a method for diagnosis and can store multiple molecules found in the blood such as antibodies, DNA, RNA, and proteins. As discussed in previous chapters, extraction of nucleic acids from DBSs is a process that can be done manually or with commercially available devices. RNA extraction from dried blood spots has most often been performed with HIV RNA and hepatitis virus (B and C) for viral load analyses [199], [225], [226]. To be able to use DBSs for detection and monitoring of viral load of these conditions is extremely beneficial not only for resource limited settings, but also generally to reduce the burden of sampling for patients since samples may have to be taken frequently for monitoring.

To be used for quantification, RNA extracted from dried blood spots must meet specific standards such as leading to sensitive and specific results [199]. Therefore the process for extraction must yield high quality RNA and high quality results. Though using paper is a newer method for sample storage, a systematic review by Vojnov et al. demonstrated that this method is comparable for use in determining HIV treatment failure threshold (1000 copies/mL) as plasma samples [227]. Many of these samples were analyzed with commercially available devices such as that from Abbott and Roche, which not all laboratories have access to. Therefore, some researchers have developed manual methods to extract RNA from DBSs and generally from paper.

Most of the protocols that use manual methods to extraction RNA from DBSs use the NucliSENS® magnetic bead purification protocol, which is described later in this chapter. Briefly, these protocols will agitate the sample in a buffer (such as the NucliSENS® lysis buffer) and then perform the magnetic bead purification protocol. This can be time consuming and require several types of equipment such as plate shakers and incubators.

Researchers have explored the extraction of RNA from paper without the use of multiple instruments. One of the few devices that have been designed for RNA extraction from whole blood was developed by Kolluri et al. They successfully showed recovery of malaria genomic DNA and HIV RNA in an instrument-free manner, but this method required several steps and reagents [228]. Thus, we aimed to similarly simplify the process of RNA extraction from paper by investigating the application of Electro-DBS to RNA extraction from whole blood DBSs.

8.2 Materials and Methods

8.2.1 Human whole blood

Human male whole blood was purchased from Research Blood Components, LLC (Watertown, Massachusetts, USA). The blood samples contained either sodium heparin or disodium EDTA anticoagulants. We refer to disodium EDTA anticoagulated blood as "EDTA"**.** The samples were tested for common infectious diseases by Research Blood Components, LLC and were stored at 4 ^{o}C upon arrival.

8.2.2 HIV-1 RNA and HIV-1 RT-qPCR probe

Quantitative Synthetic Human immunodeficiency virus (HIV-1) RNA (item #VR-3245SD) was purchased from ATCC (Manassas, Virginia, USA). This RNA is sold to have a viral load of ≥ 1 x 10^5 to 1 x 10^6 copies/µL.

8.2.3 Preparation of Dried Blood Spots

Dried blood spots were prepared by pipetting 75 µL of whole blood onto each circle onto Whatman™ 903 protein saver cards (Millipore Sigma, St. Louis, Missouri, USA). Then, they were left to dry overnight at room temperature. After drying, each card was placed into a bag with a desiccant and stored at -20 ^{o}C or -80^{o}C. To reduce the impact of storage time on the results, the DBSs used in this manuscript were not stored for longer than 1 month before use.

8.3 Reagents, Instruments, and Protocols for DBS gDNA extraction (Protocols 1-5)

For nucleic acid extraction from DBSs, protocols typically require punched-out spots or the entire circle to be shaken for various amounts of time in a solution. Heat or other kinetic factors may also be applied to increase the extraction yield. Following the shaking, a nucleic acid extraction protocol is typically performed to purify the eluted nucleic acids, which are necessary not only to increase gDNA yield, but as we investigated, to also isolate the gDNA in a solution that is PCR-compatible.

Protocols 1-5 used in this chapter were also used in in Chapter 6 (Table 8.1), they each followed a similar procedure in which a dried spot punch was placed into a solution and agitated on a plate shaker for a given time, which are specified in Table 8.1. Protocol 1 required Tween® 20 and sodium azide which were obtained from Millipore Sigma (St. Louis, Missouri, USA). Protocols 1, 2, and 3 [198]–[201] required 1X Gibco™ Phosphate buffered saline (PBS), pH 7.4 was obtained from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA). Protocols 4 and 5 [201]–[204] required a Lysis Buffer and the NucliSENS® Lysis Buffer (Biomérieux, Marcy-lÉtoile, France) was used. After shaking, all these protocols required DNA purification, which was done using the NucliSENS® Magnetic Extraction Reagents (Biomérieux, Marcy-lÉtoile, France). The elution volume of the gDNA was 80 µL for all samples. As originally described, Protocol 1 was developed to obtain DBS eluates for the analysis antigens and antibodies of infectious diseases [198], [199] and thus did not include a nucleic acid purification step, but we used one here. When testing Protocols 1-5, either 2 x 6mm punches or the entire circle were used for each biological replicate (Chapter 6). For example, for 2 x 6 mm punches, the two punches would be placed into a singular tube, which was one biological replicate.

Table 8.1: 5 protocols for DBS nucleic acid extraction.

Protocol number and source	***Solution***	***Agitation Time***	***Agitation temperature***
1 [198], [199]	1mL 1X PBS/0.05% Tween-20/0.08% sodium azide	Overnight	RT
2 [201]	150 µL 1X PBS/600 µL Lysis Buffer	2 h 30 min	RT
3 [200]	1 mL 1X PBS	30 min	RT
4 [202], [203]	2 mL Lysis Buffer	1 h	RT
5 [204]	2 mL Lysis Buffer	30 min	37 °C

8.3.1 Electro-DBS

The Electro-DBS device was used as described in Chapter 7 to explore extraction of RNA from the dried blood spot paper. Briefly, a 200 µL of a buffer is pipetted into one well of a lo-bind 96-well plate; here we used either 1X PBS or RNA Elution Buffer #6 from the chemagic™ RNA Blood Kit Special H96 (PerkinElmer, Waltham, Massachusetts, USA) [229]. The Electro-DBS device is then placed into that well. Then, 1 x 6 mm punch

of a dried spot is placed between the two electrodes of Electro-DBS and a voltage is applied for 5 minutes. After 5 minutes qPCR is performed with the sample.

8.3.2 Human Peripheral Blood Leukocytes Total RNA – Purified RNA

Human Peripheral Blood Leukocytes Total RNA (ThermoFisher Scientific Inc., Waltham, Massachusetts, USA) was used for various experiments and to make a standard curve RT-qPCR.

8.3.3 Reverse transcription quantitative PCR (RT-qPCR)

Reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA) and was performed the day of RNA extraction, and the resulting cDNA was stored at -20^{O}C. Real-time PCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA). TaqMan™ Gene Expression Assay ID# Hs00168719_m1, a primer for the peptidylpropyl isomerase B (PPIB) gene was used, which is a good reference gene for human peripheral blood (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) [157]. The Taqman™ Gene Expression Master Mix (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) was used for the assay. The TaqMan™ probe used for RT-qPCR of the HIV-1 RNA was custom developed for the p24 antigen by ThermoFisher Scientific Inc. To design this, the sequence of the p24 antigen was given to a representative at ThermoFisher Scientific who developed a custom Taqman probe for our use. For each reaction, a standard curve was made with either purified human leukocyte RNA or HIV-1 RNA, depending upon the experimental RNA of interest. 3 technical replicates were averaged for each for biological replicate analyzed.

8.3.4 Determination of RNA yield & integrity

RNA yield and integrity were found using the Agilent Bioanalyzer 2100 and Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, California, USA). RNA Integrity Numbers (RINs) were calculated by the Agilent Technologies 2100 Bioanalyzer 2100 Expert software, version B.02.11.SI811.

8.3.5 Statistical analyses

Statistical analyses were performed in GraphPad Prism 8 (San Diego, California, USA) with alpha = 0.05. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$. Further information regarding statistical comparisons can be found in the Supplementary Material.

8.4 Results and Discussion

8.4.1 Exploration of the elimination of nucleic extraction cleanup steps for RNA DBS extraction

8.4.1.1 Positive controls: Protocols from across the literature

There are several dried blood spot extraction protocols in the literature for various molecules, and vary based on time for elution, temperatures used, if agitation is required, buffers used, and more. The five protocols tested here began with agitation in different solutions for varying amounts of time, followed by manual nucleic acid purification steps with reagents from NucliSENS® which have been used successfully for HIV viral RNA extraction and viral DNA extraction [203], [204], [230], [231]. Following the same protocols from Chapter 6 (Table 8.1), we measured the samples for RNA extraction. That is, total RNA and gDNA were extracted at the same time. The cycle threshold (C_q) values measured for all the protocols' eluates are shown in Figure 8.1. We chose to measure C_q values to compare the ability of the samples to amplify during PCR, which has implications in the sample quality, starting concentration, and for downstream processing [208]. There

was no significant difference in the C_q values between EDTA and heparin DBSs for Protocols 1-5, so their results were combined (Figure 8.1).

We found that when comparing the use of the 2 x 6 mm punches versus the whole circle for extraction, there was only a significant difference for Protocol 2 ($p = 0.04$). Within 2 x 6 mm punches, RNA extracted via Protocol 3 had a higher C_q value than RNA extracted via Protocol 4. For the whole circle, RNA extracted via Protocol 3 varied the most between samples and had a higher C_q value than RNA extracted via Protocol 2. From this we concluded that there were not large differences in C_q value when using 2 x 6 mm punches and a whole circle, nor between protocols. As discussed in Chapter 6 this work is important for comparing commonly varied aspects of DBS extraction protocols to work towards a standardized method for DBS extraction. This work, to our knowledge, was the first to show that gDNA and total RNA can be extracted simultaneously from one whole blood DBS sample. Simultaneous gDNA and total RNA extraction could lead to simplified protocols and reduced resources for nucleic acid DBS extraction and means that a single sample can have a maximized impact for diagnostic testing.

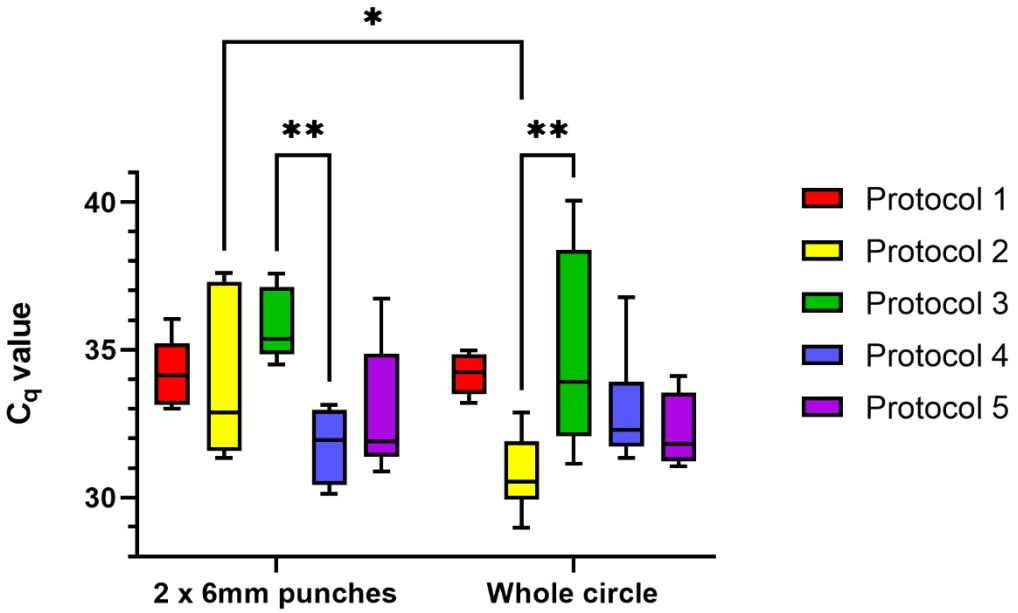

Figure 8.1: Average C_q value of RNA extraction from DBSs using Protocols 1 – 5. *6 replicates were tested for each protocol and DBS sample input type. All samples were from the same donor. Comparison of C_q values for Protocols 1-5 for 2 x 6 mm punches and whole circles.*

8.4.1.2 Viability of RT-qPCR using the buffers used in Protocols 1-5

After testing the five protocols, we wanted to see if the nucleic acid extraction steps could be skipped and perform RT-qPCR directly after the sample was agitated in its respective buffer. Thus, we mixed purified RNA with the respective buffer and performed RT-qPCR to see if the RNA would be amplified. We found that only 1X PBS led to a successful RT-qPCR reaction (Table 8.2). KOH/Trizma® is not included as this was shown to also not be compatible with RT-qPCR when tested for Figure 8.1. This showed that with 1X PBS we could begin to investigate a method to simplify the RNA DBS extraction protocol.

Table 8.2: Compatibility of buffers with RT-qPCR.

Solution (Protocol Number)	Compatible with RT-qPCR?
Tween-20 /Sodium Azide/ 1X PBS (1)	No
NucliSENS® Lysis Buffer and 1X PBS (2)	No
1X PBS (3)	Yes
NucliSENS® Lysis Buffer (4, 5)	No

8.4.1.3 Direct RT-qPCR from DBSs using Protocol 3

To investigate whether RT-qPCR could be performed from Protocol 3 despite the nucleic acid purification steps not being performed, we tested various agitation times with 1 x 6 mm punch of EDTA DBSs and then performed RT-qPCR (Figure 8.2). We tested agitation times of 5, 15, and 30 minutes (the protocol's shake time is 30 minutes) and also compared this to no agitation with the same lengths of time. We found that the C_q values of the total RNA differed greatly for 30 minutes of "no shaking" versus 15 minutes of "no shaking", and not significantly when comparing all the other conditions to each other. However, not all samples amplified within the 40 qPCR cycles, indicating that low repeatability for some conditions. For 30 minutes of shaking only two of the six samples amplified.

As is known regarding PCR, there are several inhibitors to RNA extraction in blood such as hemoglobin and IgG [127]. When using PBS, the red blood cells are not lysed as this buffer preserves red blood cells. It is not immediately clear, though, if red blood cells are lysed upon drying on DBS paper [232]. In terms of using agitation for DBS extraction, 30 minutes of agitation leads to a release of the most of the blood sample off the DBS compared to the other time points. This is clear visually when performing the experiments, but also due to the longer time placed in the buffer, the more sample that is released into the solution due to diffusion. Interestingly, the time points and shaking conditions do not differ greatly in terms of RNA yield when comparing C_q values.

The data shown in Figure 8.2 may make one consider only agitating or not agitating their samples in PBS for 5 minutes to simplify their workflows. While this is what this data demonstrates, it should also be noted that PBS will likely not be sufficient store RNA for long periods of time, as the best buffers for RNA storage are TE buffers or nuclease free water. Also, this data does not imply that this extremely simple method would be feasible for samples in which RNA is less abundant like in virally suppressed HIV patient samples. What this data does show, however, is that it could be possible to extract total RNA from DBSs in 5 minutes without agitation and just a simple “soaking” of the sample in PBS for whole blood samples, if reverse transcription is performed immediate (within a few hours of extraction). Interestingly, this did not apply for gDNA. When extracting gDNA from these samples, the yield was lower or gDNA was not successfully extracted (see link in Data Availability 8.6). Overall, this data indicated that we could explore the use of the Electro-DBS method for RNA extraction to 1) increase yield of total RNA, and 2) eventually explore the use of another buffer in which RNA is more suited to be stored in for longer times if necessary.

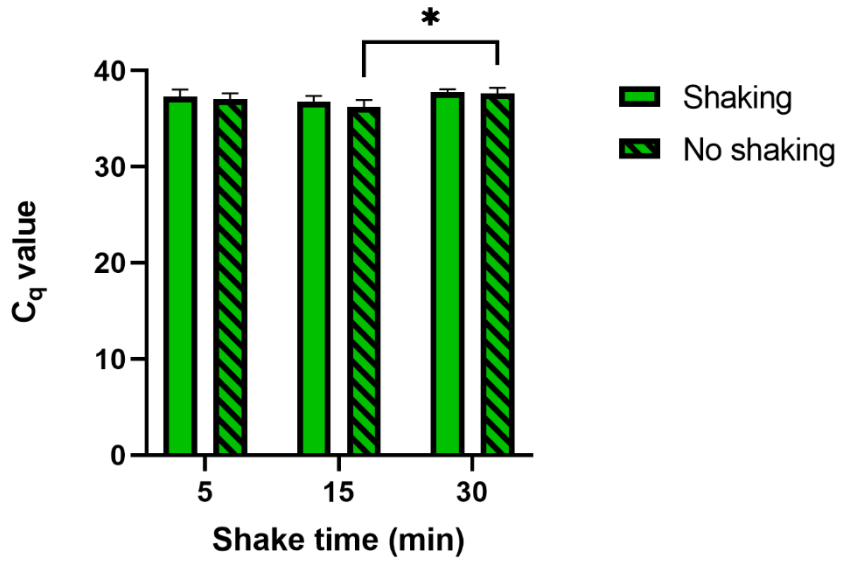

Figure 8.2: C_q values of samples eluted without nucleic acid purification using Protocol 3. *RT-qPCR was performed after 5, 15, or 30 minutes of agitation at room temperature in 1X PBS. 6 biological replicates were tested per condition, using EDTA whole blood DBSs. Not all samples amplified, the number of samples that amplified over the total number of samples that were tested are as follows: 5 minutes, shaking: 5/6; 15 minutes, shaking: 6/6; 30 minutes, shaking: 2/6; 5 minutes, no shaking: 3/6; 15 minutes, no shaking: 5/6; 30 minutes, no shaking: 5/6.*

8.4.2 Investigation of the Electro-DBS protocol with purified RNA that was not spotted onto paper

We first wanted to examine how the electrical setting used with gDNA would affect RNA in terms of degradation and/or concentration. We began with pure HIV-1 RNA, not spotted onto dried blood spot paper. However, before beginning to experiment, we first had to figure out the limit of detection of the machine we used for qPCR and of the probes we custom designed (Figure 8.3). This showed that the limit of detection was 10^4 copies/mL.

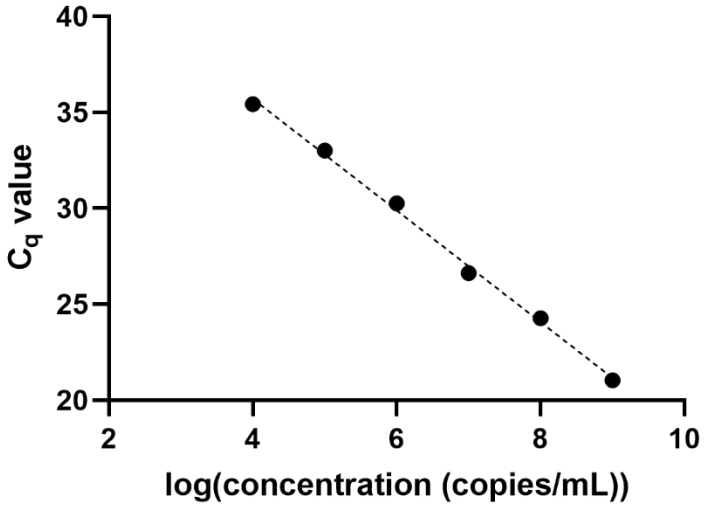

Figure 8.3: Viral load curve for HIV-1 probe. *We found that concentrations 10^9, 10^8, 10^7, 10^6, 10^5, and 10^4 copies/mL could be detected using our test conditions. R^2 = 0.997.*

We then tested the HIV-1 RNA, at 10^5 copies/mL to see if there was a specific aspect of the Electro-DBS protocol that affected RNA yield. That is, did the lo-bind 96-well plate, the electrodes, or the electrodes and electric field affect RNA yield (Figure 8.4). We initially found that the use of a lo-bind versus a not lo-bind plate was essential for RNA extraction (data not shown). We found that the electric field increased the C_q value, while the other factors did not affect the C_q value of the samples. This means that the electric field negatively impacts the RNA yielded. We then examined the use of PDMA, an agent to reduce non-specific binding of RNA to surfaces (Figure 8.5). We found that PDMA did improve the C_q value of the sample, by ~2 cycles when compared to the control. When compared to without PDMA for the 6V Electro-DBS, though the difference in C_q values was 0.7 cycles. This means that reducing the binding does have some effect on the RNA yield, but it is not the main contributing factor to the higher C_q values that were obtained when the electric field was applied.

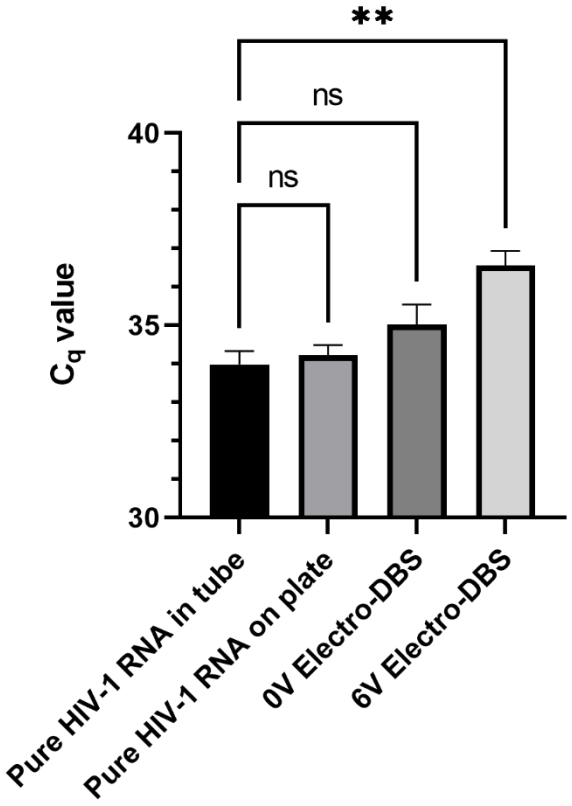

Figure 8.4: Electro-DBS effect on 10^5 copies/mL HIV-1 RNA. *Using the Electro-DBS lo-binding plate, we tested which aspect of the Electro-DBS protocol influenced RNA yield. 2 samples tested per condition.*

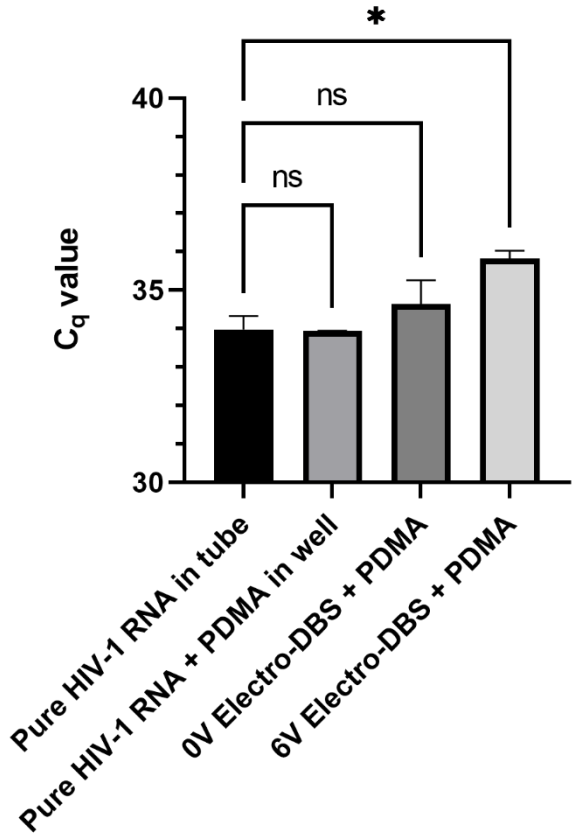

Figure 8.5: Electro-DBS effect on 10^5 copies/mL HIV-1 RNA, with 0.5% PDMA. *Using the Electro-DBS lo-binding plate, we tested if PDMA improved HIV-1 RNA yield. Only 2 samples tested per condition.*

We wanted a closer look at the RNA via the Bioanalyzer, but the concentration of the HIV-spiked samples were too low to yield quantitative results. Thus, we made larger concentrations of RNA with purified leukocyte total RNA. We made a 100 µL-solution of 40ng/µL of pure leukocyte RNA diluted in the RNA Elution/storage Buffer #6 from the Chemagic kit, pipetted the solution into a 96-well plate, and placed the Electro-DBS device into it. A lower volume than is typically used with Electro-DBS (typically 200 µL, we used 100 µL) was used to conserve the volume of the pure RNA sample. We tested similar conditions as in Figure 8.4). and Figure 8.5, by comparing C_q values of the leukocyte RNA

in tube, in a well on the plate, with the Electro-DBS device in the well at 0V and at 6V for 5 minutes (Figure 8.6). We found similar results to that of Figure 8.4). and Figure 8.5 in which the leukocyte RNA seems to be negatively affected by the electric field. We also found that the Electro-DBS device without the voltage applied reduced the quality of RNA that was eluted.

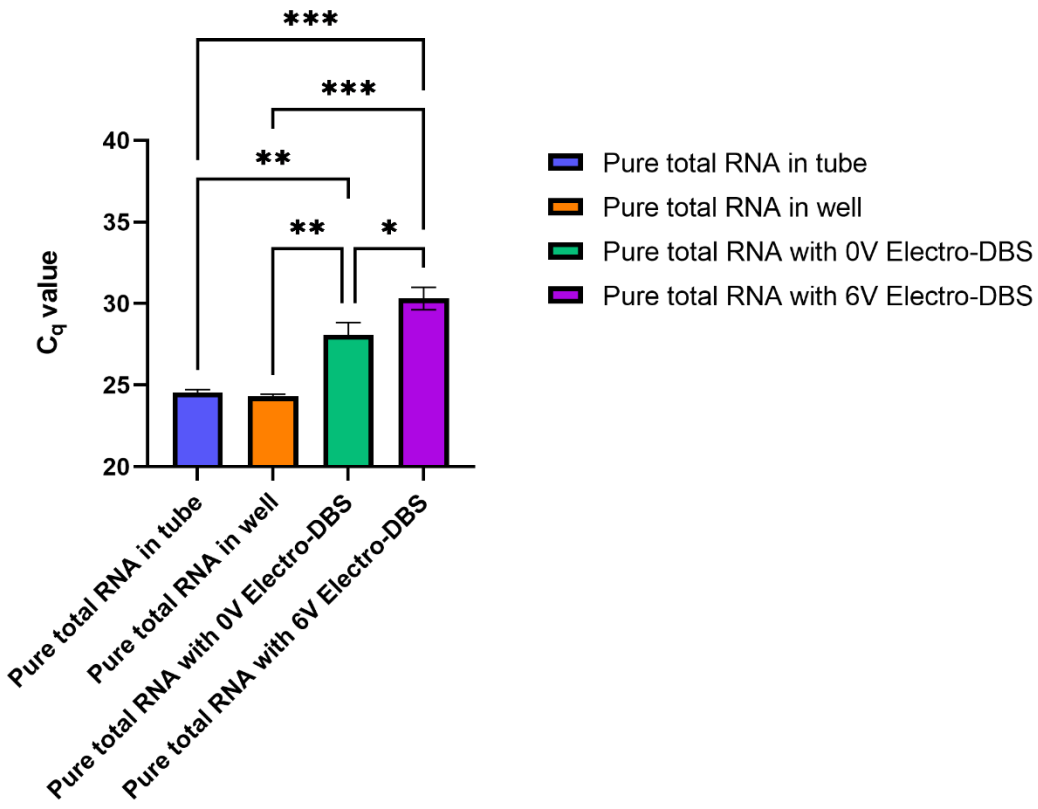

Figure 8.6: Electro-DBS protocol effect on 40ng/µL solution of leukocyte RNA. *Using the Electro-DBS on a lo-binding plate, we tested the effect of the Electro-DBS protocol on leukocyte RNA. "Pure total RNA in tube" (n = 3); "Pure total RNA in well" (n = 2); "Pure total RNA with 0V Electro-DBS" (n = 2); "Pure total RNA with 6V Electro-DBS" (n = 2).*

We then compared the Bioanalyzer concentrations and RINs of the samples to further understand why the C_q values are impacted when the electric field is used (Figure 8.7). Bioanalyzer results showed that there was not a difference in concentration nor in RIN despite the C_q values being different between experimental groups. These results have, admittedly, have very mixed concentration results, which is expected when using the Bioanalyzer (Chapter 5). Since there are limited replicates, it is difficult to make conclusive conclusions, but it appears that the voltage does not affect the RIN or concentration of the RNA eluted. In a less robust experiment, similar results were found (see link in Data Availability 8.6). Thus, it could be concluded (though not strongly) that the electric field and the Electro-DBS device itself negatively affects an aspect of reverse transcription or qPCR to result in an increase the C_q values that were yielded (Figure 8.6); the C_q values are not negatively impacted by concentration or RNA integrity.

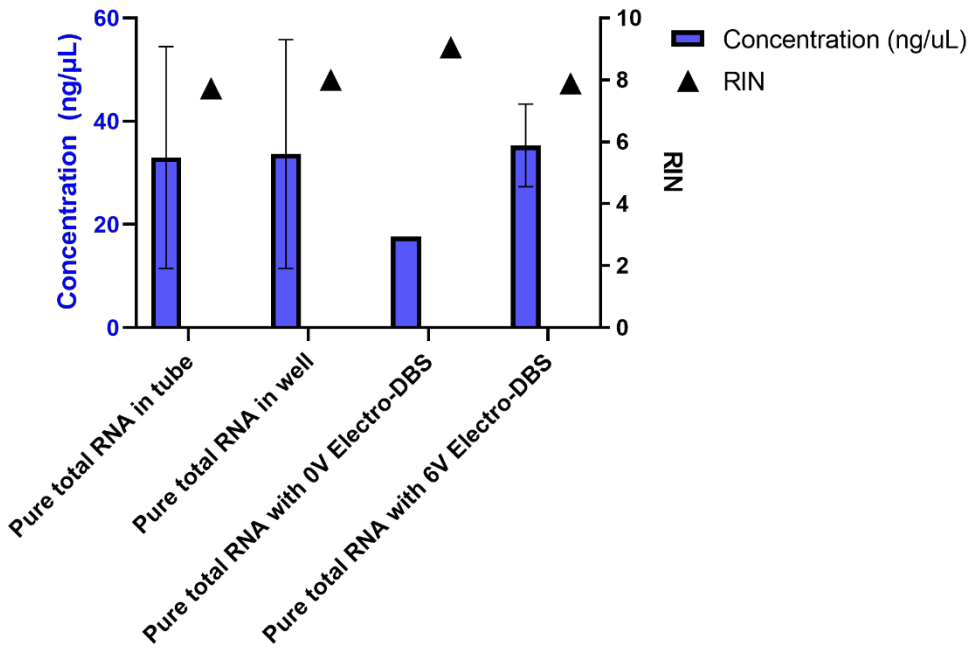

Figure 8.7: Bioanalzyer analysis of concentration and RIN of leukocyte RNA tested with the Electro-DBS protocol. *Measured concentration and RIN for samples from Figure 8.6. Only*

included measurements in which both concentration and RIN could be calculated. For some samples there was a critical error that didn't allow for the RIN to be calculated. "Pure total RNA in tube" (n = 3); "Pure total RNA in well" (n = 2); "Pure total RNA with 0V Electro-DBS" (n = 1); "Pure total RNA with 6V Electro-DBS" (n = 2). There was no statistically significant difference between trials for concentration or RIN.

8.5 Conclusions and Future Directions

In this work, we sought to understand the applicability of the Electro-DBS method to RNA extraction from DBSs. We found first that gDNA and RNA could be extracted simultaneously with the protocols we tested across the literature. Then, we found that 1X PBS or an RNA Elution Buffer would be best for use with the Electro-DBS protocol for RNA extraction. Next, we investigated specific parameters of the Electro-DBS protocol to see if they would reduce RNA yield. We found that the electric field and the Electro-DBS device in general lower RNA yield, when RNA yield was measured with RT-qPCR. With the Bioanalyzer, however, there was no significant difference in yield and RIN when using the Electro-DBS on pure RNA not on the dried blood spot paper.

This work added significant insight into the use of Electro-DBS for RNA extraction from dried blood spots. There are several future directions for this work. Firstly, with these experiments, several types of DBSs were created with: whole blood, plasma, plasma spiked with various concentrations of HIV RNA, white blood cells, and just HIV RNA. Therefore, several experiments can be done with these samples in the laboratory to investigate other parameters of DBS extraction. That is, with the white blood cells placed onto the DBS paper, some of which were made to be twice as concentrated as one would expect from a whole blood spots, one could investigate if the yield increases with more concentrated WBCs or if not. This would give insight into if there is a maximum of nucleic acids that can be extracted from these samples.

Another future direction is understanding the discrepancy between the C_q values the Bioanalyzer results for the Electro-DBS device. Since they do not seem to be related, the Electro-DBS could be causing a problem with reverse transcription and/or qPCR reactions, since the Bioanalyzer results reflect the RNA that was obtained before RT-qPCR. This could be investigated via performing the reverse transcription and then using the Bioanalyzer DNA 10,000 kit to see the integrity of sample. Similarly, after performing qPCR and verifying reverse transcription was not harmed, one could test the DNA that is obtained with the Bioanalyzer DNA 10,000 kit, should it be able to analyzed in that way.

Lastly, with the Electro-DBS device various electrical conditions should be tested to see if they improve the yield of the RNA. Testing parameters such as voltage and frequency of the electric field. Overall, this work has a lot of potential to be successful, but requires some careful experimentation to understand the barriers to obtaining RNA from DBSs.

8.6 Data Availability

https://doi.org/10.7910/DVN/CV4OAH

8.7 Supplemental Information

This section describes several other experiments that were performed as a part of this study in which there was not RNA yield.

8.7.1 Detailed statistical analyses for each figure

Figure 8.1: Average C_q value of RNA extraction from DBSs using Protocols 1 – 5. *To compare the mean differences between average C_q values for the number of punches as well as the protocols, two Šídák's multiple comparisons test was performed. For 2 x 6mm punches: Protocol 1: $\overline{C_q}$ = 34.24; Protocol 2: $\overline{C_q}$ = 34.12; Protocol 3: $\overline{C_q}$ = 35.79; Protocol 4: $\overline{C_q}$ = 31.76; Protocol 5: $\overline{C_q}$ = 32.88. For whole circle: Protocol 1: $\overline{C_q}$ = 34.18; Protocol 2: $\overline{C_q}$ = 30.79; Protocol 3: $\overline{C_q}$ = 34.87; Protocol 4: $\overline{C_q}$ = 32.92; Protocol 5: $\overline{C_q}$ = 32.19. There was a statistically significant difference the sample type for Protocol 2, with p = 0.04. When comparing each protocol to each other, there was a statistically significant difference in the means for Protocol 3 and Protocol 4 for 2 x 6mm punches (p = 0.009) and for Protocol 2 and Protocol 3 (p = 0.008).*

Figure 8.2: C_q values of samples eluted without nucleic acid purification using Protocol 3. *To compare the mean differences between average C_q values for the conditions tested, Šídák's multiple comparisons test was performed. For shaking: 5 minutes: $\overline{C_q}$ = 37.31; 10 minutes: $\overline{C_q}$ = 36.75; 30 minutes: $\overline{C_q}$ = 37.79. For no shaking: 5 minutes: $\overline{C_q}$ = 37.06; 15 minutes: $\overline{C_q}$ = 36.25; 30 minutes: $\overline{C_q}$ = 37.67. There was a statistically significant difference in the means for 15 minutes no shaking and 30 minutes no shaking, p = 0.03.*

Figure 8.4: Electro-DBS effect on 10^5 copies/mL HIV-1 RNA. *To compare the mean differences between average C_q values for the various conditions to the "Pure HIV-1 RNA in tube" condition, Dunnett's multiple comparisons test was performed. For "Pure HIV-1 in tube": $\overline{C_q}$ = 33.98; for "Pure HIV-1 RNA on plate": $\overline{C_q}$= 34.22; for "0V Electro-DBS": $\overline{C_q}$ = 35.02; for "6V Electro-DBS": $\overline{C_q}$ = 36.56. There was a statistically significant difference in the means for "Pure HIV-1 RNA" in tube and "6V Electro-DBS", p = 0.006.*

Figure 8.5: Electro-DBS effect on 10^5 copies/mL HIV-1 RNA, with 0.5% PDMA. *To compare the mean differences between average C_q values for the various conditions to the "Pure HIV-1 RNA in tube" condition, Dunnett's multiple comparisons test was performed. For "Pure HIV-1 in tube": $\overline{C_q}$ = 33.98; for "Pure HIV-1 RNA on plate + PDMA": $\overline{C_q}$= 33.94; for "0V Electro-DBS + PDMA": $\overline{C_q}$ = 34.64; for "6V Electro-DBS + PDMA": $\overline{C_q}$ = 35.82. There was a statistically significant difference in the means for "Pure HIV-1 RNA" in tube and "6V Electro-DBS + PDMA", p = 0.02.*

Figure 8.6: Electro-DBS protocol effect on 40ng/µL solution of leukocyte RNA. *To compare the mean differences between average C_q values for the various conditions to each other, Šídák's multiple comparisons test was performed. For "Pure total RNA in tube": $\overline{C_q}$ = 24.57, for "Pure total RNA in well": $\overline{C_q}$ = 24.32, for "Pure total RNA with 0V Electro-DBS": $\overline{C_q}$ = 28.09, and for "Pure total RNA with 6V Electro-DBS" $\overline{C_q}$ = 30.31. There were statistically significant differences in the mean of "0V with Electro-DBS" with the means of: "Pure total RNA in tube" (p = 0.003), "Pure total RNA in well" (p = 0.003), and Pure total RNA with 6V Electro-DBS" (p = 0.03). There were statistically significant differences in the mean of "Pure total RNA with 6V Electro-DBs" and the means of: "Pure total RNA in tube" (p = 0.002) and "Pure total RNA in well" (p = 0.0003).*

Figure 8.7: Bioanalzyer analysis of concentration and RIN of leukocyte RNA tested with the Electro-DBS protocol. *To compare the mean differences between average C_q values for the various conditions to each other, Šídák's multiple comparisons test was performed. For average concentration: "Pure total RNA in tube" = 33ng/µL, for "Pure total RNA in well" = 33.67ng/µL, for "Pure total RNA with 0V Electro-DBS" = 17.67ng/µL, and for "Pure total RNA with 6V Electro-DBS" = 35.33ng/µL. For average RIN: "Pure total RNA in tube" = 7.7, for "Pure total RNA in well" = 8, for "Pure total RNA with 0V Electro-DBS" = 9.1, and for "Pure total RNA with 6V Electro-DBS" = 7.9. There were no statistically significant differences in the means for all comparisons.*

8.7.2 Testing with KOH/Trizma® protocol.

We evaluated the total RNA yield from DBSs in which the KOH/Trizma® protocol was performed but using PBS, as done in Chapter 7. There was no quantifiable RNA amplification of the samples within 40 cycles of RT-qPCR.

8.7.3 Positive controls: Evaluation of different kits for purification

We tested the use of different nucleic acid purification kits (the Qiagen QIAmp Blood RNA Mini Kit and the QIAmp Viral Mini Kit instead of the NucliSENS kit) after agitation following Protocol 3 and did not get any yield. We evaluated Cq values yielded for whole blood DBSs, HIV-spiked dried plasma spots, and HIV-spiked plasma. There was no quantifiable RNA amplification of the samples within 40 cycles of RT-qPCR.

8.7.4 Alternative sample types: FTA™ cards, spiked HIV dried plasma spots, and white blood cell spots for Electro-DBS extraction

We tested the use of other types of cards as well with the Electro-DBS protocol. We first tested a different type of DBS paper by pipetting 75 µL of whole blood onto QIAcard™ FTA™ Classic cards (Millipore Sigma, St. Louis, Missouri, USA). These cards are specifically for nucleic acid extraction and have been used for DNA and plasmid extraction from DBSs. Additionally, we tested different types of samples to evaluate if red blood cells were inhibiting the elution of RNA from the DBSs.

We made HIV-1 RNA-spiked plasma using either the 903 or FTA paper with a similar protocol as for DBSs. To isolate plasma, whole blood was centrifuged at 2,500rpm for 15 minutes then the plasma was pipetted into a separate tube to be spiked with HIV-1 RNA.

We also made white blood cell spots by first isolating white blood cells (WBCs), Millipore Sigma's Red Blood Cell Lysis Buffer (RLB) (Burlington, Massachusetts, USA)

Protocol was used. The WBCs were then resuspended in 1X Gibco™ Phosphate buffered saline (PBS), pH 7.4 was (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) in the original blood volume they came from (i.e., if the WBCs were isolated from 2mL of blood, they would be resuspended in 2mL of 1X PBS). From there, 50-75 µL of each mixture onto each circle of 903 or FTA paper.

There was no quantifiable RNA amplification of the samples within 40 cycles of RT-qPCR. For the WBC spots, there was gDNA yield but no RNA yield.

8.7.5 Heparin DBSs using the procedure performed in Figure 8.2

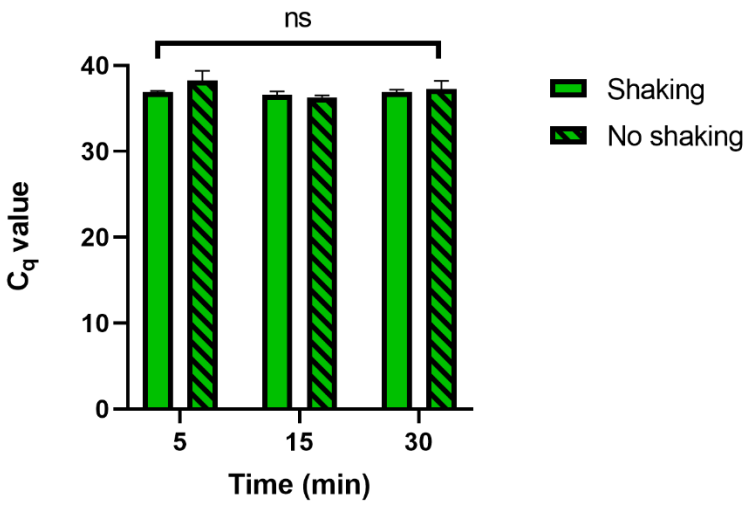

Figure SI 8.1: C_q values of samples eluted without nucleic acid purification using Protocol 3. *RT-qPCR was performed after 5, 15, or 30 minutes of agitation at room temperature in 1X PBS. 2 biological replicates were tested per condition, using heparin DBSs. To compare the mean differences between average C_q values for the conditions tested, Šídák's multiple comparisons test was performed. For shaking: 5 minutes: $\overline{C_q}$ = 36.93; 10 minutes: $\overline{C_q}$ = 36.60; 30 minutes: $\overline{C_q}$ = 36.92. For no shaking: 5 minutes: $\overline{C_q}$ = 38.26; 15 minutes: $\overline{C_q}$ = 36.30; 30 minutes: $\overline{C_q}$ = 37.25. There was no statistically significant difference in the means for all conditions.*

8.7.6 Increased time of the electric field applied with Electro-DBS

To see if, based on the experiments in Figure 8.2, increasing the amount of time that the electric field was applied would yield total RNA from DBSs, we tested

application of the electric field for 10 min, 15 min and for 5 minutes after 5 minutes of incubation in the well (with the Electro-DBS device). We performed these experiments with DBSs in 1X PBS and therefore applied 3V. There was no quantifiable RNA amplification of the samples within 40 cycles of RT-qPCR.

CHAPTER 9: CONCLUSION

9.1 Summary

This dissertation began with a qualitative assessment of the needs around obtaining a diagnosis in the Dominican Republic. This work provided insights into various barriers and facilitators to sample collection and analysis from political, economic, social, and technological points of view. The descriptions of what participants would like to see in a new point of care device informed and provided further evidence of the importance of this dissertation work. Overall, this dissertation aimed to reduce the burden of sample extraction from blood for point of care use. Microfluidic devices were designed, optimized, and validated for use in isolating plasma and for purifying human genomic DNA and total RNA. With these devices, the input sample volume was greatly reduced, the amount of reagents used for extraction and purification was greatly decreased, and the protocols were generally simplified when compared to commercially available nucleic acid extraction kits. The latter half of this dissertation focused on dried blood spots, and similarly trying to simplify nucleic acid extraction from this sample type to increase access to nucleic acid point of care testing. gDNA extraction from DBSs was first characterized and investigated to work towards a standardized manual protocol for extraction. Most notably, we developed a device, Electro-DBS, to extract high quality gDNA from DBSs simply using a buffer and a low voltage power supply. The dissertation concluded with the investigation of application of this device for the more clinically relevant RNA extraction from DBSs.

9.2 Conclusions and Future Directions

9.2.1 Qualitative approaches for engineering solutions

In Chapter 2, we explain our findings of several barriers and facilitators to obtaining a diagnosis in the Dominican Republic, at several levels of society. This work showed most importantly the significance of obtaining user input using rigorous qualitative methods for

user-centered design methods. Next, we identified the challenges and opportunities for improving sample collection and analyses processes at a large public hospital and other similar contexts. Lastly, we collected information on what characteristics participants would like to see in a point of care device.

As for the future of this work, we plan to submit this work for publication to an academic journal. We will translate this publication in Spanish hopefully to be published alongside the English publication, but also to be distributed among the participants who were interested in the results. We will also see if news agencies would be interested in disseminating this work to a lay audience, or at least some of the messages and advice that clinicians have for their patients. I believe a secondary analysis could be performed with this data, looking specifically at the experience of Haitian and other immigrants to the Dominican Republic when it comes to healthcare. Next, I think this work could be expanded upon in a few ways. First, interviews could be done with a similar population in the US, to compare and contrast responses in a different context. Similarly, this work could be done in another context to also investigate the similarities and differences. Lastly, there are several points that require deeper investigation qualitatively and also are points for collaboration with engineering. Understanding more about the global supply chain and how it affects reagent access at the hospital, delving deeper into the role of psychologists in delivering a diagnosis, understanding patient experiences, and exploring which types of analyses devices development should be prioritized for are some examples of further qualitative research questions. In terms of engineering collaborations, there could be devices that are developed and tested for use at the hospital that focus on technologies that could be used on each floor of the hospital, expansion of the use of dried blood spots or lyophilized reagents and improving patient experiences with sample collection.

There are a few limitations of this work. The social ecological model was not initially going to be used; therefore, the interview guide was not developed for the specific levels that were identified. Thus, some levels have stronger evidence than others. The study was performed at a single hospital; however, this hospital did serve a wide range of patients. Similarly, there was only one interview from the private sector perspective. Participants were asked to compare the private and public sectors if they had experience in them, but they were not probed in depth.

This work is at the intersection of public health and biomedical engineering, which is beginning to and continuing to interest several scientists in both fields. I am proud to be a part of this interdisciplinary work and forging new paths by which the fields can collaborate and converge.

9.2.2 Microfluidic point of care devices for nucleic acid extraction from blood

Chapters 3, 4, and 5 describe the development, optimization, and validation of microfluidic devices to extract and/or purify plasma and nucleic acids from blood. These devices used minimal equipment, minimal reagents, minimal steps, and minimal time to perform the extractions and yielding comparable nucleic acids to those obtained with gold standard methods. This work demonstrated the importance of several biotransport mechanisms including capillary flow, electrokinetic flow, and the role of drag in obtaining purified nucleic acids or plasma.

Future work in this area should evaluate the protocols developed here with patient samples for relevant diseases or conditions. Additionally, these protocols and methods should be integrated into a device that can perform amplification of nucleic acids and eventually diagnosis of a condition. For the plasma microfluidic device and more standardized method for manufacturing this device should be established to yield more

consistent results. Additionally, a deeper collection well would be beneficial for analyses to be performed on the microfluidic device. With the DNA microfluidic chip, exploring the adjustment of the number of magnetic beads could be interesting to see if a non-diluted blood sample could be used. For the RNA extraction method, ways to incorporate protection of the RNA from degradation on the microfluidic device should be investigated, as well as further understanding and optimization of electrokinetic flow for optimal RNA elution.

With this work there are few limitations that have identified in each chapter, but overall, this work did not show the yield from samples that may have less abundant quantities of DNA, RNA, or plasma. Additionally, not all steps in the extraction process could be performed on the microfluidic devices, but these methods were all a large step towards building automated devices for point of care sample extraction from blood.

9.2.3 Dried blood spots: towards standardization of manual nucleic acid extraction protocols

Chapters 6, 7, and 8 explore several aspects of dried blood spot nucleic acid extraction. I first identified several factors that inhibit and enhance nucleic acid extraction from dried blood spots. The inhibitors included lower viscosity of the sample, red blood cells, and a non-lysing buffer. The enhancers included red blood cell lysis buffer, a more viscous sample, and a lysing buffer in combination with heat or agitation for extraction. Then, we developed a device to rapidly extract gDNA from dried blood spots using minimal equipment. We showed that our device could obtain purer gDNA than the manual method. This device was then investigated for use with RNA, and we found that there is potential for RNA extraction but to do so, would require several steps of optimization.

There are several future directions for this work as I believe the true mechanisms in which dried blood spots operate are not well explored in the literature. For example, it is

unclear in cells are lysed on the protein saver (Whatman® 903) paper or not. Therefore, understanding if cells are lysed on the paper would provide insight into developing a standardized protocol as manual protocols are developed based on what makes logical sense for nucleic acid extraction but not based on what is best for dried blood spot paper. Since the dried blood spot cards are proprietary, this provided some challenges in understanding the mechanisms in which they work. Next, for our work specifically, the Electro-DBS system could be expanded to use for multiple wells at once for extraction. For RNA, as well as gDNA, extraction, investigation of altering the electric field in terms of frequency, length of time applied, and voltage applied could improve the yield. Most importantly, understanding in more depth how the electric field affects the eluted gDNA and RNA should be explored.

Overall, the dried blood spot work in this dissertation began an important line of work in our laboratory and for the field, as dried blood spots have the potential to change the landscape of diagnostics, but need to be better understood before fully transitioning into standard clinical care into many countries.

9.3 Contributions of this dissertation

9.3.1 Contributions to the field

To the field of molecular diagnostics, specifically blood nucleic acid-based diagnostics, this dissertation had several contributions:

- Challenge engineers and scientists to think beyond the technology but the wider context in which that device is deployed
- Describe the user needs of a population that has not been collaborated with often, those in the Caribbean setting, laboratory personnel (instead of clinicians or “patients”)

- Understanding of the limitations of microfluidic chips with magnetic beads in terms of transport of nucleic acids
- Knowledge of the specific components of blood that affect nucleic extraction from dried blood spots
- Development of several methods for nucleic acid extraction and purification with novel devices
- Utilization of several nucleic acid quantification techniques to demonstrate quality of nucleic acids

Overall, I believe that this dissertation demonstrates the importance of the work at the benchtop and away from it. There are several aspects to consider for successful implementation of a device to a variety of settings, which includes scientific rigor and understanding the context in which a device will be used.

9.3.2 Contributions to the scientific community

To the scientific community, this dissertation has contributed to a variety of aspects. Firstly, this work has been shared via several conference presentations and departmental presentations to the wider scientific community. Additionally, this work has been explained to a lay audience, such as in this article (https://engineering.brown.edu/news/2022-11-18/kiara-lee-student-spotlight). Two students were mentored to develop this work, which aided in developing their research, communication, and experimental skills. Overall, this work provides several approaches to understanding biotransport processes, device development, and improving access to blood-based point of care diagnostics. I hope this work inspires scientists, especially Black girls and women, to bring their passions into their work, even if they do not fit within the traditional bounds of their field. It is amazing to see what you can achieve and contribute when you cross boundaries.

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