

# The Exploration and Development of Focused Ultrasound Extraction (FUSE) for the Rapid Release of DNA from Complex Tissue Matrices

Alexia Danielle Stettinius

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Eli Vlasisavljevich, PhD, Chair

Hal Holmes, PhD

Adam Maxwell, PhD

Jason Holliday, PhD

John Robertson, PhD

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ACADEMIC ABSTRACT

Over the past two decades, molecular detection platforms have seen rapid advancement, reshaping the way we monitor the safety and security of our environment and human health. One of the key drivers of this transformation has been the development of simpler, faster, and more accessible nucleic acid amplification tests (NAATs) that have enabled point-of-contact (POC) DNA testing, delivering real-time results in resource-limited settings. Despite these advancements, the DNA sample preparation process is laborious, resource-intensive, and often requires hazardous chemicals, preventing the performance of DNA extraction at the POC and severely limiting the potential of POC NAATs. Thus, DNA sample preparation methods have been the primary bottleneck restricting the widespread use and applicability of POC NAATs.

To overcome this bottleneck, focused ultrasound extraction (FUSE) was recently introduced as a novel DNA extraction method capable of rapidly releasing DNA from complex tissue matrices without labor-intensive techniques or strong chemicals. This technology utilizes high-pressure focused ultrasound pulses that disintegrate tissue and release DNA through the control of acoustic cavitation. An initial feasibility study demonstrated the potential of FUSE for simple biological tissues, but the use of FUSE for preparing complex tissues has not been explored previously. Understanding the potential of the FUSE technology with diverse sample types is essential for developing versatile DNA preparation methods that can effectively protect both the environment and human health. This dissertation investigates the performance of FUSE in complex tissue matrices and evaluates the utility of a miniaturized FUSE system for streamlined DNA sample preparation. Specifically, this work addresses (1) the feasibility of FUSE in robust sample types with physical and chemical complexities that hinder DNA release, (2) the optimization of FUSE pulsing parameters to enhance the time efficiency of FUSE processing and improve the quality of released DNA, and (3) the development of a compact, accessible device for the performance of FUSE DNA sample preparation in resource-limited settings. The completion of this work will introduce a novel DNA sample preparation method to enable the use of POC NAATs.

# The Exploration and Development of Focused Ultrasound Extraction (FUSE) for the Rapid Release of DNA from Complex Tissue Matrices

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## GENERAL AUDIENCE ABSTRACT

Over the past two decades, DNA testing platforms have seen rapid advancement, reshaping the way we monitor the safety and security of our environment and human health. One of the key drivers of this transformation has been the development of simpler, faster, and more accessible genetic technologies that have enabled point-of-contact (POC) DNA testing, delivering real-time results in resource-limited settings. Despite these advancements, the DNA sample preparation process is laborious, resource-intensive, can take hours or days to complete, and often requires hazardous chemicals, preventing the performance of DNA extraction at the POC and severely limiting the potential of POC DNA testing platforms. Thus, DNA sample preparation methods have been the primary bottleneck limiting the full potential of POC DNA testing platforms.

To overcome this bottleneck, focused ultrasound extraction (FUSE) was recently introduced as a novel DNA extraction method capable of rapidly releasing DNA from complex tissues without labor-intensive techniques or strong chemicals. This technology uses sound waves to generate a cloud of microbubbles capable of breaking down tissue and releasing DNA. An initial feasibility study demonstrated the potential of FUSE for simple biological tissues, but the use of FUSE for preparing complex tissues has not been explored previously. Understanding the potential of the FUSE technology with diverse sample types is essential for developing versatile DNA preparation methods that can effectively protect both the environment and human health. This dissertation investigates the performance of FUSE in complex tissue matrices and evaluates the utility of a miniaturized FUSE system for streamlined DNA sample preparation. Specifically, this work addresses (1) the feasibility of FUSE in robust sample types with physical and chemical complexities that hinder DNA release, (2) the optimization of FUSE pulsing parameters to enhance the time efficiency of FUSE processing and improve the quality of released DNA, and (3) the development of a compact, accessible device for the performance of FUSE DNA sample preparation in resource-limited settings. The completion of this work will introduce a novel DNA sample preparation method to enable the molecular detection of complex samples at the POC.

## **Dedication**

*To the trees!! I hope this work contributes in some small way  
to their preservation and protection.*

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*Alexia Stettinius*

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**Chapter 4:** Dr. Hal Holmes, Ph.D, an employee of Conservation X Labs contributed to the conceptualization of this study. Annika Griggs and Gabby Lopez, undergraduate students in the

Virginia Tech Department of Biomedical Engineering and Mechanics at Virginia Tech, assisted with data collection. Misa Winters, MS, an employee of Conservation X Labs, and Dr. Jason Holliday, PhD, a professor in the Department of Forest Resources and Environmental Conservation at Virginia Tech contributed to analyzing data from the molecular experiments. Dr. Adam Maxwell, Ph.D research associate professor in the Department of Biomedical Engineering and Mechanics at Virginia Tech, contributed to the development of the methodology for the focused ultrasound experiments.

**Chapter 5:** Dr. Adam Maxwell, Ph.D research associate professor in the Department of Biomedical Engineering and Mechanics at Virginia Tech, contributed to the development of the methodology for the focused ultrasound experiments and design of the focused ultrasound device. Dr. Hal Holmes, Ph.D, an employee of Conservation X Labs contributed to the conceptualization of this study. Kayleigh Canavan, Annika Griggs, and Gabby Lopez, undergraduate students in the Department of Biomedical Engineering at Virginia Tech, assisted with data collection. MoonWon Jeon and Daniel Yang, undergraduate students in the Department of Mechanical Engineering at Virginia Tech, assisted with the fabrication of the focused ultrasound device. Misa Winters, MS, an employee of Conservation X Labs, and Dr. Jason Holliday, PhD, a professor in the Department of Forest Resources and Environmental Conservation at Virginia Tech contributed to analyzing data from the molecular experiments.

# **|Chapter 1: Introduction**

The field of genomics has expanded rapidly over the past 20 years, and recently, one of the main areas of advancement has been the development of simpler, faster, and cheaper methods to broaden the accessibility of DNA testing [1, 2]. To expand the accessibility of DNA testing and analysis tools, one strategy has been to develop more compact and automated nucleic acid sample preparation, amplification, sequencing, and detection platforms with process flows that do not require extensive equipment, space, training, or cost requirements [2-4]. With broadened accessibility, fields such as environmental security, conservation, plant science, and healthcare will benefit from the development of portable tools for DNA-based detection and point-of-care diagnostics [5-7]. Focused ultrasound extraction (FUSE) has recently been developed as a novel DNA extraction technology that utilizes high-pressure ultrasound pulses to generate focused cavitation bubble clouds capable of tissue disintegration and DNA release [8]. This dissertation aims to expand on the development of FUSE by investigating its feasibility in complex tissue matrices and evaluating the utility of a miniaturized FUSE system for streamlined DNA sample preparation. This chapter covers an overview of the uses of DNA testing in environmental security, plant science, and healthcare, current DNA sample preparation methods, and a background on the use of FUSE for DNA sample preparation. This introductory chapter concludes with an outline of this dissertation with an overview of the included chapters.

## **1.1. DNA Testing in Environmental Security, Plant Genetics, and Healthcare**

DNA testing and analysis platforms provide a means for rapid detection of genetic markers to reveal important features about a sample including information relevant for diagnostics, pathogenicity determination, or identification of individual traits for applications ranging from agriculture, healthcare, plant sciences, environmental security, and forensics [7, 9-11]. The

accessibility of DNA testing has progressed in recent years due to advancements in automation and portability that allow for testing to be performed by non-scientists outside of a laboratory [3, 12]. The development of portable tools has been driven by the need for point-of-contact (POC) testing, such that sample preparation and analysis can be performed at the site of collection, eliminating the need to transfer samples to off-site laboratories for analysis. POC DNA testing provides real time detection of genetic markers and rapid results. While the clinical applications of POC testing have gained the most attention, POC testing is also important for environmental security, wildlife forensics, and agricultural applications such as plant breeding or pathogen identification. Here, the uses of DNA testing for environmental security, plant sciences, and healthcare will be discussed.

The versatility of DNA testing for forensic identification has increased its prevalence in wildlife forensics and has improved environmental security surveillance measures. Crimes against protected plant and animal species include instances of illegal trade, poaching, or illegal harvest of plants, and to investigate these cases, there is often a need for species identification, individualization, or determination of geographic origin [10, 13]. DNA testing is a powerful tool in wildlife forensics and conservation because of its ability to differentiate samples based on individuality, species, and geographic origin [13, 14]. DNA profiling is used for the identification of individuals. This test involves the comparison of microsatellite DNA markers from source and suspect samples to determine if the suspect sample can be connected to the crime [15, 16]. DNA testing can also be used for species determination [17, 18]. For species identification, DNA barcoding is performed by identifying short DNA sequences that are expected to differentiate at the rate of speciation [19]. The mitochondrial cytochrome oxygenase subunit 1 (COI) gene is the accepted barcode for animal species, and for plants the designation of a universal barcode has not

been as straightforward, but currently the maturase K (matK) and ribulose biphosphate carboxylase (rbcL) genes are commonly used for plant species identification [20]. For determination of geographic origin, short nucleotide polymorphisms (SNPs) or short-tandem repeats (STRs) are commonly used to spatially differentiate populations [21, 22]. Several studies have validated the utility of DNA testing in plant and animal species for forensic identification of individuals, species, and geographic origin, demonstrating the importance of DNA testing for environmental protection.

DNA testing has been utilized in plants for more efficient plant breeding and disease control [23, 24]. In agriculture, crop production is optimized by plant breeding and the manipulation of molecular markers that control morphological, cytological, or biochemical traits [25]. Through control of these traits, plants can be selectively bred for disease resistance, growth in certain temperatures, or yield, among other factors [26]. DNA testing is necessary for the identification of genetic markers that control the expression of these traits. Additionally, DNA testing can be used for the early detection of pathogens to prevent the spread of diseases among plant populations [24]. Similar to disease detection in humans, plants need to be monitored for disease, and DNA testing provides a method of detection that is more reliable and capable of earlier diagnosis than visual methods that have been used traditionally [24]. Overall, DNA testing in plants has been used to better understand the expression of traits to improve crop productivity and limit the spread of disease.

In healthcare, DNA testing provides a means for disease diagnostics, biomarker discovery, and personalized medicine. Nucleic acid amplification tests (NAATs) are portable DNA detection platforms that have been established as a method for disease and virus detection that utilize PCR or isothermal amplification techniques to identify disease specific molecular markers [27-29]. The

value of NAATs became more well-known during the onset of the COVID-19 pandemic, where they provided fast and reliable diagnostics [30]. In recent years, research has focused on improving these platforms for enhanced accessibility and performance in low-resource settings [31]. Additionally, DNA testing can be used to uncover biomarkers that contribute to disease susceptibility and treatment response. By utilizing tissues stored in biobanks, researchers are able to perform molecular pathology with genome sequencing studies to map genetic variants and link them to complex diseases [32]. With this information, there is the potential to discover biomarkers that can be utilized in personalized medicine approaches to predict disease progression or treatment response and help physicians develop the most appropriate treatment plan. With its applications in diagnostics and personalized medicine, DNA testing has the potential to transform healthcare.

## **1.2. Current DNA Sample Preparation Methods**

The uses of DNA testing span a variety of disciplines and its utility is evident in several applications. However, in order to use genetic material for DNA testing, there must be a reliable method of DNA sample preparation that can be performed rapidly and efficiently. DNA sample preparation describes the process of sample collection, tissue homogenization, cell lysis, and DNA purification, and these steps can be performed with a number of different physical and chemical processes. Tissue homogenization is the first step of the DNA sample preparation process, and the methods used for tissue homogenization are highly dependent on the sample type. Tissue homogenization can be done using grinding, shearing, beating, or sonication methods that result in complete disruption of the tissue matrix, making cells more readily available for cell lysis [33]. Cell lysis and DNA purification are then performed to isolate the DNA. A few commonly used methods include silica column-based kits, magnetic beads, and cetyltrimethylammonium bromide (CTAB) extraction [34, 35]. In silica column-based kits, cell lysis is performed by suspending

homogenized samples in a cell lysis buffer and incubating at 60 °C until no visible tissue fragments remain [34]. DNA purification is then facilitated by a silica gel-based filter that binds the DNA, such that the DNA lysate can be washed with a series of buffers while the DNA remains bound to the filter. This process ends with elution of clean DNA. The magnetic bead protocol is similar in that samples are suspended in lysis buffer and incubated for cell lysis, but for purification, the DNA specifically binds to magnetic beads instead of the silica based filter [36]. Then the DNA is washed and eluted. For the CTAB methods, samples are added to the CTAB solution for cell lysis and the precipitation of DNA and polysaccharides, followed by the addition of solution with a high salt concentration for DNA purification and the removal of polysaccharides [34]. The outcome of each of these processes is an isolated DNA sample that can be used for DNA testing.

While the described DNA sample preparation methods have been successful for a variety of sample types, they cannot be easily translated to portable systems for POC DNA testing [27, 37-39]. Each of the reported tissue homogenization, cell lysis, and DNA purification methods require the use of laboratory equipment and several steps that must be performed by the user. With the recent push to translate to POC systems, alternative processes utilizing microfluidics or one step DNA extraction buffers have been investigated [12, 37]. These alternatives have demonstrated their utility for rapid cell lysis and purification, but without a means for portable tissue homogenization, they are unable to prepare samples with complex tissue matrices for POC DNA testing. Therefore, tissue homogenization continues to be a step that has not yet been integrated with cell lysis and DNA purification for the development of a fully automated DNA sample preparation workflow that can be used for POC DNA testing.

### **1.3. Focused Ultrasound Extraction (FUSE) for DNA Sample Preparation**

To address the limitations of DNA sample preparation from complex tissue matrices, focused ultrasound extraction (FUSE) has recently been developed as a technique capable of rapid tissue homogenization and cell lysis [8]. FUSE utilizes high-pressure ultrasound pulses to produce cavitation bubbles at the acoustic focus, and repeated delivery of these pulses results in a sustained cavitation bubble cloud. The cavitation bubble cloud induces stress and strain in its target tissue, resulting in mechanical disintegration of the tissue on the cellular and extracellular level, which has been shown to facilitate the release of DNA [8]. This mechanism is based off histotripsy, a non-invasive tissue ablation technique being developed for clinical applications including cancerous tumor ablation, kidney stones, thrombosis, neurological disorders, congenital heart disease, and biofilm ablation [40-46]. The capacity of FUSE to simultaneously homogenize tissue and lyse cells gives it the potential to overcome the barriers preventing the development of a comprehensive DNA sample preparation platform. FUSE proof-of-concept studies investigated the feasibility of FUSE for piscine tissue homogenization and DNA release, followed by PCR amplification and sequencing [8]. Results show that FUSE can release significant quantities of DNA with integrity suitable for amplicon and next generation sequencing. Additionally, FUSE performed tissue homogenization and cell lysis in only 6 minutes and 40 seconds, compared to conventional methods that required 45 minutes of tissue processing for significant DNA release. Based on the results of this study, we have the long-term goal of developing fully integrated FUSE platforms that can be used to enable DNA sample preparation from complex tissue matrices for POC DNA testing.

## **1.4. Research Outline**

This dissertation expands on initial feasibility studies to characterize the utility of FUSE in more robust tissue types and development of miniaturized FUSE platforms for more streamlined

and simplified DNA sample preparation. The first part of this dissertation (Chapters 2-3) investigates the feasibility of FUSE for rapid DNA release from different tissue types. Chapter 2 evaluates FUSE in American chestnut, tulip poplar, red maple, and chestnut oak leaf species to determine the feasibility of FUSE for tissue disintegration and the release of high-quality DNA from plant tissues. Chapter 3 examines the utility of FUSE in white oak timber, a more complex plant tissue, by investigating tissue breakdown efficiency, DNA yield, and the success of PCR amplification and sequencing. The second part of this dissertation explores the potential of optimizing and scaling FUSE DNA sample preparation. Chapter 4 examines the effect of pulse repetition frequency (PRF) and dose on tissue homogenization, DNA release, and DNA quality to determine if increased pulsing rates can be used to improve the time efficiency of FUSE. Chapter 5 focused on the design of a miniaturized FUSE device to enable POC DNA testing and broaden the accessibility of molecular detection platforms. Finally, Chapter 8 will summarize the findings presented in this dissertation and will outline a projected timeline for the proposed projects.

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## |Chapter 2: DNA Release from Plant Tissue using Focused Ultrasound Extraction (FUSE)

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Co-authors: Hal Holmes, Qian Zhang, Isabelle Mehochko, Misa Winters, Ruby Hutchison, Adam Maxwell, Jason Holliday, Eli Vlaisavljevich

### **Abstract**

**Premise:** Sample preparation in genomics is a critical step that is often overlooked in molecular workflows and impacts the success of downstream genetic applications. This study explores the use of a recently developed focused ultrasound extraction (FUSE) technique to enable the rapid release of DNA from plant tissues for genetic analysis.

**Methods:** FUSE generates a dense acoustic cavitation bubble cloud that pulverizes targeted tissue into acellular debris. This technique was applied to leaf samples of American chestnut (*Castanea dentata*), tulip poplar (*Liriodendron tulipifera*), red maple (*Acer rubrum*), and chestnut oak (*Quercus montana*).

**Results:** We observed that FUSE can extract high quantities of DNA in 9-15 minutes, compared to the 30 minutes required for control DNA extraction methods. FUSE extracted DNA quantities of  $24.33 \pm 6.51$  ng/mg and  $35.32 \pm 9.21$  ng/mg from American chestnut and red maple, respectively, while control methods yielded  $6.22 \pm 0.87$  ng/mg and  $11.51 \pm 1.95$  ng/mg, respectively. The quality of the DNA released by FUSE allowed for successful amplification and next-generation sequencing.

**Discussion:** These results indicate that FUSE can improve DNA extraction efficiency for leaf tissues. Continued development of this technology aims to adapt to field-deployable systems to increase the cataloging of genetic biodiversity, particularly in low-resource biodiversity hotspots.

## 2.1. Introduction

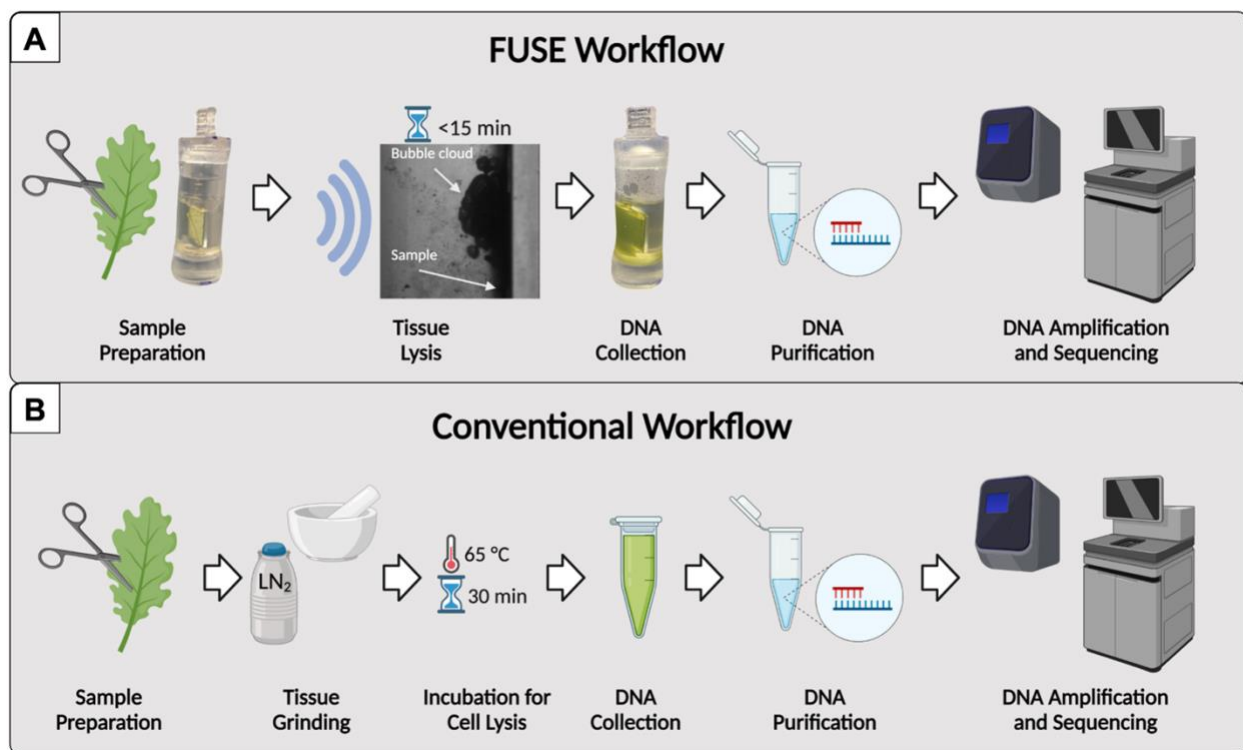
Over the past two decades, developments in genome sequencing technologies have enabled researchers to provide an unprecedented scope and depth of genetic information. Emerging technologies have equipped researchers with the tools to perform DNA and RNA sequencing in the field [2], which could allow for new genetics research to be carried out by non-scientists in a variety of settings that had not previously been feasible [3-5]. Despite technological advancements, many plant species are poorly represented in genetic databases, which limits the applicability of field-deployable sequencing platforms [6]. For example, of the nearly 400,000 unique plant species estimated to exist, only 600 have nearly complete genome coverage and assembly [7]. With such little coverage of plant taxa, it is likely that many opportunities for new uses of undiscovered traits unique to species have gone unnoticed, and with extinction rates rising, we may lose some of these opportunities. Sequencing plant genomes is also essential for utilizing genetic resources in breeding programs [8], conserving plant species [9, 10], understanding their role in ecosystem function [11-13], and phylogenetic studies [14]. Therefore, continued expansion of plant genetic databases is essential to spur discovery, drive innovation, and protect crucial resources.

A robust DNA extraction protocol that yields DNA of sufficient concentration and purity is essential for success in genotyping and sequencing applications. In plants, the release of viable DNA is hindered by tough tissue matrices that are resistant to mechanical breakdown, the presence of polysaccharide-rich cell walls, and many inhibitory compounds such as polyphenolic

metabolites [5, 15, 16]. To combat these challenges, tissue pulverization with benchtop tools, such as a mixer mill, or a mortar and pestle under liquid nitrogen, is used in conjunction with plant cell lysis and purification protocols. Plant DNA extraction is often cumbersome, and despite specialized tissue breakdown strategies, releasing DNA suitable for genomic analysis is challenging for many sample types [17]. Additionally, current plant DNA extraction techniques require an advanced laboratory [18]. With a growing need for field deployable species identification tools for biodiversity conservation, the ability to translate DNA extraction protocols to the field is becoming increasingly important [4]. The simplification of DNA extraction could be pivotal in conservation efforts where researchers must rapidly and inexpensively prepare samples from biodiversity hotspots, which are often remote and far removed from centralized laboratories [19].

Our group has recently developed a technology capable of accelerating and simplifying the DNA extraction workflow, termed focused ultrasound extraction (FUSE), to address sample preparation and DNA extraction challenges. FUSE has previously demonstrated its capacity to rapidly release DNA from Atlantic salmon muscle tissue samples with intense cavitation clouds generated by focused ultrasonic transducers [20]. This technology employs dense acoustic cavitation bubble clouds similar to those used in histotripsy, a non-invasive focused ultrasound therapy currently being developed for medical applications [21, 22]. During FUSE, the rapid expansion and violent collapse of the cavitation microbubbles induce high stress on the target tissue, which causes mechanical disintegration and results in an acellular tissue lysate [23, 24]. The tissue lysate is then collected, and the released DNA is purified for downstream analyses. This process differs from conventional extraction methods that require mechanical tissue disruption

followed by elongated incubation periods varying from 10 minutes to 1 hour, depending on the plant tissue, before DNA collection and purification (**Figure 2.1**).



**Figure 2.1. Leaf DNA extraction workflow.** (A) The FUSE process begins with the trimming of leaf samples to prepare for tissue processing. The prepared sample is aligned with the cavitation bubble cloud. The tissue is disrupted and cells are lysed in 15 minutes or less, eliminating the need for incubation. The DNA is then collected and purified for amplification and sequencing. (B) In the control extraction protocol, leaf samples are trimmed in preparation for tissue processing. Tissue processing involves manual grinding under liquid nitrogen for tissue breakdown and a 30-minute minimum incubation period for cell lysis. The DNA is then collected and purified for amplification and sequencing following the same protocol as FUSE.

Here, we test the efficacy of FUSE with leaf tissue by 1) determining the feasibility of leaf tissue breakdown, 2) measuring the DNA yield, 3) amplifying the released DNA with PCR to verify DNA quality, and 4) sequencing the resultant DNA libraries to validate the utility of FUSE in whole-genome sequencing applications. While our ultimate goal is to adapt FUSE to low-cost and field-deployable systems to enable rapid sample processing and DNA extraction from various sample types, here we address the feasibility of FUSE for DNA release from leaf tissue in a laboratory setting using prototype transducers and custom acoustically transparent sample holders. We hypothesize that FUSE can pulverize leaf tissue and yield significant quantities of DNA with

a quality suitable for PCR amplification and next-generation sequencing. If successful, FUSE could streamline leaf DNA extraction workflows to improve standard laboratory practices. Further technology development could allow the miniaturization of the FUSE system to bring this technology to the field to expand the scope of opportunities.

## **2.2. Materials and Methods**

To demonstrate the ability of FUSE to rapidly provide amplifiable DNA fragments for genetic analysis, we used fresh leaf samples collected from American chestnut (*C. dentata*), tulip poplar (*L. tulipifera*), red maple (*A. rubrum*), and chestnut oak (*Q. montana*) trees found on Virginia Tech campus in Blacksburg, VA, USA that were stored at -20 °C and thawed before use. These species were selected because they were locally available and represented a wide range of angiosperm taxonomic diversity that may correspond to variations in physical properties and secondary metabolite composition. Three leaves of American chestnut, tulip poplar, red maple, and chestnut oak were collected. Half of each leaf was used for FUSE, and the other half was used for control methods (**Supplementary Figure 2.1**) (**Appendix A**). Three samples were acquired from each half. The tissue samples were prepared and processed under the following experimental conditions:

### *2.2.1. FUSE Pulse Generation*

A custom 32-element 500 kHz array transducer with a geometric focus of 75 mm, an aperture size of 150 mm, and an effective f-number of 0.58 was used for all experiments in this study [25]. A custom high-voltage (~1 kV/element) pulser was used to drive the transducer and generate a short single cycle ultrasound pulse. This short pulse length results in low duty cycles (typically <1%) and, combined with the impedance of the transducers, results in power requirements on the order of milliwatts, that can be efficiently and safely supported by battery powered electronics, even with the voltage driving requirements. The pulser was connected to a

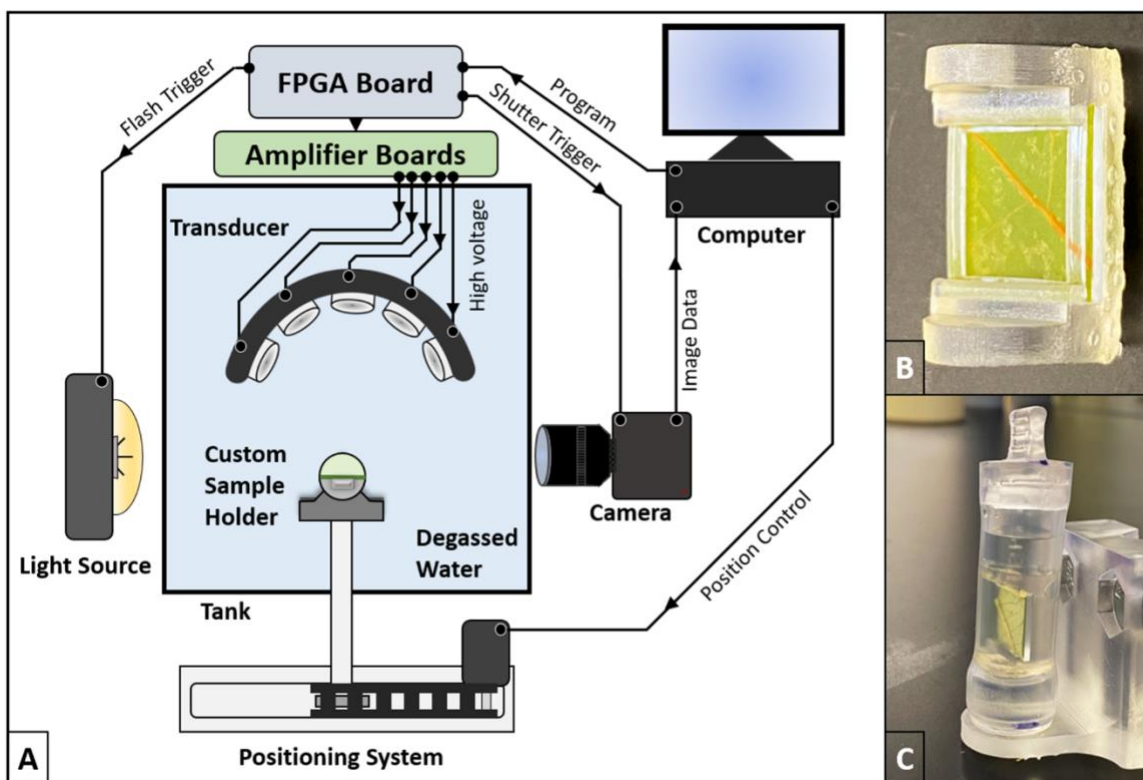
field-programmable gate array (FPGA) board (Altera DE0-Nano Terasic Technology, Dover, DE, USA), which was explicitly programmed for histotripsy therapy pulsing. A custom-built fiber-optic probe hydrophone (FOPH) [26] was used to measure the acoustic output pressure of the transducers. The FOPH was cross-calibrated at low-pressure values ( $p < 2$  MPa) using a reference hydrophone (Onda HNR-0500) to ensure accurate pressures were measured from the FOPH. The lateral and axial full width half maximum (FWHM) dimensions at the geometric focus of the transducer were measured to be 2.3 mm and 7.1 mm, respectively. The acoustic pressures used for all experiments were measured in degassed water at the focal point of the transducer, which was identified using a 3D beam scan. The acoustic output could not be directly measured at higher pressure levels ( $p > 16$  MPa) due to cavitation at the fiber tip. These pressures were estimated by summing the output focal  $p$ - values from individual transducer elements.

### *2.2.2. Visualization of FUSE Tissue Disintegration*

For all focused ultrasound experiments, high-speed optical imaging was done using a machine-vision camera (Blackfly S 3.2MP Mono USB3 Vision, FLIR Integrated Imaging Solutions, Richmond, BC, Canada) that was aligned with the focal zone of the transducer using a 100 mm F2.8 Macro lens (Tokina AT-X Pro, Kenko Tokina Co., LTD, Tokyo, Japan) and backlit by a custom-built pulsed LED strobe light capable of high-speed triggering with 1  $\mu$ s exposures. As done in previous studies, the camera and the strobe light were triggered individually by the amplifier box, with the camera shutter opening at the time of pulse generation and the strobe acting as the shutter [27]. The camera was triggered to capture one image every 50<sup>th</sup> pulse. The exposures were centered at delay times of 6, 48.5, and 98.5  $\mu$ s after the pulse arrived at the focus to allow visualization of bubble cloud formation, coalescence, and collapse.

### *2.2.3. FUSE Sample Preparation and Experimental Configuration*

All samples processed with FUSE were prepared as 12 mm squares using a sterile scalpel blade. The mass of the samples ranged from 10-30 mg. Leaf samples were secured in a custom-designed sample holder. The sample holder supported a 12.5 mm x 12.5 mm x 1 mm sapphire glass window, the leaf sample, and a polyethylene terephthalate glycol (PETG) square frame that secured the sample on the surface of the glass backing. The assembled sample holder was placed inside an optically transparent and acoustically permeable tube with an inner diameter of 9.525 mm and a wall thickness of 1.59 mm (Tygon PVC E-1000, McMaster-Carr, Douglasville, GA, USA). When placed in the tube, cylindrical appendages at the top and bottom of the sample holder created a controlled volume chamber. The upper appendage featured a small circular opening for the application of lysis buffer into the chamber. The lysis buffer was composed of 1 mL of 1% PVP-40 Buffer AP1 solution and 8  $\mu$ L of RNase A (Qiagen DNeasy Plant Kit Qiagen Inc, Hilden, Germany). A custom-built mount suspended the tube assembly in the water tank for tissue processing, and a stopper was designed to seal the other end of the tube. A robotic positioning system controlled by custom MATLAB scripts was used to align samples with the focus of the ultrasonic transducer (**Figure 2.2**).



**Figure 2.2. Experimental FUSE set-up.** (A) Ultrasonic transducers are driven by an FPGA board and amplifier. High-speed imaging is performed using a strobe and camera controlled by signals from the FPGA board. Custom scripts are delivered to the FPGA board, and imaging data is recorded by a computer. A robotic positioning system, controlled by the computer using MATLAB, is used to align the sample in the focus of the transducer array. (B) A custom sample holder designed to support a sapphire glass backing, the leaf sample, and a PETG frame is used. (C) The sample holder assembly is housed in an acoustically permeable tube for DNA extraction experiments.

The configuration of the sample and sapphire glass backing in the focus of the transducer was chosen to maximize the efficiency of tissue sonication with FUSE. When ultrasonic pulses generate a cavitation bubble cloud near a rigid boundary, high-pressure collapse is expected to occur toward the surface of the boundary [28-30]. **Supplementary Figure 2.2 in Appendix A** demonstrates this effect. As the time after pulse arrival increased, microbubble coalescence became more evident, and the concentration of bubbles near the sample surface increased. Sapphire glass was chosen because it is hydrodynamically strong and has a high acoustic impedance. The hydrodynamic strength of the sapphire glass provided an unyielding surface to support the sample when exposed to high-pressure fluid flow caused by cavitation. The high acoustic impedance increased the pressure near the boundary and induced the cavitation bubbles

to grow larger and collapse more violently. Overall, this effect maximized the impact pressure felt by the sample. In preliminary experiments, FUSE was tested without including the sapphire glass backing and sample holder. With this configuration, the sample was free to move outside of the focal zone, which decreased the tissue disintegration efficiency of FUSE and caused inconsistencies in tissue breakdown success.

#### *2.2.4. FUSE Tissue Disintegration and DNA Extraction*

The prepared leaf tissue samples ( $n = 9$ ) were processed using single cycle ultrasound pulses delivered at a pressure of 34 MPa and a pulse repetition frequency (PRF) of 500 Hz. These pulse parameters, particularly the PRF, were selected following preliminary tissue breakdown experiments that revealed this to be the most time efficient pulsing regimen before residual cavitation shield effects were observed. This effect is further discussed in section 3.2. Measurements made with a fiber optic hydrophone determined that pressure loss was negligible ( $<1\%$ ) when pulses were delivered through the sample tube. Before tissue processing, the acoustic focus was directed at the center of the sample. To completely disintegrate the leaf tissue sample, MATLAB scripts controlling the positioning system were designed to move the sample in a spiral square pattern such that each point within the  $100 \text{ mm}^2$  disintegration zone was exposed to the focal bubble cloud for 0.5 s. Using this approach, a single scan of the applied pattern delivered 250 pulses per point, with multiple scans used for each sample to achieve sufficient tissue breakdown. After FUSE tissue disintegration and cell lysis, sample purification was performed in silica columns using the standard protocol as recommended by the manufacturer (Qiagen DNeasy Plant Kit Qiagen Inc, Hilden, Germany).

The number of scans required for complete tissue disintegration was initially characterized for American chestnut, tulip poplar, red maple, and chestnut oak samples ( $n = 3$ ) backed with sapphire glass suspended in an open water bath. Images of the sample were taken after each scan.

Each image was converted to grayscale, then to binary using the Otsu method [31]. The targeted tissue area was mapped as an ROI with dimensions of 10 x 10 mm to represent the area exposed to ultrasonic pulses. The disrupted tissue area inside and outside the ROI was quantified by counting the number of pixels using custom MATLAB scripts. Pixel counts were converted to tissue disintegration area. The significance of the area measurements was determined using an unpaired student's t-test with unequal variance. Values less than 0.05 ( $p < 0.05$ ) were considered significant.

### *2.2.5. Control Sample Tissue Disintegration and DNA Extraction*

Control samples ( $n = 9$ ) were obtained by cutting the leaf tissue into 100 mg segments, and samples were disrupted by grinding with mortar and pestle under liquid nitrogen [32]. 100 mg control samples were put in the mortar; liquid nitrogen was added to freeze the samples and cool the mortar, pestle, and spatula. To begin, grinding with the pestle was done slowly, and once the liquid nitrogen was mostly evaporated, more vigorous grinding was performed to reduce the tissue to a fine powder. The tissue powder was then transferred to a 1.5 mL centrifuge tube where 1% PVP-40 Buffer AP1 solution and RNase A were added (Qiagen DNeasy Plant Kit Qiagen Inc, Hilden, Germany). The lysis buffer volume varied depending on the quality of the leaf tissue sample, which was determined by assessing the color and age of the sample. For older samples with a dark green or brown-green color, 1 mL of 1% PVP-40 Buffer AP1 solution and 8  $\mu$ L of RNase A (0.8 mg) were used. For younger samples with a yellow-green color, 500  $\mu$ L of 1% PVP-40 Buffer AP1 solution and 4  $\mu$ L of RNase A (0.4 mg) were used. This was done because the leaves with a lower water content yielded a larger sample volume after grinding with mortar and pestle under liquid nitrogen and therefore required a greater buffer volume for proper cell lysis. After the addition of the buffer, cell lysis was carried out with an initial vortex of the tube to homogenize the solution before incubation at 65 °C for 30 minutes with a short vortex every 5

minutes. Subsequent sample purification was performed in silica columns using the standard protocol as recommended by the manufacturer (Qiagen DNeasy Plant Kit Qiagen Inc, Hilden, Germany).

#### *2.2.6. DNA Quantification*

The robustness of FUSE was investigated by quantifying the released DNA. FUSE and control lysates were analyzed with a Qubit<sup>TM</sup> 4 Fluorometer (ThermoFisher, Waltham, MA, USA) and a Nanodrop<sup>TM</sup> One (ThermoFisher, Waltham, MA, USA) to determine the quantity and quality of DNA released with FUSE and control samples. DNA yield was reported as the quantity of DNA released per milligram of tissue to normalize input sample mass using the DNA concentration values from the Qubit<sup>TM</sup>. Lysates were also visualized with gel electrophoresis to assess DNA fragmentation. For data acquired from Nanodrop<sup>TM</sup> and Qubit<sup>TM</sup> measurements, an unpaired student's t-test with unequal variance was used, with values less than 0.05 ( $p < 0.05$ ) considered significant.

#### *2.2.7. Library Preparation and PCR Amplification*

To compare the two methods for downstream genotyping, genotyping by sequencing (GBS) was performed on American chestnut genomic DNA extracted by FUSE and control methods. Library preparation involved restriction digestion followed by ligation of sequencing adapters and PCR amplification [32]. American chestnut samples processed with FUSE and control methods were normalized to 55 ng, then digested with 1  $\mu$ L of ApeKI (New England BioLabs, Ipswich, MA, USA). This restriction enzyme recognizes a 5 bp degenerate sequence GCWGC, where W is an A or T [33]. For one of the American chestnut samples processed with FUSE, the quantity of eluted DNA did not reach 55 ng, so 36.2 ng of DNA was used in the digestion reaction. The restriction fragment ligation was performed as described in previous studies [32]. Briefly, the DNA fragments were ligated to Illumina-compatible adapters with 1.6

μL of T4 DNA ligase. P1 adapters contained a unique barcode region for each adapter immediately upstream of the ligated DNA fragment, and the P2 adapter was consistent for all samples. PCR amplification of the ligated fragments was performed using the primers and protocol reported previously [33]. The thermal cycling conditions were as follows: 95 °C for 1 minute, followed by 18 cycles of 95 °C for 30 seconds, 63 °C for 20 seconds, and 68 °C for 30 seconds. Lastly, samples were brought to 68 °C for 5 minutes and kept at 4 °C. Ligation and amplification were assessed by gel electrophoresis and viewed with a 2100 Bioanalyzer instrument (2100 Bioanalyzer, Agilent, Santa Clara, CA, USA) for all samples to determine if FUSE processing affected DNA integrity in a manner that affected library preparation. DNA samples were purified before and after PCR with the Monarch® PCR and DNA Clean-Up Kit (New England Biolabs, Ipswich, MA, USA). Individual sample libraries were pooled, and fragments ranging from 250-550 bp were selected using BluePippin™ (Sage Science, Beverly, MA, USA). The resulting library was visualized using a 2100 Bioanalyzer instrument.

### 2.2.8. Sequencing Analysis

The American chestnut GBS libraries were sequenced with the NovaSeq 6000 instrument (Illumina, San Diego, CA, USA) in 2x150 bp paired-end mode at Duke University Center for Genomic and Computational Biology. Raw fastq files were demultiplexed and quality filtered with the STACKS process\_radtags function. Specifically, reads were trimmed of provided adapter sequences, removed if they lacked an intact restriction cut site, and removed if they had a mean quality score less than or equal to 10 over more than 15% (in this case, 23 base-pairs) of their length [34]. Filtered reads were aligned to v.1.1 of the *C. dentata* reference genome (NCBI taxonomy ID: 134033) using the Burrows-Wheeler Aligner (BWA) mem algorithm and subsequently converted to BAM format with SAMtools [32]. Heterozygous sites were called with

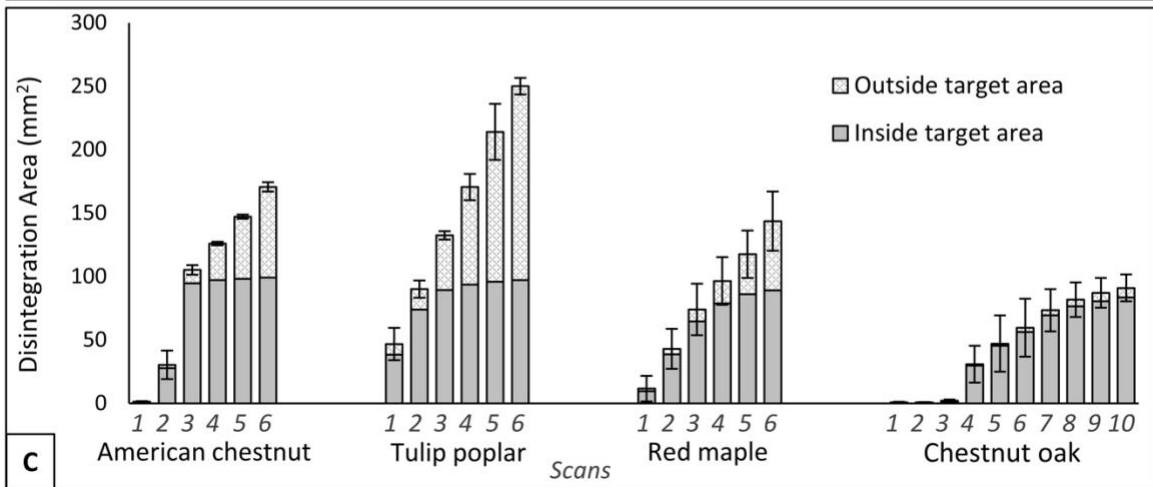
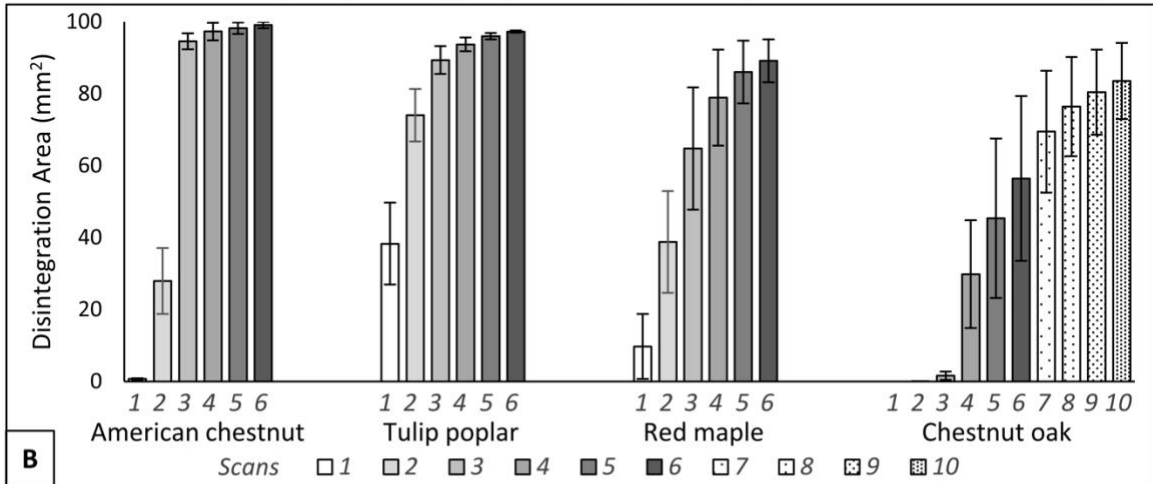
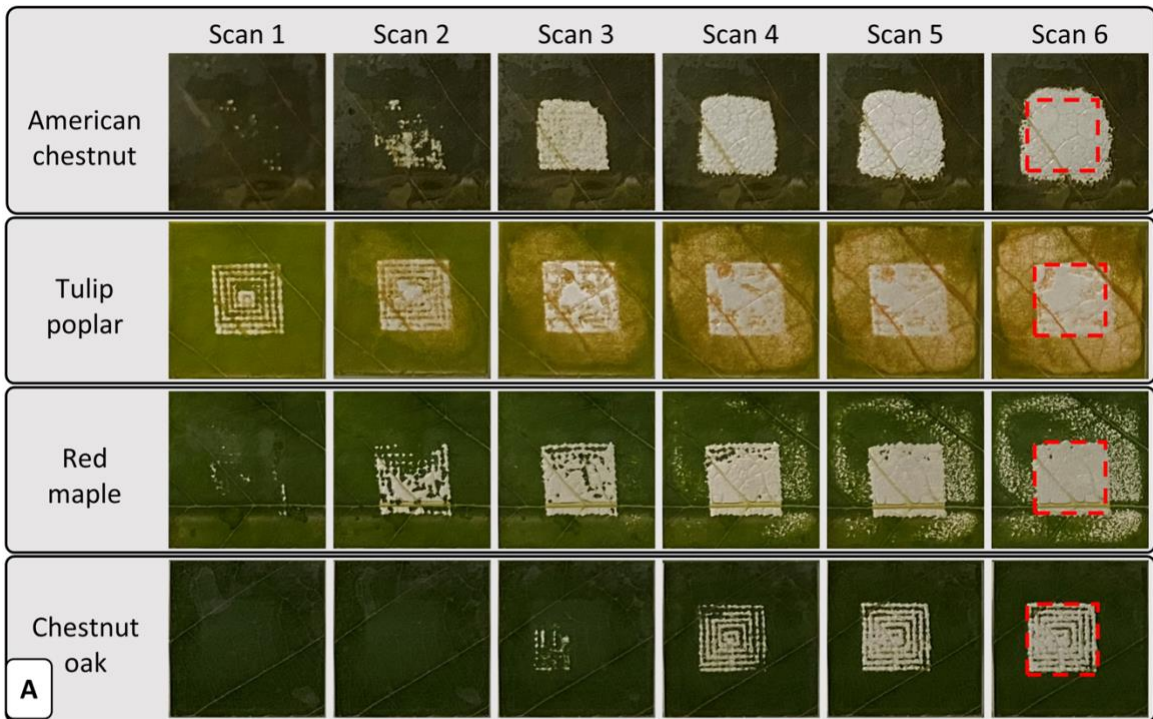
the Genome Analysis Toolkit (GATK) HaplotypeCaller algorithm [35, 36], and these GVCFs were then merged using the GenotypeGVCFs function. Variants were flagged and removed as low quality if they had the following characteristics: low map quality ( $MQ < 40$ ); high strand bias ( $FS > 40$ ); differential map quality between reads supporting the reference and alternative alleles ( $MQRankSum < -12.5$ ); bias between the reference and alternate alleles in the position of alleles within the reads ( $ReadPosRankSum < -8.0$ ); and low depth of coverage ( $DP < 5$ ). Coverage depth per sample was calculated using the SAMtools depth function. Statistical analysis of coverage depth was performed using a Wilcoxon rank-sum test with values less than 0.05 ( $p < 0.05$ ) considered significant.

## 2.3. Results & Discussion

### 2.3.1. FUSE Tissue Disintegration

The feasibility of FUSE for leaf tissue disintegration was examined with American chestnut, tulip poplar, red maple, and chestnut oak leaves by characterizing tissue breakdown after each FUSE scan (**Error! Reference source not found.A**). For all species, the damaged tissue area increased with increasing scan number, but the number of scans required to achieve significant tissue breakdown differed among species. Tulip poplar leaves were the most vulnerable to breakdown, as they were the only species with notable tissue disintegration after one scan. For American chestnut, tulip poplar, and red maple samples, tissue breakdown beyond the bounds of the targeted disintegration zone was observed. This effect was likely due to dispersed cavitation occurring outside the focal point of the converging pressure fronts. Surface inhomogeneities at solid-liquid interfaces result in the growth of cavitation nuclei that can induce cavitation at thresholds below the intrinsic threshold [37, 38]. Previous work has also shown that leaves are more susceptible to cavitation-induced tissue disruption when gas channels are present in the tissue [39, 40]. Therefore, it is possible that the surface architecture and distribution of gas channels

within the tissue matrices created cavitation nucleation sites outside the targeted area. It is also possible that residual gas bubbles from preceding pulses diffused outside the focus and served as cavitation nuclei. This would induce cavitation below the intrinsic pressure threshold and expose a larger area of the leaves to cavitation [41]. Lastly, off-target leaf tissue disintegration could result from acoustic shielding, such that the residual bubbles in the acoustic focus increased the likelihood of acoustic scattering [42, 43]. The trends in tissue breakdown for chestnut oak differed from the other three species. It is expected that variation in tissue breakdown across species was due to differences in physical properties, such as water content and tissue strength.



**Figure 2.3. FUSE tissue disintegration.** (A) Leaf tissue disintegration increases after each FUSE scan. The red square in the top right identifies the targeted tissue region. Tissue breakdown beyond the target area is the result of peripheral cavitation damage. Image data suggests that the leaf species affect FUSE tissue disintegration efficiency. (B) The disintegration area within the target area increases after each scan for all species. (C) The total disintegration area shows that tissue outside of the target area is also disintegrated by FUSE. Six scans are used for processing American chestnut, tulip poplar, and red maple samples. Chestnut oak samples required ten scans for processing due to a reduction in disintegration efficiency.

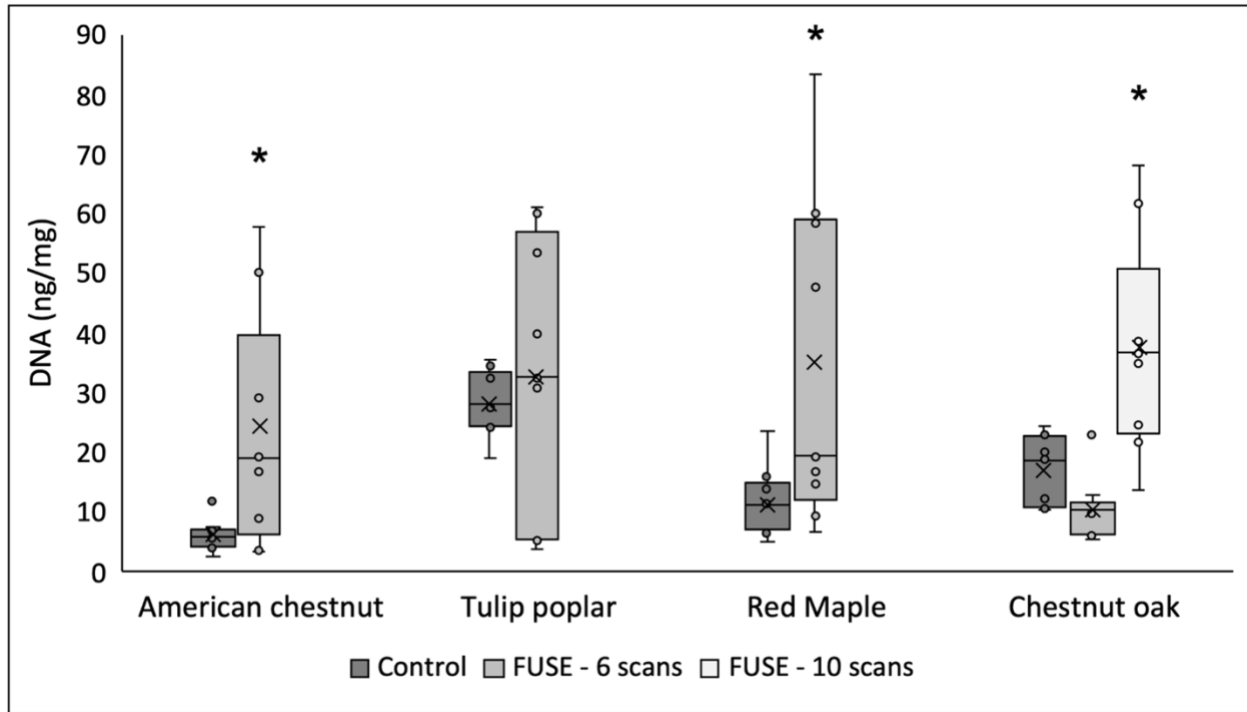
The observed efficiency of FUSE tissue disintegration was assessed quantitatively by plotting the disintegration area inside the targeted region and the total disintegration area as a function of scan number (Error! Reference source not found.**B-C**). The initial breakdown occurred the most rapidly in tulip poplar leaves, as after scan one,  $38.4 \pm 11.4\%$  of the targeted tissue region was disintegrated. In comparison,  $<10\%$  of target tissue was disintegrated after scan one for American chestnut, red maple, and chestnut oak. The targeted tulip poplar tissue region was significantly processed after two scans ( $p < 0.05$  compared to zero scans). The initial breakdown of American chestnut leaves did not occur as rapidly as tulip poplar, but American chestnut quickly approached complete tissue breakdown, as American chestnut leaves were significantly processed after three scans ( $p < 0.05$  compared to zero scans). Red maple leaves were more resistant to breakdown than tulip poplar and American chestnut, as four scans were required to achieve significant breakdown ( $p < 0.05$  compared to zero scans). Although tulip poplar, American chestnut, and red maple samples achieved significant breakdown in less than six scans, increasing the scan number decreased the margin of error in the disintegration area. Therefore, six scans were used to process American chestnut, tulip poplar, and red maple samples to allow more consistent comparisons. Six scans resulted in a 9-minute tissue processing time and a total of 1,500 pulses per point. Chestnut oak was the most resistant to breakdown, as these samples required eight scans to achieve a significant breakdown ( $p < 0.05$  compared to zero scans). Since increasing the scan number increased the area of disintegration and reduced the margin of error in the disintegration

area, ten scans were used for chestnut oak tissue processing. Ten scans resulted in a 15-minute tissue processing time and 2,500 pulses per point.

Leaf tissue breakdown outside of the targeted area was also quantified to examine the effects of dispersed cavitation. Trends in tissue breakdown outside of the targeted area were comparable to those observed within the targeted area, such that the tissue disintegration area increased with increasing scan number. Additionally, the extent of American chestnut, tulip poplar, and red maple tissue disintegration outside the targeted region was greater than chestnut oak. These results suggest that differences in the physical properties among leaf species also affect the extent of collateral tissue breakdown. However, collateral tissue breakdown is not of central importance for this study because the samples are restricted to the size of the targeted region in DNA extraction experiments.

### *2.3.2. DNA Extraction Feasibility*

The determined number of FUSE scans required to disintegrate each leaf species was applied for DNA extraction experiments to characterize the quantity of DNA released by FUSE compared to the control protocol (**Figure 2.4**). Overall, FUSE was able to release greater quantities of DNA than control methods in a fraction of the processing time. Notably, FUSE increased the DNA yield with less than half of the input sample mass required by the control protocol. The quantity of DNA released with FUSE and controls varied with species. The DNA yield provided by six FUSE scans was significantly greater than controls for American chestnut and red maple samples. No significant differences were observed in the quantity of DNA released from tulip poplar samples between six FUSE scans and controls. For chestnut oak leaves, the DNA yield provided by ten FUSE scans was significantly greater than six FUSE scans and controls for chestnut oak samples, showing that the capacity of FUSE to release DNA from tough tissues improves with an increased number of processing scans.



**Figure 2.4. FUSE and control DNA yield results.** DNA extraction results show that FUSE releases DNA from leaf tissue. A significant increase in DNA release from American chestnut and red maple samples is observed when processed with 6 FUSE scans compared to controls. DNA release from chestnut oak samples is significantly higher when 10 FUSE scans are used for processing than controls. After 6 FUSE scans, DNA release from tulip poplar samples is comparable to controls. \*Indicate significant ( $p < 0.05$ ) differences between FUSE and control samples.

The control protocol applied manual grinding under liquid nitrogen for tissue disruption and the Qiagen DNA extraction kit for cell lysis and DNA purification. Other tissue disruption techniques, such as homogenizers, rotor-stators, blenders, or bead beaters, and DNA extraction techniques, such as CTAB based methods, have also been used for DNA extraction from leaf tissue [44]. Here, mortar and pestle grinding under liquid nitrogen for tissue disruption and the Qiagen DNA extraction kit were used in the control protocol to provide an initial point of reference to demonstrate the feasibility of FUSE for leaf DNA extraction. Further research will need to be done to investigate the utility of FUSE compared to other conventional DNA extraction protocols.

The DNA yield results suggest that FUSE is as effective at DNA extraction as the control methods. **Supplementary Tables 2.1-2.4** include images of the leaf samples after FUSE processing to demonstrate the relationship between FUSE tissue disintegration and DNA release

for each species (**Appendix A**). The results suggest that the degree of tissue disintegration influenced the concentration of DNA release for samples processed with FUSE. Therefore, it is likely that inconsistencies in tissue breakdown efficiency were a source of variation in the FUSE DNA yield results. The tissue disruption outside the targeted area observed in **Figure 2.3** suggests the leaf tissue was seeding cavitation, which may be the result of bubble nuclei persisting on leaf tissue particles and causing effects similar to particle-mediated histotripsy [45]. These residual bubble nuclei on the tissue surface could have influenced the behavior of subsequent pressure fronts and altered the cavitation efficacy at the acoustic focus, leading to inconsistencies in the efficiency of tissue breakdown and the DNA yield. Despite the observed variability in leaf tissue disintegration and DNA yield, the DNA extraction results confirm that FUSE can effectively release DNA from leaf tissue at quantities comparable to control protocols. However, variability in the results presents a need for further cavitation kinetics research to improve the consistency of FUSE processing.

The DNA extraction results show that leaf species influenced the DNA yield. Since the leaves chosen represented a range of angiosperm taxonomic diversity, it was expected that differences in physical and chemical properties would affect the quantity of DNA released. The control DNA yield data was examined to determine the effect of species on DNA release. The American chestnut DNA yield was significantly lower than the average DNA yield from the control samples. The tulip poplar DNA yield was significantly greater than the average DNA yield from the control samples. The American chestnut leaves were the only samples described as brown-green (**Supplementary Table 2.1**), while the tulip poplar leaves were the only samples characterized as yellow-green (**Supplementary Table 2.2**) (**Appendix A**). These sample characteristics suggest that the American chestnut samples were more mature than the tulip poplar

samples at the time of collection [46]. It is common for older leaves to have greater amounts of secondary metabolites, which often cause low yield and poor quality DNA [16]. Therefore, it is likely that the age of the sampled leaves influenced inconsistencies in the quantity of the released DNA. Different leaf tissue types such as frozen, lyophilized, or herbarium specimens could be used to investigate the effect of leaf degradation on DNA release with FUSE. Tissue is often frozen, lyophilized, or stored as herbarium to reduce tissue degradation and allow for long term storage. Since greater DNA yield was observed from samples with less degradation, we expect that FUSE can be applied to frozen, lyophilized, or herbarium specimens, but may require some additional preparation, such as pre-soaking to re-hydrate the tissue, and further research is necessary to confirm the applicability of other leaf tissue types.

The 260/280 and 260/230 ratios were measured to assess the quality of the DNA extracted with FUSE and control methods (**Table 2.1**). For American chestnut and chestnut oak, the 260/280 ratio was significantly higher for samples processed with six FUSE scans than for control samples. The 260/280 ratio for six FUSE scans was significantly lower than control methods for red maple leaves. No discernible trends were observed in 260/230 ratios, with values in expected norms for leaf tissue. However, the 260/30 ratio was significantly higher after ten FUSE scans than controls for chestnut oak leaves. This significant difference is expected to be the result of increased tissue breakdown releasing more carbohydrates from the leaf tissue. Some species processed with FUSE showed high standard error in 260/230 ratios. This result is likely due to incomplete tissue disintegration for some samples within these groups.

**Table 2.1. Leaf DNA purity ratios.** The quality of DNA released by FUSE is comparable to DNA released by control methods. FUSE requires less processing time than control methods and releases greater quantities of DNA. DNA quantification measurements are reported from Qubit™ Fluorometer measurements, and 260/280 and 260/230 ratios are reported from Nanodrop™ measurements.

Sample Type	FUSE				Control Methods			
	Time (mm:ss)	DNA (ng/mg)	260/280	260/230	Time (mm:ss)	DNA (ng/mg)	260/280	260/230

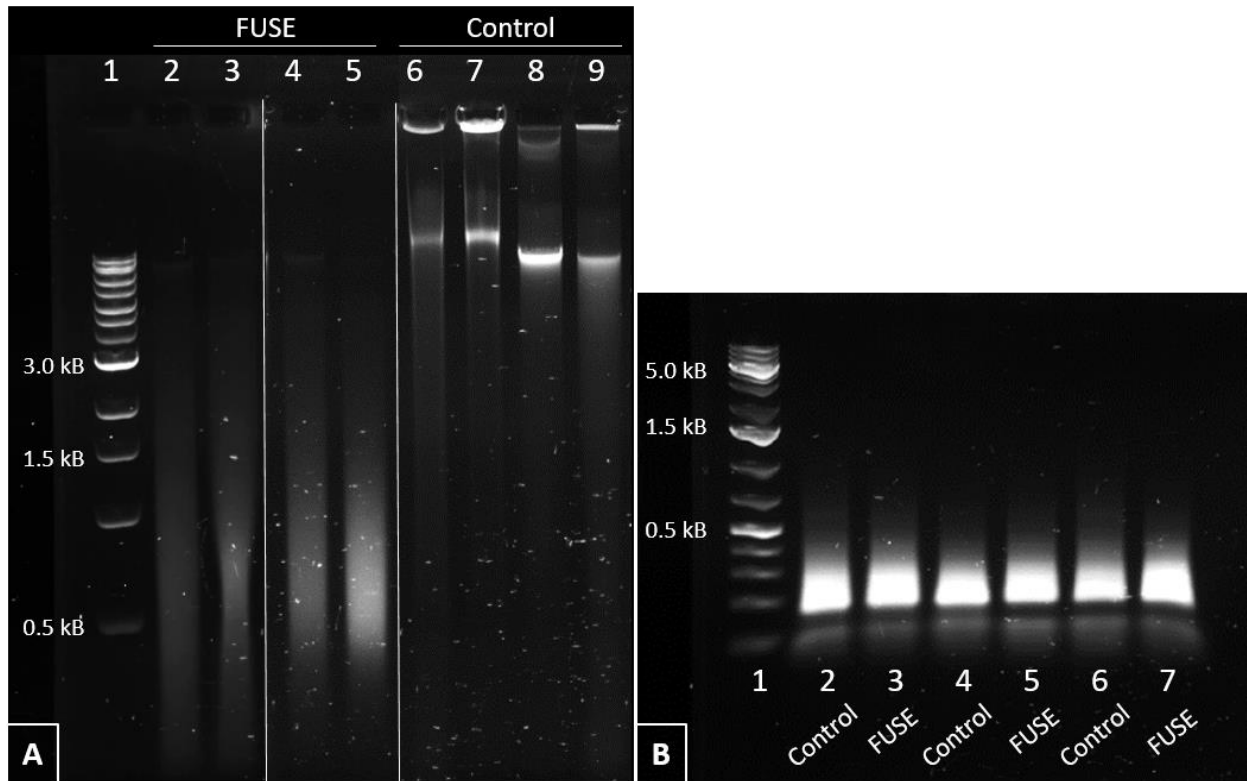
American chestnut	9:00	24.33 ± 6.51	2.34 ± 0.31	2.68 ± 3.51	30:00	6.22 ± 0.87	1.45 ± 0.01	0.52 ± 0.01
Tulip poplar	9:00	32.61 ± 7.82	1.77 ± 0.05	1.41 ± 0.34	30:00	28.37 ± 1.78	1.83 ± 0.01	1.99 ± 0.06
Red maple	9:00	35.32 ± 9.21	1.63 ± 0.04	1.95 ± 0.60	30:00	11.51 ± 1.95	1.85 ± 0.02	2.53 ± 0.19
Chestnut oak 6 scans	9:00	10.65 ± 1.74	1.84 ± 0.11	0.17 ± 3.00	30:00	17.17 ± 1.98	1.52 ± 0.01	0.66 ± 0.02
Chestnut oak 10 scans	15:00	37.85 ± 5.93	1.76 ± 0.03	2.28 ± 0.20	30:00	17.17 ± 1.98	1.52 ± 0.01	0.66 ± 0.02

The FUSE protocol used in this work involves a non-thermal tissue lysis process that has been shown to reduce the time required for DNA release [20]. The acoustic parameters used in this study, particularly the PRF of 500 Hz, were chosen for this initial feasibility study based on preliminary tissue breakdown experiments. In previous work, Atlantic salmon muscle tissue was processed with FUSE using 10,000 pulses delivered at 25 Hz, which resulted in a total processing time of 6 minutes and 40 seconds [20]. In this study, 270,000 pulses were applied at 500 Hz to complete 6 FUSE scans, and 450,000 pulses were applied at 500 Hz to complete 10 FUSE scans, resulting in total processing times of 9 and 15 minutes. At 500 Hz PRF, the time efficiency of FUSE was improved without inducing thermal effects. However, further increasing the PRF is likely to result in cavitation shielding effects that lower the effectiveness of each pulse [42, 43], so future work will be necessary to explore the optimal pulsing parameters for the implementation of higher PRFs. The optimization of pulsing parameters to implement FUSE at higher PRFs will allow FUSE to be applied to more robust tissue types that require more pulses for tissue breakdown without increasing the tissue processing time.

### 2.3.3. DNA Amplification

American chestnut samples were selected for amplification and sequencing. The samples processed with FUSE and control methods amplified successfully, demonstrating that FUSE can yield high-quality DNA from leaf tissue suitable for PCR amplification. A subset of the amplified

DNA lysates and libraries was visualized using gel electrophoresis (**Figure 2.5**) and a 2100 Bioanalyzer instrument (**Supplementary Figure 2.3**) (**Appendix A**). Visualization of the lysates shows that large genomic DNA fragments were fractionated by FUSE processing, but no differences in the prepared GBS libraries were observed.

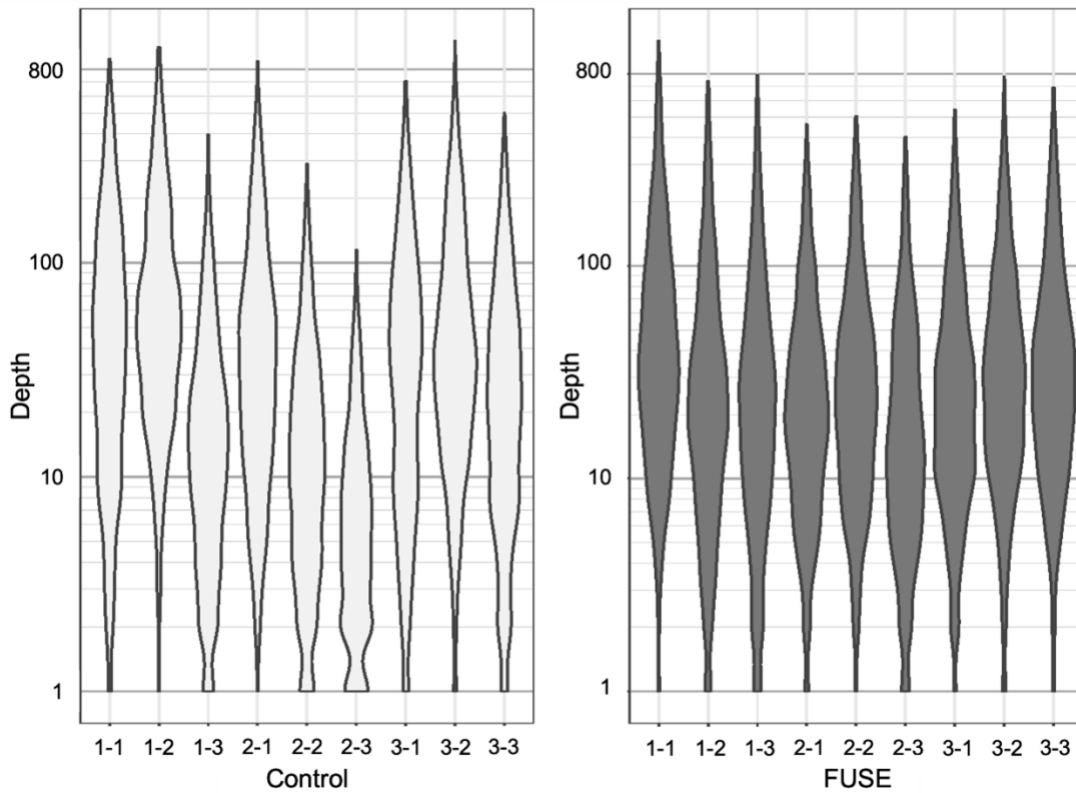


**Figure 2.5. Visualization of DNA products.** (A) Visualization of DNA extracts resulting from FUSE and control methods shows that FUSE fractionates large genomic DNA fragments. (B) The genomic DNA fractionation facilitated by FUSE did not affect GBS library preparation. \*Gel A contains images from different parts of the same gel. Rearrangements are marked with white lines.

#### 2.3.4. Sequencing

All American chestnut samples processed with FUSE and control methods provided high-quality next-generation sequencing reads (Accession Number: PRJNA837224). Because downstream applications of this technology are expected to be focused on identifying genetic variants from sequence data for population genetics and systematics, we estimated read depth for variable sites, which showed that FUSE samples had a depth comparable to controls. Read depth was moderately correlated between the two extraction methods (**Supplementary Figure 2.4**)

(**Appendix A**), and mean depth was not significantly different (25.7 for FUSE and 27.1 for controls;  $P = 0.155$  based on a Wilcoxon rank-sum test). Among FUSE and control samples, read depth was consistent for those processed with FUSE and control methods (**Figure 2.6**). Overall, these results indicate that FUSE processing is suitable for short-read NGS analysis. Observations of large DNA fragment fractionation may suggest further optimization of FUSE may be necessary to enable long-read sequencing. However, these results present the need for future studies investigating the underlying mechanisms of DNA fragmentation under FUSE processing.



**Figure 2.6. Next generation sequencing results.** FUSE provides DNA suitable for short-read next-generation sequencing. The uniformity of read depth across conventional and FUSE samples is comparable. The x-axis labeling represents the leaf and sample number, such that 1-2 identifies the read depth for leaf 1, sample 2.

## 2.4. Conclusion

This study assessed the efficacy of our recently developed FUSE protocol in plant tissues by testing samples from American chestnut, tulip poplar, red maple, and chestnut oak leaves. The success of the FUSE protocol was determined by visualizing the extent of tissue breakdown

observed after FUSE sonication, measuring the quantity and quality of the released DNA, and evaluating the suitability of DNA extracts for short-read sequencing. PCR amplification and NGS were performed to determine if the quality of the DNA was acceptable for these analyses. In accordance with previous work that established the effectiveness of FUSE for releasing DNA from Atlantic salmon muscle tissue [20], the results of this study demonstrate that FUSE can provide high quantities of DNA suitable for amplification and short-read sequencing in less time than conventional plant extraction methods that apply grinding under liquid nitrogen for tissue disruption and the Qiagen DNA extraction kit. Additionally, these results suggest that the input sample mass required by FUSE is less than what is necessary for the control extraction methods applied in this study, which could be advantageous in future work that aims to develop field-deployable FUSE systems for conservation efforts. Overall, this study shows that the applications of FUSE can be extended to plant tissue, a robust tissue that is more resistant to mechanical breakdown and has a chemical composition that has made DNA accessibility more challenging [5, 15, 16]. In conjunction with previous findings [20], these results suggest that FUSE could be used as a novel platform for DNA extraction capable of accelerating workflows for a variety of sample types.

## **2.5. Acknowledgements**

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# **|Chapter 3: Timber DNA Release using Focused Ultrasound Extraction (FUSE) for Genetic Species Identification**

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Co-authors: Hal Holmes, Isabelle Mehochko, Annika Griggs, Qian Zhang, Misa Winters, Adam Maxwell, Jason Holliday, Eli Vlasisavljevich

## **Abstract**

The use of genetic data for timber species and population assignment is a powerful tool for combating the illegal timber trade, but the challenges of extracting DNA from timber have prevented the routine use of genetics as a supply chain management tool. To overcome these challenges, we explored the feasibility of focused ultrasound extraction (FUSE) for rapid DNA release from timber. Using high-pressure ultrasound pulses, FUSE generates a cavitation bubble cloud that disintegrates samples into acellular debris, resulting in the mechanical release of DNA. In this work, FUSE was applied to white oak (*Quercus alba*) timber shavings to test the feasibility of using FUSE for timber DNA extraction for the first time. Results showed that FUSE processing disintegrated the tissue samples and released significant quantities of DNA. After five minutes of tissue processing DNA quantities of  $0.21 \pm 0.02$  ng/mg,  $0.99 \pm 0.32$  ng/mg, and  $0.14 \pm 0.01$  ng/mg, were released from medium, coarse, and combination shaving groups, respectively. Amplification and sequencing of regions within the matK and rbcL chloroplast genes confirmed that the quality of DNA prepared with FUSE was suitable for PCR and short-read sequencing applications. Overall, these results show that FUSE can serve as a DNA sample preparation method capable of releasing high-quality DNA from timber in a fraction of the time required by conventional

extraction methods. Based on the improved efficiency of DNA release with FUSE, ongoing work aims to develop this technology into portable systems that can be used to rapidly prepare timber samples for genetic species identification.

### **3.1. Introduction**

Illegal harvest of protected timber species is a critical driver of rising deforestation rates and threatens the health of forest ecosystems worldwide, particularly in biodiversity hotspots [2]. Moreover, habitat loss from deforestation fragments ecosystems and is a critical driver of species extinctions [3]. Deforestation also impacts climate change, accounting for an estimated one-fifth of global carbon emissions [4]. The financial impact of illegal logging annually is estimated to be US \$30 billion and accounts for 15-30% of total logging worldwide [5]. The threats of illegal logging have been recognized by many countries and efforts have been made to outlaw the trade of illegally harvested timber [6]. Laws such as the Lacey Act in the United States, the EU Timber regulation, and the Illegal Logging Prohibition Act in Australia, require the common name, species when applicable, and origin of timber products to be tracked through the supply chain. Adherence to legislation is monitored primarily through the verification of shipping manifests and macroscopic anatomical inspections by front-line officers to determine if an expert forensic analysis is necessary [6, 7]. However, wood anatomy cannot always provide species-level resolution and cannot delineate the geographic origin of the wood [6, 8, 9]. Timber identification technologies, including genetics, spectroscopy, stable isotopes, and machine vision models have the potential to serve as rapid field identification techniques to better assess compliance with timber regulations [7, 10-12].

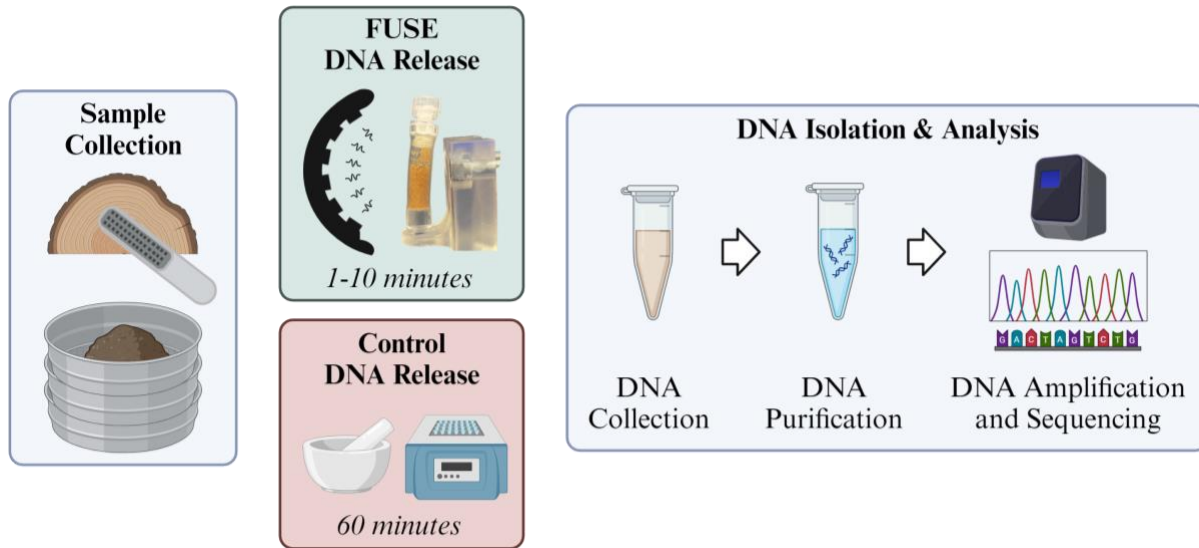
Genetic methods are the only identification tool capable of determining species, geographic origin, and individual tracking, making it an attractive alternative to the current standard of

anatomical inspections [6, 10]. Despite its advantages, genetic timber identification is not widely used due to incomplete reference databases and the difficulty of obtaining DNA of sufficient quantity and quality from wood to identify genetic species [10, 11]. As wood does not contain many living cells, DNA extracts often contain sheared DNA of low concentration, which restricts the selection of forensic markers to short sequence lengths [9, 13-15]. There are also complications associated with liberating the DNA. Physically, timber is a robust tissue that must be disrupted into fine particulates to access the DNA without overheating the tissue and damaging the DNA by inducing single or double-stranded breaks [13, 15-18]. Chemically, the composition of timber differs from other tissue types in that it contains compounds that can inhibit DNA extraction or PCR amplification [13, 19-21]. Despite the difficulties associated with the use of genetics for timber identification, prior work has demonstrated the feasibility of using timber for 1) DNA barcoding to identify species, 2) population genetics for geographic origin determination, and 3) genotyping for tracing individuals [22-24]. As a result, DNA barcoding, population genetics, and DNA profiling are all considered standard methods for forensic timber identification [7]. However, DNA extraction methods have not yet been standardized, and the difficulties associated with DNA extraction have prevented the use of genetics as a front-line compliance tool [7].

The development of a rapid, simple timber DNA extraction technique that could be integrated into supply chain and trade verification processes would aid in the establishment of genetics as a front-line timber identification tool. To simplify the DNA sample preparation process and overcome extraction challenges, our group has developed focused ultrasound extraction (FUSE) technologies for tissue homogenization, cell lysis, and DNA release [25, 26]. FUSE employs high-pressure ultrasound pulses to generate a non-thermal, focused cavitation bubble cloud. The expansion and collapse of cavitation microbubbles result in rapid tissue disintegration

and cell lysis. Previous studies have demonstrated the potential of FUSE in preparing Atlantic salmon muscle tissue and leaves for DNA testing, exemplifying the utility of FUSE in varying sample compositions for applications ranging from wildlife conservation to forest population genomics. Furthermore, we expect that this DNA sample preparation method can detect genetic markers for other uses including diagnostics, personalized medicine approaches, and agriculture [27-29].

In conventional timber sample preparation protocols, tissue homogenization is done with a mortar and pestle under liquid nitrogen or using benchtop homogenizers such as bead beater or mixer mills, followed by thermal incubation with an enzymatic lysis buffer for cell lysis and silica-based DNA purification [22, 30, 31]. With FUSE, tissue homogenization and cell lysis steps are combined to allow for more efficient workflows. DNA purification is then performed using commercially available DNA extraction kits that employ silica-based purification methods (**Figure 3.1**). We expect that FUSE will improve the efficiency of timber DNA sample preparation by reducing the time and labor required for the release of DNA suitable for genetic species identification.



**Figure 3.1. FUSE experimental workflow compared to controls.** All experiments began with collection and separation of shavings from live edge slices of timber. For FUSE DNA release, samples were suspended in DNA lysis buffer and processed with focused ultrasound for 1-10 minutes. FUSE was compared to controls that used mortar and pestle under liquid nitrogen for tissue homogenization and thermal incubation with lysis buffer for cell lysis requiring 60 minutes. DNA collection and purification were performed using silica column-based methods for both FUSE and controls. DNA quality was validated with qPCR amplification and sequencing. Created with BioRender.com

Here, the feasibility of FUSE to prepare timber DNA samples is assessed by measuring tissue breakdown efficiency, determining DNA yield and quality, and performing qPCR amplification and sequencing to detect short DNA barcoding regions to evaluate the use of this technology for the identification of genetic markers. Based on previous FUSE studies, we hypothesize that FUSE will induce timber tissue pulverization, increase the DNA yield compared to conventional methods, and release DNA with quality suitable for amplicon sequencing [26]. Overall, we aim to create a streamlined DNA extraction methodology that will help guide the adoption of genetics as a standard front-line timber identification method. Advancement of this technology will focus on the development of a field-deployable DNA sample preparation platform to be used in conjunction with portable molecular identification tools to allow for complete genetic workflows outside of the laboratory.

## 3.2. Materials and Methods

### 3.2.1. Timber Sampling

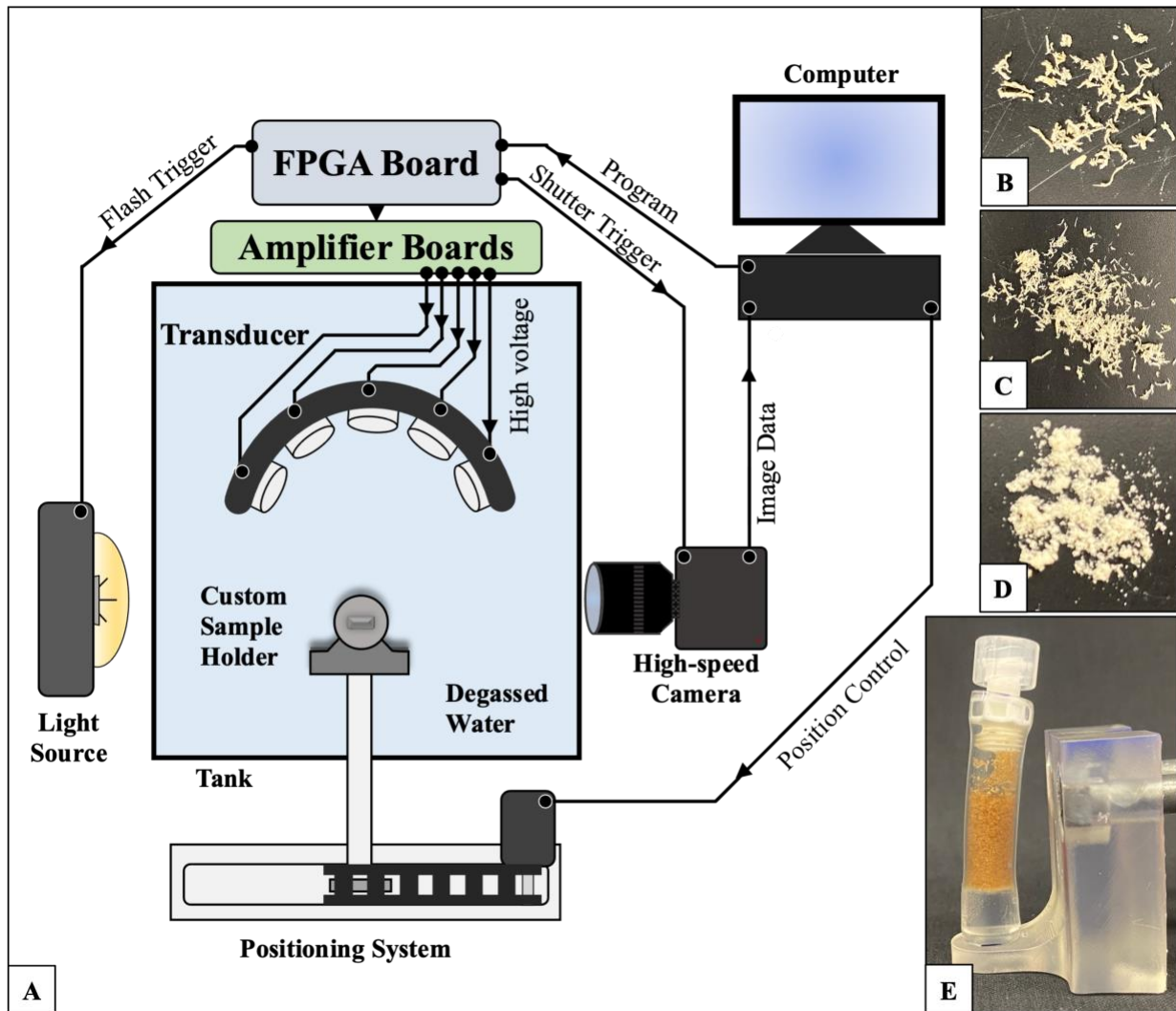
Live edge crosscut slabs of *Quercus alba*, which were stored at room temperature for approximately 2 years after harvest, were used to investigate the feasibility of FUSE for timber tissue disruption and DNA release. To prepare the tissue samples, crosscut slabs with a diameter of 28 cm were cut in half to collect shavings from the sapwood and heartwood regions. Shavings were collected using a file and rasp and passed through a series of ISO test sieves (Gilson Company Incorporated, Middleton, WI, USA) with square openings of 1 mm, 500  $\mu\text{m}$ , and 100  $\mu\text{m}$  to separate the shavings based on size. Shaving groups were defined as coarse, medium, fine, or combination groups. Coarse shavings had an area ranging from  $10^6$ - $2.5 \times 10^5$   $\mu\text{m}^2$ , medium shavings ranged from  $2.5 \times 10^5$ - $10^4$   $\mu\text{m}^2$ , and fine shavings were less than  $10^4$   $\mu\text{m}^2$ . For the combination group, shavings were only passed through the 1 mm sieve, so all shavings in this group were less than  $10^6$   $\mu\text{m}^2$ . All sieves were cleaned with 10% bleach before use to remove any contaminants.

### *3.2.2. FUSE Pulse Generation*

The ultrasonic transducer and pulsing parameters used in this study were the same as those described previously [25, 26]. Briefly, a 32-element 500 kHz array transducer with a geometric focus of 75 mm, an aperture size of 120.5 mm, and a f-number of 0.62 was driven by a custom high-voltage pulser to deliver single-cycle ultrasound pulses. The pulser was connected to a field-programmable gate array (FPGA) board (Altera DE0-Nano; Terasic Incorporated, Hsinchu, Taiwan) that was programmed for FUSE pulsing. All acoustic peak negative pressure measurements were collected using a fiber-optic probe hydrophone [32] and cross-calibrated using a reference rod hydrophone (Onda HNR-0500; Onda Corporation, Sunnyvale, California, USA). All pressure measurements were recorded in degassed water at the focal point of the transducer.

### *3.2.3. FUSE Sample Preparation and DNA Extraction*

During FUSE tissue processing, samples were contained in a custom-designed sample holder that has been used in previous studies [25]. Briefly, the holder featured a mount for the suspension of an optically transparent and acoustically permeable tube with an inner diameter of 6.35 mm and a wall thickness of 1.59 mm (Tygon PVC E-1000, McMaster-Carr, Douglasville, GA, USA). For DNA extraction experiments, 75 mg of timber shavings were suspended in a lysis buffer consisting of 500  $\mu\text{L}$  of 1% PVP-40 Buffer AP1 solution and 4  $\mu\text{L}$  of RNase A (Qiagen DNeasy Plant Kit; Qiagen Incorporated, Hilden, Germany) in the sample tube. A locking top was used to seal the top end of the tube, such that the sample could be suspended in a water tank for tissue processing. The sample holder was supported by a robotic positioning system controlled by custom MATLAB scripts. To align the sample within the focus of the ultrasound transducer, high-speed optical imaging was performed using a machine-vision camera (Blackfly S 3.2MP Mono USB3 Vision; FLIR Integrated Imaging Solutions, Richmond, British Columbia, Canada) with a 100 mm F2.8 Macro lens (Tokina AT-X Pro; Kenko Tokina Co., Tokyo, Japan) and backlit by a custom-built pulsed LED strobe light capable of high-speed triggering with 1  $\mu\text{s}$  exposures. As done in previous studies, the camera and the strobe light were triggered individually by an amplifier box, with the camera shutter opening at the time of pulse generation and the strobe acting as the shutter [26] (**Figure 3.2**). After FUSE processing, DNA purification was done using silica columns as recommended by the manufacturer (Qiagen DNeasy Plant Kit) with a final elution volume of 80  $\mu\text{L}$ .



**Figure 3.2. FUSE experimental set-up for timber processing.** (A) During FUSE processing, a 32-element transducer was driven by FPGA and amplifier boards. High-speed imaging was performed with a camera and light source. A robotic positioning system was used to align the sample with the focus of the transducer. The computer controlled the delivery of ultrasound pulses, image collection, and positioning system movements using custom MATLAB scripts. (B) Coarse, (C) medium, (D) fine, and a combination of shaving sizes were collected from live edge white oak timber slices. (E) Shavings were transferred to the FUSE sample holder before FUSE processing.

#### 3.2.4. FUSE Acoustic Parameters

Single-cycle ultrasound pulses were delivered to the center of the sample tube at a pressure of 40 MPa and a pulse repetition frequency (PRF) of 1 kHz. Measurements made with a fiber optic hydrophone showed that delivery of pulses through the sample tube resulted in a negligible (<1%) pressure loss. During FUSE tissue processing, a robotic positioning system was programmed with custom MATLAB scripts to vertically move the sample holder to scan the acoustic focus through

the length of the tube. This was done to ensure that the entirety of the sample volume was exposed to FUSE pulses. To determine the effect of FUSE processing time on tissue homogenization, DNA yield, and DNA quality, the duration of FUSE processing was varied. Processing times of one, five, and ten minutes were evaluated by delivering 60,000, 300,000, and 600,000 pulses, respectively to fine, medium, coarse, and combination shaving groups (n = 6).

### *3.2.5. Timber Particle Size Analysis*

The effectiveness of FUSE for timber tissue fractionation was evaluated by measuring the number and size of timber shavings after FUSE processing. Groups of fine, medium, coarse, and combination timber shavings were suspended in 500  $\mu$ L of deionized water and processed for zero, one, five, and ten minutes. Three replicates (n = 3) of each treatment group were performed. After processing, the shavings were imaged under a confocal microscope (Zeiss, Jena, Germany). Samples were diluted with 2 mL of deionized water prior to imaging to reduce the shaving concentration and prevent particle overlap and misinterpretation of the imaging data. Using a 50  $\mu$ L transfer pipette, three suspensions from each sample were prepared on microscope slides and visualized using 10x magnification. Ten images were taken at different locations in each slide to collect representative sample data, resulting in a total of 30 representative images per sample and 90 images per treatment group (**Supplementary Figure 3.1**) (**Appendix A**).

Images from each microscope slide were analyzed together, such that the resulting image data from each microscope slide was identified as one of nine samples within each treatment group. All images were converted to grayscale, then to binary using the Otsu method [33]. The binary image was then inverted. Timber particles were identified as regions with three or more connected pixels, and the number of timber particles in each image was counted. The size of the particles was determined by counting the number of pixels within the detected region and converting pixel size

to microns. The particle count and size data from each treatment group were pooled, and the detected particles were grouped into size ranges of 200  $\mu\text{m}^2$  increments. The shaving size distribution data was reported as the normalized area covered by particles within each size range, and the total number of detected shavings was reported for each group. The significance of the size distribution and total counts was determined using an unpaired student's t-test with unequal variance comparing zero minutes of FUSE processing to one, five, and ten minutes. Values less than 0.05 ( $p < 0.05$ ) were considered significant.

### *3.2.6. Conventional DNA Extraction using a Mortar and Pestle*

To compare FUSE to conventional extraction methods, fine, medium, coarse, and combination shavings ( $n = 6$ ) were homogenized using a mortar and pestle under liquid nitrogen to determine the efficacy of FUSE compared to conventional methods. Approximately 100 mg of shavings were transferred to the mortar, and then liquid nitrogen was added to freeze the samples and cool the mortar and pestle. Samples were ground vigorously for approximately 30 seconds to fractionate the shavings. Once the liquid nitrogen was completely evaporated, shavings were transferred to a 1.5 mL centrifuge tube where 500  $\mu\text{L}$  1% PVP-40 Buffer AP1 solution and 4  $\mu\text{L}$  of RNase A were added. Tubes were vortexed to homogenize the solution and then incubated at 65  $^{\circ}\text{C}$  for one hour with a short vortex every ten minutes. Additionally, negative control groups with fine, medium, coarse, and combination shavings ( $n = 6$ ) were suspended in 500  $\mu\text{L}$  1% PVP-40 Buffer AP1 solution and 4  $\mu\text{L}$  of RNase A (100 mg/mL) for ten minutes at room temperature. Purification of the mortar and pestle control and negative control samples was done using silica columns as recommended by the manufacturer (Qiagen DNeasy Plant Kit).

### *3.2.7. DNA Quantification*

The quantity of released DNA was measured for all treatment groups to determine the efficacy of FUSE compared to conventional methods and controls. DNA samples were analyzed with a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a NanoDrop One (Thermo Fisher Scientific). The DNA yield was reported as the quantity of DNA released per milligram of tissue to normalize input sample mass using DNA concentration values from the Qubit. The quality of the DNA was evaluated using the 260/280 and 260/230 ratios reported from the NanoDrop. DNA samples were also visualized using gel electrophoresis to compare DNA fragmentation across treatment groups. 300 ng of DNA sample was stained with 1x GelRed (Millipore Sigma, Burlington, Massachusetts, USA) and was run on the electrophoresis system for 1 hour at 100 V in a 1% agarose gel in 1x TBE buffer (Thermo Fisher Scientific). The molecular weight of the DNA was estimated by comparison with the GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific). Gel images were collected using the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). For data acquired with the Qubit and NanoDrop, an unpaired student's t-test with unequal variance was used, with values less than 0.05 ( $p < 0.05$ ) deemed significant.

### 3.2.8. Quantitative PCR Amplification

qPCR amplification of short DNA barcoding regions within the chloroplast genome was performed on DNA samples extracted from the combination group of shavings. Primers were designed to target regions within the maturase K chloroplast gene (*matK*) and the ribulose biphosphate carboxylase chloroplast gene (*rbcL*) of the *Quercus* genus. The *matK* forward primer, 5'-TTTCCGGTCATCCCATGCTTT-3', and reverse primer, 5'-TGCAGGATTCGTCGAACACT-3', targeted a 243 bp region of the *matK* gene. The *rbcL* forward primer, 5'-ACGATGCTACCACATCGAGC-3', and reverse primer, 5'-GAGGCGGACCTTGAAAGTT-3', targeted a 212 bp region of the *rbcL* gene. qPCR mixtures

consisted of 1X PowerUp SYBR Green Master Mix (Applied Biosystems; Thermo Fischer Scientific), 0.5  $\mu$ M of each primer (Integrated DNA Technologies, Coralville, Iowa, USA), 5  $\mu$ L of template DNA, and nuclease-free water to a total volume of 40  $\mu$ L. All reactions were performed on the Applied Biosystems 7300 (Thermo Fisher Scientific). Thermal cycling conditions for the matK primers were as follows: 50 °C for 2 minutes, 95 °C for 2 minutes, and 60 cycles of 95 °C for 15 seconds, 57 °C for 15 seconds, and 72 °C for 1 minute. For the rbcL primers, thermal cycling conditions were: 50 °C for 2 minutes, 95 °C for 2 minutes, and 45 cycles of 95 °C for 15 seconds, 57 °C for 15 seconds, and 72 °C for 1 minute. Amplifications were performed in triplicate for all samples, and a negative control containing nuclease-free water instead of template DNA was included with each qPCR run. Amplification was considered to be successful if threshold cycle (Ct) values were below 45 cycles for matK amplicons and 40 cycles for rbcL amplicons.

### 3.2.9. Sanger Sequencing

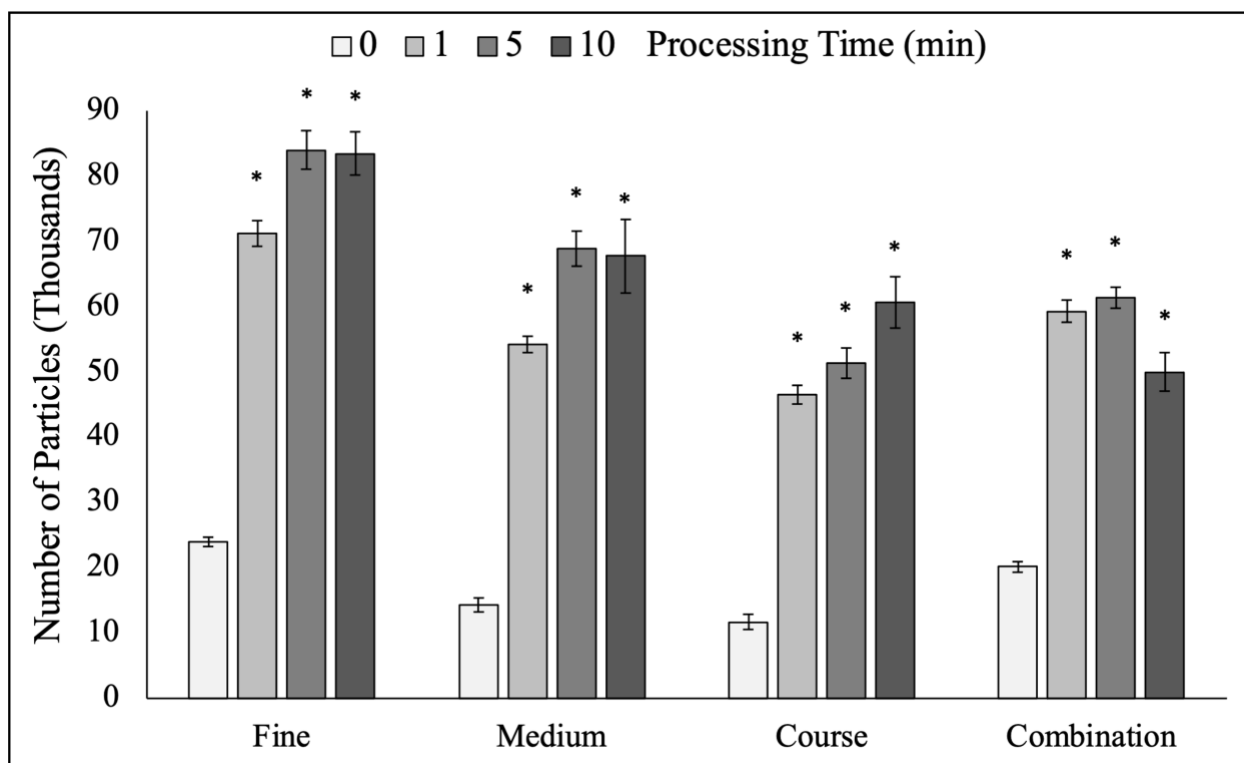
Representative amplification products generated from each primer set prepared with five minutes of FUSE processing were selected for amplicon sequencing. Samples were sent to GENEWIZ (South Plainfield, New Jersey, USA) for primer extension sequencing using Applied Biosystems BigDye version 3.1 (Thermo Fischer Scientific). The reactions were then analyzed on Applied Biosystem's 3730xl DNA Analyzer (Thermo Fischer Scientific). Sample sequences were compared to *Q. alba* matK and rbcL sequences from the National Center for Biotechnology Information (NCBI) database using the BLAST tool to confirm the identity of *Q. alba*.

## 3.3. Results

### 3.3.1. Timber Tissue Breakdown

Experiments were performed to determine the feasibility of timber tissue fractionation with FUSE. Tissue breakdown was measured by determining the number and size of white oak timber

shavings after one, five, and ten minutes of FUSE processing compared to a negative control. We hypothesized that FUSE would fractionate the timber tissue, increasing the number of particles and decreasing their size, resulting in an increase in the surface area available for DNA to escape into solution. Quantification of the particles after one, five, and ten minutes of FUSE processing revealed that there was a significant increase ( $p < 0.05$ ) in the total number of particles compared to the negative control for all shaving size groups (**Figure 3.3**). As FUSE processing time increased from one to five minutes, there was a significant increase ( $p < 0.05$ ) in the total number of particles for the fine and medium shaving groups. The effect of FUSE on timber fractionation plateaued after five minutes of tissue processing, as there was no significant difference ( $p < 0.05$ ) in the total number of particles detected between five and ten minutes of processing for the fine, medium, and coarse treatment groups. Furthermore, examination of timber particle size distribution after FUSE processing revealed a decrease in particle size compared to negative controls (**Supplementary Figure 3.2**) (**Appendix A**). Similar to the particle quantification data, the decrease in particle size plateaued after five minutes of FUSE processing. Overall, these results demonstrate that FUSE resulted in a greater number of smaller-sized particles compared to the negative control, suggesting that FUSE pulverized timber tissue.

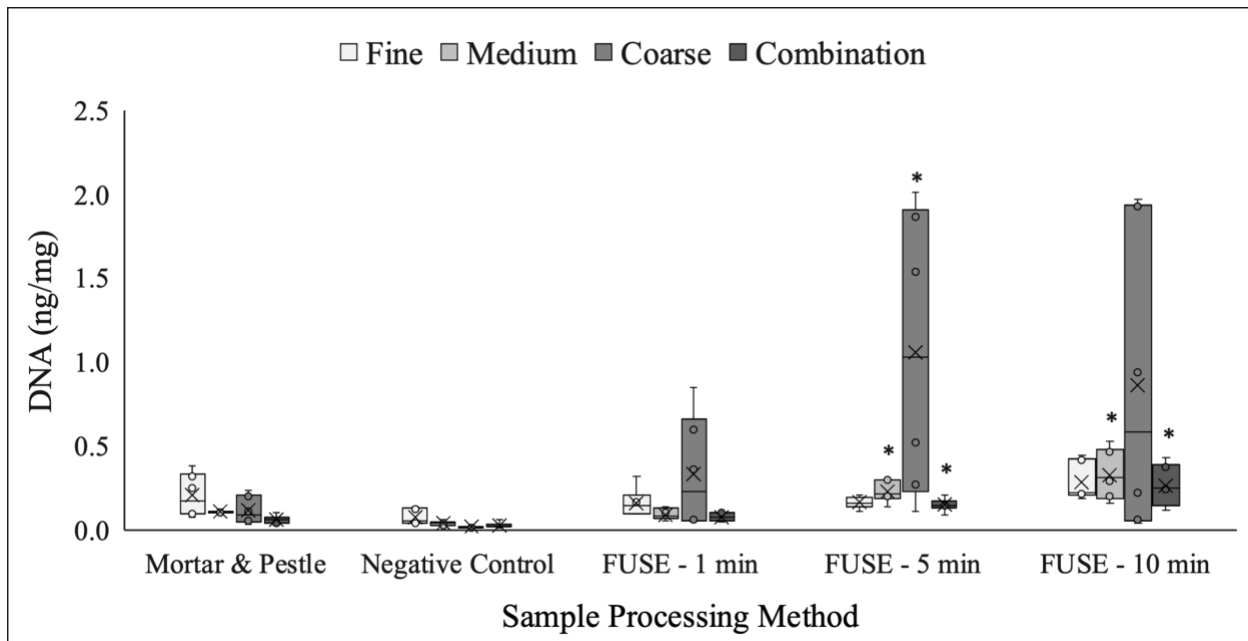


**Figure 3.3. Number of timber shavings before and after FUSE processing.** Quantification of the detected timber particles after zero, one, five, and ten minutes of FUSE processing (n = 3) shows that after at least one minute of FUSE processing, significantly more particles were detected for all shaving size groups. This result suggests that FUSE fractionates timber shavings. \*Indicates significant ( $p < 0.05$ ) differences between FUSE processing and negative controls.

### 3.3.2. DNA Extraction Feasibility

FUSE processing was compared to conventional DNA extraction methods that utilize mortar and pestle for tissue homogenization and heated incubation for cell lysis to determine the feasibility of FUSE for DNA release from timber tissue (**Figure 3.4**). Results demonstrate that FUSE released DNA from timber tissue. Five minutes of FUSE processing released significantly more DNA from timber than conventional methods that utilized mortar and pestle and one hour of heated incubation ( $p < 0.05$ ). Results show that the DNA yield was influenced by FUSE processing time and initial timber shaving size. Five minutes of FUSE processing yielded significantly more DNA than mortar and pestle controls for the medium, coarse, and combination shaving groups, while ten minutes of FUSE processing yielded more DNA than mortar and pestle controls for

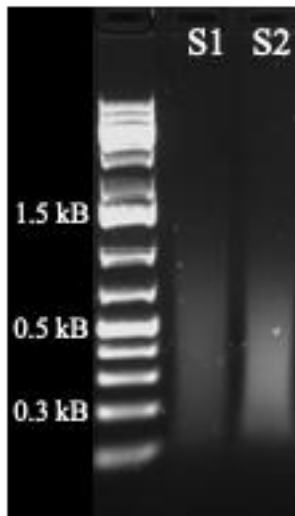
medium, and combination shaving groups ( $p < 0.05$ ). A comparison of DNA yield results after one and five minutes of FUSE processing shows that increasing the FUSE processing time increased the DNA yield for medium and combination shaving groups, while ten-minute FUSE treatments did not have a significant effect on the DNA yield compared to five-minute treatments ( $p < 0.05$ ). Additionally, trends within treatment groups revealed a relationship between shaving size and DNA yield. For samples prepared with mortar and pestle, DNA yield decreased as shaving size increased, but for samples prepared with five or ten minutes of FUSE processing, DNA yield increased as shaving size increased. Lastly, DNA purity ratios were measured to evaluate the quality of timber DNA released with FUSE and control groups (**Supplementary Table 3.1**) (**Appendix A**). The purity ratios were within the expected norms for timber DNA, suggesting that FUSE does not alter the quality of DNA released from timber tissue.



**Figure 3.4. Timber DNA yield after FUSE.** DNA yield results demonstrate that FUSE released DNA from white oak timber tissue. Five and ten minutes of FUSE processing yield significantly greater quantities of DNA than mortar and pestle processing. \*Indicates significant ( $p < 0.05$ ) differences between FUSE and mortar and pestle processed samples.

### 3.3.3. PCR Amplification and Sequencing

DNA samples (n = 30) prepared using FUSE and controls from the combination shaving group were selected for qPCR amplification. Sanger sequencing was then performed on one representative sample from each primer set to validate the utility of DNA released by FUSE for downstream analyses. Visualization of the DNA lysates with gel electrophoresis before amplification revealed that after FUSE processing DNA fragment size ranged from approximately 0.2-1.0 kB (**Figure 3.5**). qPCR results demonstrate that the quality of timber DNA released by FUSE and conventional methods is suitable for PCR-based DNA testing (**Supplementary Figure 3.3**) (**Appendix A**). qPCR success rate of matK and rbcL gene regions, measured by the number of amplified replicates in each treatment group, increased with FUSE processing time (**Table 3.1**). After five minutes of FUSE processing, the amplification success rate for the matK gene region was comparable to mortar and pestle controls. For the rbcL gene region, the amplification success of samples prepared with ten minutes of FUSE processing was comparable to those prepared with mortar and pestle.



**Figure 3.5. Visualization of timber DNA lysates.** Visualization of timber DNA lysates prepared with FUSE shows that FUSE processing sheared large DNA fragments. DNA fragment size ranged from 0.2-1.0 kB.

**Table 3.1. qPCR amplification success after FUSE processing.** matK and rbcL genes regions can be amplified with qPCR using timber DNA released by FUSE. Results show that PCR success rate improves with increased FUSE processing time.

Processing Method	matK		rbcL	
	Replicate Success	Success Rate	Replicate Success	Success Rate
Mortar & Pestle	7/18	39%	15/18	83%
Negative Control	6/18	33%	11/18	61%
FUSE – 1 min	2/18	11%	13/18	72%
FUSE – 5 min	6/18	33%	14/18	78%
FUSE – 10 min	10/18	56%	15/18	83%

Representative samples prepared with five minutes of FUSE processing provided high-quality amplicon sequencing reads for both matK and rbcL gene regions. Sample sequences were aligned to publicly available (NCBI) *Q. alba* matK and rbcL sequences resulting in a >95% match for both genes. These results show that the quality of timber DNA released with FUSE was sufficient for qPCR amplification of target genes with accuracy and success rates comparable to conventional extraction methods using mortar and pestle.

### 3.4. Discussion

Our results demonstrate that FUSE can serve as a DNA extraction technique capable of releasing significant quantities of high-quality DNA from timber tissue after five to ten minutes of processing compared to conventional methods that require one hour for tissue processing and cell lysis. Successful amplification and sequencing of target genes shows that DNA released with FUSE is suitable for sample identification assays that utilize PCR-based DNA tests or short-read amplicon sequencing. Therefore, we expect that DNA obtained with FUSE can be used for genetic species identification in the laboratory and the field for front-line surveillance.

#### 3.4.1. Effects of sample preparation on FUSE processing

In the timber tissue breakdown study, we expected to observe an increase in the total number of particles and a decrease in the size of the particles as FUSE processing time increased. For the combination shaving group, we observed an alternate trend of a decrease in the total

number of particles and an increase in the size of the particles as FUSE processing time increased. This result may be due to variations in the distribution of size and number of shavings among samples in the combination group. While this is also the case for the fine, medium, and coarse shaving size groupings, the only criterion for the combination group was that the shavings were less than  $10^6 \mu\text{m}^2$ , making this the shaving size group with the widest range of accepted sizes. Therefore, it is expected that sample-to-sample shaving size variation affected the particle size distribution results for the combination shaving group. In future work investigating the effect of FUSE on timber tissue breakdown, individual samples should be measured before and after FUSE processing to improve understanding of how the particle size distribution is impacted by FUSE.

Tissue breakdown and DNA yield results suggest that FUSE processing time and timber shaving size are both factors that impact tissue breakdown and DNA release. The plateau in DNA yield observed after five minutes of FUSE processing correlates with the results of the timber tissue breakdown study that showed a plateau in timber breakdown after five minutes of processing. These results suggest that DNA yield is dependent on the efficiency of FUSE tissue breakdown. Furthermore, DNA yield results showed that there was more DNA release from coarse shavings than other shaving size groups after FUSE processing. It is expected that cavitation-induced fluid streaming influenced the efficiency of particle breakdown and DNA yield for different shaving size groups, as the size of particles suspended in a vessel is expected to affect the streaming behavior [34]. Previous work that characterized how particles are affected by cavitation-induced fluid streaming found that larger particles (3-4 mm) are trapped near the acoustic focus while smaller particles (between 1-2 mm) follow streamline patterns and are pushed out of the focus [34]. This finding suggests that smaller timber particles may have been pushed out of the acoustic focus more readily than the larger particles. It is expected that particles trapped near the

focus were exposed to more shear stress making them more likely to break down during FUSE. In this study, the coarse shavings were most likely to be contained near the acoustic focus and most impacted by the collapse of the bubble cloud, resulting in more extensive tissue breakdown and DNA yield. Based on this finding, tissue collection techniques will be further explored in future studies such that in practice, a collection tool could be designed to separate timber tissue shavings based on size for routine and reproducible on-site timber DNA testing.

#### *3.4.2. Suitability of FUSE processing for genetic identification*

The qPCR amplification results reveal a relationship between DNA sample preparation methods and qPCR success. For both *matK* and *rbcL* gene amplification, the qPCR success rate increased with increasing FUSE processing time. These differences in qPCR success rate may be related to variations in DNA yield. Since the quantity of DNA in each qPCR mixture was not normalized, samples with higher DNA yield had more DNA input into the qPCR. For the combination shaving group, DNA yield increased with FUSE processing time, which was also correlated with qPCR success rate. When comparing the qPCR success rates of samples prepared with mortar and pestle and FUSE, the same relationship with DNA yield was observed. Five and ten minutes of FUSE processing released significantly more DNA than mortar and pestle extraction methods, and the qPCR success rate of FUSE samples processed with five and ten minutes was comparable to or greater than mortar and pestle controls. Additionally, the qPCR results showed that *rbcL* amplification was more successful than *matK*. It is expected that the differences in success rate are due to differences in the binding kinetics of the designed primers [35, 36]. In future studies, further optimization of the *matK* primer design is necessary to improve the binding efficiency.

These results demonstrate the feasibility of FUSE as a rapid timber DNA extraction tool. Continued development of this technology will focus on translation to portable FUSE systems

capable of rapidly preparing DNA samples in the field. We expect that field deployable DNA extraction will provide a means to perform genetic timber identification as a front-line timber identification tool and enable more efficient timber supply chain and trade management. Overall, we intend to broaden the accessibility of genetic tools to aid in the fight against timber trafficking.

### **3.5. Conclusion**

This study was the first to investigate the feasibility of FUSE for DNA release from timber tissue. The feasibility of the FUSE protocol was assessed by measuring tissue breakdown, DNA release, qPCR amplification, and amplicon sequencing in *Q. alba* timber shaving samples. Tissue fractionation results demonstrated that FUSE breaks down timber tissue, resulting in an increased number of particles and a decrease in particle size. DNA release with FUSE was compared to conventional methods that utilized grinding with mortar and pestle under liquid nitrogen for tissue fractionation and one hour of heated incubation in lysis buffer for cell lysis. Results showed that FUSE released significantly more DNA than conventional methods after five minutes of processing. The quality of DNA released with FUSE was comparable to conventional methods. Lastly, qPCR amplification and amplicon sequencing were performed to demonstrate the utility of DNA released with FUSE for downstream analysis. Overall, the results of this study show that FUSE can be used for timber DNA sample preparation.

### **3.6. Acknowledgments**

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# **|Chapter 4: Focused Ultrasound Extraction (FUSE) for Streamlined DNA Sample Preparation and Downstream Reaction Efficiency: A Parameter Optimization Study**

A majority component of this chapter is excerpted from a manuscript in preparation for submission to *Frontiers in Ecology and Evolution*.

Co-authors: Hal Holmes, Annika Griggs, Gabby Lopez, Misa Winters, Qian Zhang, Adam Maxwell, Jason Holliday, Eli Vlaisavljevich

## **Abstract**

Point-of-contact (POC) DNA testing has enhanced real-time surveillance and detection capabilities, leading to faster, more accurate decision-making and responses with applications spanning forensics, healthcare, and biosurveillance. However, current DNA detection devices require prepared samples with easily accessible DNA, highlighting the need for rapid, field-deployable DNA sample preparation methods capable of preparing complex sample types. Focused ultrasound extraction (FUSE) has recently been developed as a DNA extraction technique that can release DNA from robust tissue matrices. FUSE employs high-pressure ultrasound pulses to produce acoustic cavitation that pulverizes target tissue and facilitates DNA release. Here, we used piscine muscle tissue to explore the effect of FUSE pulse repetition frequency (PRF) and dose on tissue homogenization, DNA yield, DNA quality, qPCR results, and the overall time efficiency of DNA extraction. Results indicated that PRF and dose did not affect tissue homogenization success and DNA yield. For instance, results showed FUSE could achieve effective DNA release in as little as 10 seconds when treating at a higher pulsing rate (PRF= 1,000 Hz, Dose=10,000 pulses). Evaluation of the DNA quality revealed that increasing the PRF improved DNA purity, and increasing the dose resulted in the shearing of released DNA. qPCR

results showed that amplification success and reaction efficiency improved with increasing PRF. Overall, these results show that FUSE can deliver high PRFs to accelerate DNA release and provide high-quality DNA for PCR-based detection. These advancements mark significant milestones in the development of FUSE as a field-deployable platform for preparing complex samples, enabling point-of-care genetic testing for applications in forensics, healthcare, and biosurveillance.

## **4.1. Introduction**

Point-of-contact (POC) DNA testing is transforming the current standards for genetic detection and has the potential to broaden access to genetic testing in fields including healthcare, biosurveillance, and forensics. These fields have traditionally relied on centralized laboratories for sample identification using genetic methods such as nucleic acid amplification tests (NAATs) or imaging-based approaches [1-4]. Due to the extensive turnaround times and resource requirements of laboratory-based testing, POC NAATs have been explored as a more efficient and accessible alternative [5-7]. However, the complexities of integrating DNA sample preparation, amplification, and detection have been a bottleneck in the development of POC NAATs [8-10]. DNA sample preparation describes the process of sample collection, tissue homogenization when applicable, cell lysis, and nucleic acid isolation [11, 12]. Conventional DNA sample preparation methods are laborious, resource-intensive, can take hours or days to complete, and often require hazardous chemicals, making them difficult to translate to POC platforms [5, 10]. To overcome these complexities, existing POC NAATs have integrated microfluidic, cartridge-based, or paper-based sample preparation systems [13-15]. While these methods have been successful for some applications, they can only prepare samples with easily accessible DNA and do not require tissue homogenization, such as blood, urine, buccal swabs, serum, and saliva [5]. For DNA detection in

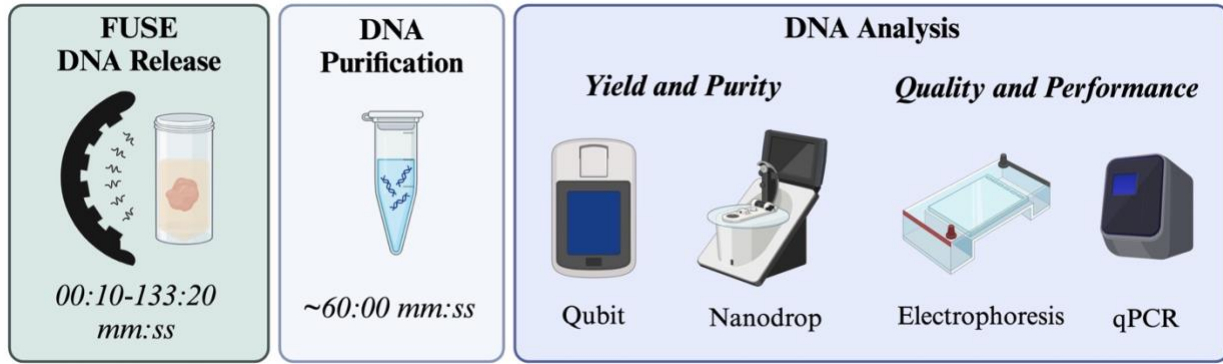
fields such as forensics, healthcare, and biosurveillance, a variety of robust sample types may be encountered, including leaves, timber, hair, teeth, bones, soft tissue, and certain bacteria [2, 7, 16-18]. For these samples, tissue homogenization is performed in the laboratory using grinding, shearing, beating, or sonication methods followed by protracted incubation periods for cell lysis and DNA release, preventing the use of POC NAATs for several applications [19, 20].

To address the limitations of POC DNA sample preparation methods, focused ultrasound extraction (FUSE) has been developed for rapid DNA release from complex tissue matrices. FUSE simultaneously homogenizes tissue and performs cell lysis by delivering high-pressure ultrasound pulses to generate a non-thermal, focused cavitation bubble cloud [21-23]. During FUSE processing, expansion and collapse of the cavitation bubble cloud disintegrates the target tissue at the subcellular level, resulting in DNA release. Previous work has demonstrated that FUSE can effectively extract DNA from piscine muscle tissue [21], leaves [22], and timber [23]. These studies showed that plant DNA extraction can be achieved in five to ten minutes compared to conventional methods that take up to an hour to complete. The sample versatility and speed of FUSE processing demonstrate its potential as a DNA sample preparation method suitable for a range of POC testing applications. Furthermore, focused ultrasound transducers can be manufactured with varying form factors and designed for portable applications [24-27], highlighting the prospect of integrating FUSE with POC testing platforms.

There have not been any studies to date exploring the effects of FUSE pulsing parameters on DNA extraction efficiency for any sample type. The FUSE process is based on a type of ultrasound tissue destruction known as histotripsy, a therapeutic ultrasound modality for non-invasive tissue ablation [28, 29]. In histotripsy, short-duration, high-amplitude focused ultrasound pulses generate a cavitation bubble cloud that precisely destroys tissue in the focal region [28, 30].

The rate of histotripsy pulsing is governed by acoustic pulsing parameters such as the pulse repetition frequency (PRF), the number of pulses delivered per second. While increasing the PRF results in faster delivery of the overall treatment dose, previous work that explored the effects of PRF on the ablation of red blood cells in a fixed tissue phantom found that high PRFs reduced treatment precision, as increasing the PRF resulted in lesions with inconsistent shapes and sizes [31]. The impact of PRF on FUSE processing has not been investigated, so in this study, we intend to determine the effect of PRF on DNA release, DNA quality, and FUSE processing time.

Here, we investigated the effect of FUSE PRF and dose on the efficiency of DNA release from piscine muscle tissue. This tissue was selected due to its relevance in wildlife forensics, as illegal, unreported, and unregulated fishing is an international issue in need of improved detection strategies [32]. To measure the impact of PRF and dose on FUSE processing, we assessed tissue breakdown efficiency, DNA yield, DNA quality, and qPCR results (**Figure 4.1**). Tissue breakdown was evaluated by visualizing tissue lysates after FUSE processing. DNA yield and DNA quality were measured using spectrophotometry and fluorometry. DNA quality was further investigated by measuring DNA fragmentation, qPCR success, and qPCR efficiency. Based on our prior work that explored the effect of dose on DNA release from plant tissues, we hypothesized that increasing the dose would result in greater tissue disintegration and increased DNA yield without affecting DNA quality [22, 23]. We do not expect that the PRF will affect the tissue disintegration efficiency, DNA yield, or DNA quality. We expect that increasing the pulsing rate will substantially reduce the time required to extract DNA quantities sufficient for genetic testing. In this work, we seek to expedite DNA sample preparation workflows by investigating the impact of pulsing parameters on FUSE processing, with the ultimate goal of developing a DNA sample preparation platform suitable for POC testing applications.



**Figure 4.1. FUSE pulsing parameter optimization.** Atlantic salmon samples were prepared with FUSE with varying dose and pulsing rates. After FUSE processing, samples were purified to isolate the released DNA. Purified DNA samples were analyzed with fluorometry, spectrophotometry, gel electrophoresis, and qPCR to determine the effects of FUSE pulsing parameters on DNA yield, quality, purity, and qPCR success.

## 4.2. Materials and Methods

### 4.2.1. Sample Preparation

Fresh filets of Atlantic salmon, *Salmo salar*, were used to study the effect of FUSE pulsing parameters on tissue disruption and DNA release. Two filets were stored at -20 °C. Samples were prepared using a sterile scalpel blade by cutting the filets into small cubes of uniform size with a mass of 25-60 mg. After sample sectioning, they were rinsed with 300  $\mu$ l of nuclease-free deionized water to remove any surface contaminants. A total of 39 samples were prepared from each filet, such that each treatment group consisted of three samples from filet 1 and three from filet 2.

### 4.2.2. FUSE Pulse Generation and Characterization

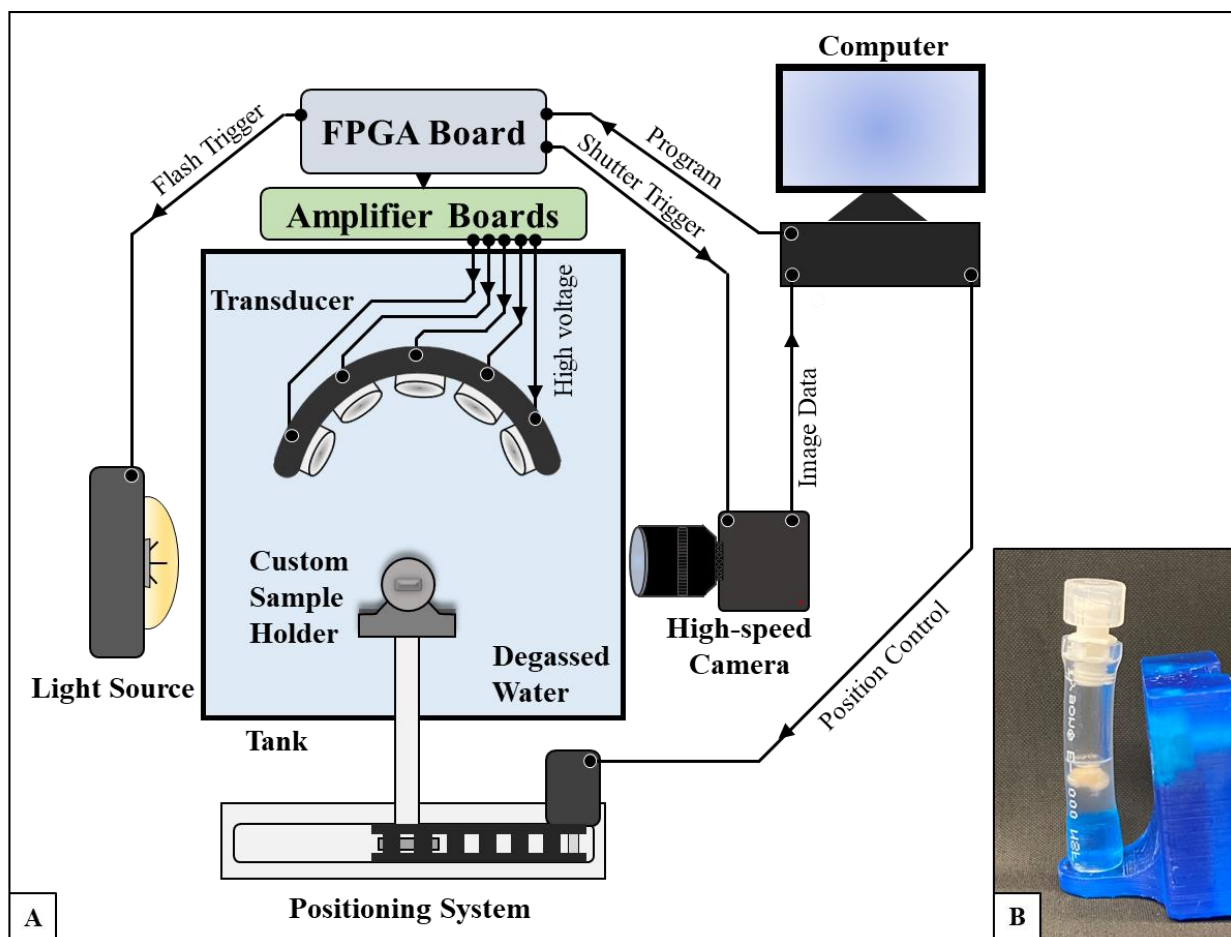
The transducer and pulsing parameters used in this study followed those of our prior work [21-23]. Briefly, a 32-element 500 kHz array transducer with a geometric focus of 75 mm, an aperture size of 120.5 mm, and a f-number of 0.62 was used for this study. The transducer was driven with a high-voltage pulser controlled by a field-programmable gate array (FPGA) board (Altera DE0-Nano; Terasic Incorporated, Hsinchu, Taiwan). The acoustic peak negative pressure of the ultrasound pulses was measured using a fiber-optic hydrophone [33] and cross-calibrated

using a reference rod hydrophone (Onda HNR-0500; Onda Corporation, Sunnyvale, California, USA). Pressure measurements were performed in degassed, deionized water at the focus of the transducer.

#### *4.2.3. FUSE Experimental Setup and DNA Extraction*

To prepare for FUSE processing, samples were suspended in a custom-designed sample holder that was used in previous studies [21, 23]. The holder was designed to support an optically transparent and acoustically permeable sample tube with an inner diameter of 6.35 mm and a wall thickness of 1.59 mm (Tygon PVC E-1000, McMaster-Carr, Douglasville, GA, USA). For all treatment groups, Atlantic salmon samples were prepared in the sample tube with lysis buffer composed of 270  $\mu\text{L}$  of Buffer ATL and 30  $\mu\text{L}$  Proteinase K (Qiagen Blood and Tissue Kit; Qiagen Inc, Hilden, Germany). The top end of the tube was sealed with a locking cap. During FUSE processing, the sample holder was mounted on a robotic positioning system controlled by custom MATLAB scripts. Samples were suspended in a water tank for FUSE tissue processing, and the positioning system was used to place the sample in the focus of the transducer. Visualization of sample alignment was performed using a machine-vision camera (Blackfly S 3.2MP Mono USB3 Vision; FLIR Integrated Imaging Solutions, Richmond, British Columbia, Canada) with a 100 mm F2.8 Macro lens (Tokina AT-ZZ Pro; Kenko Tokina Co., Tokyo, Japan) and a custom-built pulsed LED strobe light that was triggered with 1  $\mu\text{s}$  exposures. The camera and strobe light were triggered independently by the amplifier. The camera shutter was triggered to open at the time of pulse delivery while the strobe acted as a shutter and was triggered after pulse arrival to the focus to capture the resulting acoustic cavitation (**Figure 4.2**). After tissue processing, the sample holder was removed from the positioning system and images of the pulverized tissue samples were

collected. Silica-column purification was performed as recommended by the manufacturer (Qiagen DNeasy Plant Kit) with a final elution volume of 200  $\mu\text{L}$ .



**Figure 4.2. FUSE experimental set-up.** (A) An ultrasonic array transducer is driven by FPGA and amplifier boards. Movement of a robotic positioning system is guided by high-speed imaging with a camera and light source to align samples with the ultrasonic focus. A computer with custom MATLAB scripts is used to control the delivery of ultrasound pulses, image capture, and positioning system movement. (B) Atlantic salmon samples are suspended in lysis buffer in the FUSE samples holder.

#### 4.2.4. FUSE Acoustic Parameters

All samples were processed with single-cycle ultrasound pulses at a peak negative pressure of 40 MPa. Fiber optic hydrophone measurements were performed to measure pressure loss through the sample tube, and results confirmed that the tube was acoustically permeable as the pressure loss was negligible (<1%). The PRF and dose were varied to investigate the effect of FUSE acoustic parameters on the efficiency of tissue disruption, DNA yield, and DNA quality.

Doses of 10,000, 100,000 and 200,000 pulses were delivered at PRFs of 25, 250, 500, and 1,000 Hz (n = 6). With these parameters, the minimum processing time was 10 seconds with a dose of 10,000 pulses delivered at 1,000 Hz and a maximum processing time of 2 hours and 13 minutes with a dose of 200,000 pulses delivered at 25 Hz (**Table 4.1**).

**Table 4.1. Time efficiencies of FUSE pulsing parameters.** matK and rbcL genes regions can be amplified with qPCR using timber DNA released by FUSE. Results show that PCR success rate improves with increased FUSE processing time.

<b>Pulsing Parameter Time Efficiencies (mm:ss)</b>				
<b># of Pulses</b>	<b>25 Hz</b>	<b>250 Hz</b>	<b>500 Hz</b>	<b>1,000 Hz</b>
10,000	06:40	00:40	00:20	00:10
100,000	66:40	06:40	03:20	01:40
200,000	133:20	13:20	06:40	03:20

#### *4.2.5. Control DNA Extraction*

Conventional extraction methods using a mortar and pestle under liquid nitrogen were performed as a control to compare against FUSE. Tissue disruption and cell lysis of control samples (n = 6) was performed by placing 25-60 mg of tissue into a 1.5 mL centrifuge tube and adding 270  $\mu$ L of Buffer ATL and 30  $\mu$ L Proteinase K. Tubes were vortexed to homogenize the solution then incubated at 56 °C and vortexed every five minutes for 15 seconds until no tissue fragments remained. The time required for complete tissue homogenization ranged from 30-55 minutes. Control samples were purified using silica columns as recommended by the manufacturer (Qiagen Blood and Tissue Kit).

#### *4.2.6. DNA Quantification*

The released DNA was quantified for all treatment groups. DNA samples were analyzed using a Qubit 4 Fluorometer (Thermo Fischer Scientific, Waltham, Massachusetts, USA) and a NanoDrop One (Thermo Fisher Scientific). DNA yield values were reported as the quantity of DNA released per milligram of tissue to normalize the input sample mass using DNA

concentration values reported from the Qubit. The quality of the DNA was evaluated using the 260/280 and 260/230 ratios reported from the NanoDrop. DNA lysates were visualized using gel electrophoresis to determine how PRF and dose affect DNA fragmentation. 300 ng of DNA sample was run for 1 hour at 100 V in a 1% agarose gel in 1x TBE buffer (Thermo Fischer Scientific). The DNA was stained with 1x GelRed (Millipore Sigma, Burlington, Massachusetts, USA). The molecular weight of the DNA was estimated by comparison with the GeneRuler 1 kb Plus DNA Ladder (Thermo Fischer Scientific). Gel images were collected with the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). Significance of DNA yield and quality values was determined using an unpaired student's t-test with unequal variance, with values less than 0.05 ( $P < 0.05$ ) deemed significant.

#### 4.2.7. *Quantitative PCR Amplification*

PCR amplification of the cyclooxygenase subunit 1 mitochondrial gene (COI) of *S. salar* was performed on all DNA samples. The forward primer, 5'–CGCCCTAAGTCTCTTGATTTCG–3', and reverse primer, 5'–GTAGTATGGTAATGCCTGCTGC–3' were selected to amplify a 536 bp region of the COI gene [21]. Reactions were carried out in a total volume of 12  $\mu$ L with 6  $\mu$ L of 1X PowerUp SYBR Green Master Mix (Applied Biosystems), 0.5  $\mu$ M of each primer (Integrated DNA Technologies) and 1.5  $\mu$ L of template DNA. The thermal cycling conditions were 50 °C for 2 minutes, 95 °C for 2 minutes, and 30 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Amplifications were carried out in triplicate, and each qPCR run included a negative control with nuclease-free water instead of template DNA. Amplifications with threshold cycle (Ct) values below 26 cycles were considered successful. Ct values were compared across treatment groups, and significance was determined using an unpaired student's t-test with unequal variance. Values less than 0.05 ( $P < 0.05$ ) were deemed significant.

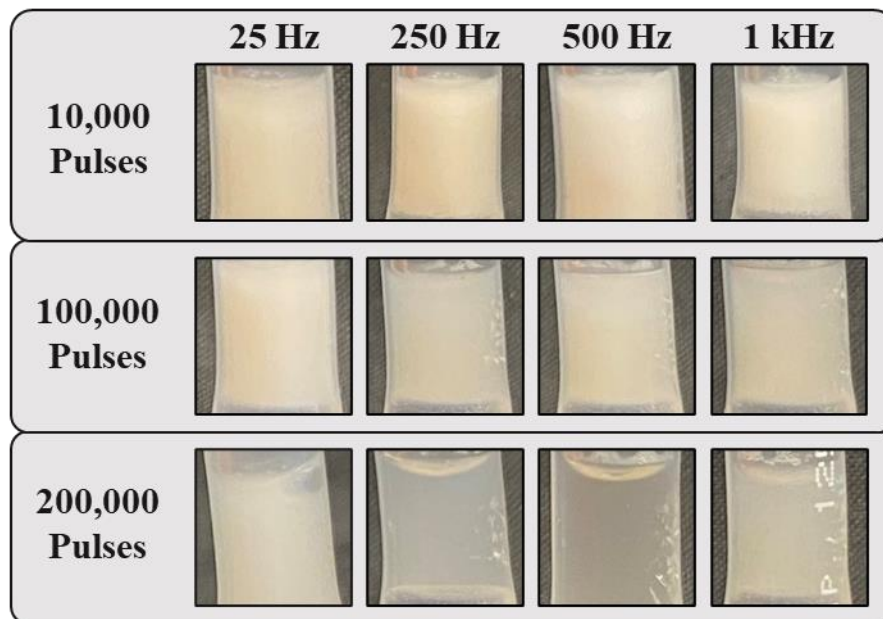
#### 4.2.8. Sanger Sequencing

Representative amplicons prepared using 10,000 pulses delivered at a PRF of 25 Hz were sequenced with Sanger sequencing. Primer extension sequencing was performed by GENEWIZ (South Plainfield, New Jersey) using Applied Biosystems BigDye version 3.1 (Thermo Fischer Scientific). The Applied Biosystem's 3730xl DNA Analyzer (Thermo Fischer Scientific) was used to analyze the results. Sample sequences were compared to reference *S. salar* COI sequences from the National Center for Biotechnology Information (NCBI) database using the BLAST tool.

### 4.3. Results

#### 4.3.1. Tissue Breakdown Efficiency

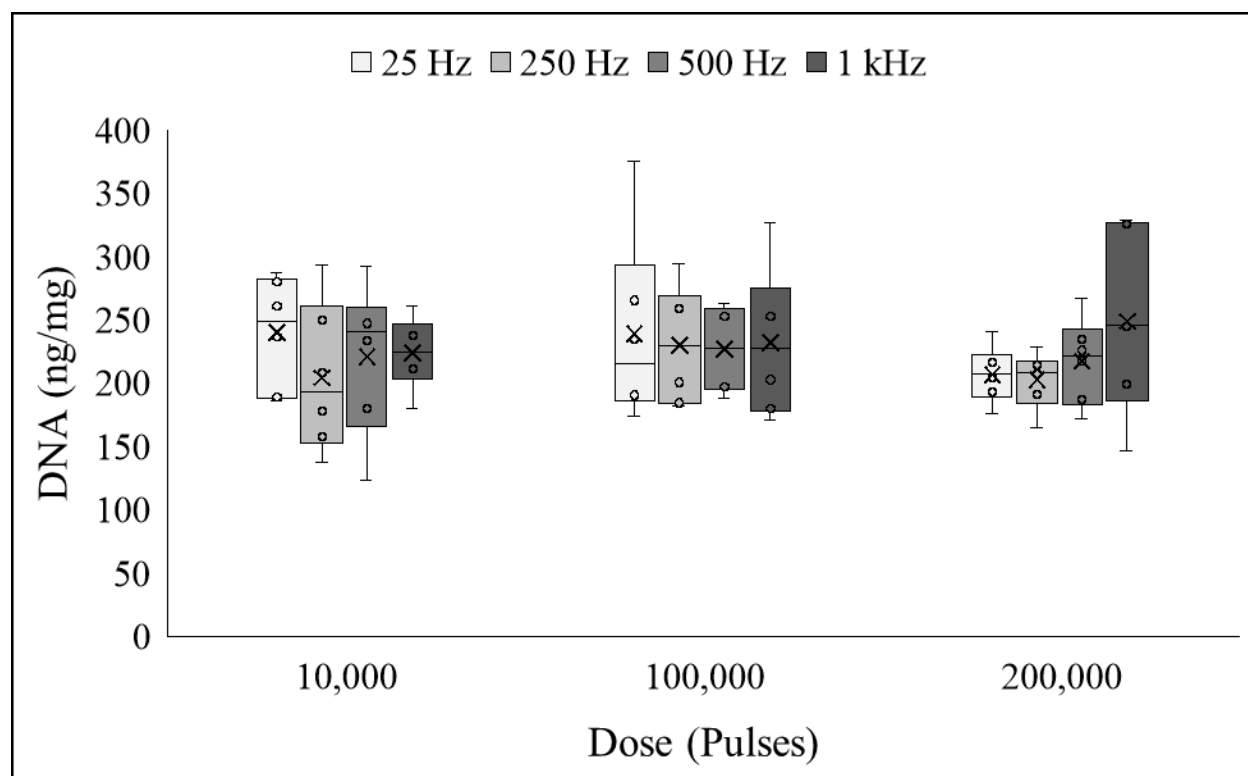
The effect of PRF and dose on tissue breakdown efficiency was determined by visualizing piscine tissue lysates after FUSE processing (**Figure 4.3**). Sample visualization revealed that all the tested pulsing parameters resulted in complete sample disintegration, such that no macroscopic tissue fragments remained after FUSE processing. While all samples showed complete tissue breakdown, a decrease in the opacity of the tissue lysate was observed with increasing dose. The observed decrease in opacity likely indicates the breakdown of microscopic tissue fragments, suggesting that increasing the dose enhances tissue breakdown.



**Figure 4.3. Representative images of Atlantic salmon tissue breakdown with FUSE.** Atlantic salmon tissue is completely disintegrated after FUSE processing. Image observation suggests that increasing the PRF and dose improved the tissue ablation efficiency.

#### 4.3.2. DNA Extraction Efficiency

PRF and dose were varied to determine the effect of FUSE pulsing parameters on DNA release (**Figure 4.4**). The results indicate that neither varying the PRF nor the dose significantly affected DNA yield, and in all cases DNA concentrations were sufficient for downstream testing. After only ten seconds of FUSE processing,  $223.91 \pm 4.79$  ng/mg of DNA was released from tissue samples, demonstrating that 10,000 pulses delivered at a PRF of 1,000 Hz can enable rapid DNA extraction. In contrast, conventional extraction methods required up to 55 minutes for tissue breakdown and cell lysis, and  $60.44 \pm 4.86$  ng/mg of DNA was released from these samples. These results show that the FUSE pulsing rate can be elevated to accelerate sample processing, leading to more time-efficient DNA extraction processes without compromising DNA yield.



**Figure 4.4. The effect of PRF and dose on FUSE DNA yield.** DNA yield results show that the quantity of DNA release is not influenced by FUSE PRF and dose. FUSE released significant quantities of DNA after only ten seconds of tissue processing by delivering 10,000 pulses at a PRF of 1,000 Hz.

The impact of PRF and dose on DNA purity was investigated by measuring the 260/280 and 260/230 purity ratios (**Table 4.2, Table 4.3**). The 260/280 ratios for all treatment groups were consistent with the expected values for DNA. For the 260/230 ratios, PRF significantly impacted the results, whereas the dose had no effect. The 260/230 ratios for samples processed with 25, 250, and 500 Hz were significantly lower than the expected range of 2.0–2.2, while samples processed with a PRF of 1,000 Hz showed 260/230 values consistent with those expected for DNA. Low 260/230 ratios were also observed for control samples, with values of  $1.54 \pm 0.11$  (**Supplementary Table 4.1**) (**Appendix A**). The rise in 260/230 ratios observed for samples processed with 1,000 Hz suggests a reduction in contaminating molecules that absorb light at 230 nm or less, including proteins, carbohydrates, or lipids remaining from the sample [34]. Therefore, it is expected that

changes in the cavitation dynamics at high PRFs enhanced the degradation of contaminants, resulting in greater DNA purity.

**Table 4.2. FUSE 260/280 DNA purity ratios.** The 260/280 ratios for FUSE samples processed with doses of 10,000, 100,000, and 200,000 pulses delivered at 25, 250, 500, and 1,000 Hz. All data was collected using the Nanodrop.

260/280				
# of Pulses	25 Hz	250 Hz	500 Hz	1,000 Hz
10,000	2.01 ± 0.01	1.98 ± 0.02	1.98 ± 0.01	2.07 ± 0.01
100,000	1.98 ± 0.02	1.94 ± 0.03	1.90 ± 0.03	2.05 ± 0.01
200,000	2.00 ± 0.01	1.96 ± 0.02	1.88 ± 0.02	2.03 ± 0.01

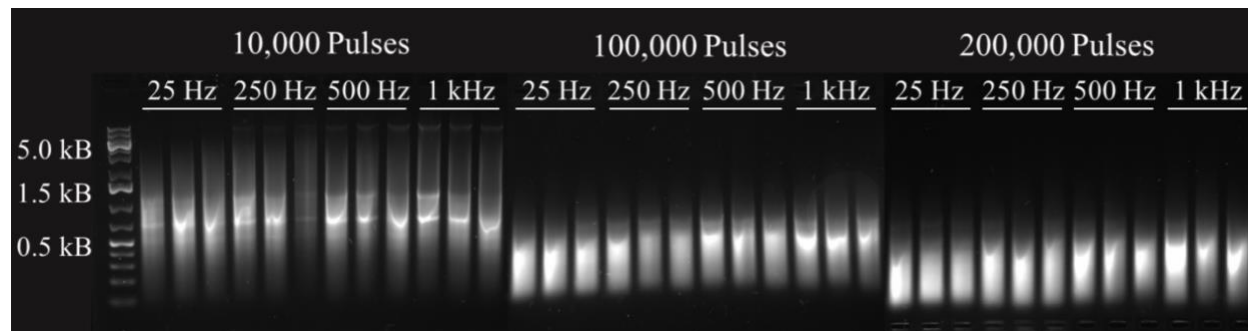
**Table 4.3. FUSE 260/230 DNA purity ratios.** The 260/230 ratios for FUSE samples processed with doses of 10,000, 100,000, and 200,000 pulses delivered at 25, 250, 500 Hz were lower than expected. Samples processed with 1,000 Hz were within the expected norms. All data was collected using the Nanodrop.

260/230				
# of Pulses	25 Hz	250 Hz	500 Hz	1,000 Hz
10,000	1.60 ± 0.10	1.51 ± 0.07	1.65 ± 0.15	2.12 ± 0.06
100,000	1.48 ± 0.04	1.55 ± 0.15	1.32 ± 0.10	2.04 ± 0.01
200,000	1.73 ± 0.09	1.56 ± 0.12	1.13 ± 0.07	2.08 ± 0.04

#### 4.3.3. DNA Fragmentation

FUSE DNA extracts were visualized with gel electrophoresis to determine the effect of PRF and dose on DNA fragmentation (**Figure 4.5**). The results demonstrated that FUSE processing resulted in DNA shearing, as smeared bands were evident for all samples, and increasing the dose reduced the size of the released DNA fragments. A shift in DNA fragment size was observed when the dose increased from 10,000 to 100,000 pulses. For samples processed with 10,000 pulses, most fragment lengths ranged from 0.3-1.5 kB, while for samples processed with 100,000 pulses or more, DNA fragments were between 0.1-0.5 kB. The effect dose on DNA fragment size plateaued when the number of delivered pulses was increased beyond 100,000 pulses. PRF did not have a discernable effect on DNA fragmentation. DNA lysates prepared with FUSE were also compared to those prepared using conventional methods (**Supplementary Figure**

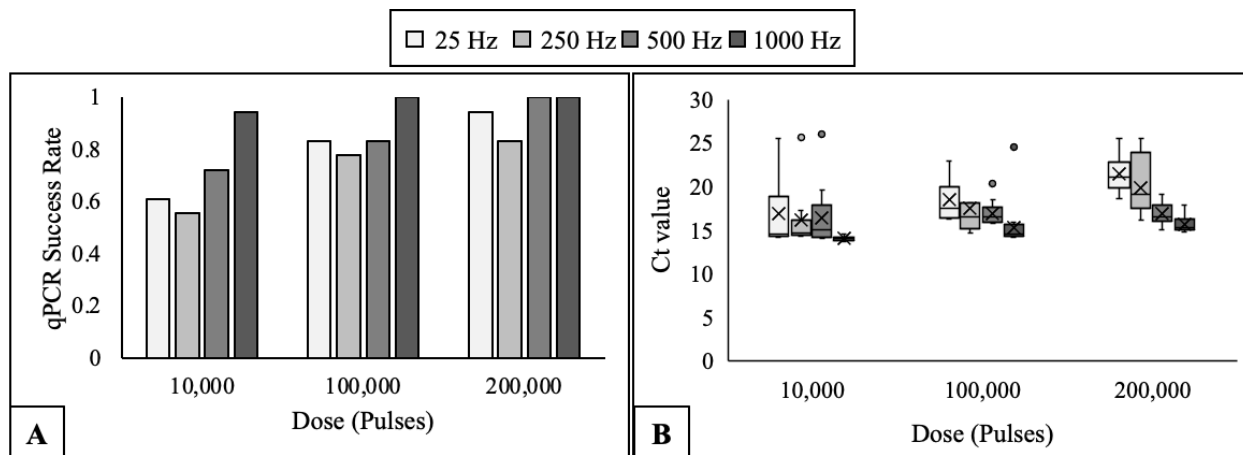
**4.1) (Appendix A).** For the control samples, distinct bands were observed, showing that conventional extraction methods did not shear the DNA. It is expected that the physical stress and turbulent fluid flow resulting from acoustic cavitation sheared the released DNA, and increasing the dose amplified this effect.



**Figure 4.5. The effect of PRF and dose on DNA fragmentation.** Visualization of DNA lysates with gel electrophoresis demonstrates that FUSE PRF and dose affect DNA fragmentation. Increasing the dose results in the release of shorter DNA fragments. For samples processed with 100,000 pulses, there is an increase in the observed length of DNA fragments when the PRF is increased.

#### 4.3.4. DNA Amplification and Sequencing

qPCR amplification results indicate that FUSE pulsing parameters influenced amplification success and reaction efficiency. For all treatment groups, greater than 50% replicates were amplified, showing that all FUSE pulsing parameters released DNA with quality sufficient for PCR amplification (**Figure 4.6A, Supplementary Table 4.2) (Appendix A)**. Trends in qPCR success rate suggest that increasing the PRF and dose resulted in greater amplification success. qPCR efficiency was also evaluated, and results demonstrate that increasing the PRF and decreasing the dose improved reaction efficiency (**Figure 4.6B, Supplementary Table 4.3) (Appendix A)**. Significance testing revealed that samples processed with 10,000 pulses had significantly greater qPCR efficiency than those processed with 200,000 pulses at PRFs of 25, 250, and 1,000 Hz. Results show that samples processed with 1,000 Hz had greater reaction efficiency than samples prepared with 25 Hz, and this was consistent across all doses. Together, these findings indicate that increasing the PRF improves qPCR success rate and efficiency.



**Figure 4.6. The effect of PRF and dose on qPCR success and efficiency.** (A) qPCR results demonstrate that increasing the PRF and dose resulted in greater amplification success, (B) and qPCR efficiency improved with increasing the PRF and decreasing dose.

## 4.4. Discussion

In this study, we expanded upon previous proof-of-concept work [21] to investigate the effect of FUSE pulsing parameters on DNA release, DNA quality, and qPCR amplification efficiency. The results demonstrate that FUSE can release high-quality DNA from piscine muscle tissue in only ten seconds, showing that the FUSE pulsing rate can be elevated for more streamlined DNA sample preparation. Furthermore, variations in the PRF and dose did not impact tissue homogenization and overall DNA yield, but, unexpectedly, an assessment of DNA purity ratios, DNA fragmentation, and qPCR results indicated that processing samples with a PRF of 1,000 Hz improved DNA quality, qPCR success rate, and qPCR efficiency. This result shows that increasing the FUSE pulsing rate enhances the time efficiency of the DNA extraction process while also improving DNA purity, making the DNA more accessible for downstream assays, such as PCR. These trends suggest that FUSE processing may also enhance the sensitivity of DNA detection assays, a critical advantage for applications with low copy number targets.

The finding that the purity of the released DNA is dependent on the pulsing rate is significant for understanding the biochemical effects of FUSE. Results revealed that the purity of samples processed with 1,000 Hz was greater than those prepared using 25, 250, and 500 Hz and

control methods. It is expected that this result is due to variations in biopolymer degradation induced by acoustic cavitation. It has been shown that ultrasound can generate polymer breakdown, and increasing ultrasound-induced shear forces can accelerate this process and aid in depolymerization [35-37]. Exploration of the effect of PRF on acoustic cavitation has demonstrated that high PRFs result in increased bubble cloud area [31]. With a larger cavitation bubble cloud, we would expect more shear stress on the target sample. Therefore, it is hypothesized that samples processed with a PRF of 1,000 Hz were exposed to greater shear stress than those processed with 25, 250, and 500 Hz, accentuating the degradation of contaminating biopolymers and leading to improved DNA purity ratios. Future work aims to perform chemical analyses such as spectroscopy or chromatography to determine the influence of FUSE pulsing parameters on the composition of the DNA extract.

In addition to its effects on DNA purity, results demonstrated that FUSE can fragment the released DNA. FUSE processing sheared the DNA, and increasing the dose resulted in greater DNA fragmentation, suggesting that at high doses, FUSE induces DNA degradation. This result suggests that FUSE generated enough ultrasound-induced shear forces to damage the released DNA. We also found that the effect of FUSE processing on DNA degradation plateaued when the dose was increased from 100,000 to 200,000 pulses. This suggests that with the applied acoustic parameters, 100,000 pulses degraded DNA to its lower limit. Interestingly, PRF did not affect DNA fragmentation, but the DNA purity ratios suggest that PRF influenced the degradation of other biopolymers. It is expected that differences in the physical properties and concentration of DNA compared to other polymers in the DNA extract resulted in DNA disintegration patterns that differed from other polymers [35]. Overall, the DNA purity and DNA fragmentation effects observed suggest that both PRF and dose have the potential to produce biochemical effects on the

processed sample by breaking down molecular components. FUSE is expected to disintegrate nucleic acids, proteins, carbohydrates, and fats, and future work aims to validate these assumptions with spectroscopy or chromatography. Based on this result, there is a need for further research into the effect of other acoustic parameters, including frequency, pulse duration, and acoustic pressure, on sample composition and DNA degradation to explore the possibility of selective contaminant depolymerization and DNA shearing.

The effects of FUSE on DNA quality were exemplified through the qPCR results. We found that both amplification success and reaction efficiency improved with increasing PRF. It is expected the increase in DNA purity observed at high PRFs made the DNA more readily accessible for qPCR. This could have been achieved by breaking apart DNA binding proteins that restrict the polymerase from accessing the DNA or by disintegrating residual proteins, carbohydrates, or lipids capable of interfering with the binding of the polymerase or primers. Dose had contrasting effects on the qPCR results, as increasing the dose improved amplification success and reduced reaction efficiency. The reduction in reaction efficiency is likely due to the increased degradation of DNA that occurred at high doses, such that fewer target copies were available, slowing the reaction kinetics. Regarding the dose dependency of the qPCR success rate, it is expected that the effect of ultrasound-induced shear forces on the sample is time-dependent, potentially affecting sample composition and influencing qPCR success rates. Although the DNA purity ratios did not show strong trends with varying doses, qPCR results suggest that increasing the dose made the released DNA more accessible for qPCR. However, the effect of dose on qPCR success was less pronounced for samples processed with a PRF of 1,000 Hz, as success rates of 94%, 100%, and 100% were reported for doses of 10,000, 100,000, and 200,000, respectively. This outcome,

coupled with the enhanced reaction efficiency at low doses, suggests that FUSE processing is most effective for qPCR sample preparation when low doses are applied at high PRFs.

## **4.5. Conclusion**

This study evaluated the effect of PRF and dose on the efficiency and quality of FUSE for DNA sample preparation. Previous feasibility studies demonstrated that 10,000 pulses could be delivered at a PRF of 25 Hz to extract DNA from piscine muscle tissue [21]. Our results show that the FUSE pulsing rate can be elevated to 1,000 Hz to significantly accelerate the extraction process without compromising DNA yield. Furthermore, increasing the pulsing rate improved DNA purity, resulting in greater qPCR success and efficiency. Overall, this work demonstrates that high FUSE pulsing rates release DNA more than 100X faster than conventional methods, optimize DNA quality, and improve the success and efficiency of downstream DNA detection assays. These findings have strong implications for the development of FUSE for POC DNA testing applications, where time efficiency, reliability, and equipment needs of DNA extraction are major bottlenecks limiting the applicability and accessibility of POC genetic testing. Ongoing work aims to develop a portable device to realize the full potential of the FUSE technology and broaden the accessibility of DNA testing for applications ranging from wildlife forensics to medical diagnostics, particularly for low copy number samples where sensitivity is critical.

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# **|Chapter 5: Development and Characterization of a Miniaturized Focused Ultrasound Extraction (FUSE) Device to Enable Point-of-Contact Genetic Testing**

A majority component of this chapter is excerpted from a manuscript in preparation for submission to IEEE Transactions on Ultrasonics, Ferroelectrics, And Frequency Control.

Co-authors: Adam Maxwell, Hal Holmes, Kayleigh Canavan, Gabby Lopez, Annika Griggs, MoonWon Jeon, Daniel Yang, Misa Winters, Qian Zhang, Jason Holliday, Eli Vlasisavljevich

## **Abstract**

Point-of-contact (POC) molecular detection has enabled rapid, accurate, and accessible testing at or near the site of need, facilitating timely decision-making in clinical, field, and industrial settings. Despite these advancements, detection platforms lack the ability to prepare DNA from complex tissue matrices, severely limiting the potential of these platforms as they currently are only able to serve a small subset of sample types. To address sample preparation inefficiencies, focused ultrasound extraction (FUSE) has been established for the rapid release of DNA from robust tissue matrices. FUSE disintegrates target tissue and releases DNA by delivering high pressure ultrasound pulses that generate a cavitation bubble cloud. In prior work, FUSE was performed using large, laboratory-based equipment. Here, a miniaturized FUSE device was designed and tested to enable POC DNA sample preparation. Design constraints were defined based on prior FUSE experiments, and based on these metrics a 750 kHz cylindrical transducer with a focal length of 19.25 mm was fabricated. The pressure output was measured, and high-speed optical imaging demonstrated that the transducer generated sustained cavitation within the focal region. The feasibility of DNA extraction from piscine and timber tissues was evaluated, and results show that the device can release DNA with quality suitable for PCR-based detection from both tissue types. These results support that this design provides a DNA sample preparation device

capable of rapidly releasing DNA from complex samples and has the potential to broaden the accessibility of molecular detection platforms.

## **5.1. Introduction**

The rapid advancement and widespread adoption of nucleic acid amplification tests (NAATs) in recent years has transformed the landscape of molecular diagnostics, particularly for point-of-contact (POC) applications [1]. NAATs can include polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), and recombinase polymerase reaction (RPA), among several other DNA amplification methods [1, 2]. These technologies have demonstrated notable versatility and can serve many applications, including healthcare and diagnostics, forensics, environmental security, and agriculture [3-7]. Factors such as rapid turnaround time, low power consumption, and design scalability enable NAATs to reach POC use cases [1, 8]. The portability of NAATs enhances their utility by enabling molecular detection in diverse environments, including at-home use, major hospitals, laboratories, mobile healthcare clinics, or remote field settings for forensic, agricultural, and environmental security use cases [9-12].

Despite the advantages of NAATs, DNA sample preparation shortcomings restrict the full potential of these platforms. While some NAATs have been designed with integrated sample processing and DNA extraction capabilities [13, 14], they can only address simple sample types with readily accessible DNA. These devices fail to provide a mechanism for preparing complex samples, such as robust tissues with tough protective structures and samples containing biomolecules that may inhibit the amplification reaction. Some sample types that fall within this category include plants, fibrous tissue, formalin-fixed paraffin-embedded (FFPE) tissue, mycobacterium, and processed tissues [15-19]. The current DNA sample preparation process for these sample types has extensive time and resource requirements, demanding a fully equipped

laboratory and can take more than 24 hours for DNA extraction [20-22]. These DNA sample preparation requirements are severely limiting the uses of NAATs in POC settings.

To overcome the shortcomings of currently available DNA extraction techniques and enable the development of NAATs integrated with robust sample preparation workflows, focused ultrasound extraction (FUSE) has been developed as a novel method for sample processing and DNA release. FUSE utilizes short-duration, high amplitude focused ultrasound pulses to generate a cavitation bubble cloud capable of breaking down samples on the tissue and cellular level for tissue homogenization and DNA liberation. Foundational studies have validated the feasibility of FUSE for preparing DNA from biological tissues (fresh and formalin-fixed) and plant tissues (leaves, timber), representing samples with a range of complexity and applications [23-26]. A recent parameter optimization study, shown in **Chapter 4**, found that with an increased pulsing rate, FUSE can extract DNA from fresh tissue in 10 seconds and improve DNA purity, suggesting that FUSE can disintegrate biomolecules contaminating the DNA extract. The time efficiency and capacity of FUSE to simultaneously homogenize tissue while lysing cells without the need for heated incubation demonstrates the value of FUSE based DNA sample preparation for NAATs. However, in all prior work, FUSE was performed using a 32-element 500 kHz array transducer that was originally designed to perform histotripsy for non-invasive medical procedures [27, 28]. The large aperture size of this transducer (~12 cm) limited its portability and also required the transducer to be suspended in a large water tank with samples positioned in the focus 7.5 cm from the face of the transducer. This setup ultimately required the use of sizeable, complex, laboratory-based equipment, demonstrating the need to scale down the FUSE experimental configuration to enable portable sample processing. There have not been any studies to date investigating the

feasibility of FUSE DNA sample preparation with alternate transducer designs and acoustic parameters.

In this study, we developed, fabricated, and tested a miniaturized, portable device to broaden the accessibility of FUSE DNA sample preparation. We hypothesized that a miniaturized FUSE device could achieve DNA release with quantities and quality sufficient for qPCR-based detection. To test this hypothesis, the device was first designed for essential implementation and acoustic specifications, ensuring that the sample holder is positioned at the focal point of a focused ultrasound transducer capable of generating cavitation. The transducer output was simulated to assess whether the resulting acoustic pressure field could achieve sufficient focal gain to sustain cavitation. The transducer was then fabricated, and the acoustic field and cavitation generation were measured to assess its performance. DNA extraction experiments were performed with two sample types to measure key DNA sample preparation metrics across tissues of varying complexity and evaluate the device suitability for FUSE compared to previous devices. Overall, this work aims to advance the FUSE technology toward a battery-powered prototype capable of performing complex DNA sample preparation in POC settings.

## **5.2. Materials and Methods**

### *5.2.1. Device Design Specifications*

A first-generation miniaturized FUSE prototype was designed to ensure robust tissue processing and efficient DNA release, adhering to the following criteria:

- 1) sufficient focal gain to generate FUSE cavitation clouds;
- 2) a focal pressure zone limited to the internal dimensions of a small test tube;
- 3) compatibility with a sample holder with simple alignment, supporting a test tube in the acoustic focus;
- 4) compactness and durability for easy portability.

The primary design objective was to develop a small transducer geometry capable of generating acoustic cavitation in a sample tube. Based on prior studies, it was assumed that the sample tube would have an inner diameter between 6.35 and 9.5 mm. The height, defined as the tube height occupied by the sample and lysis buffer, was dependent on the sample and lysis buffer volume. The height was expected to range from 11 to 16 mm, varying based on sample type and application [23, 25, 26]. For the generation of sustained FUSE cavitation clouds and to isolate cavitation to the test tube, it is necessary to restrict the focal zone (-6 dB) beamwidth to be similar to or smaller than the internal diameter and height [29-32]. To meet these constraints, the device was designed to limit the cavitation cloud dimensions to 11 mm along the major axis and 6 mm along the minor axis. A sample holder that does not interfere with the focus or acoustic window was also considered. Lastly, the overall size of the device was kept small by minimizing the focal length of the transducer to enable easy transport and versatility for POC DNA extraction applications.

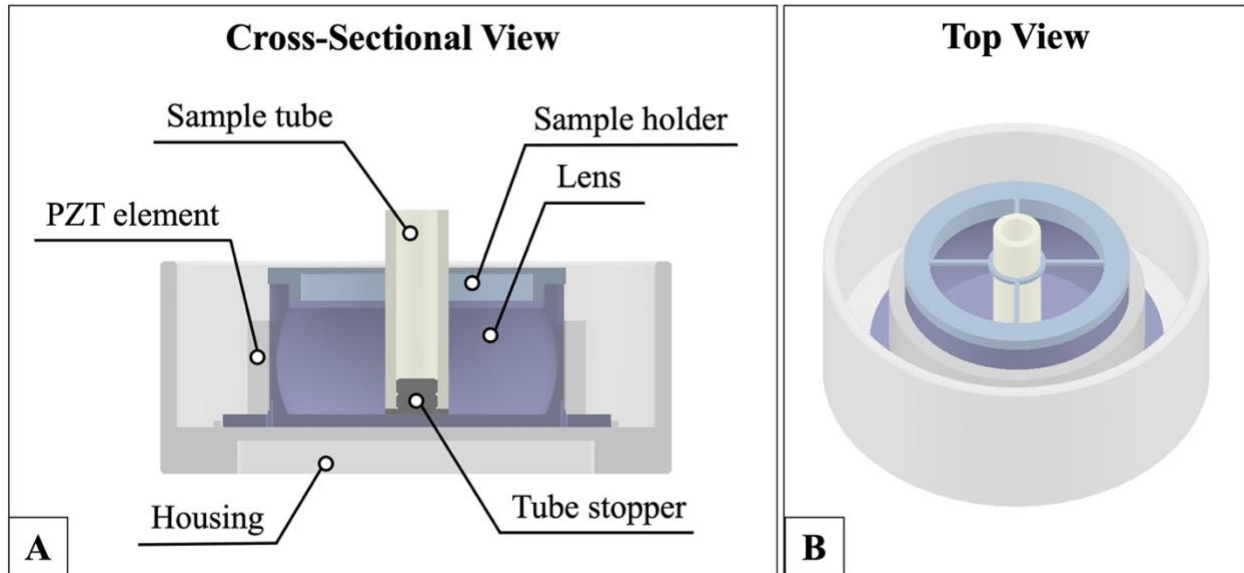
### *5.2.2. Device Simulation and Fabrication*

Based on the design constraints, a single element cylindrical transducer with a concentric focusing lens was simulated using a 1D piezoelectric element model (KLM model) [33] and the k-Wave open source toolbox [34], a k-space pseudospectral time domain method to model the acoustic output. This design enabled spherical focusing from a cylindrical source. The KLM model specified a 400 V<sub>pp</sub> square-wave signal with a frequency of 750 kHz was delivered to an L-bridge matching network with capacitance and inductance of 6000 pF and 7 μH, respectively, and then propagated to the transducer [35]. The mechanical impedance of the transducer was determined by assuming a lens with an inner radius of 19.25 mm and thickness of 2.9 mm (PerFORM, Ceramic-Like Advanced HighTemp, Protolabs, Maple Plain, MN, USA), and was concentrically

aligned with the piezoelectric element with a 3 mm thickness (Steiner and Martins, Davenport, FL, USA). This model was used to simulate source pressure output of the transducer.

The acoustic pressure field was then simulated using the k-Wave open source toolbox. The acoustic source was modeled as an axisymmetric element with a 22.5 mm radius of curvature along the height of the cylinder with a cylinder height of 14 mm. The delivery of a 40-cycle pulse with a source pressure  $p_0 = 0.2$  MPa through a coupling medium (water) was simulated. The coupling medium was bounded by air on the top surface and outer diameter of the cylinder, with a thin layer of plastic at its bottom base to contain the coupling medium in the internal cavity of the cylinder. The pressure output in the acoustic field was simulated over 200  $\mu$ s, and the temporal and spatial location of the peak negative pressure ( $p_-$ ) was determined. This simulation was performed in an axisymmetric domain with the axis of symmetry equivalent to the central axis of the cylinder. In this way, the entire cross-section of the acoustic focal zone was visualized.

A solid model of the device was developed based on simulation results. This included a focusing lens with a closed bottom placed within the cylindrical source to create an exposure chamber. Sample holders were designed to support acoustically permeable tubes of varying sizes and fit concentrically within the chamber. Lastly, an external housing was developed to contain the transducer and sample holder. A solid rendering of the FUSE device was constructed using CAD models (Autodesk Inventor, San Francisco, CA, USA) (**Error! Reference source not found.**).



**Figure 5.1. FUSE device design.** (A) Cross-sectional and (B) solid CAD models of the device showing the transducer, sample holder apparatus, and protective housing.

The transducer was fabricated using a piezoceramic cylinder element with an inner diameter of 45 mm and a height of 14 mm (Steiner and Martins, Davenport, FL, USA). The lens was designed with an elliptic geometry to produce a wave focused to the center of the height dimension of the source and in the center of the chamber. The lens was 3D printed using stereolithography with a material known to allow for sufficient energy transmission from the element (PerFORM, Ceramic-Like Advanced HighTemp, Protolabs, Maple Plain, MN, USA) [36, 37]. The element was bonded to the lens using a thin epoxy layer. Wires were soldered to the inner and outer faces of the cylindrical element and connected to a BNC port. The sample holders were 3D printed using stereolithography (Clear Resin V4, Formlabs, Somerville, MA, USA). Small and large sample tubes had inner diameters of 6.35 mm and 9.525 mm, respectively, and both had a wall thickness of 1.59 mm (Tygon PVC E-1000, McMaster-Carr, Douglasville, GA, USA). Based on prior work, the tube materials were expected to have negligible pressure loss [25]. The tubes were sealed with stoppers on either end. The outer transducer housing was fabricated using selective laser sintering 3D printing (PA12 White, Protolabs).

### 5.2.3. Transducer Characterization and Pulse Generation

The electrical impedance of the transducer with the sample holder and sample tube in place with deionized degassed water in the exposure chamber was measured by an impedance analyzer (AIM4300, Array Solutions, Sunnyvale, TX, USA) connected by a coaxial cable. This measurement informed the design of an L-bridge matching network [35] for a matched electrical impedance  $Z = 10 \Omega$  at a center frequency of 750 kHz. The transducer was driven by a multichannel class D amplifier controlled by an FPGA board (Cyclone DE1-SoC Board, Terasic, Hsinchu, Taiwan) delivering a unipolar square wave to the matching network. Custom MATLAB scripts were used to communicate with the FPGA and specify pulsing parameters.

A fiber-optic hydrophone (HF0690, Onda Corporation, Sunnyvale, CA, USA) was used to measure the acoustic pressure output of the transducer. Focal pressure waveforms were measured with deionized degassed water in the exposure chamber generating 40-cycle pulses with varying  $p$ - up to 11.6 MPa, beyond which the pressure could not be measured due to cavitation. For  $p$ - exceeding 11.6 MPa, the relationship between driving voltage and  $p$ - was quantified to linearly extrapolate  $p$ - up to the maximum amplifier output. It was determined that 15 V was needed to generate 1 MPa of pressure. An oscilloscope (TBS2000 series, Tektronix, Beaverton, OR, USA) was used to collect waveforms. The waveforms were filtered using a radiofrequency filter (BLP-1.9+, Lumped LC Low Pass Filter, DC - 1.9 MHz, 50 $\Omega$ , Brooklyn, NY, USA). The waveforms were also averaged over 512 pulses to filter noise from the hydrophone, and the final waveform was reported. In the reported waveforms, the time axis was adjusted such that  $t = 0$  represented the time of pulse delivery. Radial and axial 1D beam scans were performed along the major axes of the transducer to capture the focal beamwidths. The resulting beam profiles were recorded and normalized.

#### *5.2.4. Cavitation Cloud Characterization*

The cavitation cloud location and dimensions were determined using high-speed optical imaging. A high-speed camera (Nova S12 monochrome, Photron USA, San Diego, CA) with a 100 mm lens (Milvus 100 m f/1M ZF.2 Macro Lens, Zeiss, Jena, Germany) was used for imaging. A circular mirror was mounted to an optical rod at a 45° angle to the camera and the transducer's axial dimension to capture images within the exposure chamber. The transducer was fixed to a clear frame that was positioned over a white LED strobe light (GS Vitec Multi-LED QT Light, MultiLED G8 controller, 320 W power supply, Soden-Salmünster, Germany) for backlighting the images. The camera was mounted to an adjustable scissor jack fixed to an optics table for consistent alignment. 40-cycle pulses with  $p = 21$  MPa were delivered to the exposure chamber filled with 22 mL of deionized degassed water with a pulse repetition frequency (PRF) of 200 Hz. The camera was triggered once per pulse to collect images 2  $\mu$ s after the end of the incident pulse reached the focus.

Images were analyzed to determine the location and size of the cavitation bubble cloud within the exposure chamber. Images were converted to binary first to identify and isolate the exposure chamber from the background. The exposure chamber was defined as a circular region with a centroid and diameter, and the surrounding image was masked and cropped outside of the diameter. The grayscale images of the cropped exposure chamber were then compared to a reference image captured before pulse delivery to remove any background artifacts and isolate the cavitation nuclei. The isolated images were then binarized, and the pixels with cavitation present were signified. This process was repeated over a series of images that were stacked to generate a heat map identifying the location of cavitation in the exposure chamber over 100 pulses.

#### *5.2.5. FUSE Experimental Setup and DNA Extraction*

Piscine and timber tissue were prepared for FUSE processing to validate the performance of the device for DNA extraction. For the piscine tissue study, fresh Atlantic salmon (*Salmo salar*) filets were prepared as they were previously in **Chapter 4** [23]. Briefly, 25-50 mg cubes of tissue were sectioned and rinsed with deionized water. Samples were placed in a small sample tube (6.35 mm inner diameter) with lysis buffer composed of 270  $\mu\text{L}$  of Buffer ATL and 30  $\mu\text{L}$  Proteinase K (Qiagen Blood and Tissue Kit; Qiagen Incorporated, Hilden, Germany). For the timber tissue, live edge cuts of white oak (*Quercus alba*) were collected and prepared for FUSE processing two weeks after harvest. A file and rasp were used to collect shavings from the sapwood and cambium regions, as was done previously [26]. Shavings were passed through an ISO test sieve (Gilson Company Incorporated, Middleton, WI, USA) with square openings of 1 mm to separate any large segments from the shavings. 100 mg of shavings were placed in a large sample tube (9.525 mm inner diameter) with lysis buffer containing 1 mL of 1% PVP-40 Buffer AP1 solution and 8  $\mu\text{L}$  of RNase A (Qiagen DNeasy Plant Kit; Qiagen Incorporated). Both sample tubes had stoppers on both ends to isolate the samples during FUSE processing.

The transducer exposure chamber was filled with 22 mL of deionized degassed water, and the sample tube assembly was suspended in the exposure chamber for processing using the sample holder aligned in the center of the chamber. 40-cycle pulses with  $p = 21$  MPa and a PRF of 200 Hz were delivered to the focus for sample processing and DNA release. Doses of 5,000 and 10,000 pulses were evaluated for both tissue types ( $n = 3$ ), resulting in processing times of 25 and 50 seconds. After tissue processing, the sample holder was removed from the exposure chamber, and the tissue lysate was transferred to a 1.5 mL centrifuge tube for purification. Silica-column purification was done following the protocols recommended by the kit manufacturer (Qiagen Incorporated). The final elution volume was 200  $\mu\text{L}$  for the piscine tissue and 80  $\mu\text{L}$  for the timber.

### *5.2.6. Control DNA Extraction*

Conventional DNA extraction methods were performed as a control to compare against FUSE using methods as was done in previous studies (**Chapter 4**) [26]. For piscine tissue samples, 25-50 mg of tissue was placed in a 1.5 mL centrifuge tube with 270  $\mu$ L of Buffer ATL and 30  $\mu$ L Proteinase K (Qiagen Blood and Tissue Kit; Qiagen Incorporated). Samples were incubated at 56 °C and vortexed every five minutes for 15 seconds until all tissue was disintegrated. This process took between 20-40 minutes. For timber tissue samples, 100 mg of shavings were processed using a mortar and pestle under liquid nitrogen. Liquid nitrogen was added to cool the mortar and pestle; then the tissue was placed in the mortar and homogenized for 30 seconds. The fractionated tissue was transferred to a 1.5 mL centrifuge tube with 1 mL of 1% PVP-40 Buffer AP1 solution and 8  $\mu$ L of RNase A (Qiagen DNeasy Plant Kit; Qiagen Incorporated). Samples were incubated for one hour at 65 °C with a short vortex every ten minutes. Silica column purification was performed for both sample types (Qiagen Incorporated).

### *5.2.7. DNA Quantification*

The released DNA was quantified by measuring the DNA yield and quality using the Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and NanoDrop One (Thermo Fisher Scientific). DNA yield was normalized based on the mass of sample input using values reported from the Qubit. DNA extract purity was evaluated by measuring the 260/280 and 260/230 ratios with the Nanodrop. An unpaired student's t-test with unequal variance was used ( $p < 0.05$ ) to evaluate the significance of the data collected with the Qubit and Nanodrop. Gel electrophoresis was also performed to assess the quality of the released DNA. For these experiments, 300 ng of DNA was used for piscine samples, while 1-11 ng of DNA was input for timber samples due to low DNA yields. The DNA was stained with 1x GelRed (Millipore Sigma,

Burlington, Massachusetts, USA) and loaded into a 1% agarose gel in 1x TBE buffer (Thermo Fischer Scientific). The electrophoresis system was powered with 100 V for 1 hour. The ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) was used to capture gel images, and the GeneRuler 1 kb Plus DNA Ladder (Thermo Fischer Scientific) was included to estimate the molecular weight of the input DNA.

#### 5.2.8. *Quantitative PCR Amplification*

qPCR amplification was performed on DNA barcoding regions within the mitochondrial cytochrome oxidase subunit 1 gene (COI) of *S. salar*, as well as the maturase K (matK) and ribulose biphosphate carboxylase (rbcL) chloroplast genes of *Q. alba*. qPCR reactions were carried out as they were in prior studies for piscine (**Chapter 4**) and timber [26]. Briefly, in the piscine samples, the COI gene was targeted using a forward primer of 5'–CGCCCTAAGTCTCTTGATTTCG–3', and a reverse primer, 5'–GTAGTATGGTAATGCCTGCTGC–3' that amplified a 536 bp region (**Chapter 4**). Reactions contained 6 µL of 1X PowerUp SYBR Green Master Mix (Applied Biosystems, Thermo Fischer Scientific), 0.5 µM of each primer (Integrated DNA Technologies, Coralville, IA, USA), and 1.5 µL of template DNA. The thermal cycler was programmed as follows: 50 °C for 2 minutes, 95 °C for 2 minutes, and 30 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Amplifications with threshold cycle (Ct) values less than or equal to 25 cycles were considered successful.

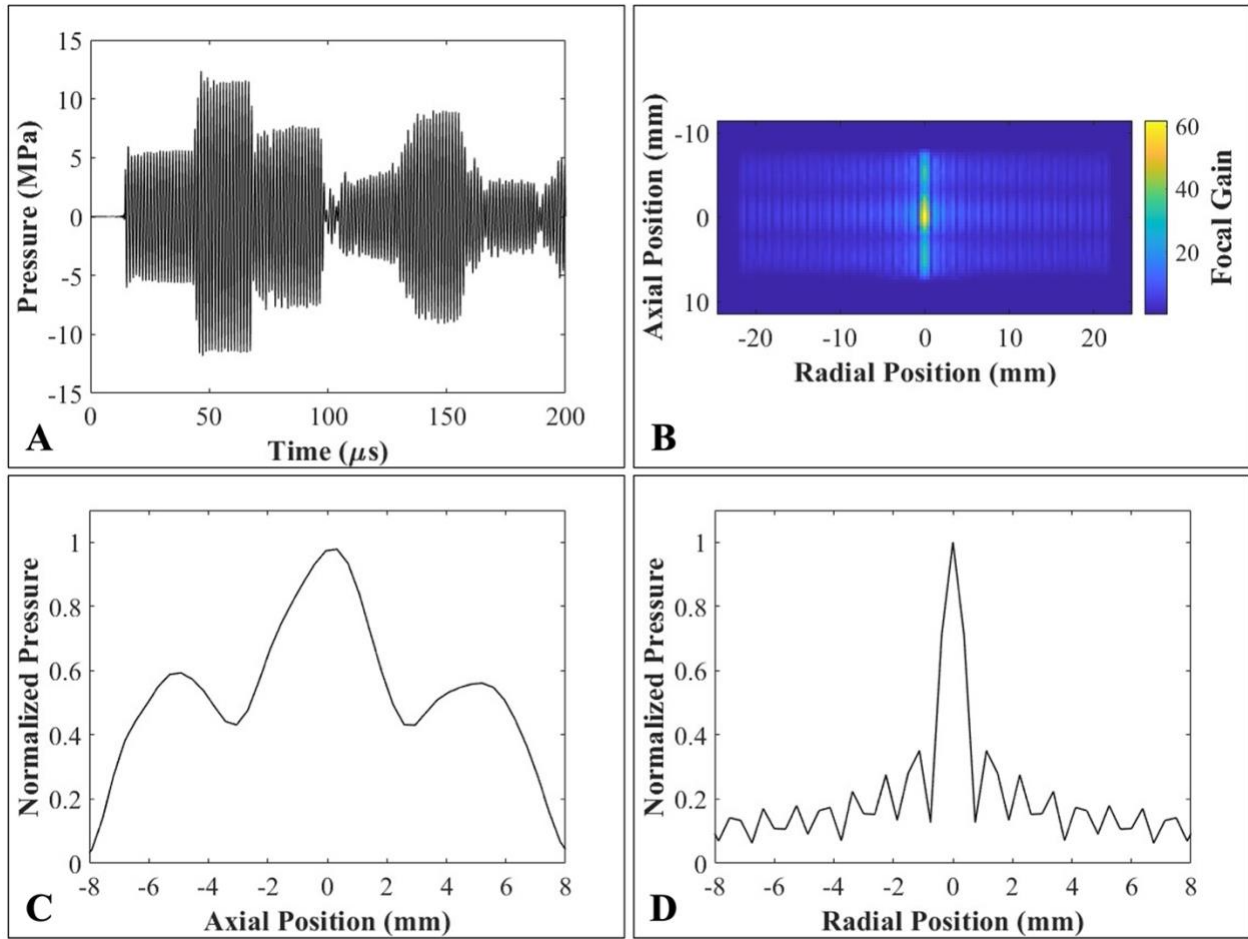
For timber samples, the matK gene was amplified using a forward primer, 5'-TTTCCGGTCATCCCATGCTTT-3', and reverse primer, 5'-TGCAGGATTTTCGTCGAACACT-3', that targeted a 243 bp region. For rbcL targeting, a forward primer, 5'-ACGATGCTACCACATCGAGC-3', and reverse primer, 5'-GAGGCGGACCTTGAAAGTT-3', were used to amplify a 212 bp region. qPCR mixture ratios followed those of the piscine

reactions, but for this case a total volume of 40  $\mu\text{L}$  was used for all reactions due to lower template DNA quantities. For both matK and rbcL primers, thermal cycling was performed using the following parameters: 50 °C for 2 minutes, 95 °C for 2 minutes, and 45 cycles of 95 °C for 15 seconds, 57 °C for 15 seconds, and 72 °C for 1 minute. Samples with Ct values less than or equal to 40 cycles were considered successful. All amplifications for both timber and piscine samples were performed in triplicate, and each qPCR run included negative controls containing nuclease-free water instead of template DNA.

## 5.3. Results

### 5.3.1. Transducer Output Characteristics

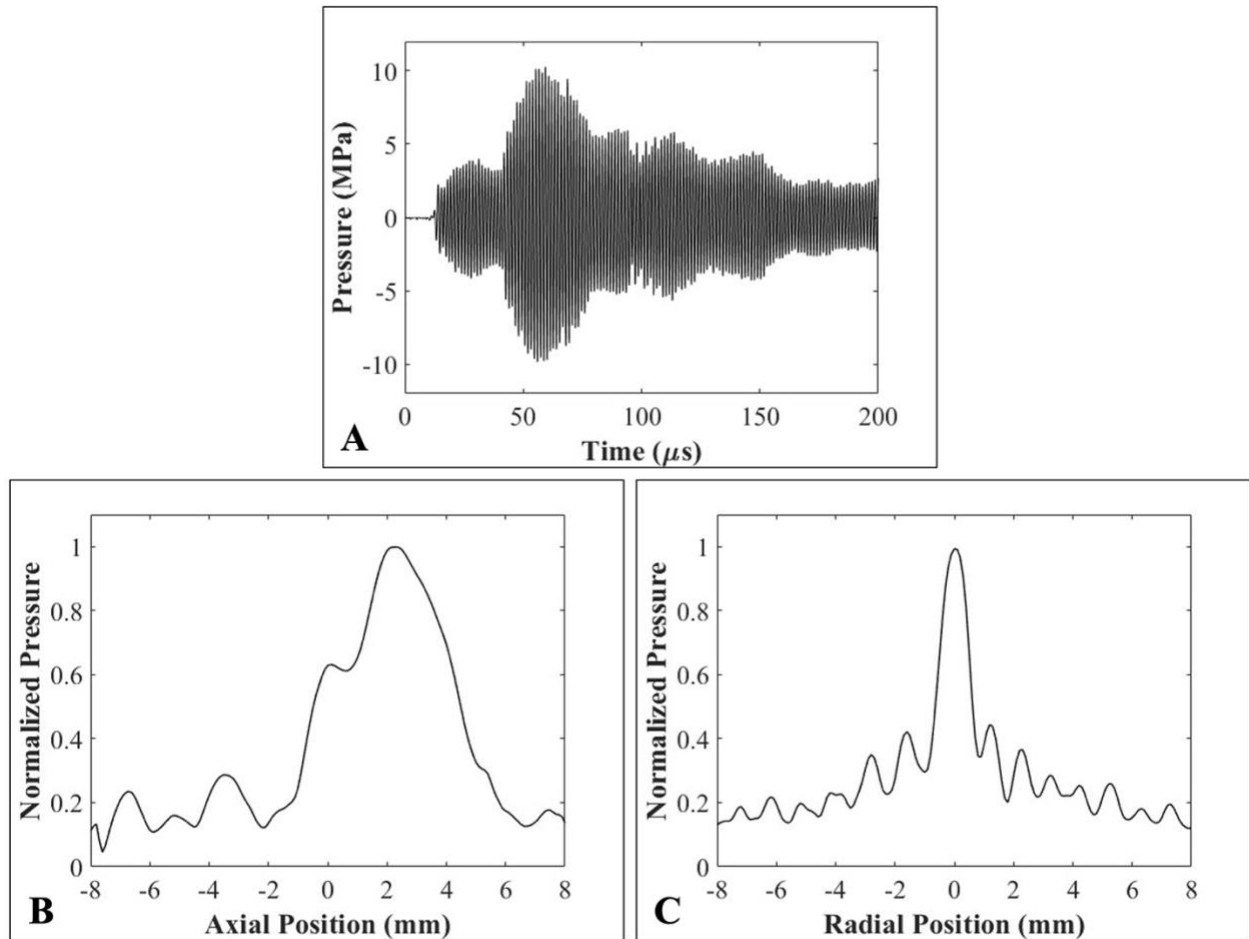
The simulated pressure waveforms reveal interference patterns resulting from the delivery of multi-cycle pulses in a reverberant exposure chamber, and beam profiles show that the acoustic focus is expected to be contained within the sample tube (**Figure 5.2**). The simulated pressure waveform demonstrates that  $p$ - occurred approximately 47  $\mu\text{s}$  after pulse delivery. This result corresponds to the expected time required for the pulse to traverse the exposure chamber and reverberate back to the focus based on device geometry and center frequency, showing that the geometry of the transducer resulted in wave interference patterns that increased  $p$ - at the focus. At  $p$ -, the simulated focal gain was 60, a pressure output comparable to other focused ultrasound devices that generate cavitation [32, 38, 39]. The simulated beam profiles reveal that the focus is expected to be centered along the radial and axial dimensions of the chamber. Results show that beam plots had -6 dB beam dimensions of 11.6 mm and 0.75 mm along the axial and radial dimensions of the transducer.



**Figure 5.2. Simulated waveform and focal pressure output.** (A) Pressure waveform in the geometric focus of the transducer assuming  $p_0 = 0.2$  MPa. (B) Cross-sectional focal gain in the geometric focus. (C) Axial and (D) radial beam profiles show that the focus is expected to be in the center of the transducer with  $-6$  dB beam dimension of 11.6 mm axially and 0.75 mm radially. The plotted focal gain is the ratio of simulated focal pressure to source surface pressure.

The fabricated transducer was cylindrical with a focal length of 19.25 mm. Measured pressure waveforms confirmed that delivering multi-cycle pulses in a reverberant exposure chamber resulted in constructive interference between emitted waveforms and reverberations that amplified  $p$ - (**Error! Reference source not found.A**). The measured beam profiles show  $-6$  dB beam dimensions of 1.2 mm and 4.8 mm were determined in the radial and axial dimensions (**Figure 5.3B-C**). Measured beamwidths were used to predict the bubble cloud dimensions [30, 31], and these results demonstrate that cavitation should be confined within the sample tube. The

measured beam profiles were approximately 60% different than simulations. It is expected that differences between the measured and simulated beamwidths are due to slight asymmetry in the fabricated transducer and simplifications in the model, which may affect the complex patterns of wave propagation and interference in the exposure chamber. Results also indicated that the acoustic focus was located in the middle of the exposure chamber in the radial dimension, but a shift in the focus toward the bottom of the chamber was observed in the axial dimension.

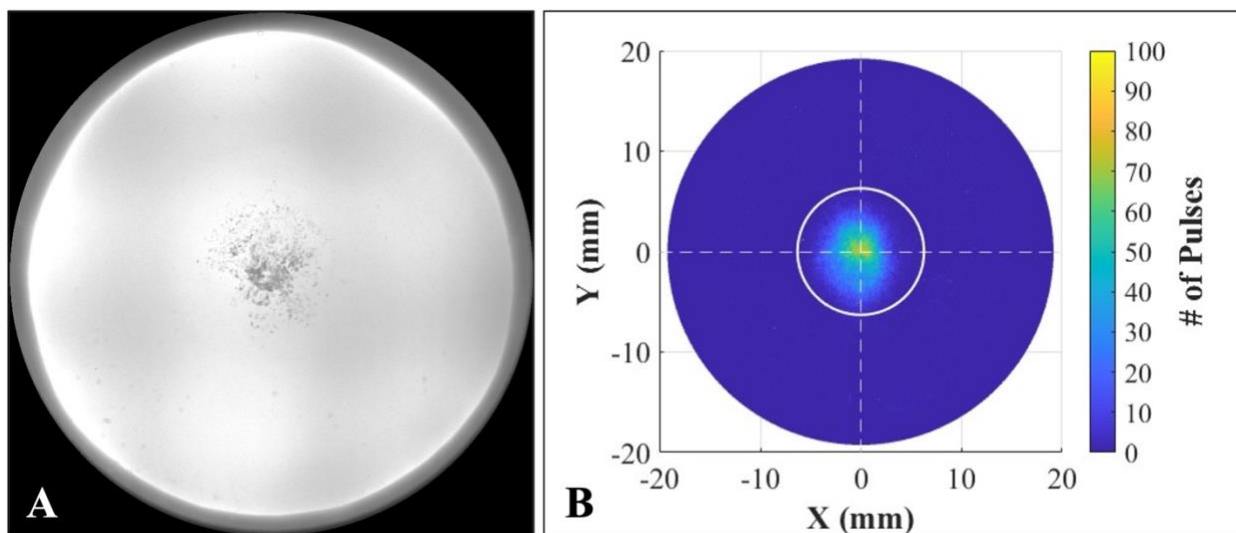


**Figure 5.3. Measured pressure output.** (A) Pressure waveform in the geometric focus of the transducer with  $p_- = 9.9$  MPa. (B) Axial and (C) radial beam profiles with -6 dB dimensions of 4.8 mm axially and 1.2 mm radially.

### 5.3.2. Cavitation Cloud Characteristics

High-speed optical imaging experiments demonstrated that the miniaturized FUSE device generated consistent cavitation (**Figure 5.4**). Imaging was done axially to characterize the

cavitation cloud in the radial and angular dimensions of the cylindrical transducer. In agreement with the simulated and measured beam profiles, cavitation was observed in the center of the chamber. Over 100 pulses, cavitation was observed 100% of the time. The cavitation cloud was dynamic and characteristic of cavitation-based focused ultrasound techniques [40]. The white circle overlaid on the heat map plot represents the inner diameter of the small sample tube. Results show that all cavitation events were contained within the bounds of the tube, demonstrating that this device can meet the requirement of inducing cavitation within the bounds of a small sample tube.

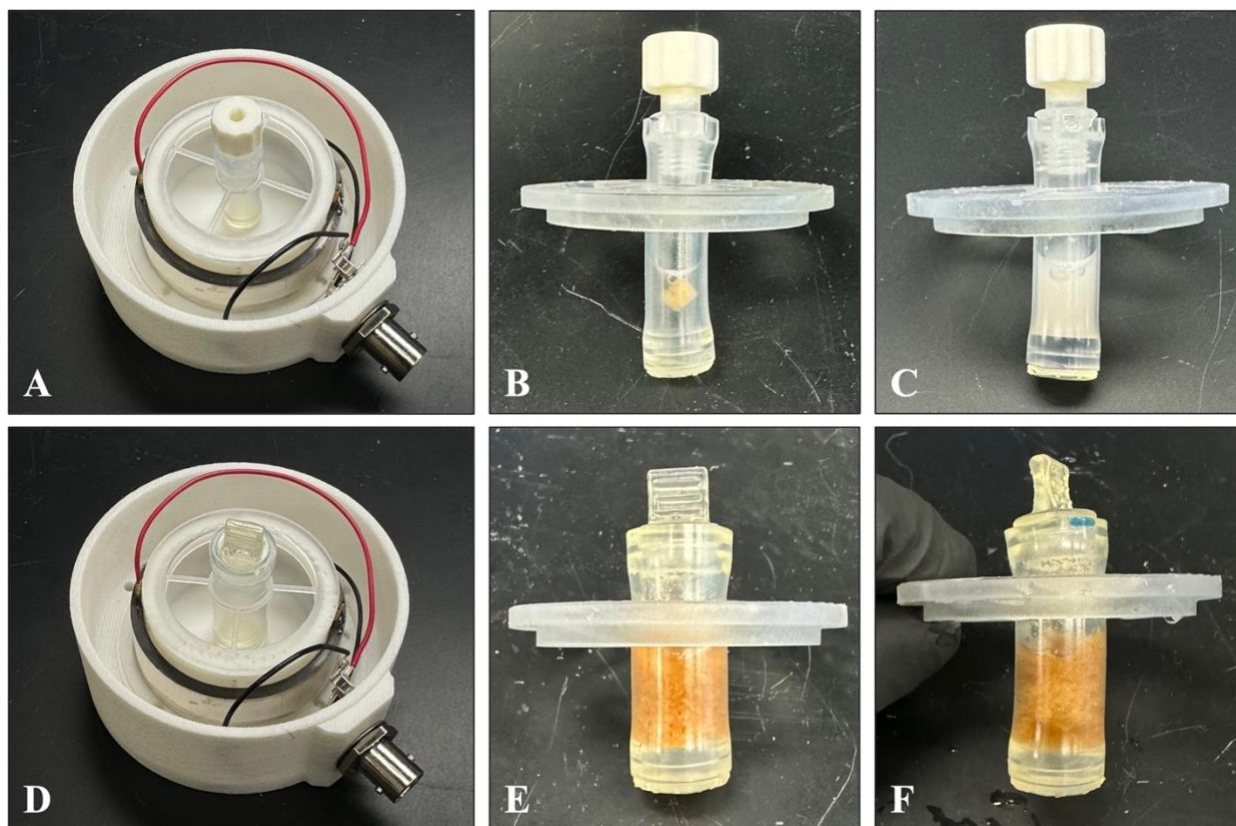


**Figure 5.4. Cavitation bubble cloud imaging.** (A) High-speed optical imaging was performed to capture the cavitation bubble cloud 2  $\mu$ s after the arrival of the final cycle of a 21 MPa, 40-cycle pulse to the acoustic focus. (B) The location of cavitation events was mapped over 100 pulses to show the size and position of the cavitation cloud within the exposure chamber. The white circle indicates the size of a small sample tube with an inner diameter of 6.3 mm. Results show that cavitation is sustained within the sample tube.

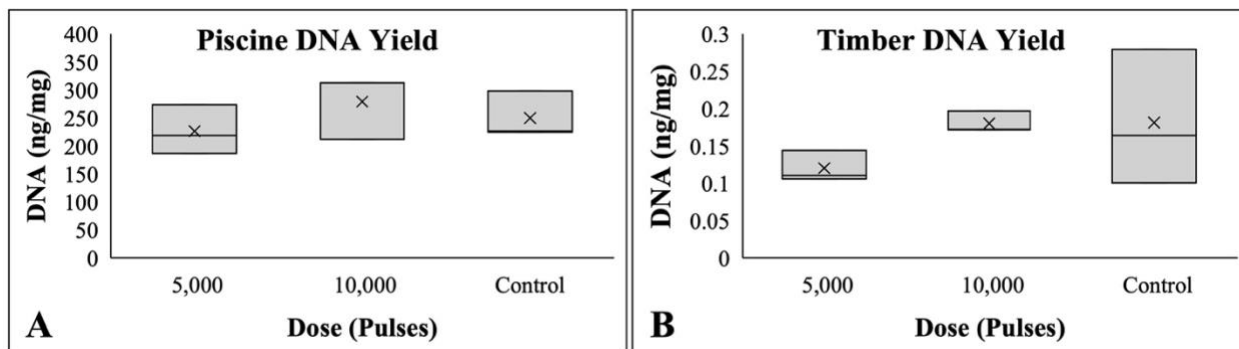
### 5.3.3. DNA Extraction and Feasibility of PCR-based Detection

The feasibility of this device for releasing DNA from piscine muscle and timber tissue was evaluated to demonstrate the potential of FUSE DNA sample preparation with a miniaturized system (**Figure 5.5**). In both cases, tissue disruption was observed after 25 and 50 seconds of FUSE processing. For the piscine tissue, complete disintegration of all visible tissue fragments

was achieved after 25 seconds of processing. For timber tissue, the extent of tissue breakdown could not be determined visually, but tissue circulation was observed during treatment, and prior work has shown that circulation is indicative of cavitation events and tissue breakdown [26]. In both cases, tissue homogenization was evidenced by the resulting DNA yield (**Figure 5.6**). For the piscine samples, high quantities of DNA were released after 25 and 50 seconds of processing, resulting in DNA yields comparable to control methods. Due to the robustness of timber tissue and the reduced amount of viable DNA present, timber DNA yields were lower than those for piscine muscle tissue. However, results demonstrate that increasing the number of pulses from 5,000 to 10,000 significantly increased the DNA yield from timber samples ( $p < 0.05$ ). Due to high variability in the control DNA yield results for timber, there were no significant differences between the controls and either of the FUSE groups.



**Figure 5.5. FUSE sample preparation and processing.** The FUSE experimental configuration for the preparation of piscine (A-C) and timber (D-F) tissue. In the first column the device is shown with a (A) 6.35 mm tube and (D) 9.525 mm tube setup. The second column shows the (B) piscine and (E) timber tissue prepared in lysis buffer. The third column demonstrates the (C) piscine and (F) timber tissue after FUSE tissue processing.



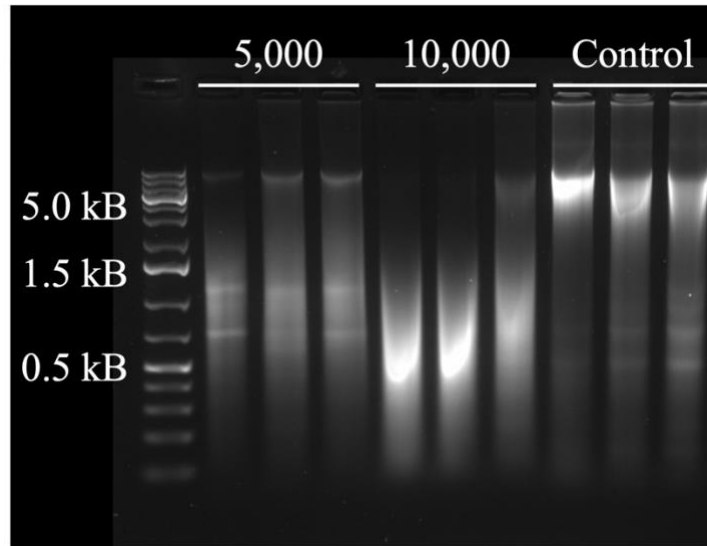
**Figure 5.6. DNA yield with a miniaturized FUSE device.** (A) Piscine and (B) timber DNA yield results demonstrate that the miniaturized FUSE device released DNA from both tissue types with yields comparable to conventional extraction methods, represented by the controls. For the timber samples, 10,000 pulses released significantly more DNA than 5,000 pulses ( $p < 0.05$ ).

DNA purity results were also reported for piscine and timber samples (**Table 5.1**). 260/280 and 260/230 ratios were within the expected norms for the piscine samples. In contrast, for the

timber samples, 260/280 and 260/230 ratios were lower than the predicted values for DNA. However, this trend was also observed in the control group, suggesting that the low purity ratios are reflective of timber samples containing high levels of DNA contaminants, not the DNA extraction method. While there were no significant differences between treatment groups for the 260/280 ratios for either sample type, timber samples prepared with 5,000 pulses had 260/230 ratios that were significantly greater than those prepared using 10,000 pulses and controls. Gel electrophoresis was also performed to assess the quality of DNA released with FUSE. Similar to previous findings in piscine tissue, results show that increasing the processing time fragmented the DNA (**Figure 5.7**). However, when samples were prepared using 5,000 pulses, high molecular weight bands were still present with sizes as great as 20 kB. Due to low quantities of input DNA, timber DNA could not be visualized.

**Table 5.1. Piscine and timber DNA purity ratios.** 260/280 and 260/230 ratios were reported for piscine and salmon samples prepared with FUSE and controls. Results show that DNA purity ratios are within the expected range for piscine samples, while timber ratios are lower than what is expected for DNA for both FUSE and controls.

<b>Sample</b>	<b>Dose (Pulses)</b>	<b>Time (mm:ss)</b>	<b>260/280</b>	<b>260/230</b>
Piscine	5,000	00:25	2.00 ± 0.03	1.72 ± 0.20
	10,000	00:50	2.02 ± 0.02	2.01 ± 0.09
	Control	20:00-50:00	2.05 ± 0.01	1.94 ± 0.16
Timber	5,000	00:25	1.26 ± 0.02	0.54 ± 0.00
	10,000	00:50	1.27 ± 0.01	0.51 ± 0.00
	Control	60:00	1.24 ± 0.01	0.51 ± 0.00



**Figure 5.7. Piscine gel electrophoresis.** Gel electrophoresis results show that increasing the FUSE dose from 5,000 to 10,000 pulses resulted in DNA shearing. The effect of 5,000 pulses on DNA shearing is comparable to controls.

For both piscine and timber samples, qPCR amplification was successful, demonstrating the potential of the miniaturized FUSE device for preparing DNA for downstream detection assays (Table 5.2, Table 5.3). The COI, matK, and rbcL genes were selected as gene targets because the COI gene is commonly used for animal species identification, while the matK and rbcL genes are typically used for plants. Both of the evaluated FUSE doses resulted in successful amplification in both sample types. For piscine samples, results suggest that increasing the dose improved qPCR success rates, similar to the results presented in Chapter 4 (Figure 4.6). The reaction efficiency across treatment groups was comparable for the piscine samples. For the timber samples, a qPCR success rate of 100% was observed for samples prepared using doses of 5,000 and 10,000 FUSE pulses, in contrast to controls that had an overall success rate of 39%. The reaction efficiency was also significantly greater for FUSE samples than controls for both matK and rbcL primer sets. Efficiencies of  $25.0 \pm 0.34$  and  $24.6 \pm 0.15$  were reported for timber samples prepared using 5,000 pulses for the matK and rbcL genes, respectively, while efficiencies of 39.2 and  $33.0 \pm 2.12$  were found for samples processed using control methods.

**Table 5.2. qPCR amplification of the COI gene in piscine samples.** The qPCR success rate and efficiency in amplifying the COI mitochondrial gene is shown. Results show that qPCR success and efficiency increased with increasing FUSE dose.

COI			
Dose (Pulses)	Success	Efficiency	Overall
5,000	7/9	16.0 ± 0.68	78%
10,000	8/9	14.6 ± 0.38	89%
Control	9/9	14.8 ± 0.42	100%

**Table 5.3. qPCR amplification of matK and rbcL genes in timber samples.** The qPCR success rate and efficiency in amplifying the matK and rbcL chloroplast genes is shown. Results show that qPCR was 100% successful in both genes after FUSE DNA release of timber samples. qPCR efficiency was not effected by dose.

Dose (Pulses)	matK		rbcL		Overall
	Success	Efficiency	Success	Efficiency	
5,000	9/9	25.0 ± 0.34	9/9	24.6 ± 0.15	100%
10,000	9/9	26.7 ± 0.70	9/9	24.9 ± 0.22	100%
Control	1/9	39.2	6/9	33.0 ± 2.12	39%

## 5.4. Discussion

This study presented the design of a portable transducer developed for the preparation of DNA samples from complex tissues in POC settings to broaden the accessibility and applicability of NAATs. The device was designed using a cylindrical transducer element and focused to deliver 750 kHz pulses with a pulse duration of 40 cycles to the center of the exposure chamber. The overall size of the device was 77 x 31 mm to allow for easy transport and versatile use. Characterization of the acoustic output showed radial and axial beamwidths of 1.2 mm and 4.8 mm, respectively. Cavitation was characterized using high-speed optical imaging, and results showed that the bubble cloud was generated in the center of the sample chamber and confined within the bounds of the desired sample tube dimensions. The performance of the device was validated by demonstrating the feasibility of FUSE for piscine and timber tissue processing, DNA release, and qPCR amplification. For both sample types, DNA with yields, purity, and quality sufficient for PCR amplification was achieved after only 25 seconds of FUSE processing. For

timber samples, the PCR performance of samples prepared by FUSE was significantly greater than controls, suggesting that FUSE improved the quality of the released DNA. Overall, the results demonstrate that a miniaturized focused ultrasound device can perform FUSE DNA sample preparation to release DNA suitable for PCR from piscine and timber samples.

The transducer design used in this study was selected primarily based on utility and ease of use as well as the size and shape of the target sample tube. In prior FUSE studies, a large 32-element hemispherical transducer was used to deliver focused ultrasound pulses to the sample of interest, and the sample tube was aligned in the focus such that the length of the tube was perpendicular to the face of the transducer [23-26]. This configuration also required robotic positioning system to expose the entire sample volume to the focus. To prevent the need for movements during tissue processing, the miniaturized transducer was designed such that the major axis of the bubble cloud spanned the height of the sample tube. This was done with a cylindrical source to increase the focusing strength and the size and density of the cavitation bubble cloud [30]. In previous FUSE studies, a transducer with an f-number of 0.62 was used, while this transducer had an f-number of 0.5.

The simulated transducer output was compared to the measured pressure waveforms, and discrepancies between the beam profiles were observed. Differences between the simulated and measured results are expected to be due to design simplifications in the simulation and experimental variability. The simulation assumed a focal length of 22.5 mm, the inner diameter of the transducer element, as the lens was not included in this model. To account for lensing effects, the source element was modeled to be elliptic with an arc position centered along the height dimension. When the device was fabricated, variations between the height of the manufactured source element and the ellipse arc location on the 3D printed lens resulted in focusing that was

slightly asymmetric. In addition to these variations between the assumptions and the fabricated device, experimental variability could have resulted from variations in the water height, gas concentration, and water temperature. Future work intends to modify the model such that the source element is flat and positioned along a curved lens. The change in the location of the source element relative to the lens arc position will also be considered to better represent wave propagations in the device.

The cylindrical design of this device created a reverberant chamber that affected the pressure output. The applied pulsing schemes and acoustic properties of the media in the exposure chamber caused interference between generated and reflected waves at the focus. This result poses the need for future experiments exploring alternate pulsing schemes to maximize constructive interference and increase the focal pressure. Designing the system to utilize reverberations and interference patterns to maximize the focal pressure, will be the most efficient way to optimize the output without excessive power and voltage requirements, a key consideration when developing a device intended for POC use cases.

The DNA extraction results confirmed that a miniaturized FUSE device effectively and rapidly releases DNA from various complex sample types. The performance of the device in breaking down and releasing DNA from timber tissue in only 25 seconds was particularly promising. In prior FUSE studies, at least five minutes of FUSE processing was required to release DNA from timber, and in these studies, a PRF of 1 kHz was used to apply the pulses, so a total of 300,000 pulses were applied to extract significant quantities of DNA [26]. In this study, the dose was 60X lower than it was previously, and DNA yields were comparable to controls. The timber qPCR results also demonstrated the power of this device in preparing DNA from robust tissues. FUSE samples were amplified with a 100% success rate for both of the evaluated primer sets, and

amplification occurred after 25-27 amplification cycles. In contrast, controls had an overall success rate of 39%, and Ct values between 33-39 cycles. In previous work that investigated the feasibility of FUSE for timber DNA sample preparation, the same matK and rbcL primer sets were used. matK success rates of 33% and 39% and rbcL success rates of 78% and 83% were observed for samples prepared with five minutes of FUSE processing and controls, respectively [26]. Therefore, in this study, the qPCR success rate increased for samples prepared with FUSE but decreased for controls. Ongoing work intends to perform additional control experiments to confirm that this trend is consistent and further optimize FUSE parameters with this system.

The DNA release and quality results for piscine samples were also positive. In all prior studies investigating FUSE, it has been shown that FUSE fragments DNA (**Chapter 4**) [24-26]. In this study, the piscine gel electrophoresis results demonstrate minimal DNA shearing after 5,000 pulses and the presence of high molecular weight bands. This result demonstrates that FUSE acoustic parameters can be modified to alter molecular disintegration patterns, similar to the work that was shown in **Chapter 4**. However, in Chapter 4, extensive DNA shearing was still observed after 10,000 pulses (**Figure 4.5**), so evidence that DNA shearing can be minimized by reducing the dose is promising and has not been demonstrated previously. Future work is necessary to better characterize this finding and determine how it relates to varying acoustic parameters.

Overall, the miniaturized transducer developed in this study shows great promise for the development of a portable FUSE platform capable of enabling rapid DNA release from complex sample types. This FUSE device has the potential to broaden the accessibility and versatility of NAATs for several applications including environmental security and global health. A rapid and reliable DNA extraction method capable of preparing robust sample types, field performance (battery-powered), and operation by non-technical users is necessary to improve the current

capabilities of NAATs [8]. The device presented in this study addresses many of the key factors necessary to enable POC use including small size, extraction in less than 30 seconds, and the ability to prepare complex samples for reliable and efficient qPCR assays. Ongoing work aims to further refine this device to enhance portability and field performance. Future studies will focus on identifying the acoustic parameters that enable optimal sample preparation while limiting power requirements to allow for the development of a battery-powered amplifier for using the device in low-resource settings.

## **5.5. Conclusion**

A miniaturized, portable device for FUSE DNA sample preparation was designed and fabricated to address the limitations of NAATs. The transducer pressure output and beam profiles were measured with focal hydrophone measurements. High-speed optical imaging was used to validate that the focal gain was great enough to generate sustained cavitation, and results show that cavitation was confined within the planned dimensions. FUSE DNA extraction with piscine and timber samples demonstrated that this device can release high quantities of high-quality DNA suitable for PCR in 25 seconds. These results demonstrate the potential of this device as a novel POC DNA extraction platform capable of preparing complex sample types, warranting the continued development of FUSE for new indications and enhanced performance.

## **5.6. Acknowledgments**

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# **|Chapter 6: Conclusions and Future Work**

## **6.1. Summary of Contributions**

This dissertation investigates the use of focused ultrasound extraction (FUSE) for DNA release from complex tissue matrices and characterizes the use of a miniaturized FUSE system to enable point-of-contact (POC) DNA testing workflows. Specifically, this research (1) establishes the feasibility of FUSE in plant tissues, both leaves and timber, (2) explores the effect of varying FUSE pulsing parameters on the quantity and quality of DNA released with FUSE, and (3) develops a miniaturized FUSE device to enable portability and accessibility of the FUSE technology. This work discovered a novel DNA extraction method capable of rapidly preparing DNA from complex sample types and designed a first-generation portable FUSE platform. The findings presented in this dissertation suggest that this technology has the potential to transform molecular diagnostics, particularly the use of nucleic acid amplification tests (NAATs) for the detection of robust tissues in POC settings.

The first half of this dissertation (Chapters 2 and 3) determined the feasibility of FUSE for preparing DNA from plant tissue to demonstrate that FUSE can release DNA from physically robust samples containing biomolecules capable of inhibiting downstream assays. The work completed in Chapter 2 of this dissertation investigated the feasibility of FUSE for leaf tissue homogenization, DNA release, DNA amplification, and sequencing. The results of this study demonstrated that FUSE can be used to prepare DNA from American chestnut, tulip poplar, red maple, and chestnut oak leaves for amplicon and next-generation sequencing. FUSE released significantly greater amounts of DNA than control methods in 9 minutes compared to the 30 minutes required for conventional DNA extraction from leaves. This study showed that the FUSE dose could be increased to increase tissue breakdown and DNA release from strong tissue types.

Overall, these results established the feasibility of FUSE for the release of high-quality DNA from leaf tissue. In Chapter 3, the utility of FUSE for DNA release from timber, a more complex plant tissue, due to the increased strength of the tissue, a higher presence of polysaccharides and secondary metabolites, and reduced DNA content. In this study, the performance of FUSE in preparing DNA from timber shavings with varying sizes was evaluated to determine the effect of timber sample preparation methods on FUSE processing and DNA release. The effect of FUSE dose on DNA yield and DNA quality was also assessed. Results showed that FUSE can pulverize white oak timber tissue, and greater DNA yields were observed when input shaving size was increased. Furthermore, 5 minutes of tissue processing resulted in significant DNA release, compared to the 1 hour required for DNA release using conventional methods. The purity and quality of the DNA were sufficient for qPCR amplification of the *matK* and *rbcL* genes that are commonly used for white oak species identification, demonstrating the potential of FUSE in preparing timber DNA for NAATs.

Chapters 4 and 5 of this dissertation explored the potential of streamlining FUSE DNA sample preparation workflows by investigating alternate pulsing parameters and developing a miniaturized, portable system. Chapter 4 expanded on prior work that established the feasibility of FUSE for DNA release from piscine samples by investigating the effect of pulse repetition frequency (PRF) and dose on the efficiency of FUSE processing and the quality of the released DNA. The results of this study showed that PRF and dose did not have a significant effect on DNA yield, demonstrating that high pulsing rates and low doses can be used to release DNA from piscine tissue in only 10 seconds. Examination of the DNA purity revealed that increasing the PRF resulted in increased 260/230 ratios, suggesting that high PRFs can break down DNA extract contaminants that have the potential to interfere with PCR or other downstream detection assays. DNA quality

evaluation showed that FUSE sheared the released DNA, and increasing the dose amplified the DNA shearing effects. The impact of DNA purity and quality results were determined by measuring qPCR amplification success and reaction efficiency, and results show that both improved with increasing PRF. Together, the DNA purity, quality, and qPCR outcomes suggest that FUSE processing is most effective in preparing piscine samples for PCR-based detection when low doses are applied at high PRFs. Overall, this study found that high FUSE pulsing rates released DNA more than 100X faster than conventional extraction methods and optimized DNA quality for improved downstream assay performance.

In Chapter 5, a transducer was designed to determine the feasibility of a portable FUSE platform for enabling POC DNA testing with complex sample types. In all prior work shown in Chapters 2-4, FUSE was performed using an array transducer with a 12 cm aperture size that was suspended in a large water tank for operation. This setup confined FUSE to a laboratory setting, so after the feasibility of the technology was established, efforts shifted to focus on the potential of downsizing this technology to make it more accessible. To do this, the device was designed to be significantly smaller than previous configurations and compact in an easy-to-use platform. A 750 kHz cylindrical transducer element with a focal length of 19.25 mm was developed to target samples contained in tubes in the center of an exposure chamber. Acoustic output characterization showed beamwidths of 1.2 mm and 4.8 mm in the radial and axial dimensions, respectively, suggesting that cavitation will be localized inside the planned sample tube with a 6 mm inner diameter and a height of 10 mm. This was confirmed with high-speed imaging that showed the presence of sustained acoustic cavitation in the center of the exposure chamber, when delivering 40-cycle pulses at a peak negative pressure ( $p_-$ ) of 21 MPa. Lastly, the utility of this device for DNA extraction was determined using piscine and timber samples. Results show that the device

generated robust cavitation capable of homogenizing tissue and releasing DNA. The yield and quality of the released DNA were sufficient for repeatable and efficient qPCR amplification. This study validated the potential of the FUSE technology to be translated into a portable device capable of rapidly releasing DNA from complex sample types.

## **6.2. Significance and Future Work**

The work presented in this dissertation explores the potential of the novel FUSE method for preparing DNA from complex tissues with a focus on the uses of this technology for environmental security applications. Initial feasibility studies demonstrated that FUSE could rapidly release DNA from plant tissue, a physically and chemically complex sample type, for use in downstream molecular detection assays. This work was expanded through parameter optimization studies characterizing that the FUSE pulsing rate can be elevated to enable DNA release with greater time efficiency and improved performance in downstream reactions. Lastly, it shows the ability to develop a miniaturized, portable, focused ultrasound transducer for performing FUSE DNA sample preparation in resource-limited settings.

This dissertation presents foundational studies that established the potential of the FUSE technology and laid the groundwork for continued advancement and development. While the effect of pulsing rate and dose have been investigated, other acoustic parameters, including center frequency and pulse duration, have not yet been explored. The miniaturized FUSE system is capable of operating at lower frequencies near 120 kHz and higher frequencies near 1.2 MHz. Future studies should investigate the performance of these frequencies at varying pulse durations, to better understand how pulsing parameters influence cavitation dynamics and impact the release and quality of DNA. In addition to alternate pulsing schemes, different transducer coupling media should also be explored to improve the user experience with the miniaturized FUSE device. The

current coupling medium of deionized, degassed water is not readily available and is not well suited for field use. Future work should explore the development of solid coupling materials that allow for sufficient pulse transmission and can be integrated into the FUSE device. This has the potential to improve device performance while also enhancing usability.

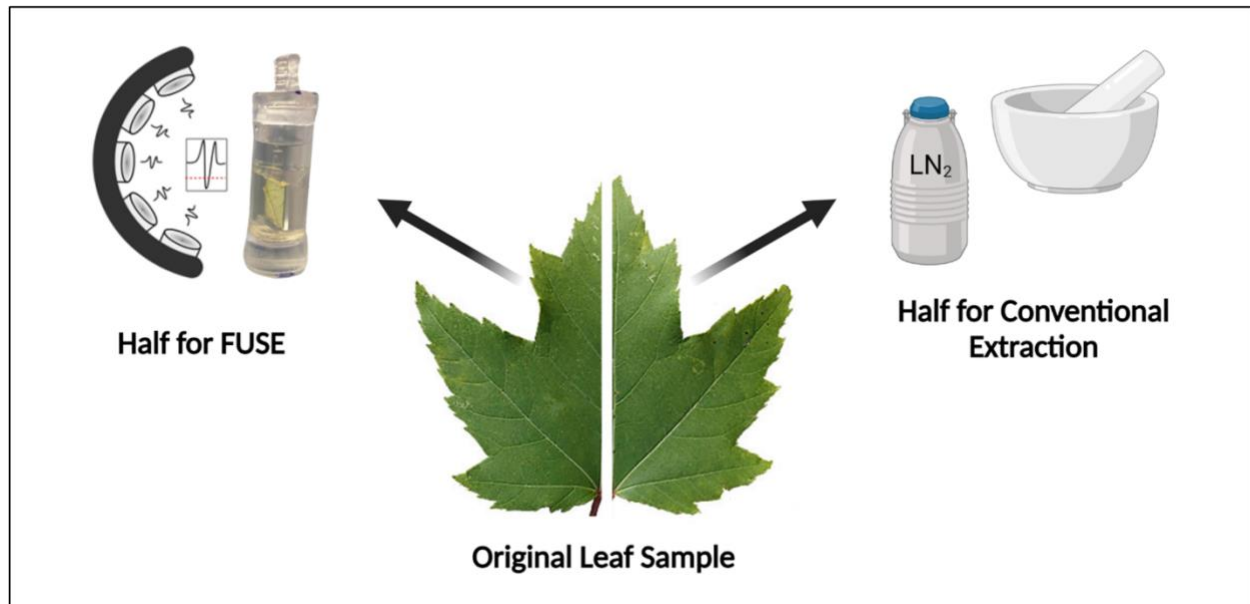
Additional studies are also needed to determine whether the performance of FUSE is dependent on DNA lysis buffer composition. Alternate buffer chemistries could be formulated specifically to enhance DNA isolation and the removal of contaminating molecules to enable direct-to-PCR workflows. One major drawback of the FUSE technology is that it currently does not address DNA purification. With the development of buffers designed specifically to target and remove PCR contaminants during FUSE processing, FUSE could provide greater DNA sample preparation efficiency. Furthermore, the results presented in this dissertation suggest that FUSE may alter the molecular composition of targeted samples, and it is possible that buffer composition influences biochemical reactions induced by FUSE. Future work is necessary to determine the role of buffer composition on DNA release and the breakdown of other biomolecules by FUSE. These findings in addition to the potential benefits of direct-to-PCR workflows, continued research on the role of DNA extraction buffer composition on FUSE processing will inform further refinement of FUSE workflows for optimal efficiency and DNA extract quality.

In addition to advancements to FUSE pulsing schemes and sample preparation methods, future work should also investigate the performance of FUSE with other complex sample types. The findings in this dissertation primarily discuss the potential of FUSE for addressing DNA sample preparation and detection challenges for environmental security and conservation applications. However, DNA sample preparation bottlenecks are not limited to this field. Other use cases, particularly in biosurveillance and healthcare, would benefit from the rapid and portable

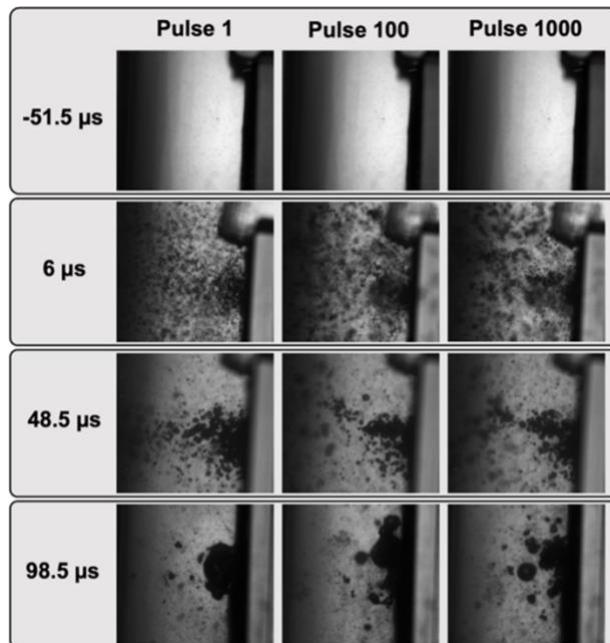
aspects of the FUSE technology. In parallel to the work presented in this dissertation, FUSE has been tested in formalin-fixed, paraffin-embedded (FFPE) tissues and a *Mycobacterium tuberculosis* model to determine its potential in molecular pathology and infectious disease diagnostics. Preliminary results show the promise of FUSE for these indications, demonstrating the feasibility of DNA release and PCR amplification. Future work should further validate the utility of FUSE with these sample types by investigating the use of the miniaturized FUSE platform in preparing these sample types in POC settings. The performance of FUSE testing in resource-limited settings is crucial for validating the expected utility of this device for addressing the shortcomings of currently available DNA sample preparation platforms.

Overall, this dissertation presents the discovery of a novel DNA extraction method utilizing focused ultrasound to enable the rapid release of high-quality DNA from complex sample types. This work was done with the intent to overcome the limitations of conventional DNA extraction methods by providing a technology capable of preparing robust tissue types in resource-limited settings to broaden the accessibility and sample applicability of molecular detection platforms. This work specifically demonstrated the potential of FUSE for uses in environmental security, particularly to address illegal logging and fishing, and has established the potential of using FUSE to address other applications in biosurveillance and healthcare. Continued exploration of FUSE is essential for portable molecular diagnostics to reach their full potential. With FUSE, we envision the performance of complete DNA sample preparation and detection workflows outside of the laboratory to enable POC DNA-based diagnostics.

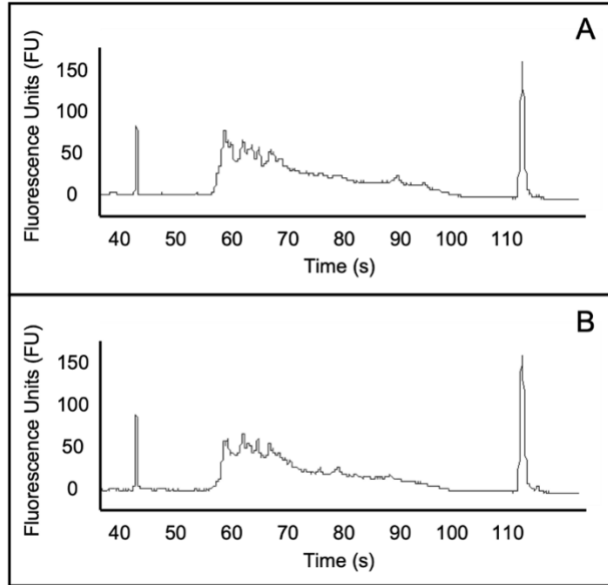
## Appendix A



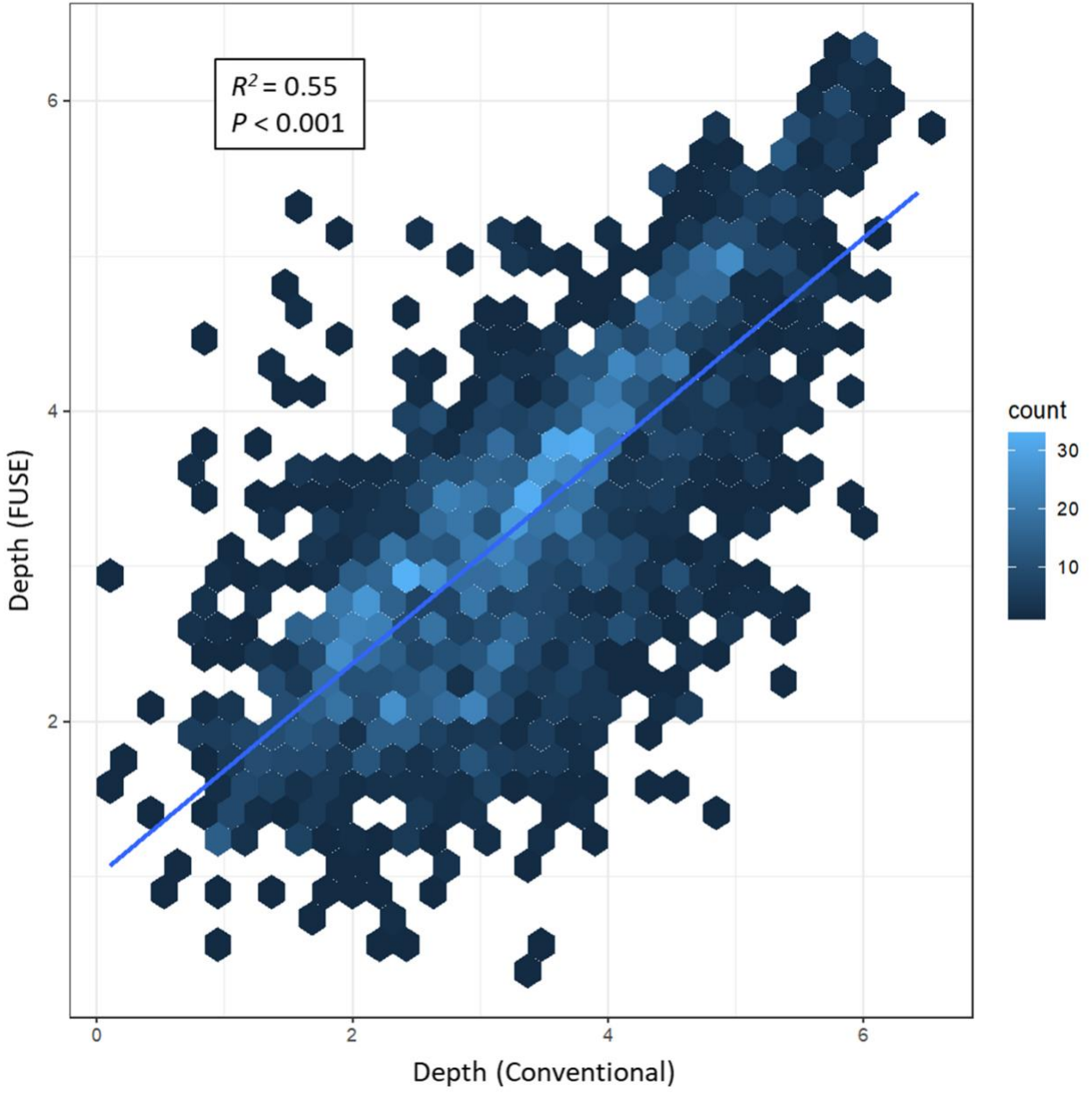
**Supplementary Figure 2.1. Leaf sample allocation.** Each leaf used in this study was divided into two, providing a matched pair to compare FUSE and conventional extraction yields.



**Supplementary Figure 2.2. The cavitation bubble cloud collapses toward the surface of the leaf tissue.** 51.5 μs before pulse arrival (row 1), the sample and sapphire glass backing are imaged. 6 μs after pulse arrival (row 2), the cavitation cloud is visible and contains many microbubbles that have not substantially expanded or coalesced. As time progresses (rows 3), the microbubbles begin to coalesce and are concentrated near the sample's surface. 98.5 μs after pulse arrival (row 4), the microbubbles are near collapse and situated adjacent to the sample.












**Supplementary Figure 2.3. Visualization of DNA fragment sizes using a Bioanalyzer.** The distribution of DNA fragment sizes for an American chestnut sample processed with conventional methods (A) is comparable to the DNA fragment size distribution for a sample processed with FUSE (B). This result confirms that the integrity of DNA provided by FUSE is suitable for PCR amplification.

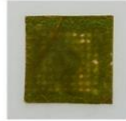
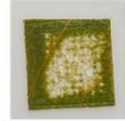
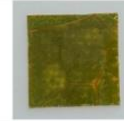

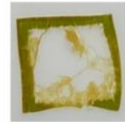

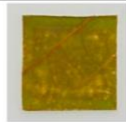
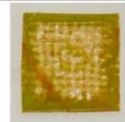



**Supplementary Figure 2.4. Relationship between  $\log_{10}$  sequencing depth at variable sites with FUSE and control samples.** Results show that FUSE sequencing depth is comparable to controls, demonstrating that DNA released with FUSE is suitable for next-generation sequencing.




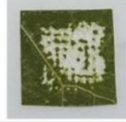
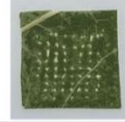




**Supplementary Table 2.1. *Castanea dentata* tissue breakdown and DNA yield results.** DNA quantification measurements are reported from Qubit fluorometer measurements, and 260/280 and 260/230 ratios are reported from NanoDrop measurements.

Leaf Species	Description	Data	Sample 1	Sample 2	Sample 3
<i>Castanea dentata</i> Leaf 1	Large, thick, dark green	6 scans			
		DNA (ng/mg)	29.48	29.32	50.14
		260/280	1.75	1.66	1.72
		260/230	2.16	1.72	2.01
<i>Castanea dentata</i> Leaf 2	Large, dry, brown-green	6 scans			
		DNA (ng/mg)	16.68	19.30	58.11
		260/280	3.43	4.27	2.04
		260/230	26.65	-4.40	1.72
<i>Castanea dentata</i> Leaf 3	Large, dry, brown-green	6 scans			
		DNA (ng/mg)	3.31	3.65	8.95
		260/280	2.60	1.64	1.91
		260/230	-0.94	-2.22	-5.77


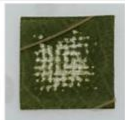
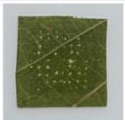

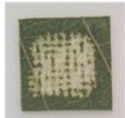
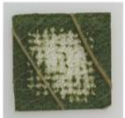
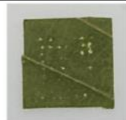
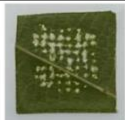




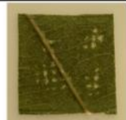

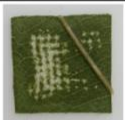

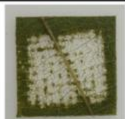

**Supplementary Table 2.2. *Liriodendron tulipifera* tissue breakdown and DNA yield results.** DNA quantification measurements are reported from Qubit fluorometer measurements, and 260/280 and 260/230 ratios are reported from NanoDrop measurements.

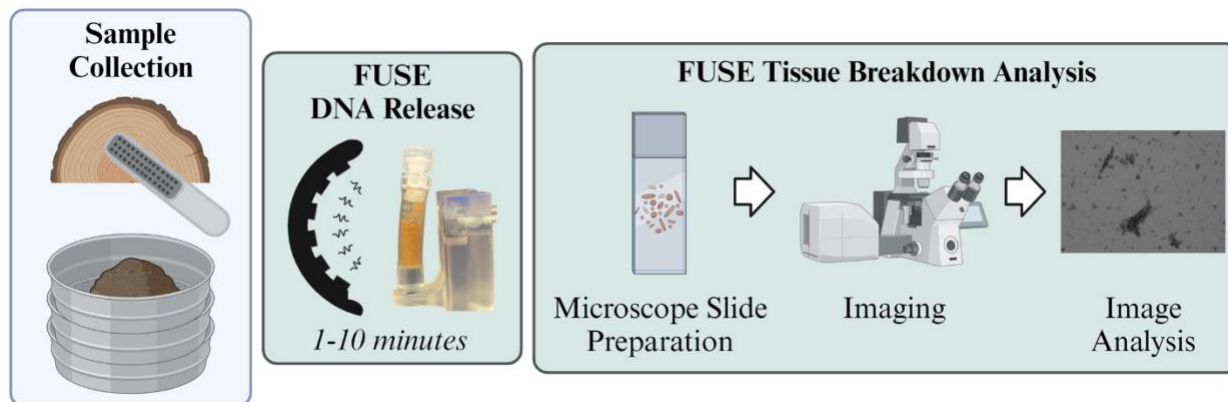
Leaf Species	Description	Data	Sample 1	Sample 2	Sample 3
<i>Liriodendron tulipifera</i> Leaf 1	Large, thick, dark green	6 scans			
		DNA (ng/mg)	5.25	30.89	5.65
		260/280	1.53	1.82	1.60
		260/230	2.59	2.12	1.16
<i>Liriodendron tulipifera</i> Leaf 2	Small, wet, yellow-green	6 scans			
		DNA (ng/mg)	53.64	60.28	61.32
		260/280	1.83	1.85	1.75
		260/230	1.50	1.88	1.69
<i>Liriodendron tulipifera</i> Leaf 3	Small, wet, yellow-green	6 scans			
		DNA (ng/mg)	3.71	32.70	40.06
		260/280	2.09	1.70	1.72
		260/230	-1.06	1.23	1.58

**Supplementary Table 2.3. *Acer rubrum* tissue breakdown and DNA yield results.** DNA quantification measurements are reported from Qubit fluorometer measurements, and 260/280 and 260/230 ratios are reported from NanoDrop measurements.

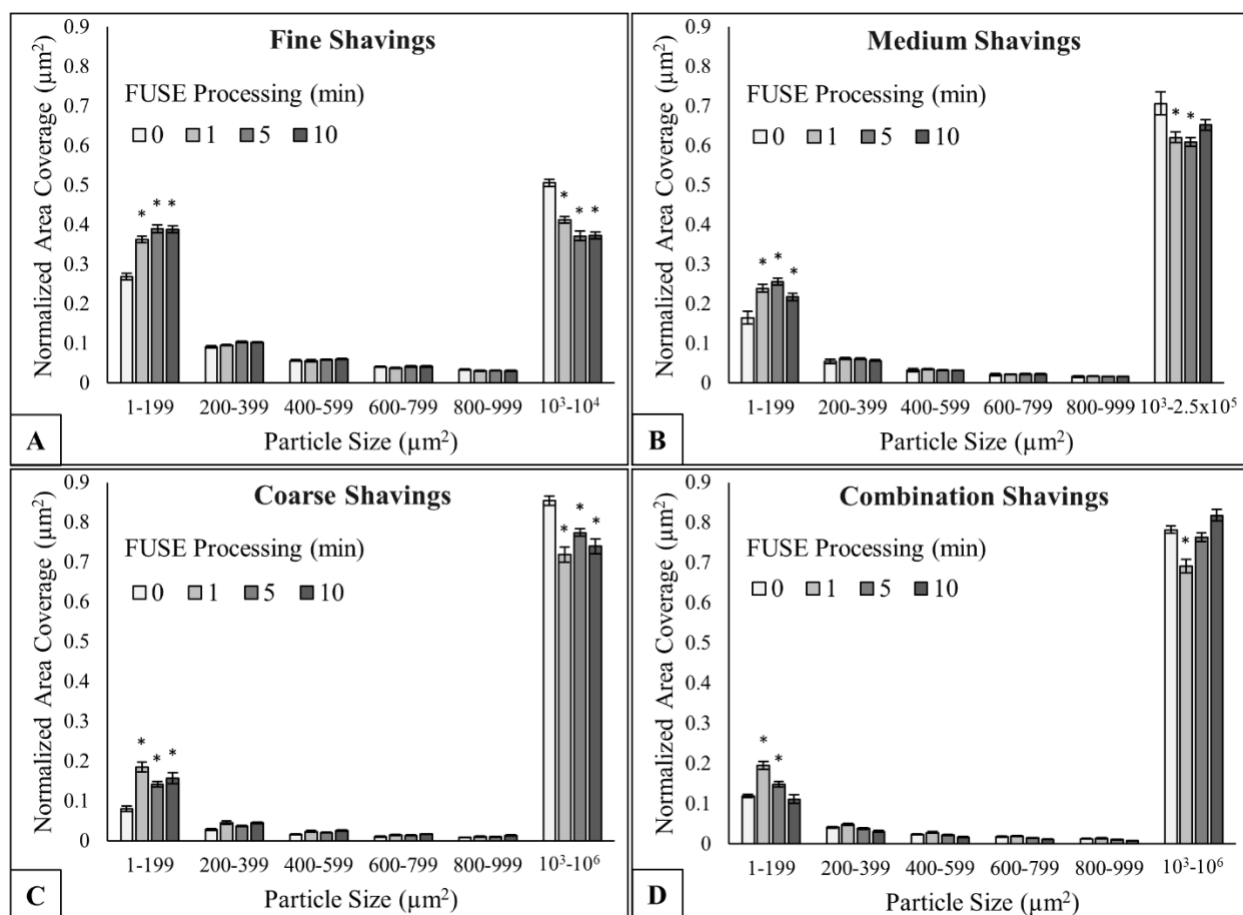
Leaf Species	Description	Data	Sample 1	Sample 2	Sample 3
<i>Acer rubrum</i> Leaf 1	Large, thick, dark green	6 scans			
		DNA (ng/mg)	47.84	60.12	19.48
		260/280	1.66	1.68	1.48
		260/230	1.73	1.89	1.15
<i>Acer rubrum</i> Leaf 2	Small, thick, dark green	6 scans			
		DNA (ng/mg)	58.61	16.85	83.51
		260/280	1.80	1.55	1.77
		260/230	2.09	1.59	1.94
<i>Acer rubrum</i> Leaf 3	Small, thick, dark green	6 scans			
		DNA (ng/mg)	9.55	14.98	6.96
		260/280	1.66	1.46	1.65
		260/230	-1.14	2.43	5.86

**Supplementary Table 2.4. *Quercus montana* tissue breakdown and DNA yield results.** DNA quantification measurements are reported from the Qubit fluorometer. 260/280 and 260/230 ratios are reported from the NanoDrop.

Leaf Species	Description	Data	Sample 1	Sample 2	Sample 3
<i>Quercus montana</i> Leaf 1	Large, thick, dark green	6 scans			
		DNA (ng/mg)	10.37	12.90	10.92
		260/280	1.60	1.69	1.64
		260/230	3.63	2.79	5.13
		10 scans			
		DNA (ng/mg)	24.55	21.78	13.69
		260/280	1.66	1.68	1.62
		260/230	1.74	1.86	1.79
<i>Quercus montana</i> Leaf 2	Large, thick, dark green	6 scans			
		DNA (ng/mg)	6.46	10.51	6.23
		260/280	2.67	1.84	1.90
		260/230	6.41	-21.12	9.13
		10 scans			
		DNA (ng/mg)	39.91	35.08	61.87
		260/280	1.86	1.87	1.89
		260/230	2.18	2.01	2.07
<i>Quercus montana</i> Leaf 3	Large, thick, dark green	6 scans			
		DNA (ng/mg)	5.69	22.90	9.90
		260/280	1.69	1.77	1.75
		260/230	-2.97	2.46	-3.94
		10 scans			
		DNA (ng/mg)	68.18	38.81	36.83
		260/280	1.78	1.77	1.74
		260/230	2.36	3.08	3.42



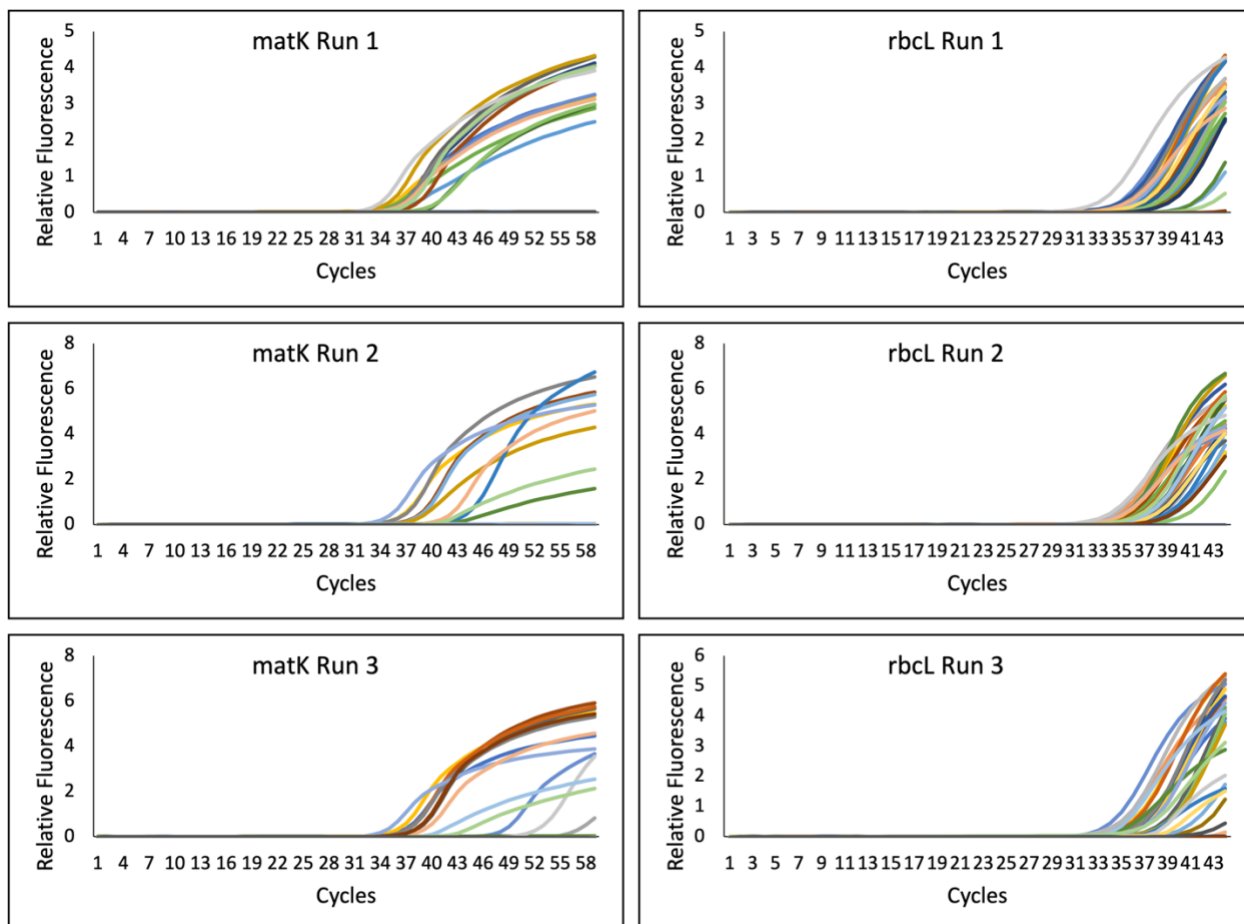
**Supplementary Figure 3.1. Timber particle size analysis workflow.** To measure the feasibility of timber tissue breakdown with FUSE, timber shavings were processed for 1, 5, and 10 minutes with FUSE and compared to a negative control. Shavings were then prepared for imaging under a microscope and images were collected. Images were analyzed to determine the number and size of timber shavings after FUSE processing.



**Supplementary Figure 3.2. Size of timber particles after FUSE processing.** FUSE processing results in a shift in the timber particle size distribution toward smaller particles for (A) fine, (B) medium, (C) coarse, and (D) combination shaving groups (n = 3). Results suggest that FUSE processing breaks down timber shavings, resulting in the detection of more small particles. \*Indicates significant (p < 0.05) differences between FUSE processing and negative controls.

**Supplementary Table 3.1. Timber DNA purity ratios.** The 260/280 and 260/230 purity ratios for white oak timber DNA released by FUSE are comparable to DNA released by mortar and pestle controls, suggesting that FUSE does not affect the quality of the released DNA. 260/280 and 260/230 ratios are reported from Nanodrop measurements.

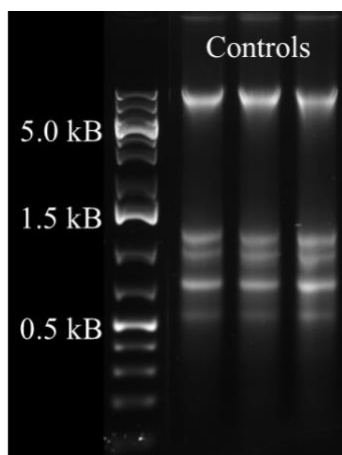
Processing Method	DNA Purity Ratios				
	Ratio	Fine	Medium	Coarse	Combination
Mortar & Pestle	260/280	1.29 ± 0.02	1.29 ± 0.03	1.17 ± 0.11	1.32 ± 0.02
	260/230	0.65 ± 0.04	0.76 ± 0.10	0.25 ± 0.16	0.81 ± 0.12
Negative Control	260/280	1.26 ± 0.02	1.06 ± 0.04	1.11 ± 0.06	1.23 ± 0.02
	260/230	0.58 ± 0.02	0.76 ± 0.03	1.59 ± 0.51	0.86 ± 0.07
FUSE – 1 min	260/280	1.05 ± 0.03	0.96 ± 0.06	1.13 ± 0.07	1.21 ± 0.03
	260/230	-0.90 ± 0.81	0.30 ± 0.55	0.94 ± 0.04	0.60 ± 0.01
FUSE – 5 min	260/280	1.12 ± 0.07	1.06 ± 0.02	1.16 ± 0.04	1.20 ± 0.03
	260/230	1.01 ± 0.27	0.98 ± 0.22	0.82 ± 0.04	0.56 ± 0.01
FUSE – 10 min	260/280	1.08 ± 0.00	1.13 ± 0.03	1.17 ± 0.03	1.22 ± 0.03
	260/230	0.62 ± 0.10	0.64 ± 0.09	0.69 ± 0.06	0.52 ± 0.01



**Supplementary Figure 3.3. qPCR amplification curves.** The qPCR results demonstrate that FUSE and conventional extraction methods yield DNA that can be amplified with qPCR. Amplifications were performed in triplicate for *matK* and *rbcL* gene regions.

**Supplementary Table 4.1. Control DNA purity ratios.** 260/280 ratios for samples prepared using conventional methods were within the expected norms. 260/230 ratios were lower than expected for DNA. All data was collected using the Nanodrop.

260/280	260/230
2.02 ± 0.04	1.54 ± 0.11



**Supplementary Figure 4.1. Control DNA fragmentation.** Visualization of DNA lysates prepared with gel electrophoresis demonstrates that conventional DNA extraction methods do not shear DNA.

**Supplementary Table 4.2. qPCR success rate.** Variation of the FUSE PRF and dose influenced amplification success. Results show that increasing the PRF and dose improved the amplification success rate.

PRF (Hz)	Dose (Pulses)	Time (mm:ss)	Replicate Success	Success Rate
25	10,000	06:40	11/18	61%
	100,000	66:40	15/18	83%
	200,000	133:20	17/18	94%
250	10,000	00:40	10/18	56%
	100,000	06:40	14/18	78%
	200,000	13:20	15/18	83%
500	10,000	00:20	13/18	72%
	100,000	03:20	15/18	83%
	200,000	06:40	18/18	100%
1,000	10,000	00:10	17/18	94%
	100,000	01:40	18/18	100%
	200,000	03:20	18/18	100%
Controls	Controls	30:00-55:00	18/18	100%

**Supplementary Table 4.3. qPCR Ct values.** Variation of the FUSE PRF and dose influenced the number of cycles required to generate an amplification product. Results show that increasing the PRF and decreasing the dose reduced Ct values.

PRF (Hz)	Dose (Pulses)	Time (mm:ss)	qPCR Efficiency
25	10,000	06:40	16.0 ± 1.1
	100,000	66:40	18.4 ± 0.6
	200,000	133:20	21.5 ± 0.5
250	10,000	00:40	16.0 ± 1.1
	100,000	06:40	17.5 ± 0.9
	200,000	13:20	19.9 ± 0.8
500	10,000	00:20	16.4 ± 0.9
	100,000	03:20	16.9 ± 0.3
	200,000	06:40	16.8 ± 0.3
1,000	10,000	00:10	14.0 ± 0.1
	100,000	01:40	15.3 ± 0.6
	200,000	03:20	15.7 ± 0.2
Controls	Controls	30:00-55:00	16.6 ± 0.4