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FINAL TECHINICAL REPORT

Quantifying and Qualifying the Influence of Standard Laboratory Procedures on Aged, Degraded, and/or Low Copy Number DNA (Award 2017-DN-BX-0139)

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ORGANIZATION OF THIS FINAL TECHINICAL REPORT…WITH HIGLIGHTS

This project was sub-divided into four distinct phases. Under each of these phases we describe the rationale for the experiments designed and conducted and have reported each collection of experiments as discrete "sub-projects." Each sub-project addresses specific issues related to the outlined problem(s). Cross reference between these sub-projects is noted, particularly by referencing specific data from outside sub-projects. Each sub-project report serves as a basic draft of a paper we will be submitting for peer-reviewed publication.

Each sub-project is supported by figures and tables located at the end of each sub-project report. Supplemental files of raw data are provided as excel files. A single bibliography was produced from the report and located at the end of the entire report.

At the time of preparing this final technical report, we are not completed with Phase IV [How much contaminating DNA is routinely encountered in the laboratory and how much (and/or in what ways) does it exhibit characteristics of aged, degraded, and/or LCN DNA?]. An addendum to this report will be filed with the National Institute of Justice when Phase IV is complete.

We are excited to share our observations with others. Support from the NIJ allowed us to think deeply about the challenges of better studying aged, degraded, and/or low copy number (LCN) DNA samples.

Some highlights:

- *"'Buyer beware' sums up this sub-project very well…DNA extraction kit/methods evaluated here were associated with variable losses of concentrations of DNA standards and their inadvertent fragmentation."* (Sub-Project 1.1 "Influence of DNA extraction methods on the quantity and quality of retained genetic material")

- *Regarding a silica-based extraction, we measured abundant molecules "lost" during extraction on found both 1) on the silica column (i.e., not eluted off during elution) and 2) in the flow-through (i.e., DNA molecules not bound to the silica). This suggests that improvements in both binding and elution are critical.* (Sub-Project 1.2 "Where, Oh Where, Has the DNA Gone?")

- *Storage of DNA standards over the course of ~10 months at room temperature (20-22°C), 4°C, -20°C, and -80°C was met with substantial stability of quality and quantity of the DNA standards. Degradation was no more notable at room temperature than compared to storage at -80°C* (Sub-Project 2.1 "Influence of Long-Term Storage at Room Temperature (20-22°C), 4°C, -20°C, and -80°C on the quality and quantity of DNA, as Well as Freeze Thaw Cycles")

- *Degradation of DNA by heat treatment is related positively to temperature exposed as well as its duration*. (Sub-Project 2.2 "Influence of Heat Treatment and Vortexing one the Quality and Quantity of DNA")

- *Increasing PCR extension times may subdue the influence of PCR inhibition.* (Sub-Project 3.1 “Extended Extension PCR: a Simple Technique for Improved Amplification of Aged, Degraded, and Low Copy Number DNA Samples Compromised by PCR Inhibitors”)

- *We conducted 500 cycle PCRs, and they did not fail!* (Sub-Project 3.2 “Why Don’t We Run 100 Cycle PCRs?”)

- Employing lower than standard denaturing temperature in PCR was a largely ineffective approach to studying degraded DNA, however we demonstrated that PCR can be conducted with denaturing as low as 85°C, but not at ≤80°C (Sub-Project 3.3 “Influence of a Range (Especially Outside) of Standard Denaturing Temperatures on the Amplification of Aged, Degraded, and/or Low Copy Number DNA.”)

PROJECT ABSTRACT

The analysis of aged, degraded, and/or low copy number (LCN) DNA is complicated largely by: (1) the presence of modern contaminating DNA, (2) the co-extraction of impurities (PCR inhibitors) along with DNA, and (3) the degree of damage accumulated by endogenous DNA post-mortem or from the time of deposition of the biological material. These problems make the analysis of DNA from aged, degraded, and/or LCN sources far more challenging than working with modern DNA.

Objectives of our research are to evaluate and develop methods that show promise to increase the net yield of DNA and its purity. These include: (1) evaluating DNA loss during its extraction and purification against standards (Phase I), (2) exploring means to mitigate DNA loss and/or further damage to the molecules in the standards (Phases I & II), and (3) further improving and/or development of novel methods to remove PCR inhibitors from DNA elutes and/or subdue their influences within the forensic workflow (Phase III).

Moreover, there are still several poorly understood aspects of how aged, degraded, and/or LCN DNA "behaves" during routine laboratory methods and, critically, whether some or all those behaviors truly differ from that of modern contaminating DNA. Moreover, there are hypotheses that newly observed damage patterns from ancient & endogenous DNA molecules are consistent and, thus, predictable. If these hypotheses are accurate, presumably there would be measurably different expectations for the behavior of – and damage accrued in – modern exogenous contaminating DNA due to the reduced time since creation and deposition of the biological material. To be clear, if the damage patterns observed in contaminating DNA mirror those routinely observed in authentic aged and degraded DNA, then damage patterns cannot be a used as a reliable means by which to discriminate between contaminating and endogenous DNA.

Our proposed research employs state-of-the-art technology for the quantification and/or qualification of DNA using an Agilent Fragment Analyzer, CFX96 Touch™ Deep Well Real-Time PCR Detection System, and Illumina sequencing platforms. The combination of these technologies will permit us to uniquely evaluate the above outlined issues and hypotheses in novel ways. The observations made during our project will directly benefit forensic genetics, criminal justice, as well as the fields of ancient DNA (aDNA) and paleogenomics.

Specific Aims

Primary goals include evaluating the effect(s) that common laboratory practices have on quality (i.e., average strand length), quantity (i.e., concentration), and purity of DNA in solution. Critically, we will assess if such treatments can cause modern DNA to take on damage patterns expected for aged, degraded, and low copy number (LCN) DNA. The observations made during our project will directly benefit forensic genetics (Safir, 2007; Butler, 2010), criminal justice (Safir, 2007; United States Department of Justice, 2017), as well as the fields of ancient DNA (aDNA) and paleogenomics (Raghavan et al., 2014; Shapiro and Hofreiter, 2014).

Our proposed research employs state-of-the-art technology for the quantification and/or qualification of DNA using an Agilent Fragment Analyzer, CFX96 Touch™ Deep Well Real-Time PCR Detection System, and Illumina sequencing platforms. The combination of these technologies will permit us to uniquely evaluate: (1) amounts of DNA lost during extraction and purification, (2) the influence of standard lab practices on DNA eluates, such as heating, freeze-thawing, and/or vortexing (3) means to improve upon "standard" PCR, with the express purpose to improve amplification of DNA from aged, degraded, and low copy number sources, and (4) the quality and quantity of contamination encountered during standard laboratory practices.

Statement of the problem

The analysis of aged, degraded, and/or LCN DNA is complicated largely by the following factors:

(1) The inadvertent introduction of exogenous contaminating DNA during its analysis. Contamination can completely outcompete endogenous DNA during polymerase chain reaction (PCR) amplification (Kemp and Smith, 2005; Fregeau et al., 2008; Community et al., 2009; Barta et al., 2013; Minor, 2014; Balk, 2015). This is *particularly problematic* in the study of aged, degraded, and/or LCN *human* DNA [e.g., in producing accurate Combined DNA Index System (CODIS) profiles].

(**2)** Co-extracted PCR inhibitors, the presence of which in DNA eluates can make PCR amplification difficult, if not impossible (Alaeddini, 2011; Monroe et al., 2013; Kemp et al., 2014a; Nilsson et al., 2016).

(3) The degree to which endogenous template molecules have been damaged or chemically modified post-mortem or from the time of deposition of the biological material (Gilbert, 2006; Alaeddini et al., 2010; Meyer et al., 2013; Hanssen et al., 2017). It is typically observed that DNA recovered from aged or degraded sources is fragmented with regards to strand length (Pääbo, 1989), and carries chemically modified nucleotides (i.e., ones that can appear as "mutations" when, in fact, they are actually taphonomic artifacts). Trace or touch DNA too can be degraded and damaged in the same fashion, in addition to being LCN (Lowe et al., 2002; Hudlow et al., 2010; van Oorschot et al., 2010).

These complications make the authentication of DNA profiles/genotypes from aged, degraded, and/or LCN samples not only difficult, but also costly. As such, a *premium* should be placed on continued funding for the development and evaluation of methods that have the potential to substantially increase the yield and purity of genetic material extracted from such sources.

Moreover, there are still a number of poorly understood aspects of how aged, degraded, and/or LCN DNA "behaves" during routine laboratory methods (e.g., Cooper and Poinar, 2000) and,

critically, whether some or all of those behaviors truly differ from that of modern contaminating DNA. For example, an expectation of asymmetrical molecular behavior between DNA from aged sources and that from the ubiquitous, fresh supply constantly being introduced in the environment (i.e., modern DNA) has been proposed as a means to discriminate between the origins of the genetic material (i.e., endogenous or exogenous sources, respectively) (Pääbo, 1989; Malmstrom et al., 2007). Damage is also visible using a High Throughput Sequencing (HTS) approach, from which it has been hypothesized that damage patterns observed from ancient and degraded endogenous DNA molecules are consistent and, thus, predictable (Krause et al., 2010; Meyer et al., 2013; Prüfer and Meyer, 2014). If these hypotheses are supported, presumably there would be measurably different expectations for damage accrued in modern exogenous contaminating DNA due to the comparatively reduced time since deposition of the biological material. To be clear, if the damage patterns observed in contaminating DNA mirror those routinely observed in authentic aged and degraded DNA, then damage patterns cannot be a used as a reliable means by which to discriminate between contaminating and endogenous DNA.

Lastly, attention should be focused on the fact that we do not know how much DNA is obtainable from any source. This can be illustrated with the following equation:

> **Net yield of DNA=** Original amount – loss in sampling – loss in extraction/purification – loss due to amplification bias (e.g., due to PCR inhibitors)

Note that each of the variables that make up the equation is unknown. Obvious objectives to increase net yield of DNA include, amongst others: (1) evaluating DNA loss during its extraction and purification against a standard, (2) exploring means to mitigate DNA loss, and (3) further improving and/or development of novel methods to remove PCR inhibitors and/or subdue their influences within the forensic workflow. These ideas will be evaluated by experiments described in Phases I-III.

The more thoroughly the above-described issues are examined, understood, and possibly resolved, the more confident one can be in the authentication (i.e., strength) of DNA results recovered from such samples. Forensic genetics (Safir, 2007; Butler, 2010), criminal justice (Safir, 2007; United States Department of Justice, 2017), as well as the fields of aDNA and paleogenomics (Raghavan et al., 2014; Shapiro and Hofreiter, 2014) will all benefit from the knowledge gained from the experiments we propose to conduct. Importantly, as technology has improved, finer-scale observations of DNA quantity and quality are permitted. We will leverage that technological power to address
many of the abovementioned issues and hypotheses.

In summary:

(1) The analysis of DNA from aged, degraded, and/or LCN sources is far more challenging than working with modern DNA.

(2) DNA from such sources is expected to behave *differently* during routine laboratory methods. However, the validity of this evidence is only as strong as how well we *actually* know if, and how, they differ.

(3) Continued development and evaluation of methods that increase the yield and purity of genetic material extracted from aged, degraded, and/or LCN sources is needed.

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Phase I: Evaluating extraction methods for their influence on the quantity and quality of DNA

Background

Following some of the earliest examples of PCR amplification from ancient and forensic DNA samples (Hagelberg et al., 1989; Pääbo, 1989; Stoneking et al., 1991; Jeffreys et al., 1992), it has been commonly observed that these samples are characterized by substantial degradation and would typically yield only 1-5% of the DNA that would be expected from modern tissues (O'Rourke and Parr, 1996). *Yet, do we have a good understanding of how much DNA is actually recoverable from aged, degraded, and or LCN sources?* Since many have the *a priori* expectation of recovering very little DNA, it is particularly relevant that various methods of DNA extraction and purification can result in tremendous losses, the result being LCN (Barta et al., 2014b; Kemp et al., 2014b). This demonstrates that extraction alone can cause DNA eluates to become LCN, a characteristic that is generally agreed to be one that is diagnostic for authenticating aDNA results (Barta et al., 2014b) [in addition to using other relevant observations that are expected of ancient DNA (Cooper and Poinar, 2000; Kemp and Smith, 2010)]. Being LCN (or becoming LCN) further exacerbates the problem of subsequent PCRs initiated from such eluates, as they are particularly susceptible to contamination (Cooper and Poinar, 2000; Bunce et al., 2012).

It has been suggested that quantifying the number of template molecules that initiate PCRs can be used as a means of authenticating results as "ancient" and not that of exogenous contamination (Cooper and Poinar, 2000). This recommendation is based on the idea that the number of starting template molecules should not be *too high* (which might be indicative of contamination) nor *too low* (which might permit miscoding lesions to be directly observed in the PCR product [see Figure 3 of Pääbo et al. (2004) and Winters et al. (2011)]). This reasoning places researchers in a "Goldilocks situation", where one is looking for DNA template numbers to be "just right"—yet the boundaries on these quantities have not been well-established, especially on the upper end. In other words—*Where does aDNA copy number end and contaminating molecule copy number begin*? This is analogous to discussions on what constitutes an "LCN" sample (Budowle et al., 2009; Gill et al., 2009; Gill and Buckleton, 2010).

There are manipulations of DNA samples that result in loss and/or degradation of that source DNA. For example, loss could be due to not swabbing all DNA present on a touched object (van Oorschot et al., 2003), or losing DNA in any of the many subsequent steps during its extraction and purification (Lee et al., 2010; Dabney et al., 2013; Barta et al., 2014b; Kemp et al., 2014b). With the goal of maximizing recovery of genetic material from aged, degraded, and/or LCN sources, numerous researchers have performed comparative studies of DNA yields using various extraction techniques (e.g., Cattaneo et al., 1997; Yang et al., 1998; Hoff-Olsen et al., 1999; Castella et al., 2006; Davoren et al., 2007; Loreille et al., 2007; Rohland and Hofreiter, 2007b; Kitayama et al., 2010; Rohland et al., 2010). These studies often found one extraction method was superior to others tested under a specific set of conditions, such as the age and state of preservation of the biological material, and/or associated impurities in the samples. However, because these studies began with no knowledge of the actual DNA quantity in the samples prior to extraction, they ultimately compared the outcome of all methods *relative* to the best (e.g., see Table 1 of Rohland and Hofreiter, 2007b). Even the most optimal extraction methods, in this case, might actually perform poorly compared to 100% efficiency.

Fewer studies have quantified DNA loss during extraction and purification against DNA standards of known quantity and quality. Lee et al. (2010) artificially degraded human genomic

DNA with DNase I and diluted this fragmented DNA to 25 ng standards. On average their best DNA extraction method retained 50.8% and 38.9% of the degraded and intact standards, respectively. However, the outcome of DNAse I treatment on the genomic DNA was not qualified (i.e., the authors did not assess the resulting strand lengths contained within the standard following DNase I treatment).

To estimate the degree of DNA loss, Dabney et al. (2013) subjected a standard mixture of five NoLimit DNA fragments (35, 50, 75, 100, and 150 bps) at a concentration of 5.7 ng/μL to the extraction method of Rohland and Hofreiter (2007a) and a modified version of that protocol. The modifications included a change to binding buffer composition, buffer volume, and replacement of loose silica for a fixed silica column (Qiagen MinElute spin column). DNA loss was quantified against the standard using a BioAnalyzer with a 1000 DNA chip. Evaluated against their standard mixture, this equated to ~5.6 billion total copies/μL (or ~1.12 billion copies/μL of each sized fragment) (see discussion by Barta et al., 2014a). This makes it necessary to evaluate loss of very high copy number standards, as Dabney et al. (2013) chose to do so, starting at ~64 billion total copies/μL (or ~12.8 billion copies/μL of each sized fragment). This concentration is probably atypical of DNA recovered from aged, degraded, and/or LCN sources. Nevertheless, Dabney et al. (2013) observed that the Rohland and Hofreiter (2007a) method was associated with 72% and 22% retention of 150 bp and 35 bp fragments, respectively. The modified extraction protocol of Dabney et al. (2013) resulted in the opposite relationship, with ~84% and 95% retention of these fragments, respectively [estimated from Figure 1 of Dabney et al. (2013)]. From their report, the cause of this effect is undeterminable, as the researchers modified multiple aspects of the protocol simultaneously and failed to discuss whether the initial copy input was a factor in DNA retention.

Barta et al. (2014a) used qPCR to estimate DNA loss of single sized DNA fragments [181 bp amplicons at concentrations of 10^2 to 10^4 copies/μL (~130-50000 copies/μL)] associated with common extraction and purification methods, including phenol:chloroform, alcohol precipitation, microconcentration, and silica-based techniques. They observed ~48-99% loss associated with these extraction techniques. One drawback of the approach of the Barta et al. (2014a) study was that their standard contained fragments of a single size. Kemp et al. (2014b) added to this experimental design by observing loss of DNA fragments ranging 106-428 bps in length following purification with the Qiagen MinElute Kit. Loss of DNA observed during this study too was staggering, and in support of the findings of Barta et al. (2014a).

Critically, while all three of these research groups converged on the observation of tremendous loss of DNA, our experimental design outlined in the Pre-Phase will permit us to address the respective limitations of each study. Our experiments are used to model *what would be expected to occur* during extraction and purification of intact genomic DNA samples to one that are aged, degraded, and/or LCN.

A tangential, but nevertheless important, research question that will be addressed is —W*here has the DNA gone during extraction and purification*? In the case of purification with silica, it must be that much of the DNA either does not efficiently bind to the silica particles or cannot effectively be removed from them.

During Phase I, we:

(1) Evaluate percent retention of DNA when processed by twenty different kits and methods, especially ones marketed for — and/or are in use— for the recovery and analysis of aged, degraded, and/or LCN DNA.

(2) Document the effect of these extraction and purification kits/methods on the *quality* of the DNA (i.e., if these processes fragment the standards in a meaningful way).

(3) Explore the source or DNA loss associated with a basic-silica-based extraction method.

Sub-Project 1.1 "Influence of DNA extraction methods on the quantity and quality of retained genetic material" (NOTE: proposed title for publication)

In this set of experiments, we sought to evaluate the influence of extraction method on the integrity of DNA using a straightforward comparison of concentration and fragment size distribution of DNA standards (i.e., "DNA in") to those measures found in the standards following extraction and purification (i.e., "DNA out"). Importantly we sought to build on the deficits of studies reviewed in the Phase I background, namely, by simultaneously evaluating changes in DNA quantity [i.e., concentration (ng/μL)] and DNA quality [i.e., size distribution of DNA in the eluate) from a range of DNA standards (from high concentration and intact to low concentration and degraded), following 10 replicates of 20 extraction methods each.

The extraction methods chosen were ones: 1) we commonly employ in our investigations of ancient DNA [i.e., Kemp et al 2014 (2014a); Kemp et al. (2007) as modified in (Moss et al., 2014)], 2) in popular use by other ancient DNA researchers (e.g., Dabney et al., 2013; Rohland et al., 2018), 3) commercially available kits with names that indicate marketing towards those working with aged, degraded, and low copy number DNA sources (e.g., using terms "Forensic", "Investigator" "Micro", "Trace"), and 4) commercial available and widely used by ancient and forensic DNA researchers (e.g., Qiagen DNeasy Blood &Tissue Kit, a "standard" kit for DNA extraction).

In creating our standards, we wanted each to represent one of four possible combinations of parameter space (i.e., across concentrations and intactness of DNA molecules), to model expectations under four different "scenarios" (Sub-Project 1.1 Figure 1). First, Standard 1 (STD 1) is full genomic DNA extracted from pig (*Sus scrofa*) liver at ~100 ng/μL. Extraction of this standard is meant to simulate expectations of DNA recovered from fresh tissue. Standard 2 (STD 2) is a dilute version of STD 1 at ~1 ng/μL. This standard is meant to represent "touch" or "trace" DNA (van Oorschot et al., 2010) that might be recovered in a fairly intact state, but at low concentration. Standard 3 (STD 3) is a sonically sheared version of STD 1 at ~100 ng/μL with most molecules being under 500 bp in length. This standard does not have a real-life analogue with which we are familiar (i.e., a highly concentrated, highly degraded DNA specimen). Yet, we found it important to conduct experiment on this standard to fully explore the parameter space. Lastly, Standard 4 is a dilute version of STD 3 at ~1 ng/μL. This is meant to represent DNA recovered from an aged, degrade, and low copy number DNA specimen (e.g., aged/archaeological bones and teeth).

We recognize that even at ~1 ng/μL, our lowest standards are not near "low copy number" as defined as <100-200 pg of input (National Forensic Science Technology Center, 2007; Budowle et al., 2009; Gill and Buckleton, 2010; Word, 2010; Marshall, 2014). However, it is notable that the <100-200 pg measure is one taken after extraction and purification of DNA and, thus, is not indicative of the actual about of starting nucleic acid concentration, *which is an unknown quantity*. The Agilent Fragment Analyzer, used in this sub-project, has a low-end detectability of 50 pg/μL. With knowledge that some of the extraction kits examined here may be associated with >95% loss (Barta et al., 2014b), a starting concentration of 1 ng/μL would be needed so as the resulting eluates concentrations are still measurable. Examination of standards at lower concentrations would need to relay on a qPCR approach (which is much more sensitive to the detection of low quantities of molecules), however one that cannot produce simultaneous information about the quality of a DNA sample.

MATERIALS AND METHODS

Creation of DNA Standards

We created four standards from DNA extracted from pig (*Sus scrofa*) liver* with the Qiagen DNeasy Blood & Tissue Kit, following the manufacturer's protocol (Qiagen, 2016).Quantities and qualities of the standards were determined using an Agilent Fragment Analyzer (Sub-Project 1.1 Supplemental Table 1-Tabs B-E). For standards 1 and 2 (intact genomic DNA) we measure quality as the fraction of DNA molecules belonging to each the following eight categories: 1) 20-100 bp, 2) 100-500 bp, 3) 500-1,000 bp, 4) 1,000-3,000 bp, 5) 3,000-6,000 bp, 6) 6,000-8,000 bp, 7) 8,000-10,000 bp, and 8) 10,000-30,000 bp. The quality of standards 3 and 4 (sonicated DNA) was measured by the fraction of DNA molecules belonging to each the following seven categories: 1) 20-50 bp, 2) 50-100 bp, 3) 100-250 bp, 4) 250-500 bp, 5) 500-1,500 bp, 6) 1,500-5,000 bp, and 7) 5,000-20,000.

Standard 1: intact genomic DNA, with 42.06±8.78% of the molecules in the range of 8,000-30,000 bp (averaged over categories of % molecules 8,000-10,000 bp and 10,000-30,000 bp.), at 114.84±26.02 (averages and standard deviations of the standards as measured across all experiments; see Sub-Project 1.1 Supplemental Table 1-Tab B).

Standard 2: intact and diluted genomic DNA at 1.13±0.16 ng/µL, with 39.32±4.13% of the molecules ranging 8,000-30,000 bp (averaged over categories of % molecules 8,000-10,000 bp and 10,000-30,000 bp.) (averages and standard deviations of the standards as measured across all experiments; see Sub-Project 1.1 Supplemental Table 1-Tab C).

Standard 3: DNA sonically sheared with a QSonica, with ~75.45±7.46% of molecules ranging 100 to 500 bp at a concentration of 117 ng/µL±96.33 (averages and standard deviations of the standards as measured across all experiments; see Sub-Project 1.1 Supplemental Table 1-Tab D).

Standard 4: DNA sonically sheared and dilute, with the bulk of the molecules 66.46 ±28.07%) in the range of 100 to 500 bp at a concentration of 0.69±0.44 ng/µL (averages and standard deviations of the standards as measured across all experiments; see Sub-Project 1.1 Supplemental Table 1-Tab E).

"DNA In" compared to "DNA Out"

Volumes of each of the four standards (Sub-Project 1.1 Supplemental Table 1-Tabs B-E) was "extracted" following the protocols listed in Sub-Project 1.1. Table 1. Each kit demanded a particular volume input (Sub-Project 1.1 Supplemental Table 1-Tabs B-E) and this was considered in the calculation of the final percent recovery. As an example, the Bio-Rad InstaGene Matrix (Bio-Rad, n.d.) extraction (abbreviation BIG) required 30 µL of input DNA volume and produced 230 µL of DNA output volume. In this case the final concentration of DNA out was adjusted by a $7^2/_3$ multiplier to compensate for the dilution effect produced by this extraction kit.

* Pig liver sample graciously provided by Bart Bingham.

For each extraction kit/method, two researchers (Kristine G. Beaty and Brittany Bingham) extracted five replicas of each standard accompanied by an extraction negative control†, for which volumes of DNA free water were substituted for the volumes of DNA standard they replace, respectively.

Extraction retention efficiency was calculated as:

[(copies in-copies out)/copies in] X 100= % efficiency

Following extraction, concentration of "DNA out" was calculated with a Qubit 3.0. Those concentrations were used to dilute appropriately for analysis by the Agilent Fragment Analyzer. These dilutions too were considered in the back calculations of "DNA in" concentrations. In the case the concentration was undetectable with the Qubit, the full concentration ""DNA out"" was analyzed on the Fragment Analyzer.

RESULTS

Standard 1 (STD 1) Intact Genomic DNA, Concentrated

Twelve of 20 kits/extraction methods (60%) were associated with <50% retention of the molecules found in standard 1 (Sub-Project 1.1 Supplemental Table 1 Tab A; Sub-Project 1.1 Tables 2-3) . Six of these extractions were also associated with the noticeable loss of DNA molecules ≥6,000 bp in length (Sub-Project 1.1 Figure 2A).

Notably (as point of comparison, a kit not particularly designed for processing degraded DNA) the Qiagen Dneasy kit [QDN (Qiagen, 2016)] was associated with a high percentage of DNA retention (125.68±68.40%). Four of the ten observations of "DNA out" from this kit were associated with ~200 % retention. Removal of these data points would reduce retention percentage to 77.92±18.37%), a generally high retention percentage considering our overall observations.

The Rohland et al. (2018) method using a large volume column and binding buffer G (RCG) was associated with a particularly high percentage of retention (88.5±8.39%). The same extraction method with the substitution of binding buffer C (RCG) was also associated with a high percentage of retention (80.89%), but a large variance (standard deviation= 47.88%) driven by two extreme values (RCD 1-3 and 1-4; Sub-Project 1.1 Supplemental Table 1 Tab B)

Standard 2 (STD 2) Intact Genomic DNA, Dilute

Nine of 20 kits/extraction methods (45%) were associated with <50% retention of the molecules found in standard 2 (Sub-Project 1.1 Supplemental Table 1 Tab A; Sub-Project 1.1 Tables 2-3). Seven of these kits/extraction methods were also associated with <50% retention of the molecules found in standard 1. The Qiagen Dneasy kit [QDN; (Qiagen, 2016)] was here was associated with much lower retention of molecules (18.12±4.85%) from this standard and the Qiagen Investigator Kit [QIV; (Qiagen, 2012)] performed poorly (18.75±12.06%) compared to retention percentage of standard 1 (65.77±20.47%). The Qiagen Dneasy kit [QDN (Qiagen, 2016)] retained only 18.12±4.85% of the molecule of this standard (Sub-Project 1.1 Table 2).

† DNA found in these negative controls (representing cross contamination generated in the laboratory or contamination originating from the place or manufacture) will be scrutinized under Phase IV (which is presently in progress).

Eight of these extractions were also associated with the noticeable loss of DNA molecules ≥6,000 bp in length (Sub-Project 1.1 Figure 1B- see those extraction kits/methods in boxes to the right).

The Rohland et al. (2018) method using beads and binding buffer D (RBD) was associated with a particularly high percentage of retention (96.65±18%) with little change to the quality to the DNA. Nucleospin DNA Trace (Macherey-Nagel, 2018) (TNT) Charge Switch Forensic DNA Purification Kit Invitrogen, 2005) (ICS) also performed well (88.77±25.65% and 72.63±23.37%, respectively) with little change to the quality of the DNA standard.

Standard 3 (STD 3) Degraded DNA, Concentrated

Eighteen of 20 kits/extraction methods (90%) were associated with <50% retention of the molecules found in standard 3 (Sub-Project 1.1 Supplemental Table 1 Tab A; Sub-Project 1.1 Tables 2-3). At best, PrepFiler (Applied-Biosystems, 2008) (TPF) retained 81.15±42.6%. The second highest average retention was observed with the Dabney et al. (2013) method (DAB) at 7.35±33.05%. The variances associated with these methods are relatively high. It is notable that the variances associated with the retention of standard 3 are generally higher than those associated with retention of standards 1 or 2. The Qiagen Dneasy kit [QDN (Qiagen, 2016)] retained 13.83±10.16% of the molecule of this standard (Sub-Project 1.1 Table 2).

Two of these kits/methods (NA Investigator Kit (Qiagen, 2012) and InstaGene Matrix (Bio-Rad, n.d.) were also associated with a particularly loss of DNA molecules <500 bp in length (see right box in Sub-Project 1.1 Figure 1C).

Standard 4 (STD 4) Degraded DNA, Dilute

Fourteen of 20 kits/extraction methods (70%) were associated with <50% retention of the molecules found in standard 4 (Sub-Project 1.1 Supplemental Table 1 Tab A; Sub-Project 1.1 Tables 2-3). One of these six kits with >50% retention [Geneclean Kit for Ancient DNA (using Dehybernation Solution B) (Biomedicals, 2013); MGB] was associated with a noticeable change to the quality of the resulting eluates Sub-Project 1.1 Figure 1D. The other five kits retain well the quantity and quality of standard 4 (albeit some with large variances- i.e., RBD and RBG). As observed with experiments on standard 3, the variance associated with the retention of standard 4 is generally higher than those associated with retention of standards 1 or 2. The Qiagen Dneasy kit [QDN (Qiagen, 2016)] retained only 0.03±0.04% of the molecule of this standard (Sub-Project 1.1 Table 2).

Four of these kits/methods (K07, MGA, MGB, and PIQ) were also associated with a particular loss of DNA molecules <500 bp in length (see second to right box in Sub-Project 1.1 Figure 1D) and three kits/methods (BIG, QDN, and TN8) with the retention of ultrashort DNA 20-50 bp in length (Gutaker et al., 2017; de Filippo et al., 2018) (see right box in Sub-Project 1.1 Figure 1D).

CONCLUSIONS

“Buyer beware” sums up this sub-project very well. As expected, the DNA extraction kit/methods evaluated here were associated with variable losses of concentrations of DNA standards and their inadvertent fragmentation.

We encourage others to evaluate extraction methods as we have done here, to provide some expectation(s) for what characteristics might be observed in the resultant eluates. Given the

large degrees of DNA loss associated with kits/methods marketed toward (or cited as examples for) forensic and ancient DNA researchers.

Moreover, we are optimistic as this problem of DNA loss and fragmentation becomes more commonly held knowledge, that others will focus their efforts on optimizing extraction methods. It is notable, however, that something major still does not add up. For example, since the method we most routinely use in our laboratory (K014) is associated with large losses of degraded molecules, how is it possible for us to perform repeated silica-based extractions (Kemp et al., 2006; Kemp et al., 2014a) to remove PCR inhibition while still retaining ample genetic material for subsequent amplification. In our first study of archaeological salmonid mitochondrial DNA, we were required to perform on average 4.62±2.31 repeat silica extractions (Grier et al., 2013), to sufficiently remove PCR inhibitor from the eluates. These repeat silica extractions followed the extraction method of Kemp et al (2007) as modified by Moss et al. (2014) (extraction K07 in this present study), which is also associated with only 2.9±1.67% retention. So we could image some average sample that was under investigation by Grier et al (2013), beginning with ~2.9% of its original DNA, compounded with ~4 rounds of repeat silica extraction with losses of 2.9%, resulting in retention (0.0462X0.029X0.029X0.029X0.029=) of merely $3.26*10^{-6}$%. Thus, either typical ancient DNA specimens contain far more DNA than is commonly recognized, or the there are other compounded variable to silica-based extractions that have not sufficiently modeled.

Additional insight into the nature of best retaining short and low copy number molecules is still critical.

Sub-Project 1.1 Table 1. Extraction methods evaluated in this sub-project (with abbreviations).

Method Abbreviation	Manufacturer/ Protocol	Kit/Method	Extraction "Type"
BIG	Bio-Rad	InstaGene Matrix (Bio-Rad, n.d.)	Forensic
DAB	Dabney et al. (2013)	Dabney et al. (2013)	Ancient
ICS	Invitrogen	Charge Switch Forensic DNA Purification Kit (Invitrogen, 2005)	Forensic
K07	Kemp Working Group-"Old Method"	Modified Kemp et al. (2007) described by Moss et al (2014)	Ancient
K14	Kemp Working Group-"New Method"	Kemp et al. (2014a)	Ancient
MGA	MP Biomedicals	Geneclean Kit for Ancient DNA (using Dehybernation Solution A) (Biomedicals, 2013)	Ancient
MGB	MP Biomedicals	Geneclean Kit for Ancient DNA (using Dehybernation Solution B) (Biomedicals, 2013)	Ancient
PIQ	Promega	Promega DNA IQ System (Promega, 2016)	Forensic
QDN	Qiagen	Dneasy Blood & Tissue Kit (Qiagen, 2016)	Forensic
QIV	Qiagen	DNA Investigator Kit (Qiagen, 2012)	Forensic
QMC	Qiagen	DNA Micro Kit (Qiagen, 2014)	Ancient/Forensic/Common
QME	Qiagen	MinElute PCR Purification Kit (Qiagen, 2008)	Ancient/Forensic/Common
RBD	Rohland et al. (2018)	Rohland et al. (2018), Beads, Binding buffer D	Ancient
RBG	Rohland et al. (2018)	Rohland et al. (2018), Beads, Binding buffer G	Ancient
RCD	Rohland et al. (2018)	Rohland et al (2018), Large volume column, Binding buffer D	Ancient
RCG	Rohland et al. (2018)	Rohland et al. (2018), Large volume column, Binding buffer G	Ancient
TN8	Takara	Nucleospin Trace 8 (Macherey-Nagel, 2014b)	Forensic
TNT	Takara	Nucleospin DNA Trace (Macherey-Nagel, 2018)	Forensic
TNX	Takara	Nucleospin gDNA Cleanup XP(Macherey-Nagel, 2014a)	Forensic
TPF	Thermofisher	PrepFiler™ (Applied-Biosystems, 2008)	Forensic

Sub-Project 1.1 Table 2. Percent recovery of four DNA standards each by 20 kits/methods. Method abbreviations are detailed in Sub-Project 1.1 Table 1. See Sub-Project 1.1 Supplemental Table 1 for addition information.

		STD 1		STD 2		STD 3		STD 4	
Method	**Kit/Method**	**# measures**	**% recovered**	**# measures**	**% recovered**	**# measures**	**% recovered**	**# measures**	**% recovered**
BIG	InstaGene Matrix (Bio-Rad, n.d.)	10	0.25±0.11	10	165.53±46.71	10	43.56±7.29	10*	15.47±3.77
DAB	Dabney et al. (2013)	10	56.98±8.17	NA*		10	57.35±33.05	NA*	
ICS	Charge Switch Forensic DNA Purification Kit (Invitrogen, 2005)	10	35.01±12.31	10	72.63±23.37	6	34.94±9.79	10	49.88±10.42
K07	Modified Kemp et al. (2007) described by Moss et al (2014)	10	11.76±18.98	10	0.37±0.35	10	3.62±6.86	10	2.9±1.67
K14	Kemp et al. (2014a)	10	0.2±0.04	10	36.03±13.03	10	0.65±0.29	9	4.71±1.74
MGA	Geneclean Kit for Ancient DNA (using Dehybernation Solution A) (Biomedicals, 2013)	10	26.9±10.22	10	110.27±32.01	10	21.63±3.42	10	4.32±6.61
MGB	Geneclean Kit for Ancient DNA (using Dehybernation Solution B) (Biomedicals, 2013)	10	11.31±2.89	10	94.99±169.96	10	5.19±1.69	10	207.31±164.93
PIQ	Promega DNA IQ System (Promega, 2016)	10	21.91±11.46	10	27.76±12.1	10	4.6±1.54	9	4.36±2.97
QDN	Dneasy Blood & Tissue Kit (Qiagen, 2016)	10	125.68±68.40	10	18.12±4.85	10	13.83±10.16	9	21.73±35.65
QIV	DNA Investigator Kit (Qiagen, 2012)	10	65.77±20.47	10	18.75±12.06	10	17.6±8.73	10	29.93±13.41
QMC	DNA Micro Kit (Qiagen, 2014)	10	46.86±9.99	10	37.74±8.58	10	7.39±2.21	9	23.8±8.85
QME	MinElute PCR Purification Kit (Qiagen, 2008)	10	73.5±43.13	10	50.01±11.17	10	38.82±5.35	9	47.61±10.49
RBD	Rohland et al. (2018), Beads, Binding buffer D	10	66.92±15.92	10	96.65±18.00	10	8.56±4.17	10	284.38±218.68
RBG	Rohland et al. (2018), Beads, Binding buffer G	10	51.19±33.57	10	121.76±41.26	10	34.46±6.53	10	166.54±55.01
RCD	Rohland et al (2018), Large volume column, Binding buffer D	10	80.89±47.38	10	59.02±8.72	10	10.81±3	10	51.65±20.5
RCG	Rohland et al. (2018), Large volume column, Binding buffer G	10	88.5±8.66	10	65.63±16.83	10	9.44±1	10	131.77±19.77
TN8	Nucleospin Trace 8 (Macherey-Nagel, 2014b)	10	24.99±23.64	10	12.14±7.74	10	22.35±14.3	10	1.54±1.68
TNT	Nucleospin DNA Trace (Macherey-Nagel, 2018)	10	37.02±23.25	10	88.77±25.65	10	32.1±18.95	10	56.45±21.88
TNX	Nucleospin gDNA Cleanup XP(Macherey-Nagel, 2014a)	10	23.68±5.23	10	7.83±8.14	10	38.58±9.76	10	23.18±7.95
TPF	PrepFiler™ (Applied-Biosystems, 2008)	10	46.15±5.01	10	5.86±6.94	10	81.15±42.6	10	32.38±11.88

*no measures obtained, thus no calculation of % recovery

Sub-Project 1.1 Table 3. Summary of results by standards. Kits/methods associated with <50% average retention are noted, as well as those that impact quality of DNA eluates (i.e., disrupting original fragment size distribution, as visually assessed from Sub-Project 1.1. Figure 2).

		STD 1 -intact genomic DNA ~100 ng/ µL		STD 2 -intact genomic DNA ~1 ng/ µL		STD 3 -degraded DNA ~100 ng/ µL		STD 4-degraded DNA ~1 ng/ µL	
Method (Abbr.)	**Kit/Method**	**<50% average retention**	**Impact quality**	**<50% average retention**	**Impact quality**	**<50% average retention**	**Impact quality**	**<50% average retention**	**Impact quality**
BIG	InstaGene Matrix (Bio-Rad, n.d.)	✓	✓		✓	✓	✓	✓	✓
DAB	Dabney et al. (2013)								
ICS	Charge Switch Forensic DNA Purification Kit (Invitrogen, 2005)	✓	✓			✓		✓	
K07	Modified Kemp et al. (2007) described by Moss et al (2014)	✓	✓	✓	✓	✓	✓	✓	✓
K14	Kemp et al. (2014a)	✓		✓	✓	✓		✓	
MGA	Geneclean Kit for Ancient DNA (using Dehybernation Solution A) (Biomedicals, 2013)	✓	✓		✓	✓		✓	✓
MGB	Geneclean Kit for Ancient DNA (using Dehybernation Solution B) (Biomedicals, 2013)	✓			✓	✓	✓		✓
PIQ	Promega DNA IQ System (Promega, 2016)	✓		✓		✓		✓	✓
QDN	Dneasy Blood & Tissue Kit (Qiagen, 2016)			✓		✓		✓	✓
QIV	DNA Investigator Kit (Qiagen, 2012)			✓		✓	✓	✓	
QMC	DNA Micro Kit (Qiagen, 2014)	✓		✓		✓		✓	
QME	MinElute PCR Purification Kit (Qiagen, 2008)					✓		✓	
RBD	Rohland et al. (2018), Beads, Binding buffer D					✓			
RBG	Rohland et al. (2018), Beads, Binding buffer G				✓	✓			
RCD	Rohland et al (2018), Large volume column, Binding buffer D					✓			
RCG	Rohland et al. (2018), Large volume column, Binding buffer G					✓			
TN8	Nucleospin Trace 8 (Macherey-Nagel, 2014b)	✓		✓	✓	✓		✓	✓
TNT	Nucleospin DNA Trace (Macherey-Nagel, 2018)	✓				✓			
TNX	Nucleospin gDNA Cleanup XP(Macherey-Nagel, 2014a)	✓	✓	✓	✓	✓		✓	
TPF	PrepFiler™ (Applied-Biosystems, 2008)	✓	✓	✓				✓	

Sub-Project 1.1 Supplemental Table 1. Results from this Sub-Project 1.1.

Tab A: Summary of average retention percentages and associated standard deviations of extracting the four standards by twenty different kits/methods.

Tab B: Standard 1 (Intact Genomic DNA, Concentrated) results (quantities and qualities)

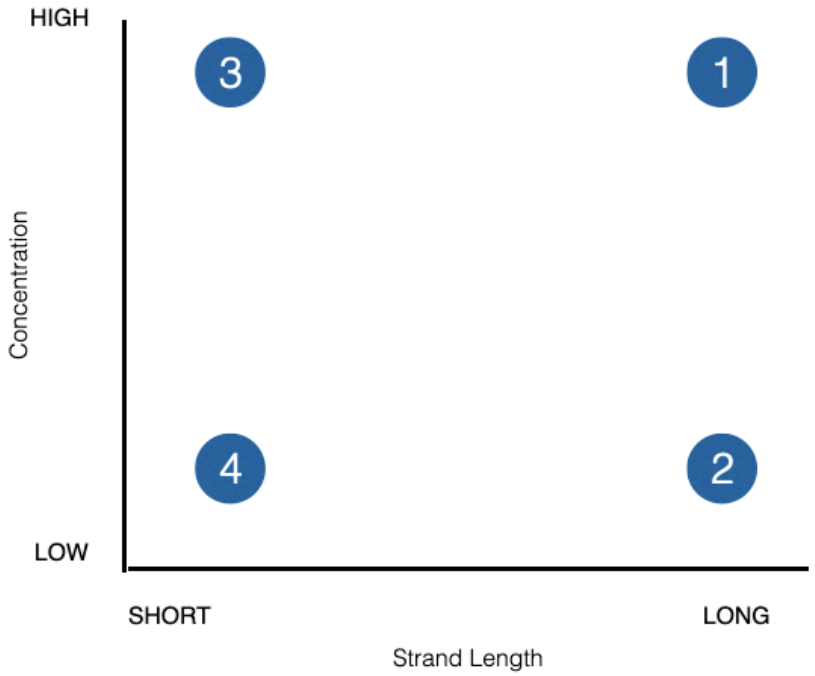
Tab C: Standard 2 (Intact Genomic DNA, Dilute) results (quantities and qualities)

Tab D: Standard 3 (Degraded DNA, Concentrated) results (quantities and qualities)

Tab E: Standard 4 (Degraded DNA, Dilute) results (quantities and qualities)

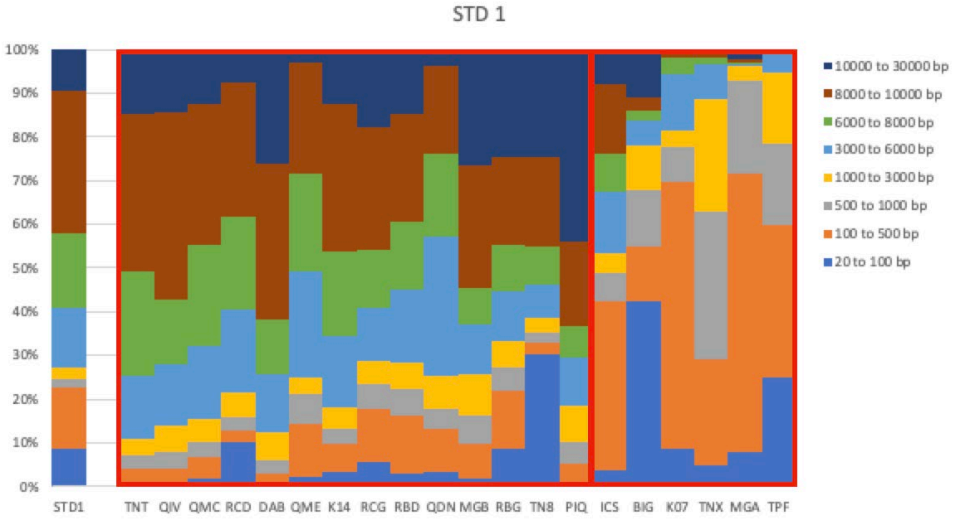
Tab F: Fragment size distributions used for ordering methods/kits in Sub-Project 1.1 Figure 2.

Sub-Project 1.1 Figure 1. Visual depiction of how standards 1-4 explore parameter space of concentration and intactness of DNA strands.

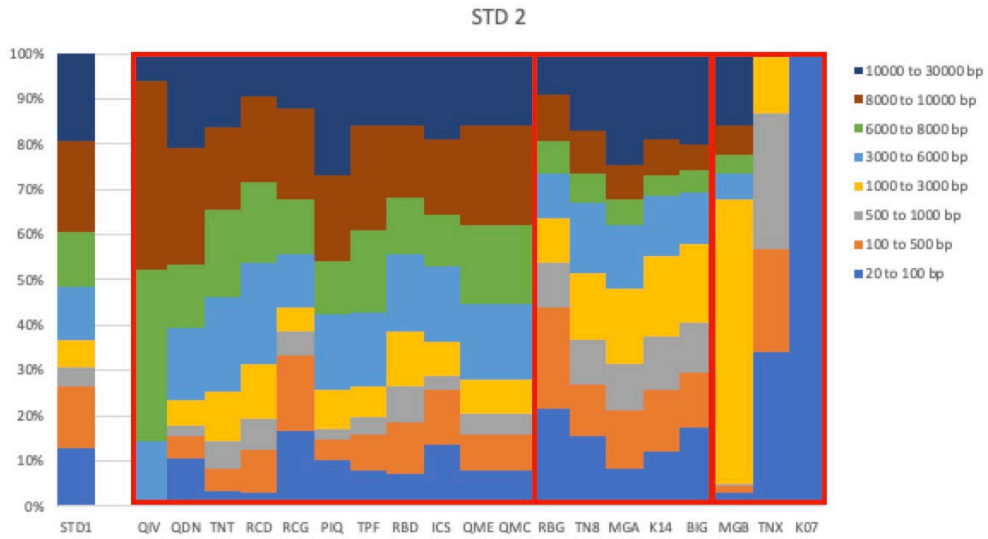


Sub Project 1.1 Figure 2. Visual depiction or fragment size distribution of the average "DNA in" standard (left) and those observed in the average of each of the "DNA out" elution from the kits/methods tested in this study. Data from Sub-Project 1.1 Supplemental Table 1 Tabs B-E. Number of measures contributing to the averages is found in Sub-Project 1.1 Table 2.

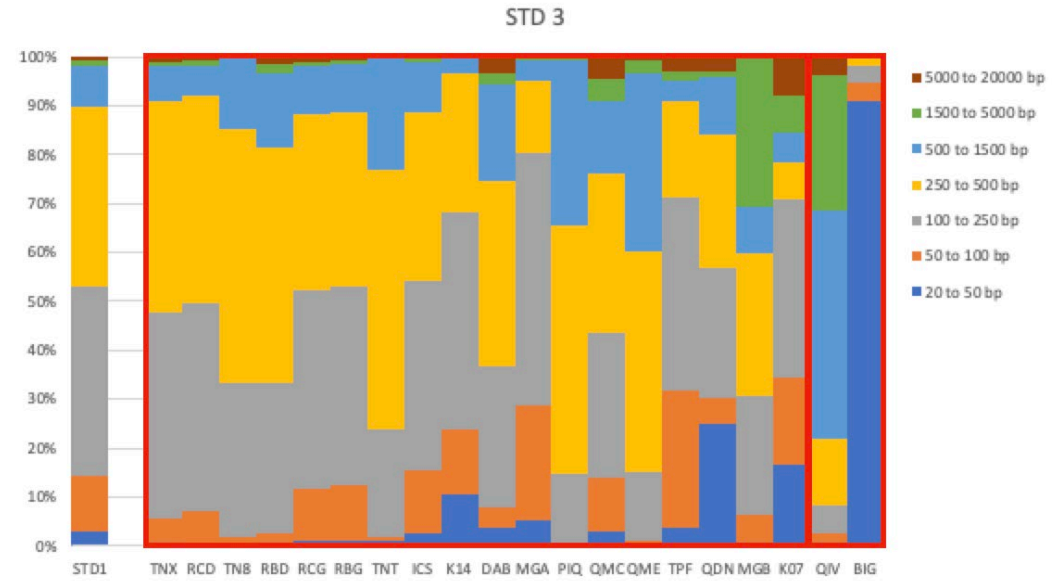
A) Fragment distribution of STD 1 (left; "DNA in") that of the standard following extraction (right; "DNA out"). Kits/methods are arranged with decreasing fraction of molecules 6,000-10,000 bp in length from left to right (see Sub-Project 1.1 Supplemental Table 1 Tab F). The six kits/methods to the right are associated loss of larger sized fragments (i.e., they retain shorter molecules with higher efficiency).



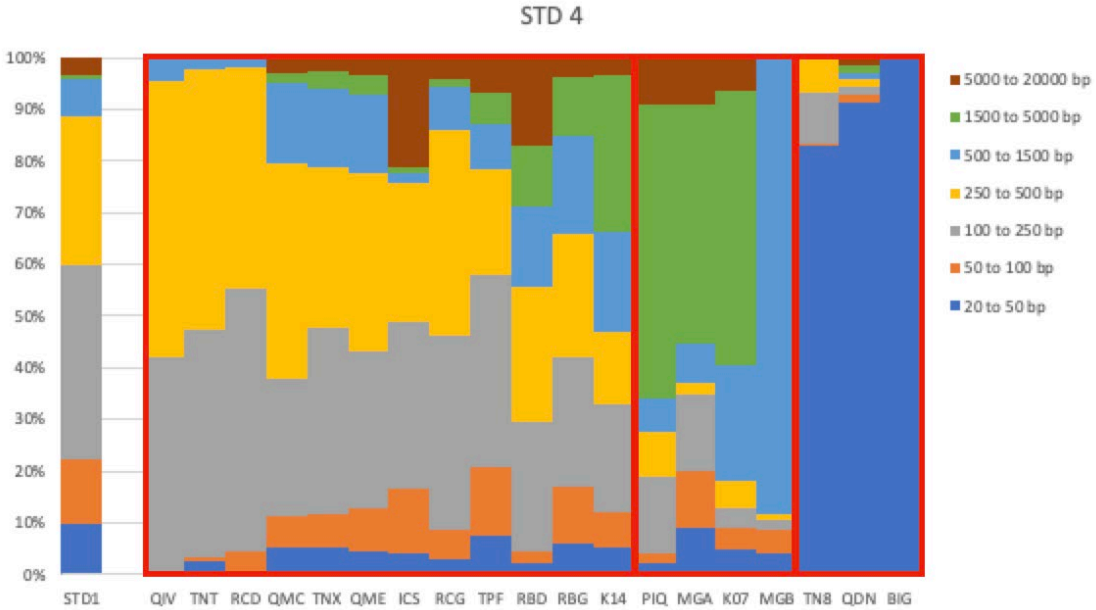
B) Fragment distribution of STD 2 (left; "DNA in") that of the standard following extraction (right; "DNA out"). Kits/methods are arranged with decreasing fraction of molecules 6,000-10,000 bp in length from left to right (see Sub-Project 1.1 Supplemental Table 1 Tab F). The Dabney et al. (2013) method did not retain molecules in this experiment (and, thus, is not depicted here). The eight kits/methods to the right are associated loss of larger sized fragments (i.e., they retain shorter molecules with higher efficiency).



C) Fragment distribution of STD 3 (left; “DNA in”) that of the standard following extraction (right; “DNA out”). Kits/methods are arranged with decreasing fraction of molecules 100-500 bp in length from left to right (see Sub-Project 1.1 Supplemental Table 1 Tab F). The four kits/methods to the right are associated loss of larger sized fragments (i.e., they retain shorter molecules with higher efficiency).



D) Fragment distribution of STD 4 (left; “DNA in”) that of the standard following extraction (right; “DNA out”). Kits/methods are arranged with decreasing fraction of molecules 100-500 bp in length from left to right (see Sub-Project 1.1 Supplemental Table 1 Tab F). Note that kit TNT average is based on 8 measures, whereas its average retention is based on ten (Sub-Project 1.1 Table 2). The four kits/methods depicted in the second box to right are associated loss of <500 bp sized fragments. The three kits to the right are associated with the retention of ultrashort DNA (20-50 bp).



Sub-Project 1.2 "Where, Oh Where, Has the DNA Gone?" (NOTE: proposed title for publication)

In the case of silica-based DNA extraction, DNA loss can conceivably occur mostly at one of two steps, or both. First, either the DNA: 1) does not bind with 100% efficiency to the silica and/or 2) cannot be eluted from the silica with 100% efficiency. This likely contriburtes to variable levels of DNA retention during extraction [e.g., see Sub-Project 1.1; Barta et al. (2014b) and Kemp et al. (2014b)].

In this sub-project, we aim to determine the location of the molecules that: 1) do not efficiently initially bind to silica or 2) do not efficiently elute off silica into the final eluate. We conducted these experiments using a common silica-based extraction method: Qiagen Dneasy Blood & Tissue Kit (Qiagen, 2016).

Many years ago, we made in-laboratory observations that DNA was continually released from a silica column[‡]. We took a DNA standard and "extracted" it. DNA was released from the column with a volume of warmed water, as is typical. Then we washed the column again with an additional volume of warmed water. We observed DNA in all the "washes" of the column out to 8 or 10 elutions. We observed roughly a proportional decrease of DNA in each elution after the next. All of this work was conducted with observations of purified amplicons used as standards at very low concentrations (Barta et al., 2014b) and counted via qPCR on a Applied Biosystems 7300 Real Time PCR System. When plotted these results produce a characteristic tail to the right.

We designed our experiment here based around those earlier experiments. But we added to them in a few important ways:

1) We tested four standards (similar to those employed in Sub-Project 1.1): 1Z) Intact Genomic DNA, Concentrated, 2Z) Intact Genomic DNA, Dilute, 3Z) Degraded DNA, Concentrated, 4Z) Degraded DNA, Dilute. These four standards again allow us to explore parameter space (see Sub-Project 1.1 Figure 1).
2) We used a very commonly employed, commercially available extraction method: Qiagen Dneasy Blood & Tissue Kit (Qiagen, 2016). While this extraction kit was was not developed to extract low quanity and/or quality DNA standards per se (and do perform very well with them, as illustarted by scrutiny under Sub-Project 1.1 as extraction "QDN"), we chose it as a standard of comparison in our experiment.
3) For two standards [1Z) Intact Genomic DNA, Concentrated and 3Z) Degraded DNA, Concentrated] we extracted them and subsequently washed the columns with 10 subsequent elutions (following the basic experimental design explained above).
4) *Notably we retained the flow-through from the initial extraction*. We reasoned that unbound DNA might be found in this 50:50 mixture of Buffer AL:absolute ethanol. To test this idea, we ran the flow-through across a fresh silica column from the Dneasy Blood & Tissue Kit. We retained this flow-through. We also tested DNA in ten subsequent elutions from that second column. We repeated this, ultimate producing 100 eluates from these initial DNA standards off ten columns (a 10X10 experiment).
5) For the other two standards [2Z) Intact Genomic DNA, Dilute and 4Z) Degraded DNA, Dilute] we did similar, while only producing 25 eluates (a 5X5 experiment).
6) In addition to DNA concentration, measures of DNA "quality" were taken. In other words, we used an Agilent Fragment Analyzer to produce fragment size distribution. Is it

[‡] Prior to publication of this sub-project, I will attempt to track down these old records. It would be very illustrative.

an “intact” sample, is it “degraded?” This can certainly be up for debate, but they are ultimately *qualifiable* distinctions: hence differences in “quality”.

Our predictions are:

1) to observe a distribution of declining quantity with each subsequent wash of the initial extraction column. This will produce a tailed distribution declining to the right.

2) that there are unbound DNA molecules in the flow-through and—importantly—if we can bind them on a fresh column of silica, we can measure them again directly. We do not know that this has been conducted prior, so we honestly were not sure it would work, but carried out a systematic test to make some novel insights into basic-silica-based extraction.

3) If we can detect DNA in the flow-through, subsequent washes of the column will produce a tailed distribution declining to the right.

We honestly had no predictions for what we might observe in the change of DNA quality across these various eluates. We conducted this portion of the experiment also to generally add to better understanding the performance a basic-silica-based extraction.

MATERIALS AND METHODS

Creation of Standards

We created four standards from pig (*Sus scrofa*) brain DNA (Zyagen; cat. PG-201). Quantities were determined using the average of three estimates produced by a Qubit 3.0. Qualities of the standards were determined with an Agilent Fragment Analyzer by the fraction of molecules binned as follows: 1) 20-100 bp, 2) 100-300 bp, 3) 300-500 bp, 4) 500-1,000 bp, 5) 1,000-5,000 bp, 6) 5,000-10,000 bp, 7) 10,000-30,000 bp, and 8) 30,000-60,000.

Standard 1Z: intact genomic DNA at 118±7 ng/µL with ~80% of molecules at 10,000-60,000 bp (Sub-Project 1.2 Supplemental Table 1-Tab A).

Standard 2Z: intact and diluted genomic DNA at 0.6±0.01 ng/µL, with 37.8% of the molecules ranging 10,000-60,000 bp (Sub-Project 1.2 Supplemental Table 1-Tab B).

Standard 3Z: sonically degraded DNA at 95.13±3.21 ng/µL with 70.39% of molecules ranging 100 to 500 bp (Sub-Project 1.2 Supplemental Table 1-Tab C).

Standard 4Z: sonically degraded DNA at 0.5 ng/µL with 73% of molecules ranging 100 to 500 bp (Sub-Project 1.2 Supplemental Table 1-Tab D).

Measuring Molecules

All extractions were conducted with the Qiagen Dneasy Blood & Tissue Kit (Qiagen, 2016). Experiments on STD 1Z will serve as an illustration for the other three standards.

First, 200 µL of STD 1Z was extracted (elution called “E1”) and the initial flow-through retained after centrifugation through the column [i.e., following step 4; Qiagen (2016)]. Following this purification, another 200 µL of DNA was subsequently eluted from the same column with 200 µL

of Buffer AE [i.e., following step 8; Qiagen (2016)]. and retained for analysis (is now sample "E2"). This was repeated eight additional times creating a total of 10 eluates (E1-E10) from the initial column.

The flow-through from the first column was passed through a fresh Qiagen column [i.e., starting with step 4; Qiagen (2016)] and the flow-through of this again retained after centrifugation. The Qiagen Dneasy Blood & Tissue Kit (Qiagen, 2016) was completed (i.e., steps 5-8) producing a 200 µL eluate (now "F1a"). As in the case of the first column, nine additional volumes of Buffer AE were in turn passed across the column and retained (now "F1b-F1j). Thus, ten new elutions were produced from the second column.

The flow-through from the second column was passed through a fresh Qiagen column and treated as just described. It produced eluates F1a-F1j.

In the end, this experiment collected 100 elutions from the extraction of STD 1Z (10 elutions off 10 columns each).

This experimental design was repeated for STD 3Z.

Standards 2Z and 4Z were treated similar, but assessed by a 5X5 experimental design (i.e., five elutions off five columns each).

DNA concentration of the 250 elates produced from these experiments was determined by the average of three measures by a Qubit 3.0. DNA quality (i.e., fragment size distribution) was determined with an Agilent Fragment Analyzer.

RESULTS

Standard 1 (STD 1) Intact Genomic DNA, Concentrated

This standard (1Z) began at 118±7 ng/µL with 77.98% of molecules at 10,000-60,000 bp (Sub-Project 1.2 Supplemental Table 1 Tab A). Following the first extraction with a Qiagen Dneasy Blood & Tissue Kit, the standard (now sample E1 in Sub-Project 1.1 Supplemental Table 1 Tab A) then contained 31.40±1.74 ng/µL with 81.34% molecules in the same range (Sub-Project 1.2 Figure 1A**)**. This equates to a 26.6% retention of the molecules with little disruption to the spectrum of fragment sizes it contains (i.e., its "quality").

Of the 100 eluates produced from experimentation with this standard, DNA was detected by Qubit in 44 of them. (Sub-Project 1.2 Supplemental Table 1 Tab A). Displayed visually in Sub-Project 1.2 Figure 2A, a trend is clear. The first extraction column contains appreciable DNA eluted out ten times by volumes of 200 µL of Buffer AE. This produced the pronounced "tail" in samples E1-E9 in Sub-Project 1.2 Figure 2A. Each of the subsequent extractions of the original flow-through (i.e., those producing samples F1-F9) are associated retention of 60.55±22.26% each prior standard. "Tails" are produced by repeated elutions from the next 8 columns. Each is simply diminished in its respective magnitude. DNA was detected by Qubit in only F9a, but none of the subsequent elutions from this 9th column.

The results from fragment analysis (Sub-Project 1.2 Figure 1A) show two trends: 1) decreasing fragment size recovery from each subsequent elution of any given column (e.g., see E1-E10), and 2) decreasing fragment size recovery in the 4th to 9th extraction of the flow-through (f1a-f9a).

Standard 2 (STD 2) Intact Genomic DNA, Dilute

This standard (2Z) began at 0.6±0.01 ng/µL, with 37.8% of the molecules ranging 10,000-60,000 bp (Sub-Project 1.2 Supplemental Table 1-Tab B). Following the first extraction with a Qiagen Dneasy Blood & Tissue Kit, the standard then contained 0.11±0.01 ng/µL (now sample E1 in Sub-Project 1.1 Supplemental Table 1 Tab B) with 14% molecules in the same range (Sub-Project 1.2 Figure 2C). This equates to a 19% retention of the molecules with noticeable disruption to its quality. Notably the E1 of standard 2Z contains 30% molecules ranging 20 to 100 bp, compared to only being representative of 4.75% of the molecules in the flow-through having been re-bound to the column. There are notable quality differences between the standard and the DNA retained through its extraction (Sub-Project 1.2 Figure 2C).

None of the other eluates contained detectable DNA by Qubit assessment.

Standard 3 (STD 3) Degraded DNA, Concentrated

This standard began at 95.13±3.21 ng/µL with 70.39% of molecules ranging 100 to 500 bp (Sub-Project 1.2 Supplemental Table 1-Tab C). Following the first extraction with a Qiagen Dneasy Blood & Tissue Kit, the standard then contained 9.35±0.33 ng/µL (now sample E1 in Sub-Project 1.1 Supplemental Table 1 Tab C) with 58% of molecules in the same range. This equates to a 9.82% retention of the molecules with some disruption to its quality.

Of the 100 eluates produced from experimentation with this standard, DNA was detected by Qubit in 16 of them. (Sub-Project 1.2 Supplemental Table 1 Tab C). Displayed visually in Sub-Project 1.2 Figure 1C, where observable (i.e., from series E, F1, F2, and F4), there is a pattern like that observed in STD 1, decreasing return of DNA concentration per each additional elution of that column.

Additionally, from E1 to F4a there was a trend towards decreasing DNA in their fragment sizes per continued elution from of the flow-through. However, this does not extend beyond that: 1) samples F4b-fFa all retain proportionally larger fragments than earlier treatments, 2) samples F8a and fFa retain predominantly fragments ≤ 5000 bp.

Standard 4 (STD 4) Degraded DNA, Dilute

This standard at 0.5 ng/µL with 73% of molecules ranging 100 to 500 bp (Sub-Project 1.2 Supplemental Table 1-Tab D). Following the first extraction with a Qiagen Dneasy Blood & Tissue Kit, the standard then contained an undetectable amount of DNA.

CONCLUSIONS

From our investigation of the nature of DNA loss associated with the Qiagen Dneasy Blood & Tissue Kit, we located abundant “lost” molecules 1) on the silica column (i.e., not eluted off during elution) and 2) in the flow-through (i.e., DNA molecules not bound to the silica).

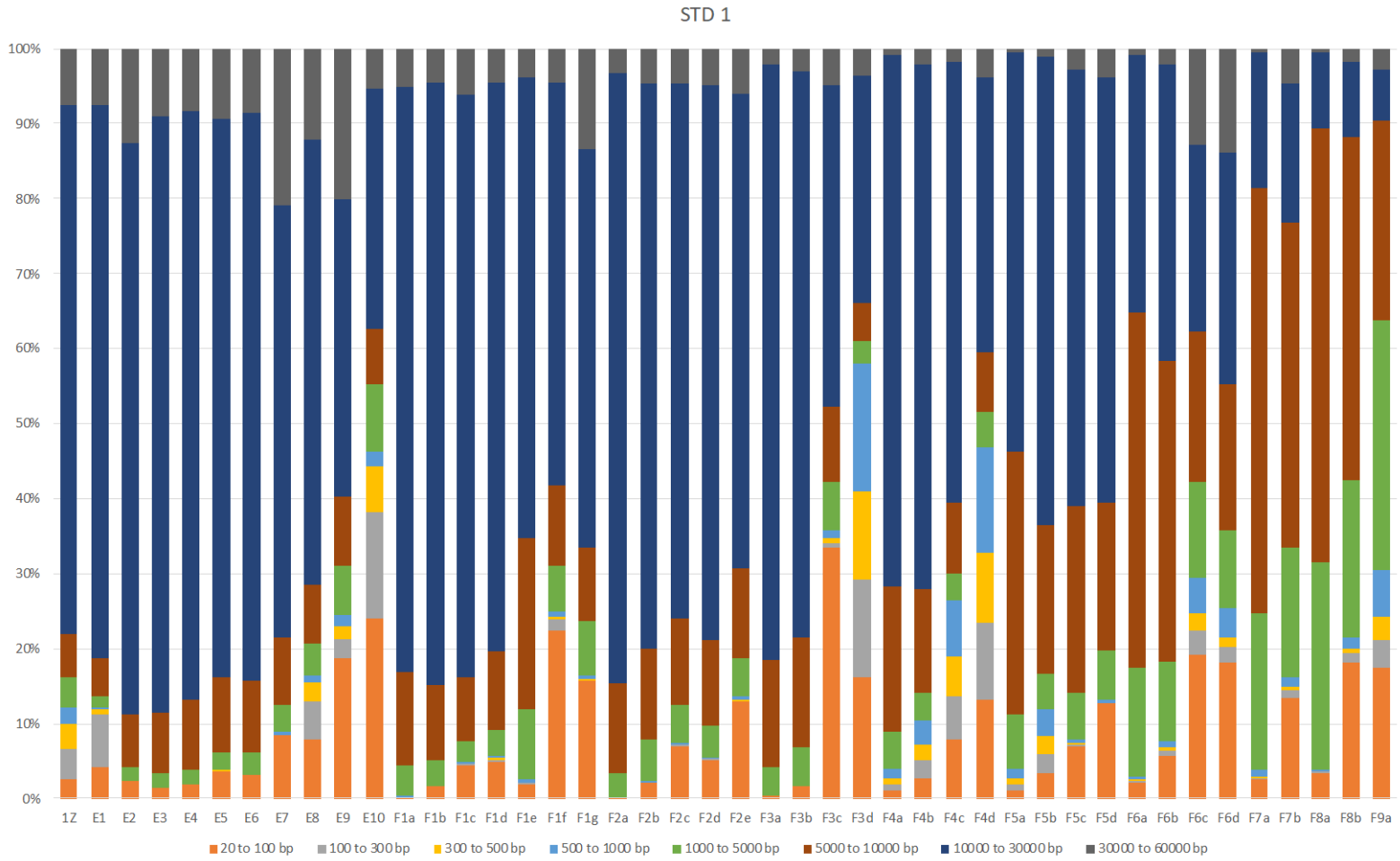
Following our initial in-laboratory experiments (described in the introduction), from which we had documented DNA still residing on the silica column, we began performing final elutions with two ½ volume washes of warmed water (vs one larger volume eluted once). The notion was to return a large amount of DNA from the column while maintain

a high concentration. The problem lies in the fact that, perhaps 10 elutions would return all the DNA from the column. But, at that point the combined elution may be at a point too low in concentration for subsequent PCR amplification.

This suggests that improvements in both binding and elution are critical and are likely to improve investigations of aged, degraded, and low copy number DNA sources.

Sub-Project 1.2 Figure 1. Fragment size distribution found in elutions by standard.

A) There were 44 eluates from Standard 1 (Intact Genomic DNA, Concentrated) in which the Qubit detected DNA. Here are depicted the fragment size distributions reported in Sub-Project 1.2 Supplemental Table 1 Tab A. The first column represents the fragment size distribution observed in the standard (i.e., “DNA in”).



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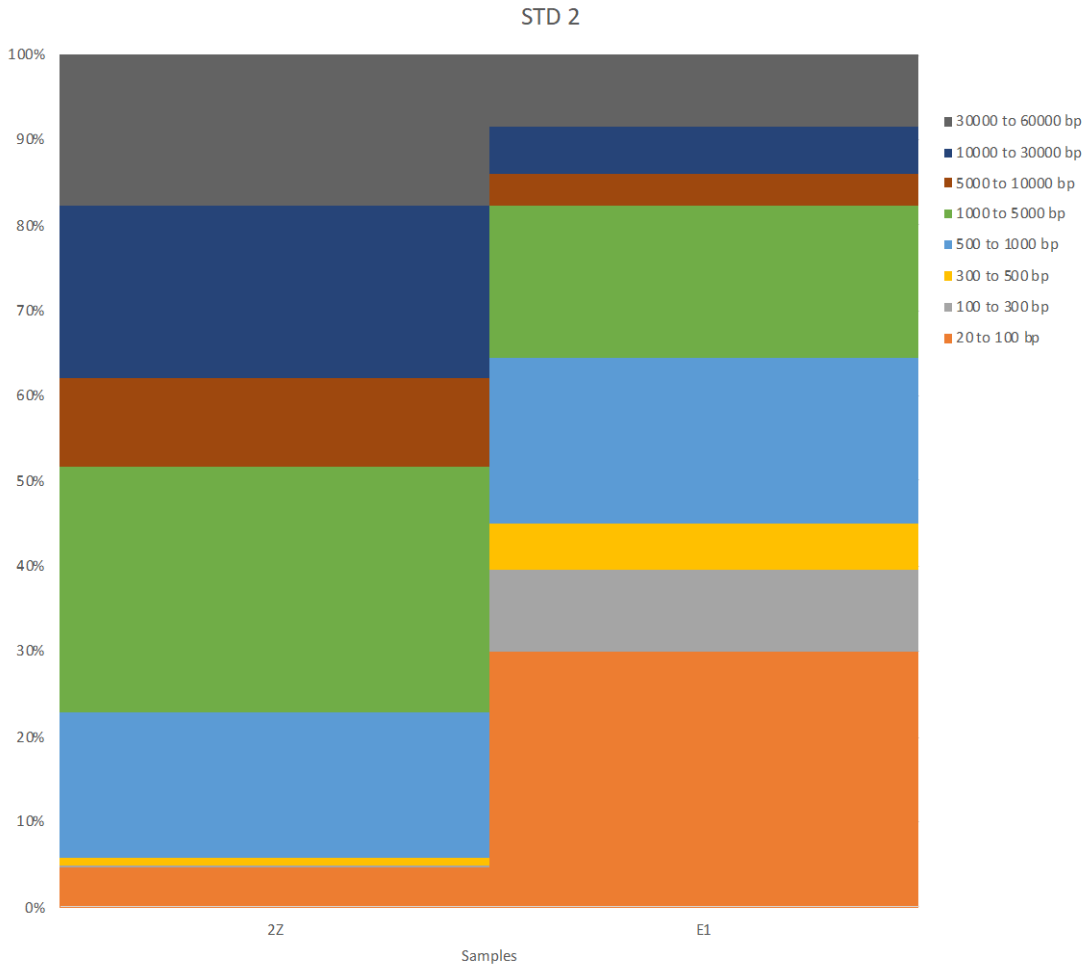
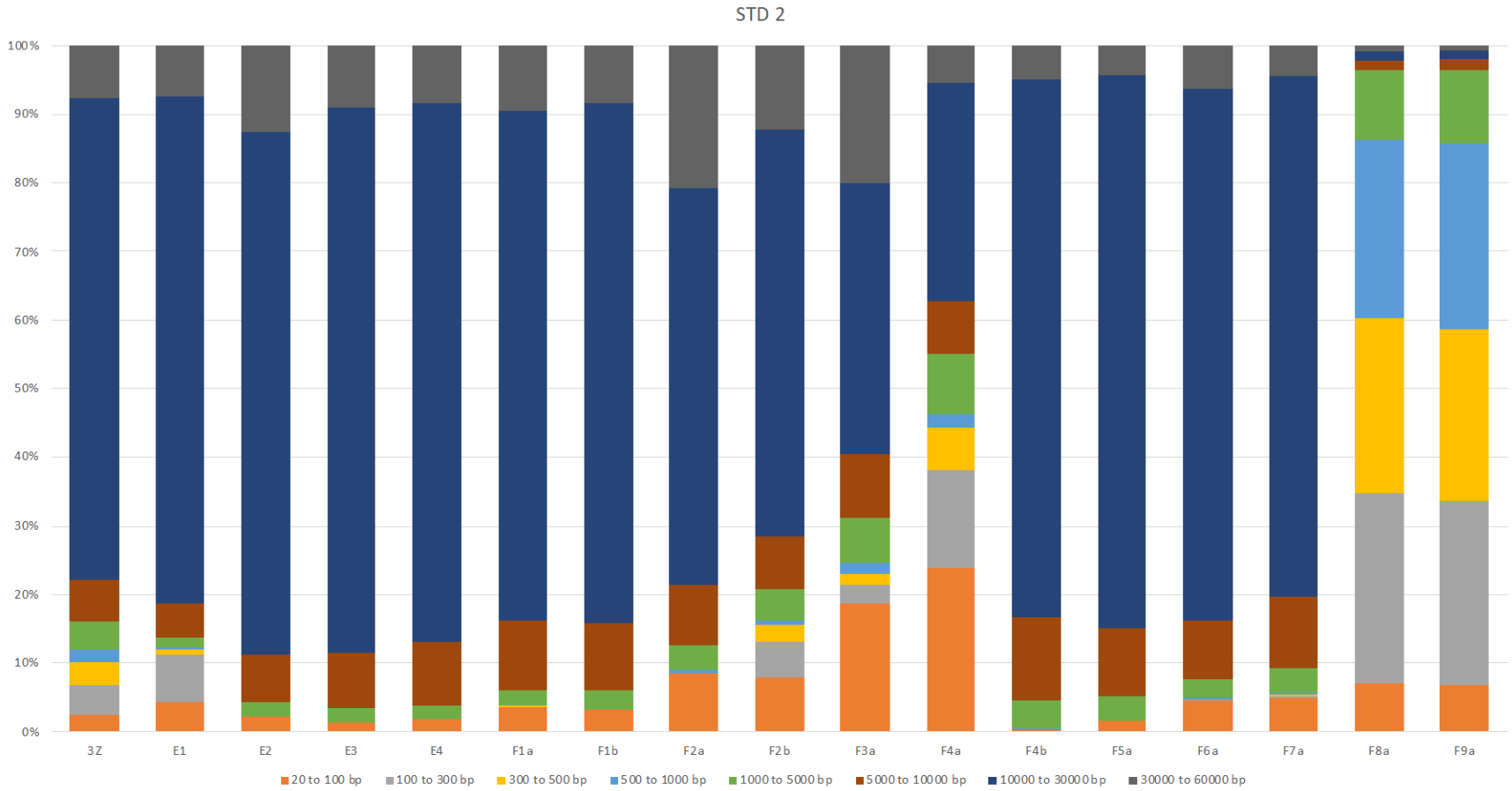
B) DNA was detected in only the first extraction of Standard 2 (Intact Genomic DNA, Dilute) in which the Qubit detected DNA. Standard 2 began with 37.8% of the molecules ranging 10,000-60,000 bp Here are depicted the fragment size distributions reported in Sub-Project 1.2 Supplemental Table 1 Tab B. The first column represents the fragment size distribution observed in the standard (i.e., “DNA in”).



C) There were 16 eluates from Standard 3 (Degraded DNA, Concentrated) in which the Qubit detected DNA. Sample 3Z began with 70.39% of molecules ranging 100 to 500 bp Here are depicted the fragment size distributions reported in Sub-Project 1.2 Supplemental Table 1 Tab C). The first column represents the fragment size distribution observed in the standard (i.e., “DNA in”).



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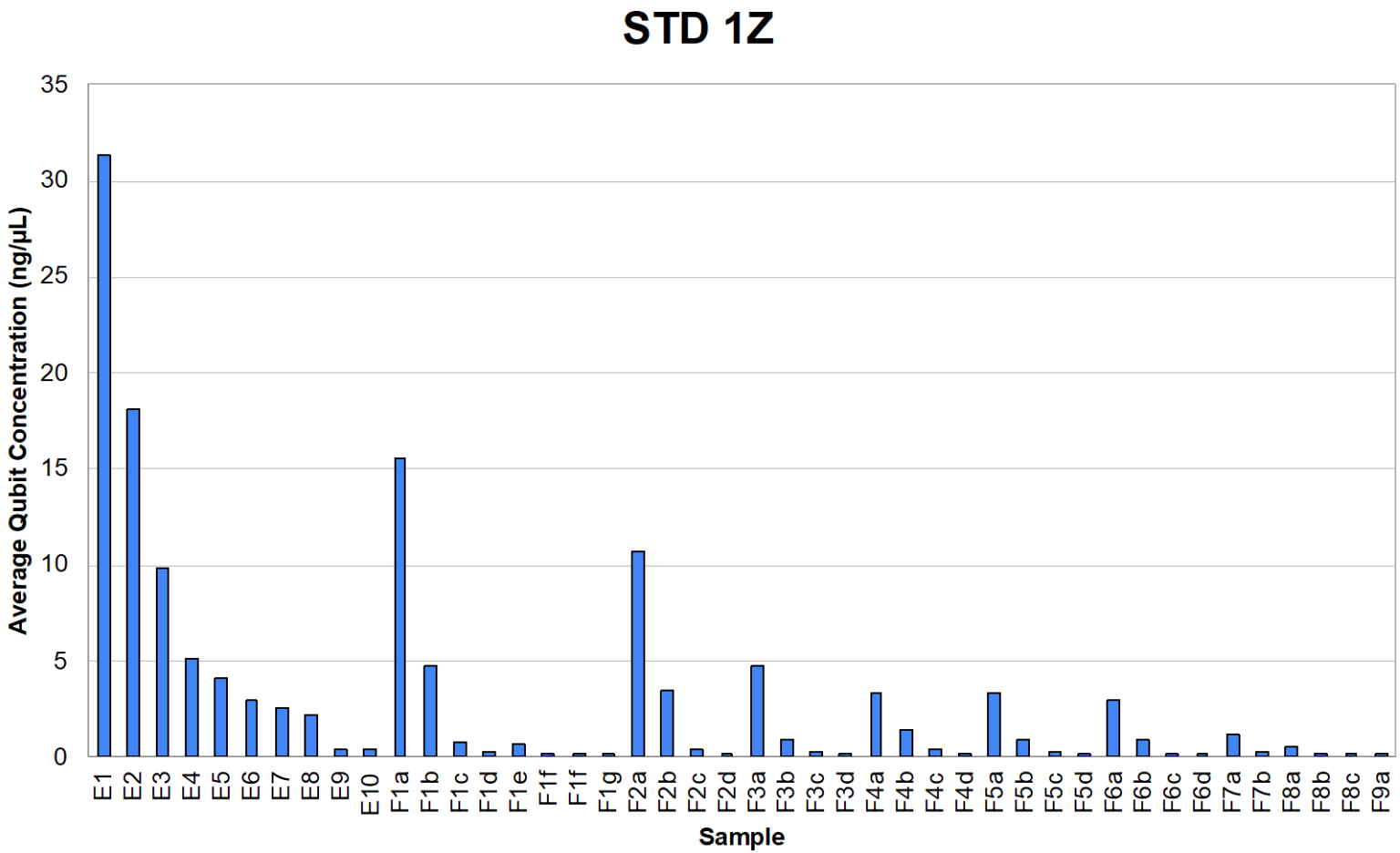
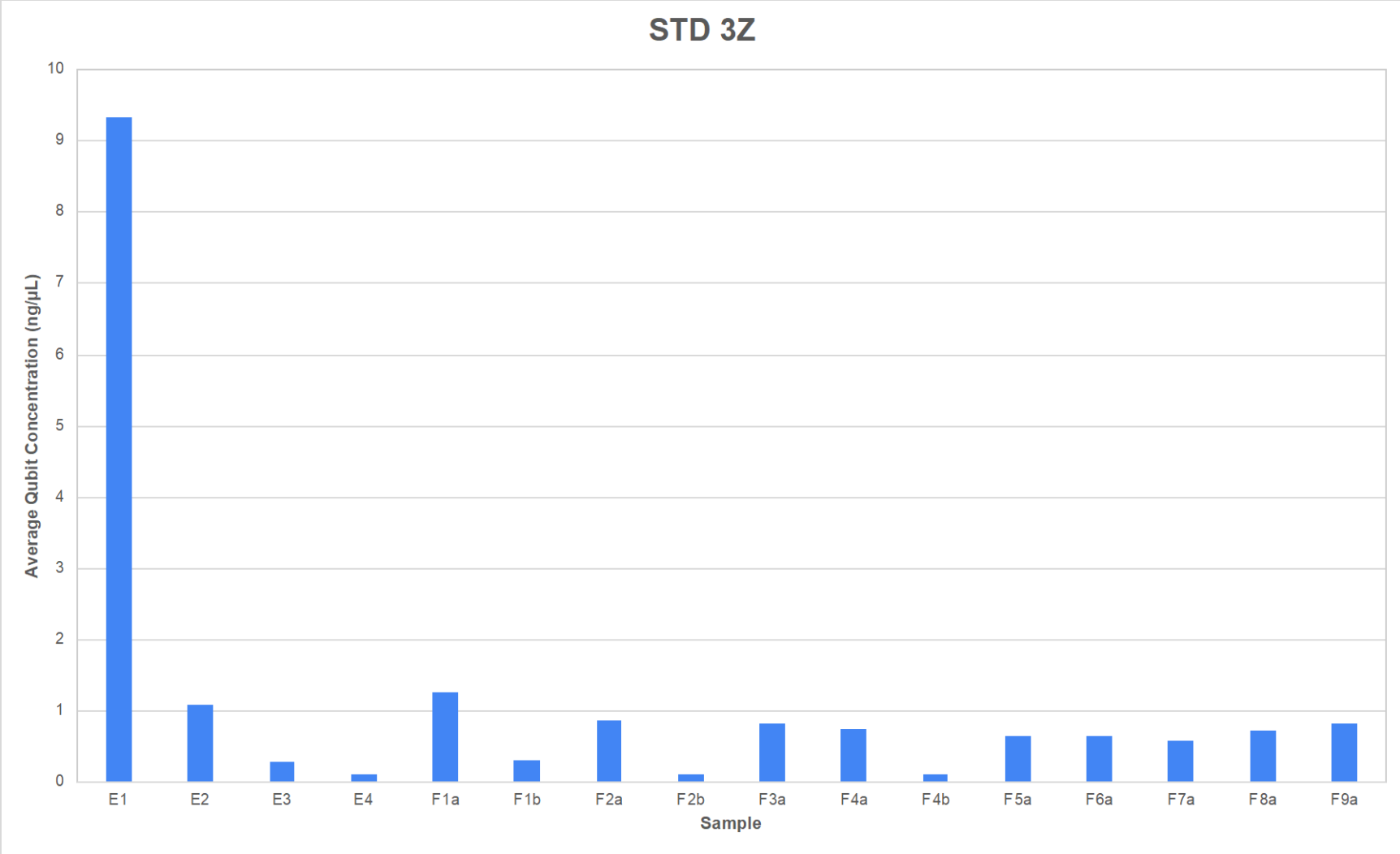
Sub-Project 1.2 Figure 2. DNA detected (ng/µL) with a Qubit in 44 elutions from STD 1Z. See Sub-Project 1.2 Supplemental Table 1 Tab A for complete data set, as well as standard deviations.

A) Standard 1Z (Intact Genomic DNA, Concentrated) prior to the first extraction was at 118±7 ng/µL



B) Standard 3Z (Degraded DNA, Concentrated) prior to the first extraction was at 95.13±3.21 ng/µL.



Sub-Project 1.2 Supplemental Table 1. Results from this Sub-Project 1.2.

Tab A: Quantities and qualities of DNA measured in the 100 elutions originating from STD 1 (Intact Genomic DNA, Concentrated).

Tab B: Quantities and qualities of DNA measured in the 25 elutions originating from STD 2 (Intact Genomic DNA, Dilute).

Tab C: Quantities and qualities of DNA measured in the 100 elutions originating from STD 3 (Degraded DNA, Concentrated).

Tab D: Quantities and qualities of DNA measured in the 25 elutions originating from STD 4 (Intact Genomic DNA, Dilute).

Phase II: Evaluating the influence of routine laboratory methods (subjection to heat, freeze-thaw treatments, and/or vortexing) on the quantity and quality of DNA

Background

During our previous NIJ grant (2011-DN-BX- K549), we found DNA capture methods [i.e., "fishing" (Anderung et al., 2008) and Primer Extension Capture (Briggs et al., 2009)] to be very inefficient, despite our hypothesis that they would substantially increase the ratio of target DNA molecules to non-target, and thus be of extraordinary value to forensic DNA investigations (Kemp, 2015; Winters et al., 2017; Winters et al., 2018). In practice, these capture methods simply result in DNA loss across the board, target and non-target molecules alike.

As a means of addressing the source of DNA loss during these capture experiments, we creatively subjected eluates to the hybridization temperatures and duration of DNA capture methods *only*. To be clear, hybridization is *essential* to DNA capture; it cannot be avoided. In this case, it is concerning that loss of over 60% of the molecules in the DNA eluate was caused by exposure to hybridization temperatures alone. As DNA cannot be "lost" in this fashion, one possible explanation is that heat treatment degrades the DNA molecules with regards to length, making them non-amplifiable as they no longer contain both priming regions, a requisite for PCR amplification and qPCR. Thus, these lost molecules might be better termed "unamplifiable" than lost.

Our research builds importantly on our previous experiments by the technical ability to simultaneously observe both quality and quantity of DNA following exposure to hybridization conditions, and heat conditions in general. Our prediction is that duration and intensity of heat treatment will be correlated with a shift towards shorter mean stand length, with no change to concentration.

The rate of DNA strand breakage is related directly to temperature (Lindahl and Andersson, 1972; Lindahl, 1993) and it has been demonstrated that the "thermal age" of an ancient specimen can be more meaningful in predicting DNA recovery than its calendrical age (Smith et al., 2003). Also, Wang and McCord (2011) exposed full genomic DNA in deionized water to 95°C temperature for 10 minutes in order to create degraded DNA conditions in their study of fishing for DNA. This demonstrates that genomic DNA will readily degrade at high temperature in little time. Visualized by gel electrophoresis, Wang and McCord (2011) were limited in their ability to measure the induced degradation and fragmentation of the standard. Use of more sophisticated technology, as proposed here, will permit us to make more fine-scale observations of damage induced following experimental treatment to heat.

Making finer scale observations of heat treatment damage has implications for improving approaches for working with aged, degraded, and LCN DNA samples. For example, polymerases that require extended hot starts might be less preferable to those that require shorter periods. In fact, an informative experiment would be to compare PCR yields wherein DNA is added to the reactions following hot start to those wherein the DNA is added prior to the hot start activation. If our presumptions are correct, the former will yield less DNA than the latter based on less template molecules available for amplification. Alternatively, an approach that combines a reduced initial hot-start activation time and increased number of cycles (with denaturing each round resulting in activation of more and more polymerase) may be preferable over extended hot start conditions alone, as described by Applied Biosystems (2010). While

this is an intriguing and logical idea, we have no knowledge of this being practiced; it certainly is not common.

Now we turn attention to freeze-thaw cycles. Many researchers and technicians may work with eluates stored in the refrigerator or stored frozen at -20°C and thawed each time for use (which can be sped up by vortexing, holding the tubes in one's gloved hands, and/or by shaking the tubes). Regarding the long-term storage of DNA eluates, many researchers archive their samples at -20°C or -80°C, with eluate subsamples either stored in the refrigerator or stored frozen -20° and thawed out as needed. *How many times should one freeze-thaw a sample? What is the effect on the quality of the DNA as a result?* These are unknown or certainly under-documented outside of anecdotal evidence passed from one laboratory member to another, or between laboratories.

During Phase II we:

(1) Stored aliquots of four degraded DNA standards at room temperature (20-22°C), 4°C, -20°C, and -80°C, sampling volumes over ~10 months to ascertain changes in their qualities and quantities (298 days, 20 instances of freeze-thaw cycles for samples at -20°C, and -80°C)

(2) Stored aliquots of four degraded DNA standards at room temperature (20-22°C), 4°C, -20°C, and -80°C, sampling volumes daily for 30 days to ascertain changes in their qualities and quantities (30 instances of freeze-thaw cycles for samples at -20°C, and -80°C.

(3) Experimental treated DNA standards to temperature exposures and vortexing periods.

Sub-Project 2.1 "Influence of Long-Term Storage at Room Temperature (20-22°C), 4°C, -20°C, and -80°C on the quality and quantity of DNA, as Well as Freeze Thaw Cycles" **(**NOTE: proposed title for publication)

Many researchers store DNA eluates in the refrigerator or store them frozen at -20°C and thaw them each time for use. Regarding the long-term storage of DNA eluates, many researchers archive their samples at -20°C or -80°C, with eluate subsamples either stored in the refrigerator or stored frozen at -20° and thawed out as needed. It is not uncommon for a genetics laboratory to have storage options at these different temperatures, the notion being to protect the long-term integrity of DNA in solution. Moreover, rules for DNA storage are often formed in-house[§], and are based largely on intuition and/or anecdotal evidence. What is viewed as proper behavior in one laboratory might be viewed as heretical in another.

Ultimately, how many times should one freeze-thaw a sample? What is the effect on the quantity and quality of the DNA as a result? These are unknown or certainly under documented outside of anecdotal evidence. Thus, we tested the integrity of DNA in solution held at various temperature over time and subject to freeze-thaw cycles.

In this experiment, we created DNA standards (similar to those employed in other sub-projects):

1) Degraded DNA, Concentrated from pig liver
2) Degraded DNA, Concentrated from pig brain
3) Degraded DNA, Dilute from pig liver.
4) Degraded DNA, Dilute from pig brain

stored them at room temperature (20-22°C), 4°C, -20°C, and -80°C, and sampled volumes of them periodically over 298 days (~10 months) to ascertain changes in their concentration (i.e., "quantity") fragment size distributions (i.e., quality). This treatment resulted in 20 freeze-thaw cycles (i.e., for those standards held below 0°C).

We conducted a similar experiment with 30 freeze-thaw cycles over a thirty-day period on .

We expect fragmentation to correlate with temperature and time of exposure. With ample time and temperature, this fragmentation may be recorded as a decrease in concentration if the standards begin to fragment into sections below 20 bp. In other words, standards stored at room temperature should accrue more damage of this form than those stored at -80°C. Based on intuition, lower temperatures should be protective of DNA fragmentation.

MATERIALS AND METHODS

For our first set of experiments, we created four standards. The quantity and quality of which were determined with an Agilent Fragment Analyzer across eight categories: 1) 20-50 bp, 2) 50-100 bp, 3) 100-200 bp, 4) 200-400 bp, 5) 400-600 bp, 6) 600-800 bp, 7) 800-1,000 bp, and 8) 1,000-3,000 bp. Two standards were derived from DNA extracted from pig (*Sus scrofa*) liver[**]

[§] I first noticed this as a Post-Doctoral researcher at the Vanderbilt University Medical Center. In 2007, their central genetics collection and processing laboratory stored all their DNA eluates in the refrigerator. I was shocked to say the least, as I was accustomed to only removing DNA eluates from -20°C for quick processing (i.e., to set up a PCR) and returning them promptly to the freezer. Since that time, in my laboratory we store samples from active projects in the refrigerator and return finished projects/samples to -20°C for long-term storage. However, this is largely done out of convenience and now tradition, not based on evidence that this is an optimal strategy. It works well for us though.

[**] Pig liver sample graciously provided by Bart Bingham.

with the Qiagen DNeasy Blood & Tissue Kit, following the manufacturer's protocol (Qiagen, 2016):

> Standard 3 (STD 3): sonically degraded DNA at ~100 ng/µL with 63.85% of molecules 100-400 bp (eluted with molecular grade water) (Sub-Project 2.1 Supplemental Table 1 Tab A)
>
> Standard 4 (STD 4): sonically degraded DNA at ~1 ng/µL with 66.33 % of molecules 100-400 bp (eluted with molecular grade water) (Sub-Project 2.1 Supplemental Table 1 Tab B)

The other two standards were created from pig brain DNA (Zyagen; cat. PG-201):

> Standard 3Z (STD 3Z): sonically degraded DNA at ~100 ng/µL with 63.85% of molecules 100-400 bp (diluted with TET) (Sub-Project 2.1 Supplemental Table 1 Tab C)
>
> Standard 4 (STD 4): sonically degraded DNA at ~1 ng/µL with 66.33 % of molecules 100-400 bp (diluted with TET) (Sub-Project 2.1 Supplemental Table 1 Tab D)

Volumes of these four standards were stored at room temperature (20-22°C), 4°C, -20°C, and -80°C and sampled for subsequent analysis on the Fragment Analyzer:

1) daily for the first week
2) weekly for the following ten weeks
3) monthly for the following eight months

In total, samples were measured in this manner from 9/9/19 to 7/6/20 (298 days, having undergone 20 cycles of freeze thaw over this time course).

For our second set of experiments, we created two standards. The quantity and quality of which were determined with an Agilent Fragment Analyzer across eight categories: 1) 20-50 bp, 2) 50-100 bp, 3) 100-200 bp, 4) 200-400 bp, 5) 400-600 bp, 6) 600-800 bp, 7) 800-1,000 bp, and 8) 1,000-3,000 bp. The two standards were created from pig brain DNA (Zyagen; cat. PG-201):

Standard 3Z (STD 3Z): sonically degraded DNA at ~100 ng/µL with 63.85% of molecules 100-400 bp (diluted with TET) (Sub-Project 2.1 Supplemental Table 1 Tab C)

Standard 4 (STD 4): sonically degraded DNA at ~1 ng/µL with 66.33 % of molecules 100-400 bp (diluted with TET) (Sub-Project 2.1 Supplemental Table 1 Tab D)

Volumes of these four standards were stored at room temperature (20-22°C), 4°C, -20°C, and -80°C and sampled every day for 30 days. Aliquots from these samples were analyzed on the Fragment Analyzer in this manner from 6/30/20 to 7/30/20 (having undergone 30 cycles of freeze thaw over this time course).

RESULTS

Over the course of 298 days of storage at room temperature (20-22°C), 4°C, -20°C, and -80°C, substantial stability of quality of the DNA standards is notable. These data are summarized and displayed visually in Sub-Project 2.1 Supplemental Table 1 Tabs A- D. Examples are shown in Sub-Project 2.1 Supplemental Figure 1.

Over the course of 30 days of storage at room temperature (20-22°C) and 4°C and freeze-thaw cycling at -20°C, and -80°C, substantial stability of quality and quantity of the DNA standards is also notable. These data are summarized and displayed visually in Sub-Project 2.1 Supplemental Table 2 Tabs A-B. Examples are shown in Sub-Project 2.1 Supplemental Figure 2.

CONCLUSIONS

In the years that we have been working in laboratories we have come to appreciate that what is viewed as proper behavior in one laboratory might be viewed as heretical in another. This is no different regarding practices for the long-term storage of DNA samples. Strongly held beliefs about the nature of DNA degradation can lead to different traditions in different laboratories. In one laboratory, the Director may choose to limit the number of freeze-thaw cycles or time subjected to above 0°C temperatures. In another, they may store samples in the refrigerator indefinitely. These traditions become based on suggestions (or certainly from under documented or anecdotal evidence) passed on from someone in the laboratory to the next a long, long time ago...the origin of what we refer to as “Laboratory Folktale”[††].

In contrast to our predictions, we observed remarkable stability of DNA standards stored from room temperature to -80°C both in water in TET buffer over ~10 months and subjected to repeated freeze-thaw cycles. This was also observed in experiments on an additional two standards subjected to 30 days of consecutive freeze-thaw cycles.

With these observations in mind, we suggest that others consider experimental evidence (or any direct and controlled observations) regarding DNA degradation, and relying less on intuition. While intuition is certainly useful in science, it can lead to origination of such laboratory folk tales, where one is certain that their story is the true fable.

Now, imaging that a one is hired to start a new genetics laboratory. How many resources should be invested in -80°C freezer, when they may not be necessary to maintain the integrity of DNA stored in solution for the long-term? Could resources be applied to more critical equipment?[‡‡]

[††] See Brunstein (2015) and da Silva et al. (2016)

[‡‡] Especially given my experience with the failure of -80°C freezers. Maintaining this low temperature is very demanding on the compressor. While this is certainly anecdotal observation, they were very costly failures.

Sub-Project 2.1 Supplemental Table 1. Results from Sub-Project 2.1 of DNA standards sampled periodically over 298 days of storage at room temperature (20-22°C), 4°C, -20°C, and -80°C.

Tab A: Standard 3 (STD 3): sonically degraded DNA at ~100 ng/µL with 63.85% of molecules 100-400 bp (eluted with molecular grade water) (Sub-Project 2.1 Supplemental Table 1 Tab A)

Tab B: Standard 4 (STD 4): sonically degraded DNA at ~1 ng/µL with 66.33 % of molecules 100-400 bp (eluted with molecular grade water) (Sub-Project 2.1 Supplemental Table 1 Tab B)

Tab C: Standard 3Z (STD 3Z): sonically degraded DNA at ~100 ng/µL with 63.85% of molecules 100-400 bp (diluted with TET) (Sub-Project 2.1 Supplemental Table 1 Tab C)

Tab D: Standard 4Z (STD 4Z): sonically degraded DNA at ~1 ng/µL with 66.33 % of molecules 100-400 bp (diluted with TET) (Sub-Project 2.1 Supplemental Table 1 Tab D)

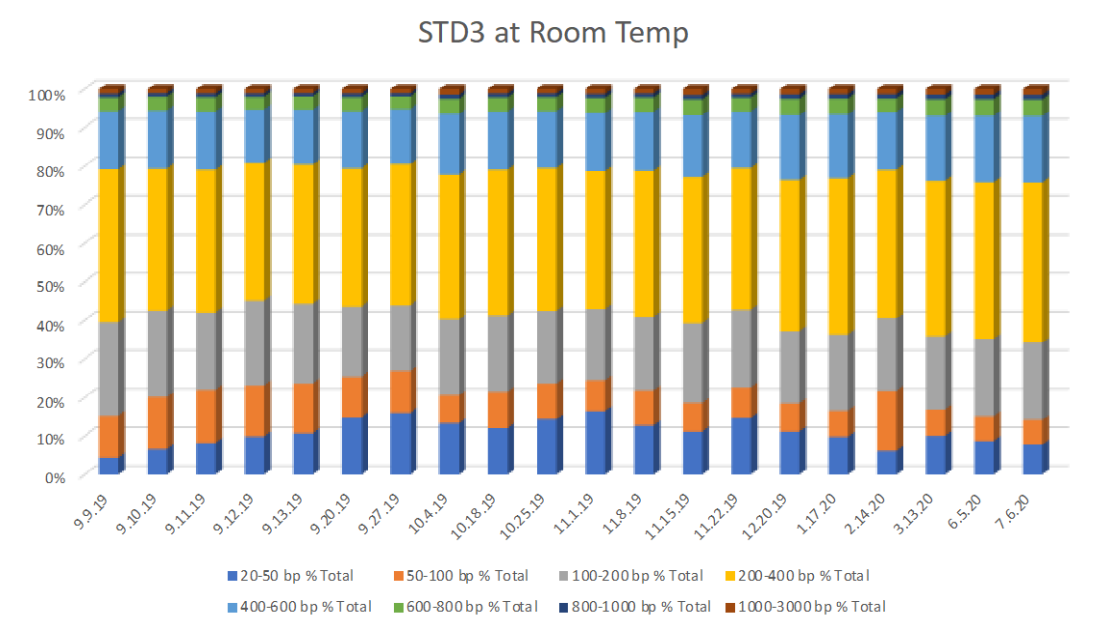
Sub-Project 2.1 Supplemental Table 2. Results from Sub-Project 2.1 of DNA standards sampled daily over 30 days of storage at room temperature (20-22°C), 4°C, -20°C, and -80°C.

Tab A: Standard 3 (STD 3): sonically degraded DNA at ~100 ng/µL with 63.85% of molecules 100-400 bp (eluted with molecular grade water) (Sub-Project 2.1 Supplemental Table 1 Tab A)

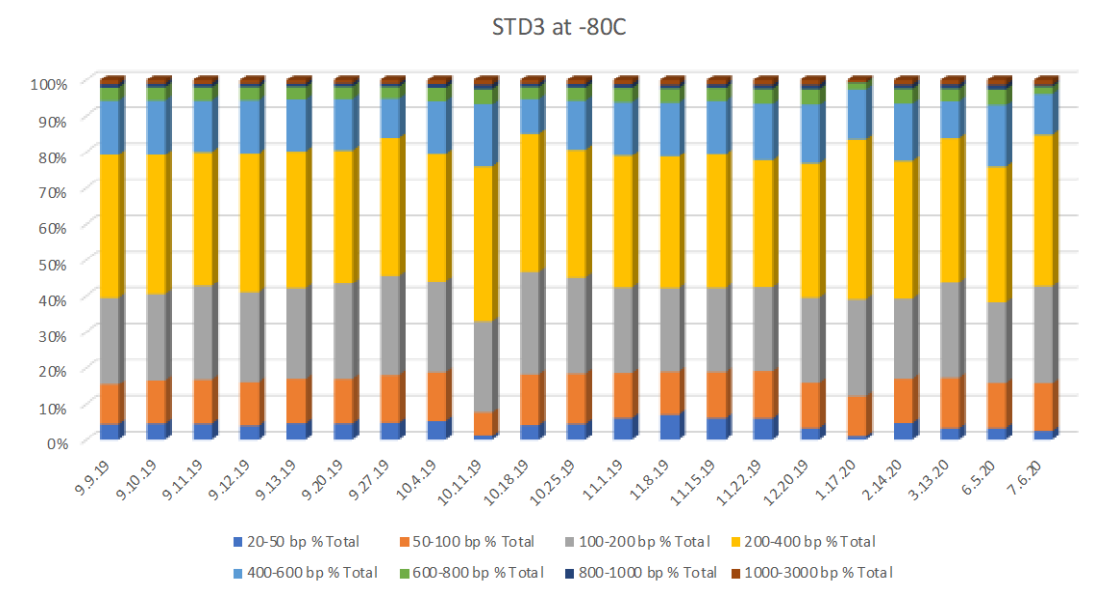
Tab B: Standard 4 (STD 4): sonically degraded DNA at ~1 ng/µL with 63.33 % of molecules 100-400 bp (eluted with molecular grade water) (Sub-Project 2.1 Supplemental Table 1 Tab B)

Sub-Project 2.1 Figure 1. DNA quality measured from 9/9/19 to 7/6/20 (298 days) over 20 cycles of freeze thaw. Data are reported in Sub-Project 2.1 Supplemental Table 1 Tabs A-D.

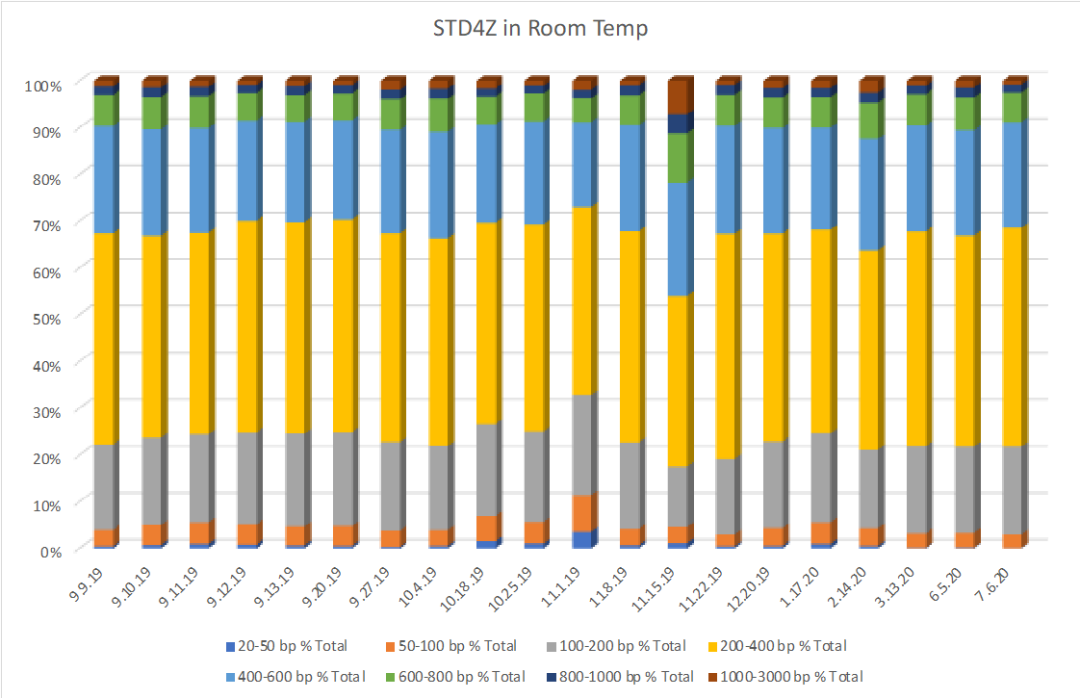
A) Standard 3 at room temperature.



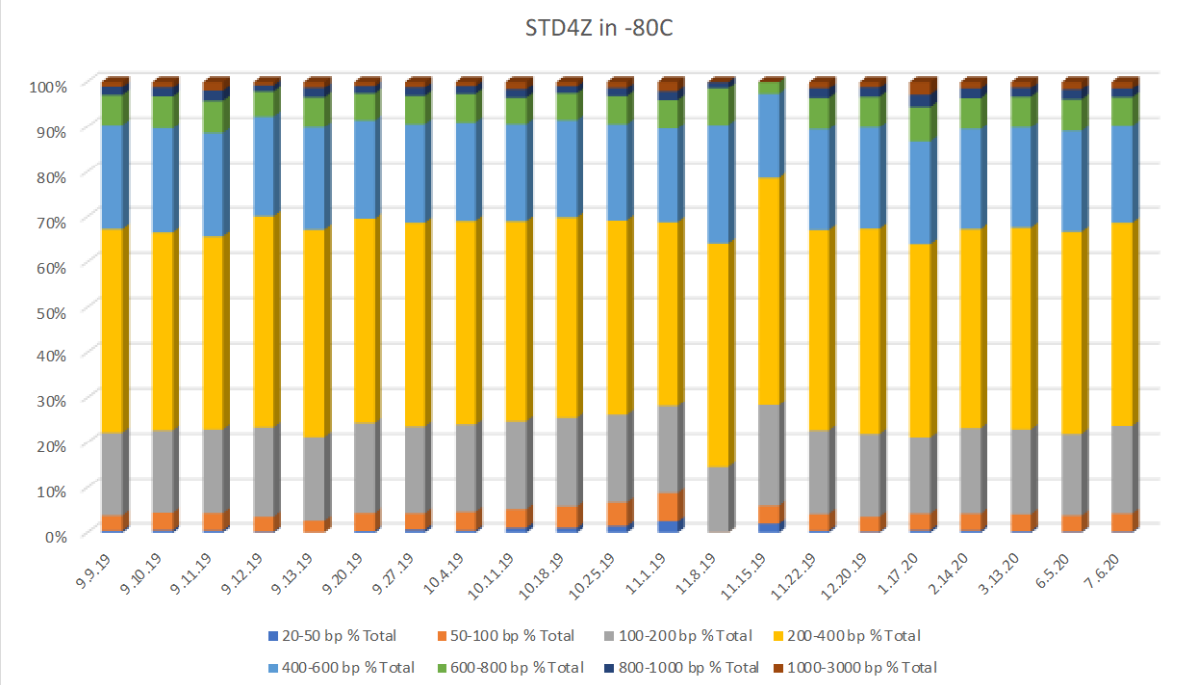
B) Standard 3 at -80°C.



C) Standard 4Z at room temperature



D) Standard 4Z at -80°C.



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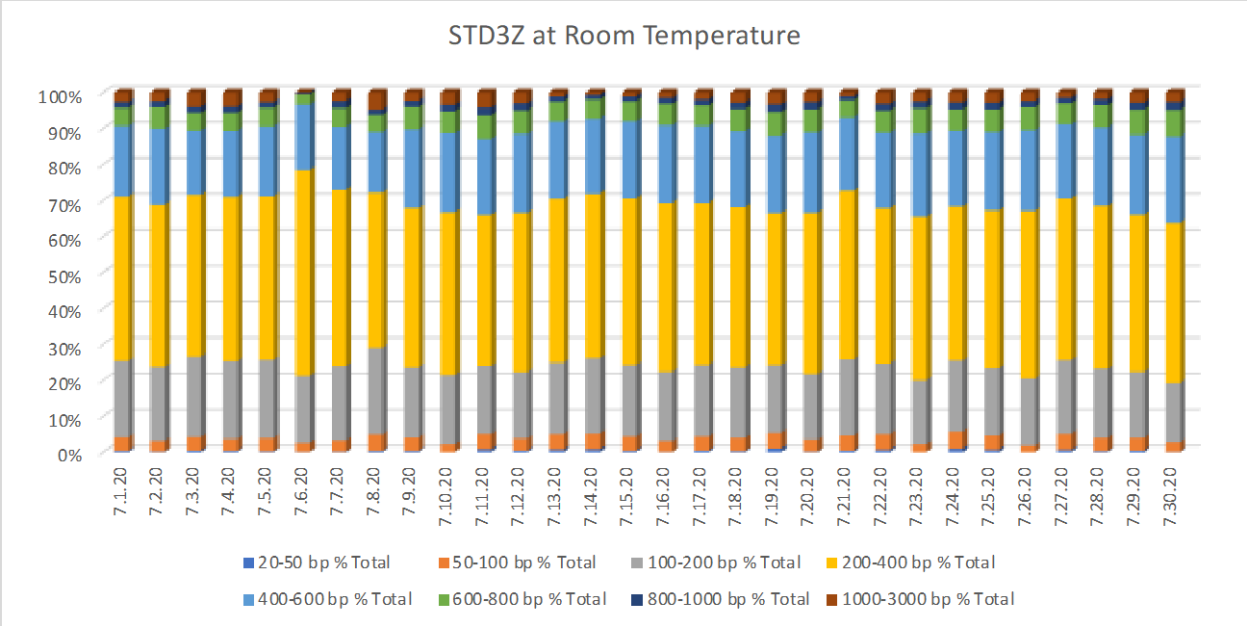
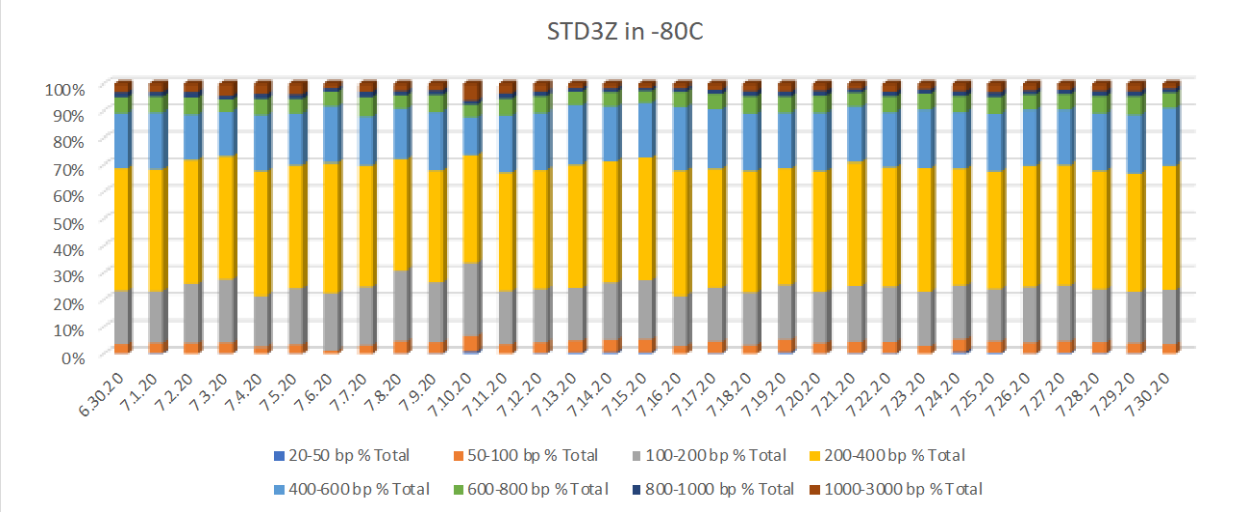
Sub-Project 2.1 Figure 2. DNA quality measured from 6/30/20 to 7/30/20 over 30 days of freeze-thaw cycles. Data are reported in Sub-Project 2.1 Supplemental Table 2 Tabs A-B.

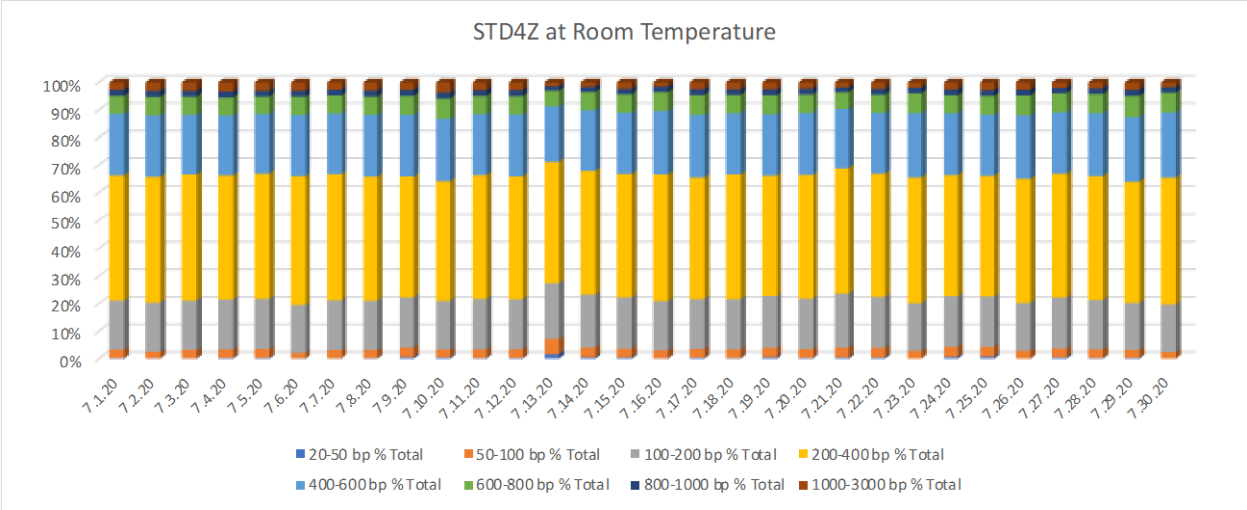
A) Standard 3Z at room temperature.



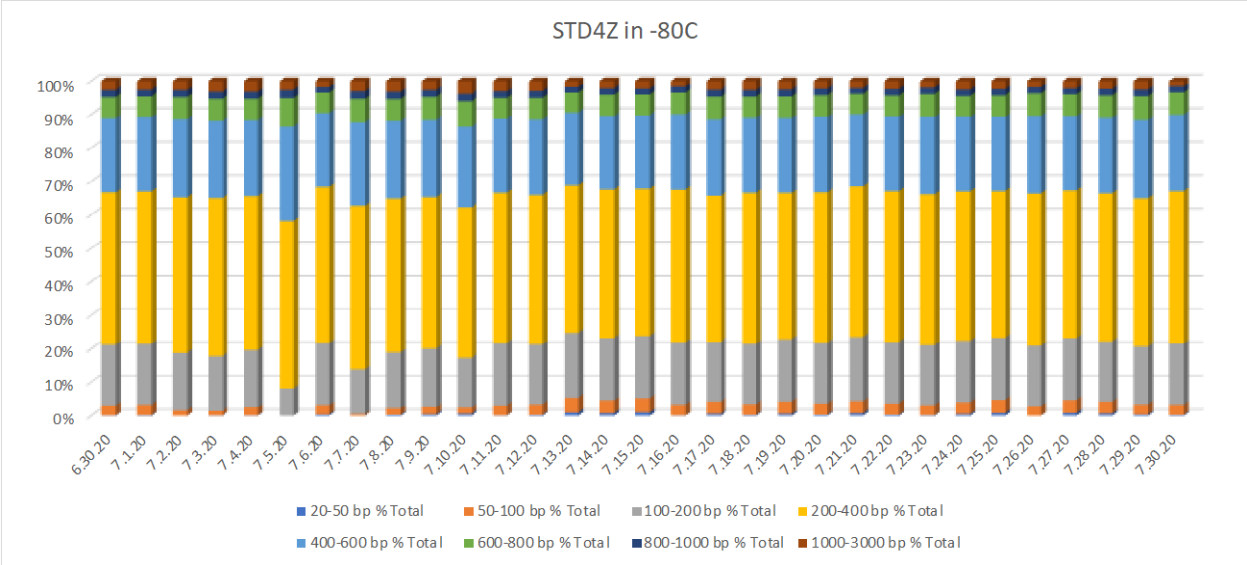
B) Standard 3Z at -80°C.



C) Standard 3Z at room temperature.



D) Standard 3Z at -80°C.



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Sub-Project 2.2 "Influence of Heat Treatment and Vortexing one the Quality and Quantity of DNA" (NOTE: proposed title for publication)

In this experiment we evaluated the influence of two standard laboratory practices, that is heat treatment and vortexing, on the integrity of DNA standards (concentration and fragment size distribution)

In this experiment, we created two DNA standards similar to those used in prior sub-projects:

"Standard 1" (STD 1): concentrated and intact genomic DNA

"Standard 4" (STD 4): degraded and low concentration

We expect fragmentation to be more intense with increasing temperature treatment and time exposures. We also predict that vortexing, if damaging to DNA, will fragment DNA proportion with increased time of treatment,

MATERIALS AND METHODS

Subjection to Temperature Holds

For these experiments we created two standards from pig liver DNA extractions. The first we call "Standard 1" (STD 1), which is akin to STD 1 in previous experiments (intact genomic DNA). This standard begat at 104.21±13.3 ng/µL with 90.1% of its molecules at 10,000-30,000 bp (Sub-Project 2.2 Supplemental Table 1-Tab A). The second standard is called "Standard 4" (STD 4), which is meant to be consistent with previous STD 4 standards (degraded and concentration) This standard began at 1.35±0.45 ng/µL with 78.37% of its molecules at 100-600 bp (Sub-Project 2.2 Supplemental Table 1-Tab B).

These standards were each treated to 37°C, 56°C, and 90°C for up to 48 hours. We sampled aliquots from these standards after 1 min, 15 min, 30 min, 1 hour, 24 hours, and 48 hours and measure their quality and quantities on an Agilent Fragment Analyzer.

Each standard began as a volume of 30 µL in 1.5 ml tubes. Tubes were heated in a Fisher Scientific Dry Bath Incubator. After removing from heat, samples were vortexed briefly, then placed in a centrifuge briefly and subsequently sampled for fragment analysis.

Subjection to Vortexing

Standards were subjected to vortexing at maximum speed (3200 rpm) on a VWR Analog Vortex: 1) "briefly", 2) for 15 sec, 3) for 30 sec, and 4) for 60 sec/). Following treatment, aliquots were removed for fragment analysis.

RESULTS

Subjection to Temperature Holds

Standard 1 (intact genomic DNA) was relatively stable at 37°C for up to 48 hours of exposure at this temperature Sub-Project 2.1 Figure 1A; Sub-Project 2.1 Supplemental Table 1 Tab A). At 56°C fragmentation of this standard begins after 15 minutes, with the accumulation of fragments

5,000-10,000 bp and shorter with time up to 48 hours (Sub-Project 2.1 Figure 1B). At 90°C fragmentation begins immediately and after 48 hours most fragments are <200-600 bp (Sub-Project 2.1 Figure 1C; Sub-Project 2.1 Figure 2).

Standard 4 (degraded and low copy number DNA) was relatively also stable at 37°C for up to 48 hours of exposure at this temperature Sub-Project 2.1 Figure 3A; Sub-Project 2.1 Supplemental Table 1 Tab B). At 56°C, noticeable fragmentation of this standard begins after 24 hours minutes, with the accumulation of fragments 50-100 bp and shorter (Sub-Project 2.1 Figure 3B). At 90°C fragmentation begins after 15 minutes with the accumulation of fragments 50-100 bp and shorter (Sub-Project 2.1 Figure 3C). However, after 24 hours the results are hard to explain as these treated eluates resemble closer the original state of the standard.

Subjection to Vortexing

Vortexing had minimal influence on the integrity of the DNA standards (Sub-Project 2.1 Figure 4A and 4B; Sub-Project 2.1 Supplemental Table 1 Tabs C-D).

CONCLUSIONS

DNA degradation occurs at high temperatures and with increasing time of exposure (this is also supported by experiments in Sub-Project 3.2 (“Why Don’t We Run 100 Cycle PCRs?”). For those working with already degraded and low copy number DNA eluates should be mindful of this, as it can further exacerbate these characteristics.

On the other hand, vortexing for less than 1 minute had minimal influence on the integrity of the DNA standards. This standard treatment is likely not one that is not of high concern when processing aged, degraded, a low copy number DNA samples.

Sub-Project 2.1 Supplemental Table 1. Change in DNA fragment size distribution and concentration of Standards 1 and 4.

Tab A: Standard 1 subjected to holds at 37°C, 56°C, and 90°C for 1 min to 48 hours.

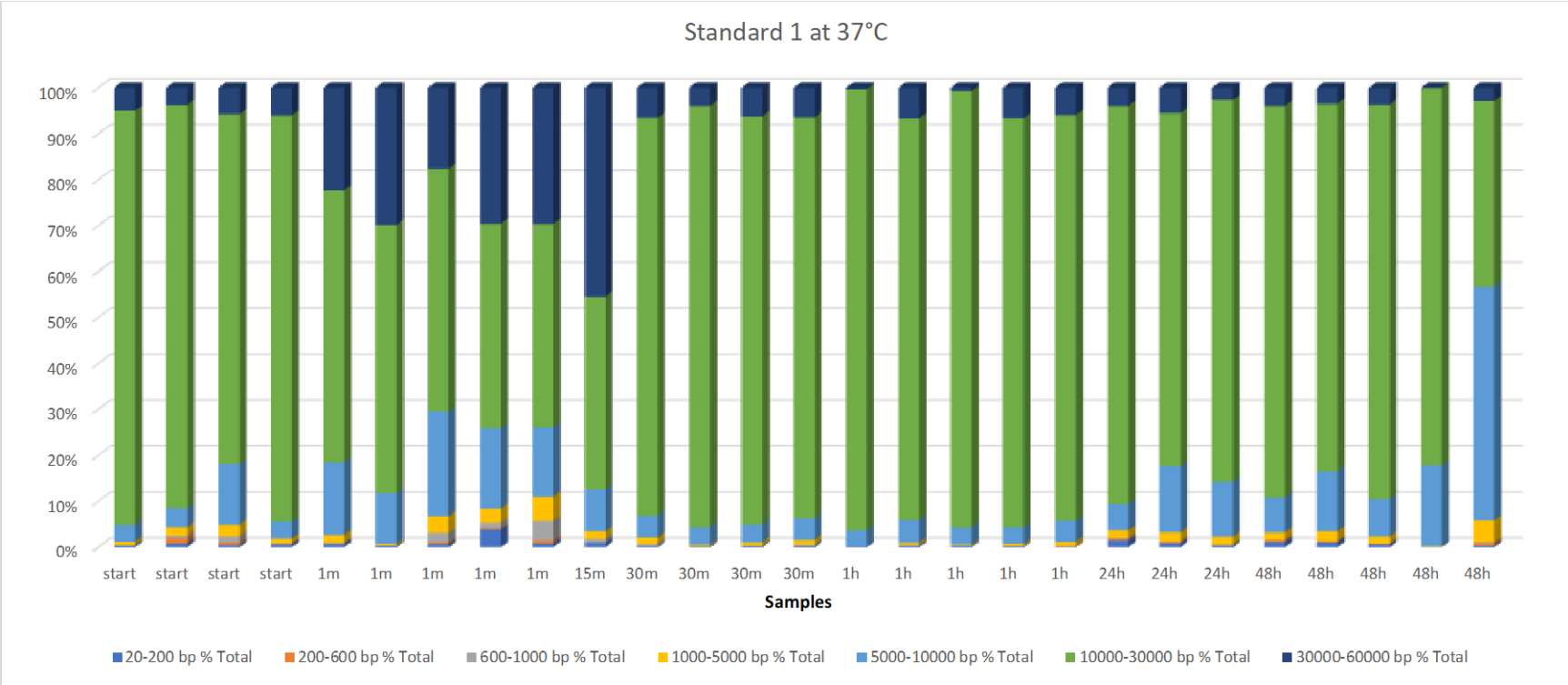
Tab B: Standard 4 subjected to holds at 37°C, 56°C, and 90°C for 1 min to 48 hours**.**

Tab C: Standard 1 subjected to vortexing from “briefly” to 1 minute.

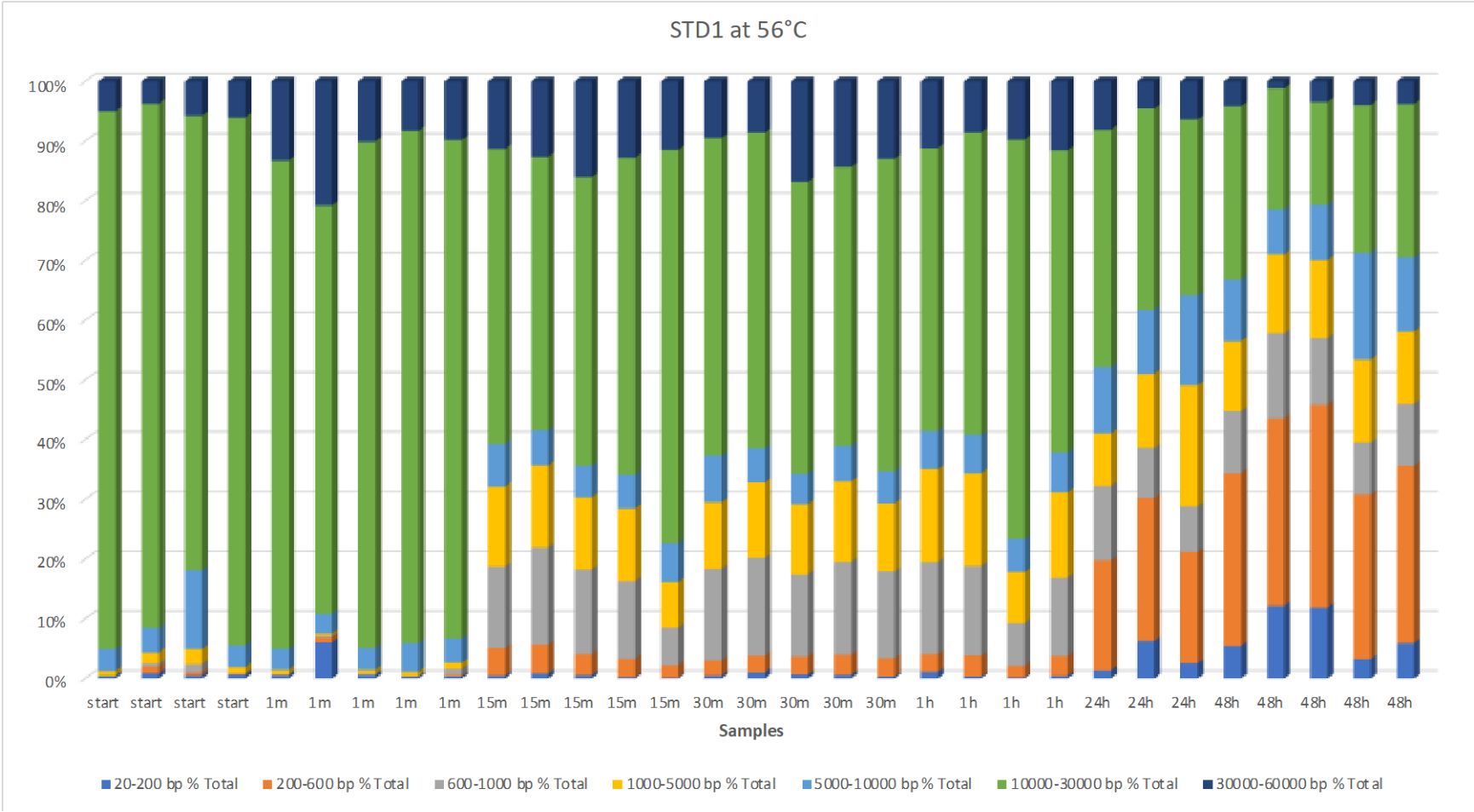
Tab B: Standard 4 subjected to vortexing from “briefly” to 1 minute.

Sub-Project 2.1 Figure 1. Change in DNA fragment size distribution of Standard 1 subjected to holds at 37°C, 56°C, and 90°C for 1 min to 48 hours. Data are reported in Sub-Project 2.2. Supplemental Table 1 Tab A.

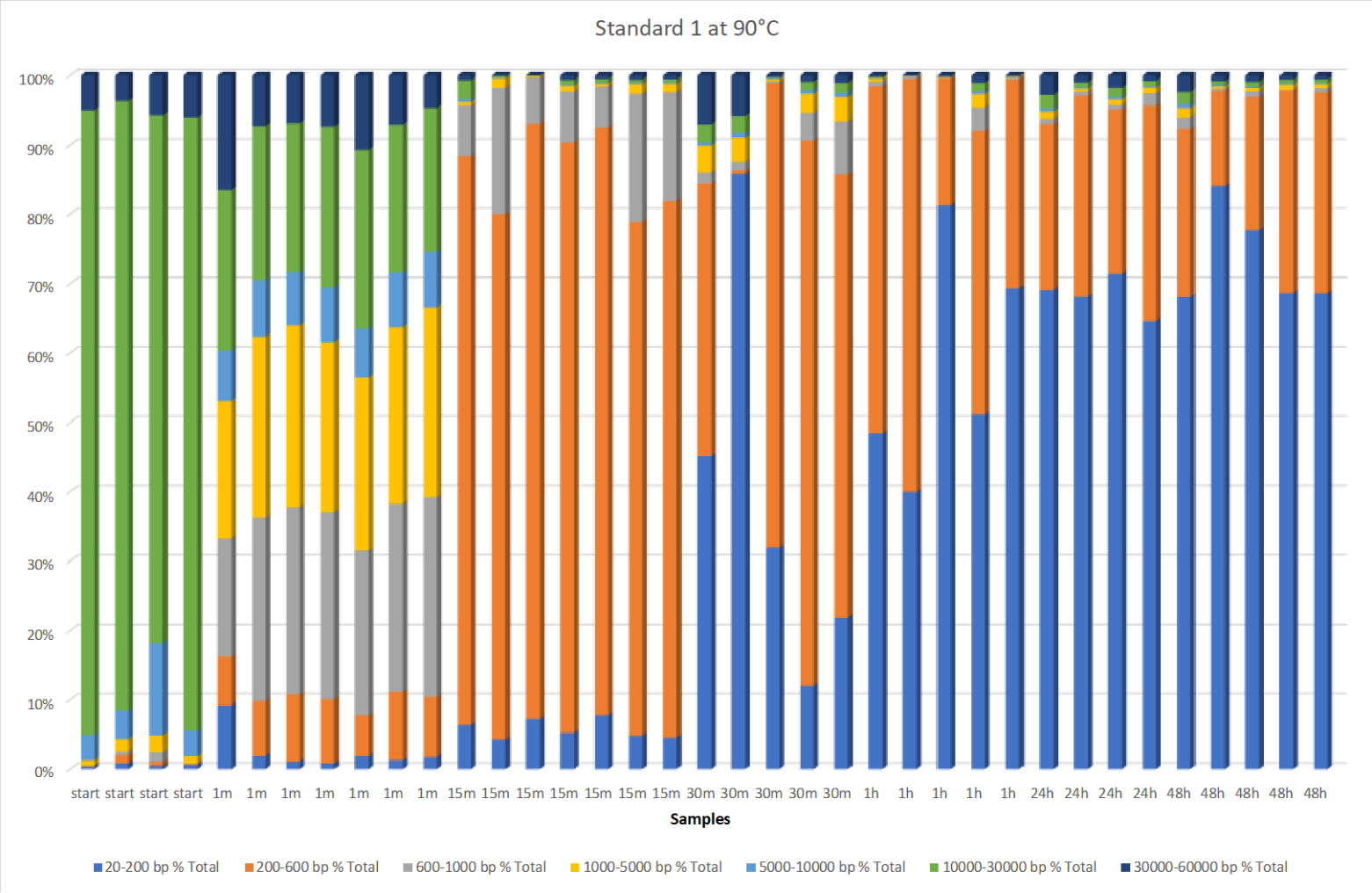
A)



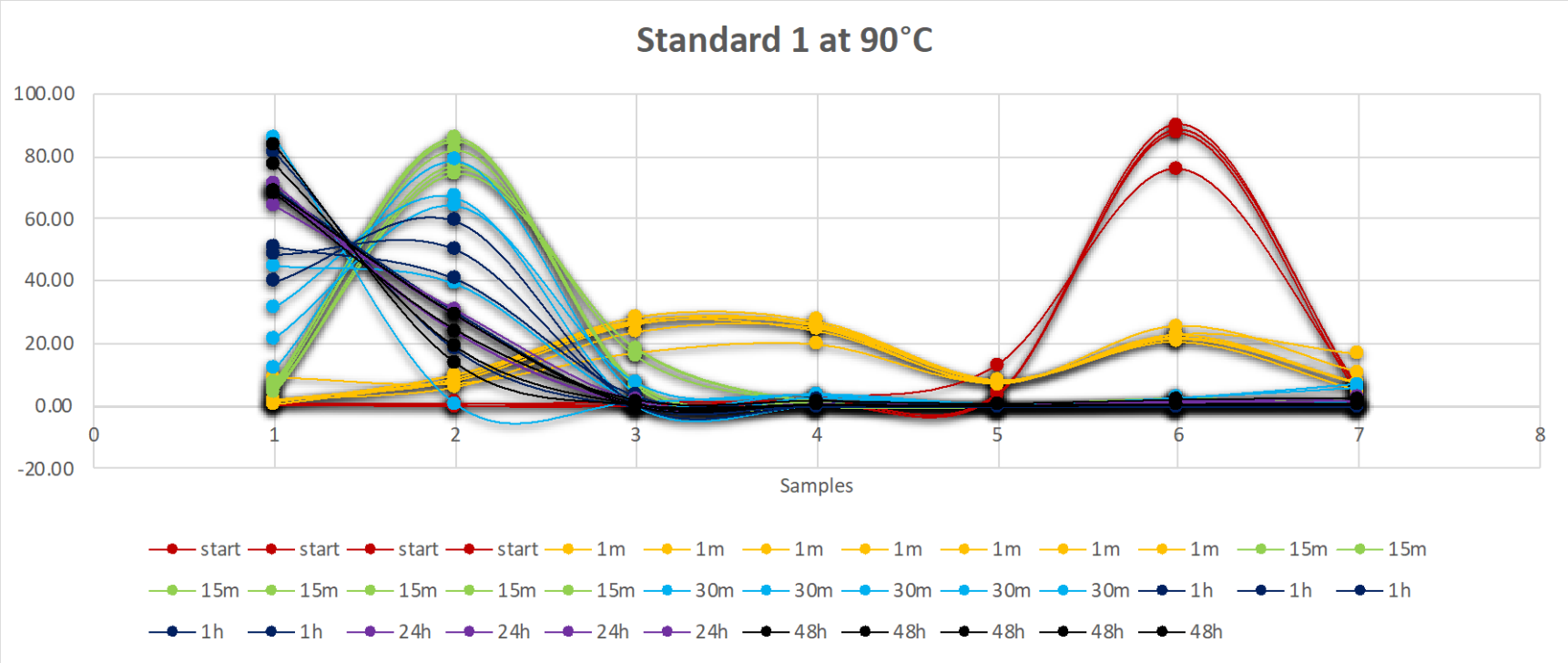
B)



C)

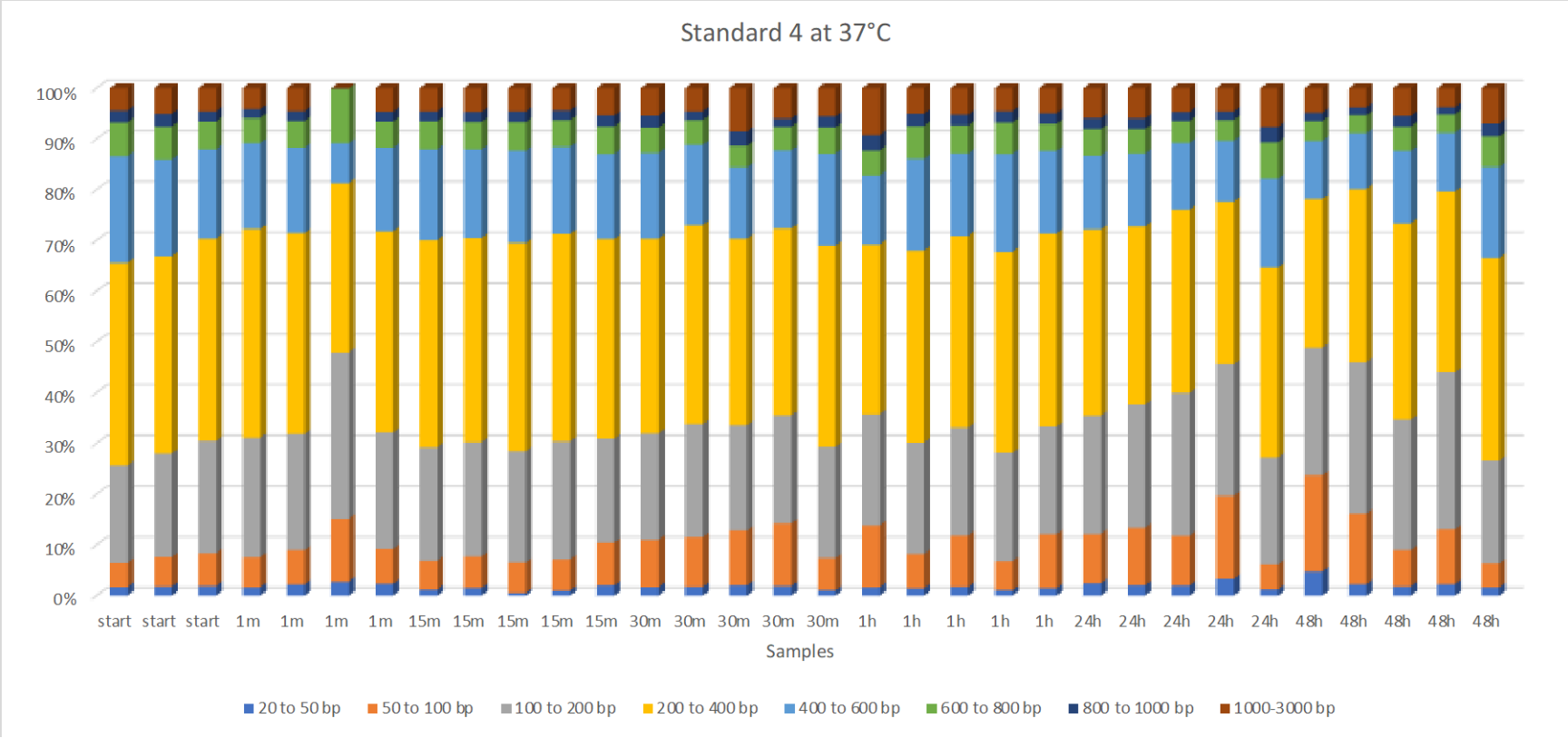


Sub-Project 2.1 Figure 2. Change in DNA fragment size distribution of Standard 1 subjected to holds at 90°C for 1 min to 48 hours. This represents an alternative depiction to the trends reported in Sub-Project 2.1 Figure 2C.

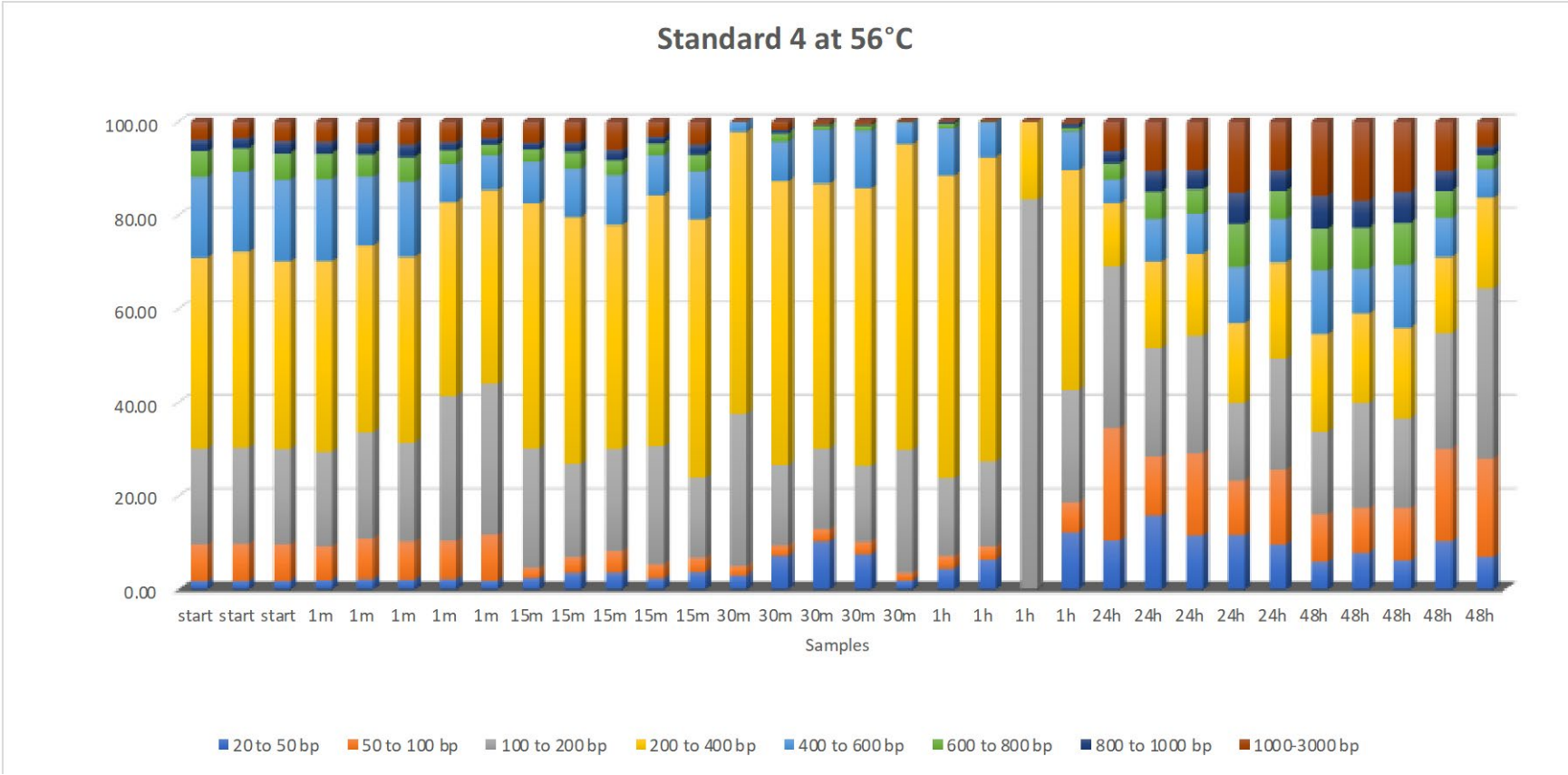


Sub-Project 2.1 Figure 3. Change in DNA fragment size distribution of Standard 4 subjected to holds at 37°C, 56°C, and 90°C for 1 min to 48 hours. Data are reported in Sub-Project 2.2. Supplemental Table 1 Tabs A-B.

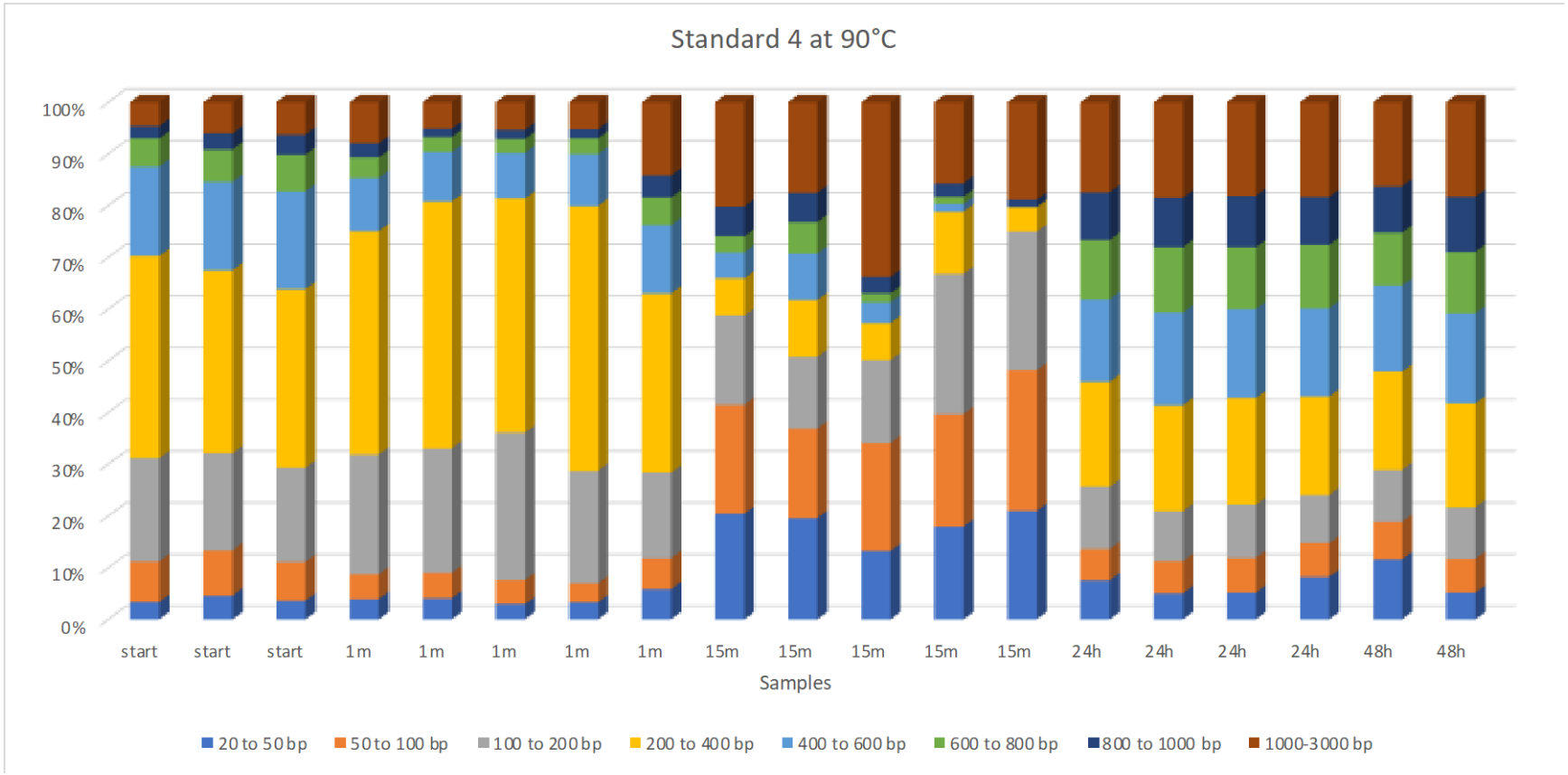
A)



B)

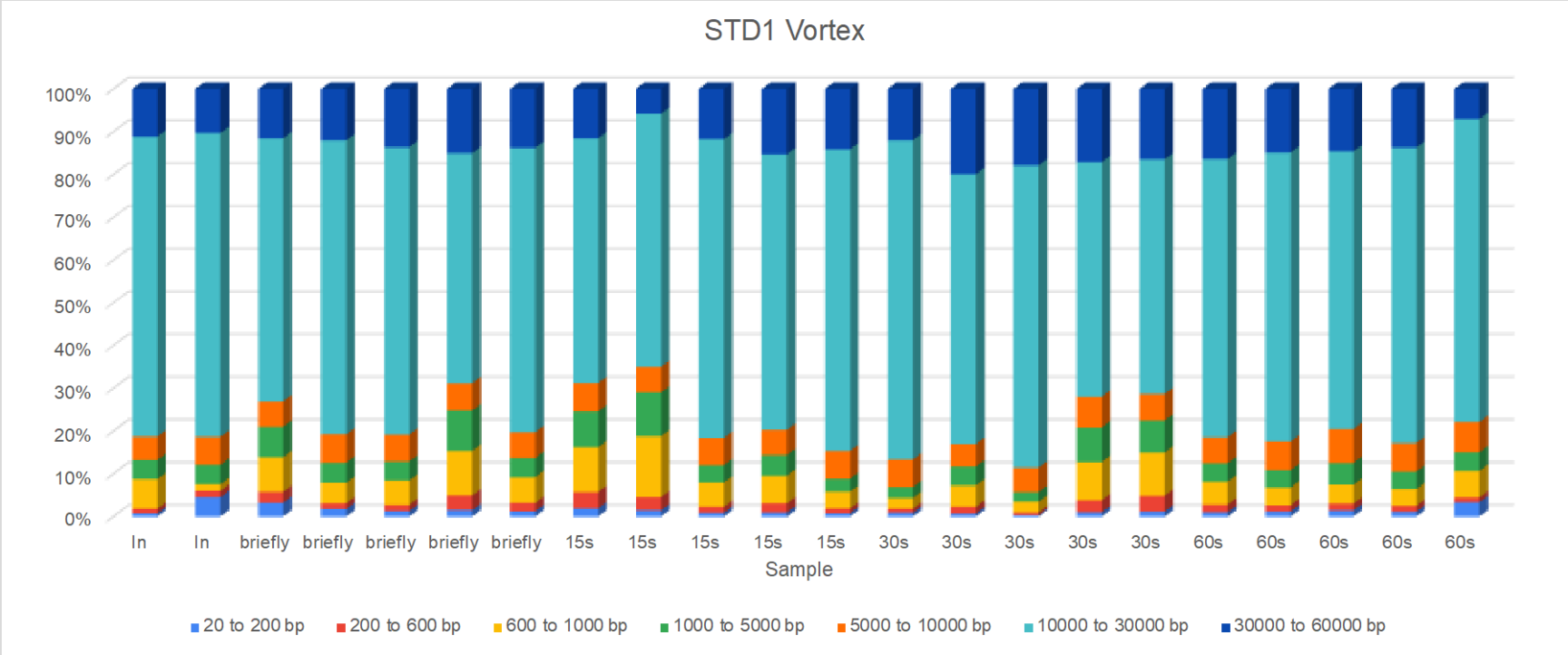


C)

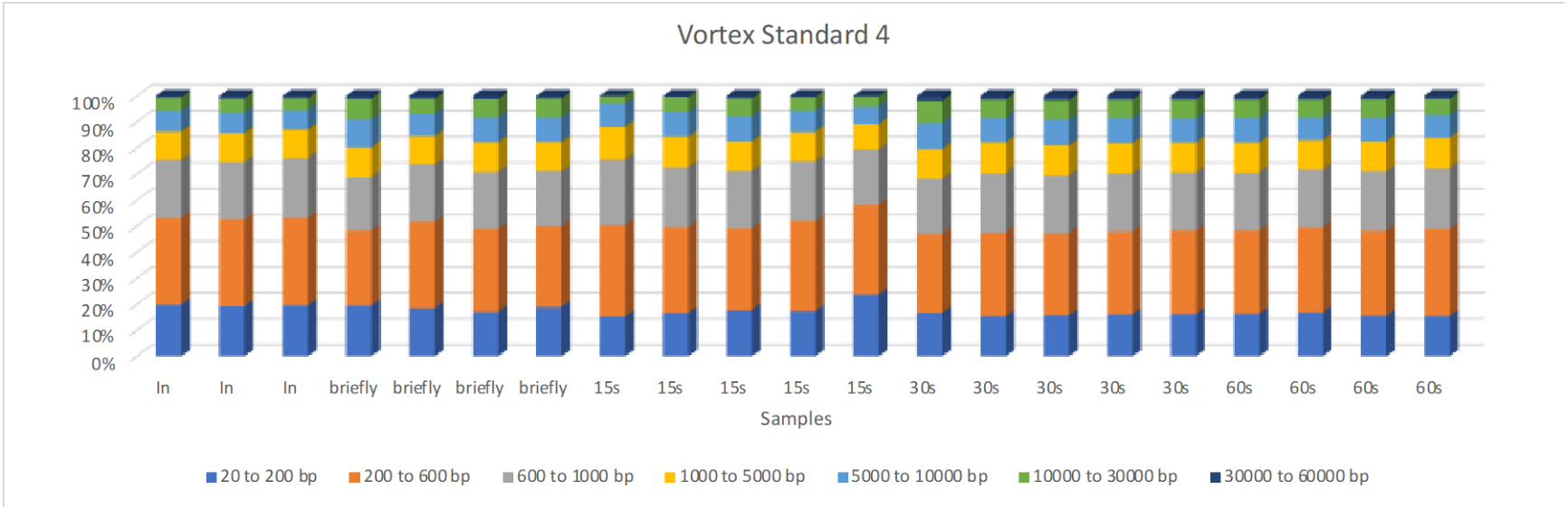


Sub-Project 2.1 Figure 4. Change in DNA fragment size distribution of Standard 4 subjected to vortexing. Data are reported in Sub-Project 2.2. Supplemental Table 1 Tabs C-D.

A) Standard 1 (intact, genomic DNA)



B) Standard 4 (degraded, low concentration)



Phase III: Going back to the basics: The polymerase chain reaction

Rather than simply a means to an end, PCR should be viewed as a tool for solving a particular problem, which is locating few template molecules and increasing their numbers exponentially. The better "standard" PCR and its derivatives are understood, the more easily that problem can be solved. Simple and cost-effective modifications to PCR that can be easily incorporated into the forensic workflow were evaluated during this phase, including: 1) extended extension times, 2) increased PCR volume, 3) increased primer concentration).

Background

PCR appears to be magical. As magical as it may seem, it is not. Yet, we turn our attention now to cases in which the "trick" fails (i.e., when PCR fails). In this context, we should view PCR as a tool that can be used to solve the problem of locating few template molecules and increasing their numbers exponentially (i.e., the trick).

Working with limited knowledge of the intricacies of PCR can makes it difficult to work out reasonable experimental design(s) to determine the source(s) of the failure. For example, negative results are often reported, as just that, negative. However, this is without appreciation for the effects that PCR inhibitors might have had on those results; samples compromised by PCR inhibitors appear to have "no" DNA, when it is simply not amplifiable (Kemp et al., 2006; Monroe et al., 2013; Kemp et al., 2014a; Johnson and Kemp, 2017).

As another example, it is useful to understand base pair complementarity, as it is how primers can "find" the right place to anneal during PCR. Beyond PCR, base pair complementarity is key to a number of other advanced methods and technologies, for example: 1) methods for enriching samples of target DNA, including "fishing" for DNA, DNA capture, primer extension capture (PEC), or pull down (e.g., Anderung et al., 2008; Briggs et al., 2009; Maricic et al., 2010; Carpenter et al., 2013), 2) genotyping arrays, 3) qPCRs (i.e., probe-based ones), and 4) binding to flow cells to initiate clonal amplification prior to HTS. The concept is the same across all these examples; complementarity causes DNA to find other specific pieces of DNA, and to do that exceptionally well.

Modifications to "standard" PCRs have already produced several useful tools, such as booster PCR (Filice et al., 1993), degenerate oligonucleotide-primed (DOP) PCR (Telenius et al., 1992), and touch-down PCR (Don et al., 1991). More recently, in their study of an approximately 50,000-year-old horse bone, Orlando et al. (2011) employed a lower than typical denaturation temperature of 80°C to amplify target DNA. The rationale is that the shorter, degraded DNA molecules will preferentially denature at this temperature over the more intact modern DNA contamination. Yet, this relies on the assumption that modern contamination is not degraded in the same manner (see Phases II and IV).

Denaturation is essential to PCR. So early, preferential denaturation of degraded DNA strands might tip the scales towards their preferential amplification. To appreciate this concept, one must consider base complementarity and its relationship to hydrogen bonding, the overall strength of which is based on the specific sequence *and* length of the molecules. In this case, shorter molecules should denature at relatively lower temperatures. Indeed, experiments that build upon the approach of Orlando et al. (2011) will be insightful, as this could be used to preferentially target fragmented DNA. Since heat treatment of DNA can be damaging (see Sub-

Project 2.2), all things being equal, subjected DNA to lower denaturing temperatures during PCR may serve to the better preserve the intactness of the template molecules.

In further regard to PCR inhibitors, Kemp et al (2006) determined that modern DNA controls mixed with an inhibited sample (i.e., one containing unknown amounts of unknown inhibitors) can be amplified, but this was not the case when aDNA controls (i.e., a degraded DNA sample) were spiked with volumes of the same inhibited sample. These observations are counter to the notion of the existence of a PCR inhibitor "threshold". If such a threshold exists and it is a hard cut-off, one would expect no amount of DNA to be amplifiable, regardless of its quantity and quality, which is clearly not the case in the above-described example. Nevertheless, techniques to remove inhibitors such as "repeat silica extraction" (Kemp et al., 2006; Kemp et al., 2014a) must work by lowering the overall concentration of PCR inhibitors at a faster rate than DNA is inadvertently lost during subsequent purifications (Barta et al., 2014b) (the influence of repeat silica extraction on DNA quality and quantity was assessed during in Phase I).

Another means to circumvent the influence of PCR inhibitors might lie in *increased* reaction volumes. Increasing PCR volumes would result in increased physical distance between inhibitors, template DNA, and the other reagents. This may aid in subduing the inhibitory influence(s) of some of the impurities, especially ones not bound to the DNA such as, calcium or tannic acids This approach may be less effective against inhibitors that are bound to the DNA, such as humic acids or melanin, but should be somewhat effective for a mixed mode inhibitor, such as collagen or phenol (Opel et al., 2010; McCord et al., 2011b).

There are many uncertainties when it comes to determining the optimal parameters for amplifying LCN DNA, including cycle number (Rameckers et al., 1997). In the case of the aDNA field, use of increasing numbers of PCR cycles has become more common. In the late 1990s and early 2000s, aDNA PCRs generally employed less than 40 cycles. For example, Yang et al. (1998) employed 32 cycles and Eshleman and Smith (2001: 4317) described their use of 40 cycles as "unusually high". For many years we have conducted 60 cycle PCRs to amplify aDNA molecules (Kemp et al., 2007; Monroe et al., 2013) which became, after much success, standard operating procedure, despite initial opposing viewpoints from colleagues over our approach (personal communication).

It is also possible that increased numbers of repeated heat treatments (required for PCR) may, in fact, subdue the influence of PCR inhibitors (Moore, 2011), but it also probably degrades DNA (see Phase II; also Sub-Project 3.2 "Why Don't We Run 100 Cycle PCRs?",) (Kemp, 2015; Winters et al., 2017; Winters et al., 2018). If both these assertions are correct, researchers attempting PCR amplification from LCN DNA samples, especially ones associated with unknown levels of unknown inhibitors, are left in a very precarious situation. This is worthy of follow-up investigations to determine if simple solutions exist that can be easily incorporated into the forensic workflow.

During this phase we conducted experiments to evaluate the following:

(1) To measure the influence of increased extension times, increased primer concentration, and increased PCR volume on successful amplification aged, degraded, and/or LCN DNA compromised by co-extracted PCR inhibitors. [Sub-Project 3.1]

(2) To document the influence of increased cycle number on successful amplification of aged, degraded, and/or LCN DNA. [Sub-Project 3.2]

(3) To further evaluate the notion of Orlando et al. (2011) that lower than standard denaturing temperature (i.e., <94-95°C) in PCR can be utilized to amplify aged, degraded, and/or LCN DNA. [Sub-Project 3.3]

Sub-Project 3.1 "Extended Extension PCR: a Simple Technique for Improved Amplification of Aged, Degraded, and Low Copy Number DNA Samples Compromised by PCR Inhibitors" (NOTE: proposed title for publication)

During this portion of the grant period, we tested the influence of increased: 1) PCR extension times, 2) PCR volume, 3) and primer concentration in the PCR master mix, on the level of successful amplification of aged, degraded, and/or LCN DNA compromised by co-extracted PCR inhibitors.

Regarding extension times during PCR, this variable is typical changed with regards to targeted amplicon size. Longer amplicons take longer to make and copy than do shorter ones. However, we hypothesized that increased extension times may compensate for the influence of PCR inhibition based on two observations. First, in our laboratory, we have routinely observed that a DNA eluate deemed to be "inhibited" [i.e., via spiking with a positive control, effectively equivalent to an internal positive control (IPC) (Kemp et al., 2014a)] will fail to produce targeted amplification of fragments ranging ~120-200 bps. Yet, in many of these reactions, the production of primer-dimers of 50-60 bps is possible. The only difference between targeted amplicons and primer-dimer is their relative length. Therefore, it is possible that PCR inhibitors are, in part, behaving in a manner as to slow the reaction speed, not simply preventing it outright from occurring. Secondly, McCord et al (2011a) reported that the successful production of larger amplicons is influenced more by the presence of PCR inhibitors than are smaller amplicons.

We hypothesized that that subduing the influence of PCR inhibitors might also lie in *increased* reaction volumes, which results in increased physical distance between inhibitors, template DNA, and the other reagents.

Lastly, we also experimented with the relationship between increased primer concentration and its ability to improve amplification of inhibited DNA eluates. This idea stemmed from non-formal observations in our laboratory that appeared worth pursuing further.

We tested these ideas on DNA recovered from various rockfish (genus *Sebastes*) vertebrae recovered from the archaeological record. DNA eluates recovered from these samples are ideal to test our ideas, as they are variably compromised by PCR inhibitors. We used qPCR and overall success rate to measure the experimental outcomes. Our expectations are that:

1) successful approaches that subdue inhibitory influences will lead to decreased Cq values, indicating more DNA is available for amplification over standard treatment.

   -and-

2) successful approaches produce statistically significant increase in successful PCR amplification.

MATERIALS AND METHODS

Samples/Archaeological Context

A total of 111 specimens used in this study were selected from the fish bone assemblages recovered from a late-19th-century Chinese diaspora archaeological site in California. Specifically, specimens originated from Market Street Chinatown, a large, urban Chinese community in San Jose, California that was home to over 1,000 permanent residents from the 1860s through 1887, before the site was destroyed by arson fire (Laffey, 1993; Yu, 2001). Fieldwork at the site took place in the 1980s, when archaeologists identified and excavated over 60 trash pit features containing refuse associated with the community's many residences and businesses (Voss, 2008). Field methods including the use of ¼" mesh screens to recover artifacts and faunal remains from excavated soils.

Prior to analysis in this study, all fish remains were analyzed by Dr. Ryan Kennedy (Research Associate, University of New Orleans) in either Indiana University's William R. Adams Zooarchaeology Laboratory or the University of New Orleans Archaeology Laboratory (e.g., Kennedy, 2017; Kennedy, 2018; Kennedy et al., 2019). This work followed standard zooarchaeological methods (e.g., Reitz and Wing, 2008), including taxonomic identification via comparative morphological analysis between unknown archaeological specimens and modern comparative skeletal specimens from common fish species found at Chinese diaspora archaeological sites. Rockfishes are common across Chinese diaspora archaeological sites and following morphological analysis Dr. Kennedy selected rockfish specimens for genetic analysis (and eventually for species level identification, but not the focus here; Kemp and Kennedy-study in progress).

DNA Extraction

All pre-PCR activities were conducted in the ancient DNA (aDNA) facility at the Laboratories of Molecular Anthropology and Microbiome Research (LMAMR[§§]) at the University of Oklahoma. This facility is a dedicated workspace for processing aged, degraded, and/or LCN DNA samples. Precautions aimed to minimize and monitor the introduction of contamination are practiced in the laboratory.

We extracted DNA from 111 vertebrae morphologically identified as rockfish (*Sebastes spp*.) in batches of seven or six, each with an accompanying extraction negative control (totaling 16 extraction negatives) to monitor for potential contamination.

Inhibition Test

We tested each DNA eluate, as well as the extraction negative controls for the presence of PCR inhibitors sufficient to prevent PCR amplification of an ancient DNA control sample using Omni Klentaq LA, a fairly robust polymerase against PCR inhibitors (Monroe et al., 2013). In this case, the control consisted of DNA extracted and pooled from various archaeological turkey (*Meleagris gallopavo*) specimens dating approximately 180-1400 years ago (Kemp et al., 2017). Pooling DNA in this manner is meant to reduce inter-sample variability of DNA concentration, as well as associated amounts of PCR inhibitors in the control pool. The control was determined to PCR amplify consistently for a 186 base pair (bp) fragment of the turkey D-loop in six PCR reactions, thus serving as a positive control.

Then, PCR reactions were set up again to amplify the control for the same reaction, but in this case, reactions were spiked with 1.5 µL of each of the rockfish DNA and extraction negative eluates. If the addition of the rockfish eluate prevented the turkey DNA from amplifying, we

[§§] lmamr.org

consider the sample to be “inhibited”. Conversely, if the rockfish aliquot did not prevent amplification of the turkey DNA, we consider the sample to be “not inhibited”. Details for this procedure and rationale are described by Lanman et al. (2021) and Kemp et al. (2014a)

Half of each sample eluate (and the accompanying extraction negative controls) that were inhibited were subject to repeat silica extraction until they were sufficiently “free” of inhibition, as described by Lanman et al. (2021) and Kemp et al. (2014a). These inhibitor free portions of the eluates were used in various experiments below to conduct genetic identification as belonging to the genus *Sebastes* (i.e., the samples are of rockfish).

Initial Species Identification

While morphological identification was initially used to determine the specimens as rockfish, it was critical for us first to genetically confirm their identification as fish belonging to the genus *Sebastes*. As described below, the quantitative PCR (qPCR) assays employed in this study were designed specifically to target and measure amplification of three sections of the rockfish mitochondrial cytochrome b (cyt b) gene. Thus, a DNA eluate from a specimen misidentified morphologically as a rockfish sample may contain amplifiable DNA but fail to amplify because it is only distantly related to those species belonging to the genus *Sebastes*.

For initial genetic identification, we amplified all eluates for a 189 bp fragment of the mitochondrial 12S gene using “universal” fish primers (Jordan et al., 2010). This was conducted on the original eluates, as well as on volumes of the same eluates that were removed and subjected to repeat silica extraction, as described above (Kemp et al., 2014a). In some cases, experimental conditions to this 12S amplification contributed to this initial genetic identification (see Sub-Project 3.2 “Why Don’t We Run 100 Cycle PCRs?”, also additional experiments by Kemp and Kennedy- study in progress)

Amplicons were sequenced at Genewiz[***] in both directions. The resulting 148 bp sequences were first evaluated for quality and base calling using Sequencher v5.4.6. and then subjected to a Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) search of the National Center for Biotechnology Information’s (NCBI) Genbank (Sayers et al., 2020).

Standard qPCR Assays

All samples identified as rockfish (*Sebastes spp*.), as well as those samples that did not produce amplicons in the 12S PCRs were subjected to the following qPCR assays. Samples falling into the latter category represent: 1) samples that contain no amplifiable DNA in the range of 189 bps required as template for the 12S reaction, 2) rockfish samples that contain well preserved DNA, but simply failed in 12S reaction, or 3) non-rockfish samples that contain well preserved DNA, but simply failed in 12S reaction.

We used three sets of primers that are useful in determining species identification of rockfish (Moss et al., In Press) (Sub-Project 3.1 Table 1). Each targets a short (<185 bp) subsection of the mitochondrial cytochrome b gene, ones that we call “cyt b1”, “cyt b2”, and “cyt b3”. Under “standard” qPCR reaction conditions, a control DNA sample extracted from a contemporary rockfish sample[†††] behaved very predictably as measured by both its intra- and inter-reaction

[***] www.genewiz.com

[†††] Contemporary rockfish samples, used as controls in our laboratory, were graciously provided to us by Anne Pollnow of Sea Level Consulting, LLC, Sitka, Alaska

quantification cycle (Cq) variability (Sub-Project 3.1 Table 2). It is also notable that these primers do not regularly produce primer-dimers, but do so more often in the absence of target DNA. This is typically observable in qPCRs that yield Cq values, but are associated melt curves that are markedly different from those that have amplified only target DNA.

As extra precautions to exclude results originating from non-target amplification that could otherwise bias our results, we: 1) separated all PCR products on 2% agarose gels stained with GelRed to visually inspect amplicons sizes, and 2) sequenced 285 of 515 (55.3%) the amplicons produced from the rockfish DNA eluates to confirm targeted amplification (i.e., cyt b sequences from fish belonging to genus *Sebastes*). All Cq values compromised by primer-dimer formation are noted in Sub-project 3.1 Supplemental Table 1 Tabs B-F and the other tables summarizing the results of this subproject (Sub-project 3.1 Tables 3-6).

Only those Cq values associated with the production of target amplicons (confirmed by sequencing in 55.3% of the cases or assumed by production of expected amplicon size alone in the other 44.7% of the cases) were subjected to analysis of the effectiveness of alternative strategies in subduing inhibitory influences.

Standard qPCR reactions of 20 µL contained 1X SsoAdvanced™ Universal SYBR® Green Supermix, 0.125 mg/mL bovine serum albumin (BSA), 0.24 µM of each primer, and 1 µL of template DNA (Sub-Project 3.1 Table 1). Quantitative PCR reaction conditions were as follows: 1) 3 min denaturation at 94°C, 2) 60 cycles of 15 s holds at 94°C, 56°C, and 72°C (with a plate read following each cycle), and 3) a final extension at 72°C for 3 min. The qPCR reactions were followed by melt curve analysis starting at 65°C and increasing the temperature by 0.5°C, after which the plate was read. This was conducted 60 times, ultimately reaching a temperature of 95°C. Reactions were cooled to 10°C. All qPCR reactions were conducted in a CFX96 Touch Deep Well Real-Time PCR Detection System.

Experimental qPCR Assays

Experimental Increase of Extension Times

qPCR reactions were modified to target cyt b1 with extension times of 1 min, 5 min, and 10 min per cycle over the standard 15 sec extension time. Targeting cyt b2 and cyt b3 by qPCR was modified with extension times of 1 min and 5 min over the standard 15 sec.

Experimental Increase of qPCR Volumes

First, standard cyt b2 qPCRs were scaled up to 40, 60, 80, 100, and 125 µL while maintaining 1 µL of template. Final concentrations of each PCR reagent were identical to the standard 20 µL reaction.

Secondly, the standard qPCR was scaled up to 40 µL and 125 µL with water while maintaining 1 µL of template. Reactions of 40 µL contained 0.5X SsoAdvanced™ Universal SYBR® Green Supermix, 0.0625 mg/mL bovine serum albumin (BSA), 0.12 µM of each primer, and 1 µL of template DNA. Reactions of 125 µL contained 0.008X SsoAdvanced™ Universal SYBR® Green Supermix, 0.001 mg/mL bovine serum albumin (BSA), 0.00192 µM of each primer, and 1 µL of template DNA.

Experimental Increase of Primer Concentration

Standard cyt b3 qPCRs were scaled up with 1 µM of each primer (4.17X concentration over standard) and 2 µM of each primer (8.33X concentration over standard) over the standard 0.24 µM of each primer.

Sequencing Cyt B1, Cyt B2, and Cyt B3 Amplicons

We sequenced 285 of 515 (55.3%) the amplicons produced from the rockfish samples. Our focus was especially on sequencing results from the most "extreme" experimental conditions (e.g., 10 min extension, the largest volume, highest primer concentration) merely out of overabundant curiosity as whether they functioned normally, producing expected results. In addition, we sequenced additional amplifications from samples suspected to show signatures of postmortem nucleotide modification [i.e., "damage", the most common form of which is deamination of cytosines which lead to artifactual C>T "transitions" (Gilbert et al., 2007)] in any given treatment. Observations of novel haplotypes that could not be confirmed via replication are presumed in our study to be the product of such damage, or any other related postmortem nucleotide degradation that, if not considered otherwise, would be misidentified as an authentic population variant. Production of damaged sequences were considered as "successes", as the damage is either inherent to the specimen or produced by polymerase error. Under either scenario, observation of such (presumed) deviant sequences suggests that amplification in these cases was initiated from very few template molecules.

We also sequenced any amplicons produced from extraction or PCR negative controls that appeared to be in the targeted size range.

Rockfish sequences were placed in the context of the study of Hyde and Vetter (2007) (see comparative data summarized in Sub-project 3.1 Supplemental Table 2). We utilized NCBI's BLAST function (Altschul et al., 1990) in search of novel haplotypes observed in our study against the records of Genbank (Sayers et al., 2020).

Amplicons were sequenced at Genewiz[‡‡‡] in both directions.

Statistical Analysis

Chi square (X^2) tests were used to compare success rates between treatments (we used a free on-line X^2 calculator[§§§]) and two-tailed t-tests were conducted to compare treatments in their average Cq values and associated standard errors (we used a free on-line statistical calculator[****]). An alpha level of 0.05 was set as the cut-off for statistical significance of the tests.

RESULTS AND DISCUSSION

Sebastes Identification, Contamination, False Positives, DNA Damage, and Amplifications without Associated Cq Values

‡‡‡ www.genewiz.com
§§§ https://www.socscistatistics.com/tests/chisquare/default2.aspx
**** https://www.medcalc.org/calc/comparison_of_means.php

Of the 111 rockfish vertebrae, results from sequencing 12S fish identification amplicons identified six non-rockfish (Sub-Project 3.1 Supplemental Table 1, Tab A; Sub-project 3.2), also additional experiments by Kemp and Kennedy-in preparation), summarized in Sub-project 3.1 Supplemental Table 1. Three specimens (R83, R84, LR20) matched 100% to Striped Bass (*Morone saxatillis*) (e.g., GenBank Accession AH013018.2) and White Bass (*M. chrysops*) (e.g., AY372812.1). Specimen R85 matched 92.62% to Common Snook (*Centopomus undecimalis*) (e.g., GenBank Accession MK616995.1), R94 matched 94.56% to Red Scorpionfish (*Scorpaena scrofa*) (GenBank Accession DQ125234.1), and R97 matched 100% to Cabezon (*Scorpaenichthys marmoratus*) (GenBank Accession KM057987.1). These samples were removed from further consideration in this study.

One extraction negative control (LR Extraction Negative Control 1) revealed the presence of sockeye sample (*Oncorynchus nerka*; e.g., 100% match to NC_008615.1) in one of the five times it is was amplified for the 12S region (from additional experiments by Kemp and Kennedy-study in preparation). This extraction negative control also failed to amplify in all other PCRs conducted in during this sub-project (i.e., across cyt b1, cyt b2, and cyt b3). No samples in this study were identified as sockeye salmon and no other negative controls (extraction negatives or PCR negatives) tested positive for this species. The origination of this contaminant is unknown. We have previously identified sockeye salmon in another archaeological-based project conducted more than three years ago (Kemp and Tushingham[††††]-study in preparation). We also use a contemporary, positive control of sockeye salmon on a regular basis during 12S amplification (added to PCRs in the PCR laboratory just prior to amplification). During this sub-project we did not, electing to use a rockfish positive control. However, this observation of contamination might have come from cross contamination from post-PCR to the aDNA laboratory, despite our best efforts to minimize such contamination. It is notable also in this case, that in our recent study of 88 salmonid vertebrae, we only observed rainbow trout/steelhead (*Oncorynchus mykiss*) and Chinook salmon (*Oncorynchus tshawytscha*) and did not observe sockeye salmon or salmonid DNA, in general, in any negative control (Lanman et al., 2021).

Forty-three specimens were identified as rockfish with 12S PCR sequencing and 62 failed to amplify with the 12S primers (Sub-Project 3.1 Supplemental Table 1). In total, these 105 specimens (or portions of them) were subjected to the various cyt b1, cyt b2, and cyt b3 qPCR reactions described below. From the sequences of a selection of those amplicons, we determined that 44 of the 62 samples that failed in the 12S reactions are, in fact, rockfish (i.e., belonging to genus *Sebastes*). This left 18 specimens for which we observed no amplification in any reaction. These samples may contain DNA strands below the length of our targeted range (i.e., 147-189 bps) or they may not be rockfish but not identified as such with a 12S sequence. Notably, our inhibition test indicated that 15 of these 18 DNA eluates are inhibited, so it is possible that not one of our experimental treatments could alleviate the inhibitory influences associated with PCR amplification from these DNA eluates.

Thus, our results in this sub-project are focused on the behavior of the DNA recovered from 87 specimens identified as rockfish. Our inhibition test indicated that 70 of these DNA eluates are inhibited (80.5%). Note, however, that we inadvertently lost the volume of sample R38 early in our experiments, so beyond a few initial experiments, we then focused largely on the remaining 86 specimens identified as rockfish. In some experiments we tested only portions of the rockfish specimens.

[††††] Dr. Shannon Tushingham, Department of Anthropology, Washington State University

None of the extraction negative controls were inhibited, suggesting that inhibition is sample specific and not largely due to inhibitory effects that might have arisen during the extraction procedure [e.g., carry over of isopropanol, see Monroe et al. (2013)]. The volume of extraction negative control 11 was inadvertently lost during these experiments. In this case, we rely on the remaining 15 extraction negative controls for our estimates of contamination originating during this sub-project in total.

Contamination and False Positives

During this sub-project we tested the 15 extraction negative controls for possibly containing rockfish DNA in a total of 187 qPCR reactions (Sub-Project 3.1 Supplemental Table 1). In one case, extraction negative control 10 produced a sequence of rockfish (i.e., genus *Sebastes*) haplotype 56 with a 567T in the cyt b2 amplification of a 40 µL (0.5X dilute) reaction. While likely a damaged sequence (the 567T mutation makes this observation unique, was not repeatable, and is not reported in GenBank). This haplotype 56 of cyt b2 is definitive of the Yelloweye Rockfish (*Sebastes ruberrimus*) [Sub-Project 3.1 Supplemental Table 2; Hyde and Vetter (2007)]. This result was not observed in: 1) any of the other reactions in this batch of experiments, 2) any other cyt b2 amplification (across all treatments), or 3) any other reactions from this extraction negative control in any other cyt b2 amplification attempts. Notably too, extraction negative control 10 did not amplify in any of the cyt b1 or cyt b3 qPCR reactions. It is likely that this observation stemmed from some form of cross-contamination, despite the extra precautions to minimize such occurrences in the LMAMR ancient DNA laboratory. The rate of which is estimated in this sub-project to be 1/187 (0.53%) in amplification of the three targeted cyt b sub-regions.

Extraction negative control 8 also produced what appeared to be a target sized amplicon for the cyt b3 qPCR reaction with 5 min extension, but the sequence of these amplicons revealed “No significant similarity found” in a BLAST (Altschul et al., 1990) of Genbank (Sayers et al., 2020). One PCR negative control amplification (of 139 total) associated with the standard qPCR reaction of cyt b1 produced a similar result. It is possible that these amplicons originated from the DNA of some unreported microorganisms that amplified somewhere during the experiments. These were, notably, not repeatable observations.

Of the 285 amplicons sequenced from the rockfish samples, the standard cyt b1 amplification of sample R54 produced a result revealing “No significant similarity found” in a BLAST (Altschul et al., 1990) of Genbank (Sayers et al., 2020). This was notably not a repeatable observation and PCR amplification of this the sample failed in all other cases.

Sequencing of three reactions failed or were compromised by background noise sufficient to exclude interpretation: 1) cyt b2 amplification of sample LR4 with 1 min extension, 2) cyt b2 amplification of simple R30 with 5 min extension, and 3) cyt b3 amplification of L24 with 5 min extension. We excluded these results from our tally of successes. Therefore, in interpreting our overall rockfish results that were not sequenced (total of 230 reactions), our best estimate for miscalling of target across this sub-project is 1/285 (0.35%) and failed sequencing is 3/285 (1.1%). Of the 281 rockfish sequences produced, 34 (12.1%) revealed evidence of post-mortem damage. We consider these damaged sequences to be “successes”, as we have previously shown that such damage does not compromise species identification though phylogenetic analysis (i.e., tree building), where that is the goal of a project (Moss et al., In Press). In other words, these results are representative of target amplification, albeit as evidence from sequencing amplicons originating from damaged template molecules. Interestingly, this is a cost of targeting LCN DNA sources to begin with, such stochasticity is

expected and needs to be controlled for before drawing a consensus of the authentic haplotype. Replication is key to authenticating abnormal, questionable, and/or unexpected results, such as those that do not meet evolutionary expectations (Gilbert et al., 2005).

In three cases, amplicons that resulted in rockfish sequences, were obtained with no associated Cq values (Sub-Project 3.1 Supplemental Table 1): 1) Sample LR4 in a 60 µL cyt b2 reaction produced a damaged haplotype 30 sequence, 2) sample R68 in a 40 µL cyt b2 0.5X reaction produced an undamaged haplotype 67 sequence, and 3) sample R56 with 1 µM of each primer (4.17X concentration over standard) produced an undamaged haplotype 49. These three observations are considered as successes below, but obvious did not contribute to tallies of average Cqs for those specific treatments.

Extended Extension Experiments

The cyt b1 standard qPCR resulted in 50.6% amplification (44/87 samples) (Sub-Project 3.1 Table 3). All uninhibited samples (17/17) amplified with an average Cq of 37.55±4.50 (standard deviation) and 27/70 inhibited samples (38.6%) amplified with an average Cq of 41.81±6.84. Uninhibited samples produced a statistically lower average Cq value (p-value= 0.0283, two-tailed t-test). Throughout this sub-project, the average inhibited Cq values were more than 3.3 over the average Cq values originating from the amplification of inhibited samples indicating that on average there was more than ten-fold or more starting templates initiating the former reactions over the latter. Under the assumption that all the samples studied in this sub-project contain roughly the same amount of DNA (note: the relationship between DNA preservation and degree of inhibition is unknown), this would indicate that the inhibitory influences are either preventing amplification or when permitted, are initiated from >10-fold less molecules).

Increasing the cyt b1 reaction extension time to 1 min resulted in 75.9% success (66/87 samples amplified) overall more success than “normal” (44/87, p-value=0.0010, X^2 test). Success with 5 min plateaus (Sub-Project 3.1 Table 3). The more limited experimentation with 10 min extension indicates a similar pattern (Sub-Project 3.1 Table 4**).** It is notable that the qPCR contemporary rockfish DNA controls failed to amplify with 10 min extension (Sub-Project 3.1 Supplemental Table 1). It is also notable that while success increased substantially with 45 seconds or more of extension times, the average Cq values were quite similar across the treatments. Despite our prediction that there would be a decrease in Cq values associated with a successful tactic such as extended extension time, this was not the case.

The cyt b2 standard qPCR resulted in 47.7% amplification (41/86 samples) (Sub-Project 3.1 Table 3) with an average Cq of 40.66±5.30. With 1 min extension time, the success rate dropped to 36% (31/86), but not significantly so (p-value=0.1222, X^2 test). However, it is notable that the Cq value associated with standard qPCR 40.66±5.30 is significantly lower than when 1 min extension was applied (45.08±7.28, p<0.0001). With 5 min extension the success rate (42/86 48.8%) was nearly identical to success with standard 15 sec extension (p-value= 0.8787, X^2 test).

The cyt b3 standard qPCR resulted in 36% amplification (31/86 samples) (Sub-Project 3.3 Table 3). Increasing the cyt b3 reaction extension time to 1 min resulted in 64% success (55/86 samples amplified), an improvement over standard condition (p-value=0.0003, X^2 test). However, with a success rate of 37.2% (32/86 samples), extension of 5 min was not an improvement over standard condition (p-value=0.8742, X^2 test).

Despite the contemporary rockfish DNA controls failing to amplify with 10 min extension in the cyt b1 qPCR (Sub-Project 3.1 Supplemental Table 1), the controls otherwise behaved quite predicably in reference to our initial assessments (Sub-Project 3.1 Table 2).

It is notable that where success increased substantially by adding 45 seconds or more to the extension times, the average Cq values were quite similar across the treatments. Despite our prediction that there would be a decrease in Cq values associated with a successful tactic such as extended extension time, this was not the case.

Experimental Volume Treatments

Of the 14 samples compared for volume treatments, the standard reaction produced a 28.6% success rate with an average Cq of 36.5±1.62 (Sub-Project 3.1 Table 5**)**. Increasing the volumes to 40, 60, 80, and 100 µL (while maintain reagent concentrations) lead to no improvement (p-values >0.05). It is notable that the qPCR standards showed increased Cq values (4-17 cycle increases; Sub-project 3.1 Supplemental Table 1) over initial behaviors in standard qPCR (Sub-Project 3.1 Table 2). None of the rockfish samples amplified in the 125 µL reaction and only the most concentrated contemporary control (at 6.33 ng/µL) amplified, but with a Cq of ~12 more cycles over standard conditions.

Only sample R68 in the 40 µL cyt b2 0.5X reaction produced a positive result (1/14, 7.1%). Here too, the qPCR contemporary controls produced Cq values of ~5-8 more cycles in comparison to standard conditions. No samples or qPCR contemporary controls amplified in the 125 µL cyt b2 0.16X reaction.

Experimental Primer Concentration Treatments

Over the standard primer concentrations of each at 0.24 µM (31/86, 36%) an increase to 1 µM of each primer (4.17X concentration over standard) resulted in an identical level of success (Sub-Project 3.1 Table 6). It is notable that the contemporary qPCR standards produced expected Cq values, but on an agarose gel showed target amplification with the addition of a larger band and smear. Increasing primer concentration to 2 µM of each (8.33X concentration over standard) performed similarity in success rate (3/22, 13.6%) over standard conditions (6/22, 27.3%) (p-value=0.2622, X^2 test).

CONCLUSIONS

We evaluated extended extension times, experimental qPCR volume increases and associated variations of increased reagent concentrations and increases in primer concentrations in their respective efficacies in amplifying DNA recovered from various rockfish (genus *Sebastes*) vertebrae recovered from the archaeological record. DNA eluates recovered from these samples were ideal to test our ideas, as they demonstrated to be variably compromised by PCR inhibitors. We used associate qPCR success rate and associate Cq to measure the experimental outcomes. Our expectations were that successful approaches will lead to decreased Cq values (i.e., indicated more DNA is available for amplification) over standard treatment and lead to statistically significant success in PCR amplification.

We hypothesized that increased extension times may subdue the influence of PCR inhibition. We found support for this in increased successes in the cyt b1 and cyt b3 qPCRs. However, there was no appreciable reduction in Cq values as we predicted. Cyt b2 reactions behaved

differently, where 1 min extension performed worse than standard conditions (and was associated with higher, not lower average Cq). We are unclear why this marker behaved differently. However, our recommendation is for those working with DNA samples compromised by PCR inhibition, an increase in extension time might be a key to their future success. The cost is only in time. Our standard cyt b reactions with 15 sec extension run for 2 hours and 4 minutes. The reactions with: 1) 1 min extension run for 2 hours and 47 minutes, 2) 5 min extension run for 6 hours and 47 min, and 3) 10 min extension run for 11 hours and 47 minutes (Sub-Project 3.1 Table 7).

We hypothesized that that subduing the influence of PCR inhibitors might also lie in *increased* reaction volumes, which results in increased physical distance between inhibitors, template DNA, and the other reagents. This prediction was unmet. At the extremes, the 125 µL reactions (i.e., at standard concentration), and 40 µL and 125 µL dilute reactions performed poorly. Thus, we do not recommend volume increases as an effective means of subduing inhibitory influences.

We also hypothesized that increased primer concentration might improve amplification of inhibited DNA eluates. We found no support for this.

Lastly, while some of these strategies aided in subduing PCR inhibition or at least produced equivocal results from ancient, degraded, and LCN DNA eluates, at least some of them cause contemporary qPCR DNA standards to behave far from “standard”.

Sub-Project 3.1. Table 1. Cytochrome b primers used in this study with targeted mitochondrial regions and amplicons lengths.

Target Region	Primers	Coordinates to *S. babcocki* (DQ678422)	Coordinates to *S. crameri* (DQ678437)	Sequence (5' to 3')	Amplicon Length
cyt b1	RockCB1F	2-20		TGGCAAGTCTACGAAAGAC	147
	RockCB1R	130-149		AATAGTCCTGTGAGGATTTG	
cyt b2	RockCB2F	502-521		TTCTCAGTAGACAATGCAAC	179
	RockCB2R	662-681		TTTGTATGAGAAGTAGGGGT	
cyt b3	RockCB3F		202-220	CATATTTGCCGGGACGTAA	185
	RockCB3R		365-386	ACGAAAGCAGTTATTATAACTA	

Sub-Project 3.1 Table 2. Intra- and inter-qPCR variability of quantification cycle (Cq) data observed from a contemporary rockfish DNA sample. Note the relatively low standard deviations, indicating that these three reactions reliably count copies of rockfish mitochondrial cytochrome b.

		qPCR 1		qPCR 2		Average	Standard Deviation
cyt b1	6.33 ng/µL	23	22.72	22.89	22.54	22.79	0.20
	1 ng/µL	25.59	25.29	25.66	25.31	25.46	0.19
	0.1 ng/µL	28.43	28	29.14	28.65	28.56	0.47
	0.01 ng/µL	31.97	31.71	31.58	31.45	31.68	0.22
	qPCR negative	na	na	na	na	na	na
cyt b2	6.33 ng/µL	23.71	23.31	24.20	24.06	23.82	0.40
	1 ng/µL	26.20	26.22	26.91	26.87	26.55	0.39
	0.1 ng/µL	29.52	29.22	30.32	29.97	29.76	0.49
	0.01 ng/µL	32.80	33.06	34.02	33.90	33.45	0.61
	qPCR negative	na	na	na	na	na	na
cyt b3	6.33 ng/µL	22.66	22.21	22.26	22.06	22.30	0.26
	1 ng/µL	25.19	25.34	24.97	24.73	25.06	0.27
	0.1 ng/µL	28.39	28.30	28.37	28.23	28.32	0.07
	0.01 ng/µL	32.21	32.02	31.92	31.65	31.95	0.23
	qPCR negative	na	na	na	na	na	na

Sub-Project 3.1 Table 3 Results from Cyt B1, Cyt B2, and Cyt B3 qPCR Reactions with Variable Extension Times (15 sec, 1 min, 5 min).

Marker		Standard, 15 sec extension	Cq	1 min extension	Cq	5 min extension	Cq
cyt b1	Overall	44/87 (50.6%)	40.17±6.34	66/87 (75.9%)	39.61±5.83	64/86 (74.4%)	39.06±6.02
	Uninhibited	17/17 (100%)	37.55±4.50	16/17 (94.1%)	37.12±4.27	16/17 (94.1%)	35.00±1.81
	Inhibited	27/70 (38.6%)	41.81±6.84	50/70 (71.4%)	40.40±6.07	48/69 (69.6%)	40.41±6.33
cyt b2	Overall	41/86 (47.7%)	40.66±5.30	31/86 (36.0%)*	45.08±7.28	42/86 (48.8%)†	42.52±6.78‡
	Uninhibited	14/17 (82.4%)	39.86±4.94	8/17 (60.0%)*	42.28±6.45	12/17 (70.6%)	40.52±5.77
	Inhibited	27/69 (39.1%)	41.07±5.52	23/69 (33.3%)	46.18±7.43	30/69 (43.5%)†	45.13±7.48‡
cyt b3	Overall	31/86 (36%)	42.20±7.78	55/86 (64%)	39.67±6.12	32/86 (37.2%)§	43.68±7.93
	Uninhibited	10/17 (58.8%)	40.58±6.23	12/17 (70.6%)	39.49±6.03	8/17 (47.1%)§	44.07±8.48
	Inhibited	21/69 (30.4%)	43.27±8.42	43/69 (62.3%)	39.72±6.22	24/69 (34.8%)	43.56±7.92

*One sample (LR4) produced a band with an associated Cq of 43.22. However, the sequencing failed. We did not consider this a success.
†One sample (R30) produced a band with an associated Cq of 57.89. However, the sequencing failed. We did not consider this a success.
‡Two the of 42 amplifications (samples R10 and R37) were associated with extra smaller, non-target bands. Thus, their respective Cq values of 36.42 and 40.51 were not included in this average.
§One sample (LR24) produced a band with an associated Cq of 52.31. However, the sequencing failed. We did not consider this a success.

Sub-Project 3.1 Table 4- Results from Cyt B1 experiments with 10 min extension.

Marker		Standard, 15 sec extension*	Cq	1 min extension*	Cq	5 min extension*	Cq	10 min extension	Cq
cyt b1	Overall	20/27 (74.1%)	37.33±4.47	22/27 (81.5%)	37.45±6.25	21/27 (77.8%)	36.19±4.52	20/27 (74.1%)	37.26±4.74
	Uninhibited	12/12 (100%)	36.42±1.99	11/12 (91.7%)	35.73±2.50	11/12 (91.7%)	34.77±1.65	11/12 (91.7%)	36.41±2.81
	Inhibited	8/15 (53.3%)	38.71±6.66	11/15 (73.3%)	39.17±8.33	10/15 (66.7%)	37.74±6.10	9/15 (60.0%)	38.29±6.42

*Comparative subsection of the overall data from standard conditions from **Sub-Project 3.1 Table 3** for cyt b1 reported here for comparison to the more limited testing of a 10 minute extension

Sub-Project 3.1 Table 5. Results of Experimental Volume Treatments Observed in the Cyt B2 qPCRs.

	20 µL*	Cq	40 µL	Cq	60 µL	Cq	80 µL	Cq	100 µL	Cq	125 µL	Cq	40 µL (0.5 X dilute)	Cq	125 µL (0.16X dilute)	Cq
Overall	4/14 (28.6 %)	36.5± 1.62	7/14 (50.0 %)	45.21± 3.81	6/14‡ (42.9 %)	53.84± 3.68	3/14 (21.4 %)	46.93± 3.68	1/14 (7.1 %)	56.6†	0/47 (0.0 %)	N/A	1/14 (7.1 %)	N/A¶	0/14 (0.0 %)	N/A
Uninhibited	2/6 (33.3 %)	36.31†	3/6 (50.0 %)	44.92± 4.92	3/6‡ (50%)	51.36†	1/6 (16.7 %)	47.03§	1/6 (16.7 %)	56.6†	N/A	N/A	1/6 (16.7 %)	N/A¶	N/A	N/A
Inhibited	2/8 (25.0 %)	36.70†	4/8 (50.0 %)	45.44± 3.58	3/8 (37.5 %)	55.49± 4.11	2/8 (25.0 %)	46.88†	0/8 (0.0 %)	N/A	N/A	N/A	0/8 (0.0 %)	N/A	N/A	N/A

*Subset of data taken from the standard cyt b2 qPCRs reported in **Sub-Project 3.1 Table 3**
†No standard deviation to report, as only two measures recorded
‡One of these success amplifications (sample LR4, identified as a damaged *Sebastes* haplotype 30) did not produce a Cq value
§Not an average, as only one measure recorded
¶The one success amplifications (sample R68, identified as an undamaged *Sebastes* haplotype 67) did not produce a Cq value

Sub-Project 3.1 Table 6 Results of Experimental Primer Concentrations in Cyt B3 qPCRs.

* data taken from the standard cyt b3 qPCRs reported in **Sub-Project 3.1 Table 3**
† one of these success amplifications (sample R56, identified as an undamaged *Sebastes* haplotype 49) did not produce a Cq value

	Standard*	Cq	1µM of each primer (4.17X standard reaction)	Cq	Standard‡	Cq	2µM of each primer (8.33X standard reaction)	Cq
Overall	31/86 (36.0%)	42.20±7.78	31/86 (36.0%)†	40.33±6.22	6/22 (27.3%)	43.54±9.44	3/22 (13.6%)	43.50±10.46
Uninhibited	10/17 (58.8%)	40.58±6.23	10/17 (58.8%)	37.57±2.69	2/2 (100%)	36.59§	1/2 (50.0%)	44.31¶
Inhibited	21/69 (30.4%)	43.27±8.42	21/69 (30.4%)†	41.70±7.04	4/20 (20.0%)	46.96¶	2/20 (10.0%)	43.16±9.84¶

‡ subset of data taken from the standard cyt b3 qPCRs reported in **Sub-Project 3.1 Table 3**
§ not an average, as only one measure recorded
¶ no standard deviation to report, as only two measures recorded

Sub-Project 3.1. Table 7. Reaction times with extended extension for cyt b1-3 amplicons.

Extension Time (s/min)	Reaction Time (Hours:Minutes)
15/0.25	2:04
60/1.0	2:47
300/5.0	6:47
600/10.0	11:47

Sub-Project 3.1 Supplemental Table 1. Results from this Sub-Project 3.1.

Tab A: Initial species identification based on 148 bp mitochondrial DNA 12S gene sequences

Color coding and symbols used in the following tabs. Green Cq values are indicators of target amplification. Red cells labeled N/A indicate failed production of targeted amplicons. Yellow cells highlight sequences likely compromised by post-mortem nucleotide damage. Orange cells demarcate sequences with no similarity found in GenBank (likely originating from microorganisms). Dark blue cells demarcate failed sequencing reactions and light blue/aqua colored cells indicated successful amplifications with no associated Cq values.

Tab B: Results from the cyt b1 extension time experiments

Tab C: Results from the cyt b2 extension time experiments

Tab D: Results from the cyt b3extension time experiments

Tab E: Results from the cyt b2 volume experiments

Tab F: Results from the cyt b3 primer concentration experiments

Sub-Project 3.1 Supplemental Table 2. Rockfish haplotype chart.

This supplemental table reports rockfish haplotypes as reported by Hyde and Vetter (2007) for the 12S, cyt b1, cyt b2, and cyt b3 amplicons. Keys are provided numerically and alphabetically to the right of the haplotype charts. This table was updated in August 2021 to included observations of these haplotypes amongst species not observed by Hyde and Vetter (2007) (these updates are highlighted in orange).

Tab A: 12S haplotypes

Tab B: cyt b1 haplotypes

Tab C: cyt b2 haplotypes

Tab D: cyt b3 haplotypes

Sub-Project 3.2 "Why Don't We Run 100 Cycle PCRs?" (NOTE: proposed title for publication)

All things being equal, increased number of PCR cycles should increase the opportunity for amplification of low copy number templates (i.e., initiated in increasingly later cycles) and simultaneously increase concentrations of amplicons produced (regardless on starting template copy number).

In other words, *why do we not routinely conduct 100 cycle PCRs*? *or those in greater cycle numbers*? Are there *associated costs*, simply beyond the (obvious) cost of time to run higher number cycle PCRs? Is there an associated cost to increased cycling, such as the production of *PCR artifacts and/or aberrant results*?

We tested these ideas as follows. First, we addressed whether PCR cycling conditions alone, were damaging to DNA standards in a master-mix sans polymerase (i.e., not allowing amplification to occur). Four DNA standards ranging from highly concentrated and intact to degraded and low copy number were subject to PCRs run in this manner for 40, 60, 80, 100, 200, and 600 cycles.

Secondly, using DNA recovered from archaeological fish remains morphologically identified as rockfish (genus *Sebastes*) we qPCR amplified, with 40-500 cycles, a short stretch of the mitochondrial 12S gene, one useful for identifying remains as rockfish (Moss et al., In Press) as well as fish potentially belonging to other taxonomic groups. These primers were called "universal" by their creators (Jordan et al., 2010) and in our experience they are robust for amplifying DNA from distantly related fish (and even some mammalian) taxa. In addition to recording their associated Cq values (i.e., indicating something about their relative starting concentration of the template), we also quantified the concentration of target DNA produced from these treatments and sequenced all target amplicons to confirm their authenticity (i.e., fish 12S mtDNA) and to observe potential discrepancies or artifacts. We recognize that a PCR will eventually hit a saturation point, where one of the reagents becomes limiting or the polymerase loose sufficient activity. After this point, we would expect degradation to the amplicons due to repeated heating of the DNA (Phase II) if amplicons are not being replace by the PCR. In any case, this area of inquiry has been inadequately explored and one of great curiosity for us.

From these investigations we sought to determine which treatments are superior in their success rate of amplification from these low copy number, aged, and degraded template molecules, especially for considering which yield more amplicons over others. In this case we equate increased concentration as success, on top of amplification success alone. There would be simply more molecules for sequencing, or resequencing in the case of failure, which happens in Sanger sequencing from time to time (Kemp-unpublished observation).

MATERIALS AND METHODS

Evaluating the Influence of PCR Conditions (Without the Addition of Polymerase) on the Integrity of DNA Standards

We created four standards from DNA extracted from pig (*Sus scrofa*) liver with the Qiagen DNeasy Blood & Tissue Kit, following the manufacturer's protocol. Quantities and qualities of the standards were determined using an Agilent Fragment Analyzer (Sub-Project 3.2 Supplemental Table 1- Tab A), binned into the following eight categories: 1) 20-100 bp, 2) 100-

300 bp, 3) 300-500 bp, 4) 500-1,000 bp, 5) 1,000-3,000 bp, 6) 3,000-5,000 bp, 7) 5,000-10,000 bp, and 8) 10,000-20,000 bp.

> **Standard 1**: intact genomic DNA, with the bulk of the molecules (62.74%) in the range of 3,000-5,000 bp, at 61.08 ng/µL
>
> **Standard 2**: intact and diluted genomic DNA at 0.78 ng/µL, with the bulk of the molecules ranging 10,000-20,000 bps (45.68%)
>
> **Standard 3**: DNA sonically sheared with a QSonica, with the bulk of the molecules (82.59%) in the range of 20 to 100 bp, at a concentration of 31.95 ng/µL
>
> **Standard 4**: DNA sonically sheared and dilute, with the bulk of the molecules (50.38%) in the range of 100 to 300 bp, at a concentration of 1.43 ng/µL

Each standard was used as template DNA in PCRs containing all components except the polymerase (for which we substituted water). Fifteen microliter PCR reactions contained: 1X Omni Klentaq Reaction Buffer (including a final concentration of 3.5 mM $MgCl_2$), 0.32 mM dNTPs, 0.24 µM of each primer, and 1.5 µL of standard DNA template. First, we measured the impact of no cycling on a sample of the standards (i.e., simply the standards as mixed with the buffer, dNTPs, and primers). Subsequently we treated 1.5 µL volumes of the of the standards to the following cycling conditions. Following denaturing at 94˚C for 3 minutes, 20, 40, 60, 80, or 100 cycles of PCR were conducted at 94˚C for 15 s, 60˚C for 15 s, and 68˚C for 15 s. Finally, a 3-minute extension period at 68˚C was conducted prior to bringing the reactions to 10˚C. After sampling the end product of 100 cycles, these reactions were subjected to the following: Following denaturing at 94˚C for 3 minutes, 500 cycles of PCR were conducted at 94˚C for 15 s, 60˚C for 15 s, and 68˚C for 15 s. Finally, a 3-minute extension period at 68˚C was conducted prior to bringing the reactions to 10˚C (in this latter case, the standard was subject to one round of hot start denaturing, followed by 100 cycles of PCR, followed by another round of hot start denaturing and 500 cycles of PCR- we simply refer to this as being treated with 600 cycles of PCR).

Following treatment, the quality and quantity of the standards were determined with the Fragment Analyzer across the same categories: 1) 20-100 bp, 2) 100-300 bp, 3) 300-500 bp, 4) 500-1,000 bp, 5) 1,000-3,000 bp, 6) 3,000-5,000 bp, 7) 5,000-10,000 bp, and 8) 10,000-20,000 bp. Changes in quality were measured as observable shifts the percentages of molecules across these eight binned categories (as compared to non-treatment controls). Changes in quantity were measured as observable shifts in ng/µL (as compared to non-treatment controls). This follows the simple comparison to “DNA in” compared to “DNA out” which served as a foundation in Phases I and II of the project.

Evaluating the Influence of Increased PCR Cycle Number on Amplification Success

Samples

We studied DNA eluates recovered from 14 samples described in Sub-Project 3.1- Tab B and their associated extraction negative controls (samples R85-R98 and extraction negative controls 13 and 14).

Quantitative PCR Assays

We amplified all eluates for a 189 bp fragment of the mitochondrial 12S gene using "universal" fish primers (Jordan et al., 2010). Note that Jordan et al. (2010). originally described their reverse primer in the wrong orientation. The corrected primers are OST12S-F (5'-GCTTAAAACCCAAAGGACTTG-3') and OST12S-R (5'-CTACACCTCGACCTGACGTT-3').

Quantitative PCR reactions of 20 µL contained 1X SsoAdvanced™ Universal SYBR® Green Supermix, 0.125 mg/mL bovine serum albumin (BSA), 1.0 µM of each primer, and 1 µL of template DNA (Sub-Project 3.2 Table 1). Reaction conditions were as follows: 1) 3 min denaturation at 94°C, 2) 40, 60, 80, 100, 200, or 500 cycles of 15 s holds at 94°C, 56°C, and 72°C (with a plate read following each cycle), and 3) a final extension at 72°C for 3 min. The qPCR reactions were followed by melt curve analysis starting at 65°C and increasing the temperature by 0.5°C, after which the plate was read. This was conducted 60 times, ultimately reaching a temperature of 95°C. Reactions were cooled to 10°C. All qPCR reactions were conducted in a CFX96 Touch Deep Well Real-Time PCR Detection System.

We note here that these 12S primers are exceptional in their ability to amplify mtDNA from a large number of distantly related fish species, including salmon (*Oncorhynchus spp*.), herring (Clupeidae), smelt (Osmeridae), snakehead fish (*Channa spp*.) as well as cartilaginous fish (Triakidae; Hound Sharks) (Palmer et al., 2018; Lanman et al., 2021; Kennedy et al., in press). However, they are prone to the production of primer-dimer and sometimes co-amplify an ~100 bp segment of microorganismal DNA most closely related to species of *Pseudomonas*[‡‡‡‡]. Moreover, as demonstrated in this sub-project, they do not perform well on contemporary DNA controls of rockfish.

Thus, while we report and evaluate Cq values here, we have included notes as to whether this indicates amplification of target alone or was influence by primer-dimer formation and/or non-target amplification (Sub-Project 3.2 Supplemental Table 1- Tab B). Success in this sub-project is measured by amplification success, considered independent of the associated Cq values, and the evaluation of whether there is a relationship between cycle number and aberrant sequencing results.

Study of Amplicons

We sequenced from sub-volumes of the amplicons produced from the qPCRs as described under Sub-Project 3.1 and were placed in the context of the study of rockfish mtDNA variation by Hyde and Vetter (2007) (see pertinent comparative 12S data summarized in Sub-Project 3.1 Supplemental Table 2 Tab A). We utilized NCBI's BLAST function (Altschul et al., 1990) in search of novel and non-rockfish haplotypes observed in our study against the records of Genbank (Sayers et al., 2020).

Additional sub-volumes of these amplicons were subject to analysis Agilent Fragment Analyzer with the High Sensitivity Genomic DNA Analysis Kit (DNF-488) for products from 40-200 cycles reactions and High Sensitivity NGS Fragment Analysis Kit (DNF-474) for those produced from 500 cycle reactions. Manufacturer's instructions were followed. This allowed us to measure the concentration of the target amplicons.

‡‡‡‡ "*Pseudomonas* is a genus of Gram-negative, Gammaproteobacteria, belonging to the family Pseudomonadaceae and containing 191 validly described species. The members of the genus demonstrate a great deal of metabolic diversity and consequently are able to colonize a wide range of niches" (Wikipedia accessed October 3, 2021).

Comparing Efficacy of 80 Cycle PCR Over 60 Cycle PCR of Natural State DNA, 1:10 Dilutions of that Natural State DNA, and DNA Eluates Subjected to Repeated Silica Extraction

Samples

We further studied DNA eluates recovered from 91 samples described in Sub-Project 3.1 Supplemental Table 1 and their associated extraction negative controls (samples R8-R98 and extraction negative controls 2-14[§§§§]). In addition, we studied DNA eluates recovered from an additional seven fish vertebrae morphological identified as rockfish (i.e., genus *Sebastes*) and an accompanying extraction negative control (samples R99-R105). Extraction, purification, and inhibitor removal followed that described in Sub-Project 3.1)

PCR

Amplification of 189 bp region of the mitochondrial 12S gene (as described above) was compared using 80 cycle PCR on volumes of natural state DNA (i.e., non-manipulated, post extraction) in comparison to: 1) natural state DNA PCR amplified for 60 cycles, 2) 1:10 dilution of the natural state DNA PCR amplified for 60 cycles, 3) repeat silica extracted (i.e., inhibitor "free") volumes of the same samples PCR amplified for 60 cycles. PCRs contained 1X Omni Klentaq Reaction Buffer, 0.32 mM dNTPs, 0.24 µM of each primer (OST12S-F and OST12S-R; see above), 0.3 U of Omni Klentaq LA polymerase, and 1.5 µl of template DNA (natural state, diluted, or inhibitor free). PCR conditions were as follows: denaturing at 94˚C for 3 minutes followed by 60 or 80 cycles of PCR conducted at 94˚C for 15 s, 55˚C for 15 s, and 68˚C for 15 s. Finally, a 3-minute extension period at 68˚C was conducted prior to bringing the reactions to 10˚C.

All targeted amplicons were sequenced as described under Sub-Project 3.1 and placed into the context of rockfish haplotypes in the study of Hyde and Vetter (2007) (Sub-Project 3.1 Supplemental Table 2).

Statistical Analysis

Chi square (X^2) tests were used to compare success rates between treatments (we used a free on-line X^2 calculator[*****]) and two-tailed t-tests were conducted to compare treatments in their average Cq values and associated standard errors (we used a free on-line statistical calculator[†††††]). An alpha level of 0.05 was set as the cut-off for statistical significance of the tests.

RESULTS AND DISCUSSION

Evaluating the Influence of PCR Conditions (Without the Addition of Polymerase) on the Integrity of DNA Standards

An initial, unexpected result was observed in our experiments aimed to determine the influence of PCR conditions (minus polymerase) on DNA integrity: the quantity and quality of the DNA

[§§§§] Volumes of sample R38 and extraction negative control 11 were studied in this sub-project, before being inadvertently lost as described in Sub-Project 3.1

[*****] https://www.socscistatistics.com/tests/chisquare/default2.aspx

[†††††] https://www.medcalc.org/calc/comparison_of_means.php

standards appeared differently according to fragment analysis when measured in a mixture of PCR reagents (but not subjected to heat) (Sub Project 3.2 Supplemental Table 1 Tab A). For example:

> **Standard 1 (alone)**: intact genomic DNA, with the bulk of the molecules (62.74%) in the range of 3,000-5,000 bp, at 61.08 ng/µL
>
> **Standard 1 (in Klentaq PCR minus polymerase)**: intact genomic DNA, with the bulk of the molecules (58.53%) in the range of 20 to 100 bp and (31.21%) in the range of 5,000-10,000 bp, at 75.37 ng/µL

Standards 2-4 were also read at greater concentration and with increased concentration of small fragments over untreated standards. All these shifts can be visually appreciated by comparing the red curves (standards alone) to light orange curves (standards in Klentaq PCR) in Sub Project 3.2 Figure YY. Presently, we do not have an explanation for this observation, but believe that comparisons between standards in Klentaq PCR minus polymerase can be made between untreated (not subjected to thermocycling) and treated (subjected to rounds of thermocycling).

Subjection of standards 1-4 all reveal the degradation of large fragments, resulting the in accumulation of smaller fragments (Sub Project 3.2 Supplemental Table 2 Tab A and Sub Project 3.2 Figure 1). This demonstrates that PCR conditions alone are damaging to DNA strands. In other words, if the PCR is not building (i.e., with polymerase and necessary substrates) it is destroying. Thus, excessive cycling may come at cost.

Evaluating the Influence of Increased PCR Cycle Number on Amplification Success

Across cycle number treatments, 80 cycles produced the greatest success, with 11/14 samples amplifying (78.6%), but not statistically so compared to 40 cycles (chi-squared test; 42.9%, p=0.5302), 60 cycles (57.1%, p=0.2248), 100 cycles (57.1%, p=0.2248), or 200 cycles (p=0.2248). Five hundred cycle PCR performed poorest, with 28.6% success (compared to 80 cycle PCR, p=0.0079). Targeted was amplification was confirmed by sequencing these amplicons, the results of which are described below.

Samples R88, R89, R92, and R94 amplified in all reactions ranging from 40 to 500 cycles. R96 and R97 amplified in all reactions except those of 500 cycles. Samples R93 and R98 amplified only with ≥60 cycles, and R85, R87, R90, and R95 amplified only with ≥80 cycles. Samples R87 and R91 did not amplify under any conditions during this sub-project, but yielded cyt b results during sub-project 3.1).

Importantly, these results support the use of higher than standard numbers of PCR cycles in the study of aged, degraded, and low copy number DNA eluates.

Study of Amplicons

Sequencing the 12S amplicons from samples R86-93, R95, R96, R98 reveal them to be rockfish (genus *Sebastes*). Sample R85 revealed a 92.62% match to *Centopomus undecimalis* (Common Snook) (GenBank Accessions: KC441979.1 and MK616995.1), R94 is a 94.56% match to *Scorpaena scrofa* (Red Scorpionfish) (GenBank Accession: DQ125234.1), and R97 is 100% match to *Scorpaenichthys marmoratus* (Cabezon) (GenBank Accession: KM057987.1).

There were no contradictions between sequencing results where >2 amplicons were produced from a specimen (e.g., R88 produced the sample haplotype across the six reactions spanning 40-500 cycles). Samples R85, R90, and R95 each produced amplicons only with 80 cycles. R85 revealed a 92.62% match to *Centopomus undecimalis* (Common Snook) (GenBank Accessions: KC441979.1 and MK616995.1). Sample R90 revealed a 12S haplotype 1 (Sub Project 3.1 Supplemental Table YY Rockfish Haplotype Chart) derived at np C162T. There is no 100% match to this haplotype on Genbank. Thus, this could represent either a novel haplotype or one that shows damaged at np 162. Since these result were unrepeatable, we cannot determined if the sequence shows signs of post-mortem nucleotide damage [(i.e., "damage", the most common form of which is deamination of cytosines which lead to artifactual C>T "transitions" (Gilbert et al., 2007)]. Lastly, sample R95 revealed a 12S haplotype 6, the most common rockfish haplotype.

Overall, these results do not reveal a relationship between increased cycling and the accrual of PCR artifacts and/or aberrant results.

Regarding the production of amplicons, 80 cycles produced the highest concentration (140±33.09 ng/µL), which was not statistically different from the production of amplicons with 100 cycles (two-tailed t-test; p=0.8116) or 200 cycles (p=0.1659) (Sub-Project 3.2. Table 1; Sub Project 3.2 Supplemental Table 1 Tab C). Eighty cycles of PCR produced higher concentrations of amplicons in comparison to that produced with 40 cycles (p<0.0001), 60 cycles (p=0.0016), and 500 cycles (p=0.0004). Visual depiction of these results in found in Sub-Project 3.2. Figure 3.

Additional Observations

An interesting and unexpected set of results were observed. Amplification of samples fell into one of two basic "behaviors" where Cq variance could be observed. Samples R87, R89, R92, R93, and R96 had standard deviations of >20% that of their respective means. Interestingly these same sample show a relationship between Cq value and total PCR cycle number (i.e., the samples that are not flat lined in Sub-Project 3.2. Figure 2**).** The other samples R88, R94, and R97 have standard deviations of <6% that of their respective means and do not show a positive relationship between Cq and cycle number. These samples are shown to be flat-lined in Sub-Project 3.2. Figure 2**.**

Comparing Efficacy of 80 Cycle PCR Over 60 Cycle PCR of Natural State DNA, 1:10 Dilutions of that Natural State DNA, and DNA Eluates Subjected to Repeated Silica Extraction

Samples

Using sixty cycles of PCR, 7 of the 91 (7.7%) archaeological fish samples amplified from raw (untreated) DNA using the 12S primer (Sub Project 3.2 Supplemental Table 1 Tab C). Sixty cycles on 1:10 dilutions of the sample DNA eluates performed similarly (6/91; 6.6%), as did increasing the cycling the number to 80 in the raw DNA eluates (6.6%). Only after inhibitor "removal" (via repeated silica purification) did the success rate increase, employing 60 cycles (19/91; 20.9%; p-value<0.05 in comparison to other treatments).

CONCLUSIONS

We designed this set of experiments around the question: “why don’t we run 100 cycle PCRs? or those in greater cycle numbers?” Beyond the obvious cost to time, are there any other costs? Are there benefits?

We tested these ideas as follows. First, we addressed whether PCR cycling conditions alone, was degrading to DNA in the master-mix sans polymerase (i.e., not allowing amplification to occur). Four DNA standards ranging from highly concentrated and intact to degraded and low copy number were subject to PCRs run in this manner for 24, 40, 60, 80, 100, 200, and 600 cycles. We found that DNA mixed in such a master mix and subjected to thermocycling conditions is subject to degradation. In other words, if PCR is not building, it can be destroying. This suggests that cycling beyond some point can be counterproductive.

Using DNA recovered from archaeological fish remains morphologically identified as rockfish (genus *Sebastes*) we qPCR amplified, with 40-500 cycles, a short stretch of the mitochondrial 12S gene, one useful for identifying remains as rockfish (Moss et al., In Press) as well as fish belonging to other taxonomic groups. According to these experiments, 80 cycles performed best as measured by percent success in amplification combined with concentration of amplicons produce. However, increasing cycling alone was not determined to alleviate the influence of PCR inhibitors over their removal from DNA eluate by repeated silica purification.

We urge other to further determine which treatments are superior in their success rate of amplification from such low copy number, aged, and degraded template molecules, especially for considering which yield more amplicons over others. In this case we equate increased concentration as success. While not evaluated here, our logic might be easily applied to STR genotyping to allow allele detection to cross the desire relative *fluorescence* units (RFU). If a sample can be genotyped with 100 cycles, but not less, this is would be incredibly valuable to know.

Sub-Project 3.2. Table 1. Results of amplification with 40-500 cycles of PCR.

		40 Cycles			60 Cycles			80 Cycles			100 Cycles			200 Cycles			500 Cycles			
Sample	Inhibited?	Amp?	Cq	Conc. (ng/µL)	Amp?	Cq	Conc. (ng/µL)	Amp?	Cq	Conc. (ng/µL)	Amp?	Cq	Conc. (ng/µL)	Amp?	Cq	Conc. (ng/µL)	Amp?	Cq	Conc. (ng/µL)	Average Cq and Standard Deviation
R85	Yes	✗	N/A		✗	N/A		✔	51.51	88.37	✗	N/A		✗	N/A		✗	N/A		51.15
R86	Yes	✗	N/A		✗	N/A		✗	N/A		✗	N/A		✗	N/A		✗	N/A		
R87	Yes	✗	N/A		✗	N/A		✔	38.61	116.97	✔	67.51	62.25	✔	90.89	77.30	✗	N/A		65.67±26.19
R88	Yes	✔	36.16	11.11	✔	35.12	96.30	✔	34.05	147.97	✔	35.21	150.64	✔	35.09	122.35	✔	36.62	63.95	35.38±0.91
R89	Yes	✔	36.48	8.88	✔	34.60	98.50	✔	34.14	163.56	✔	34.61	153.23	✔	38.02	1.19	✔	57.67	75.94	39.25±9.14
R90	Yes	✗	N/A		✗	N/A		✔	54.03	86.82	✗	N/A		✗	N/A		✗	N/A		54.03
R91	Yes	✗	N/A		✗	N/A		✗	N/A		✗	N/A		✗	N/A		✗	N/A		
R92	No	✔	35.15	13.05	✔	34.78	95.83	✔	33.78	163.84	✔	42.90	135.63	✔	55.45	134.27	✔	37.42	39.69	39.91±8.28
R93	Yes	✗	N/A		✔	46.56	27.73	✔	43.83	117.28	✔	61.83	99.97	✔	87.32	112.07	✗	N/A		59.89±19.93
R94	Yes	✔	36.07	10.74	✔	34.88	83.72	✔	32.13	171.28	✔	33.37	186.89	✔	34.82	190.39	✔	37.57	46.07	34.81±1.92
R95	Yes	✗	N/A		✗	N/A		✔	32.08	150.81	✗	N/A		✗	N/A		✗	N/A		32.08
R96	Yes	✔	31.91	24.48	✔	34.53	91.66	✔	32.63	150.88	✔	36.40	139.07	✔	57.11	116.08	✗	N/A		38.52±10.54
R97	No	✔	35.49	17.67	✔	35.98	127.73	✔	35.38	184.86	✔	35.36	162.21	✔	37.05	135.67	✗	N/A		35.85±0.72
R98	Yes	✗	N/A		✔	44.38	57.21	✗	N/A		✗	N/A		✗	N/A		✗	N/A		44.38
		6/14 (42.9%)		14.32±5.30	8/14 (57.1%)		84.83±30.12	11/14 (78.6%)		140.24±33.09	8/14 (57.1%)		136.24±38.79	8/14 (57.1%)		111.17±54.51	4/14 (28.6%)		56.41±16.58	

Sub-Project 3.2. Table 2. Reaction times employing 40-500 cycles.

Cycle Number	Reaction Time (Hours:Minutes)
40	1:30
60	2:03
80	2:36
100	3:08
200	5:51
500	13:59

Sub-Project 3.2 Supplemental Table 1. Results from Sub-Project 3.2.

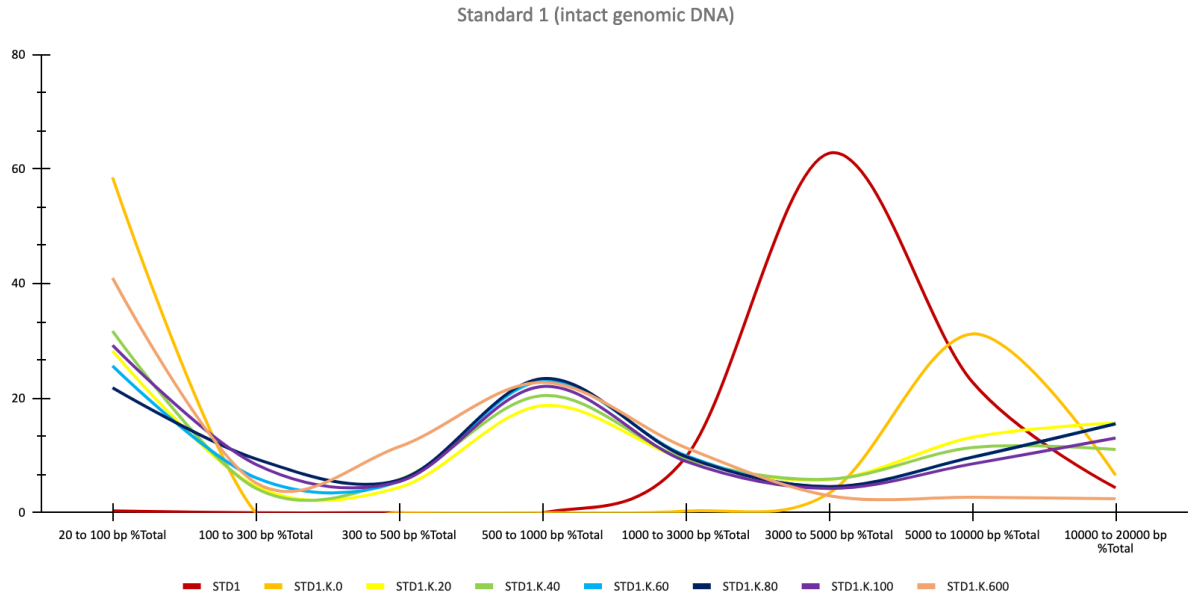
Tab A: Fragment analyzer results from standards subjected to thermocycling sans polymerase.

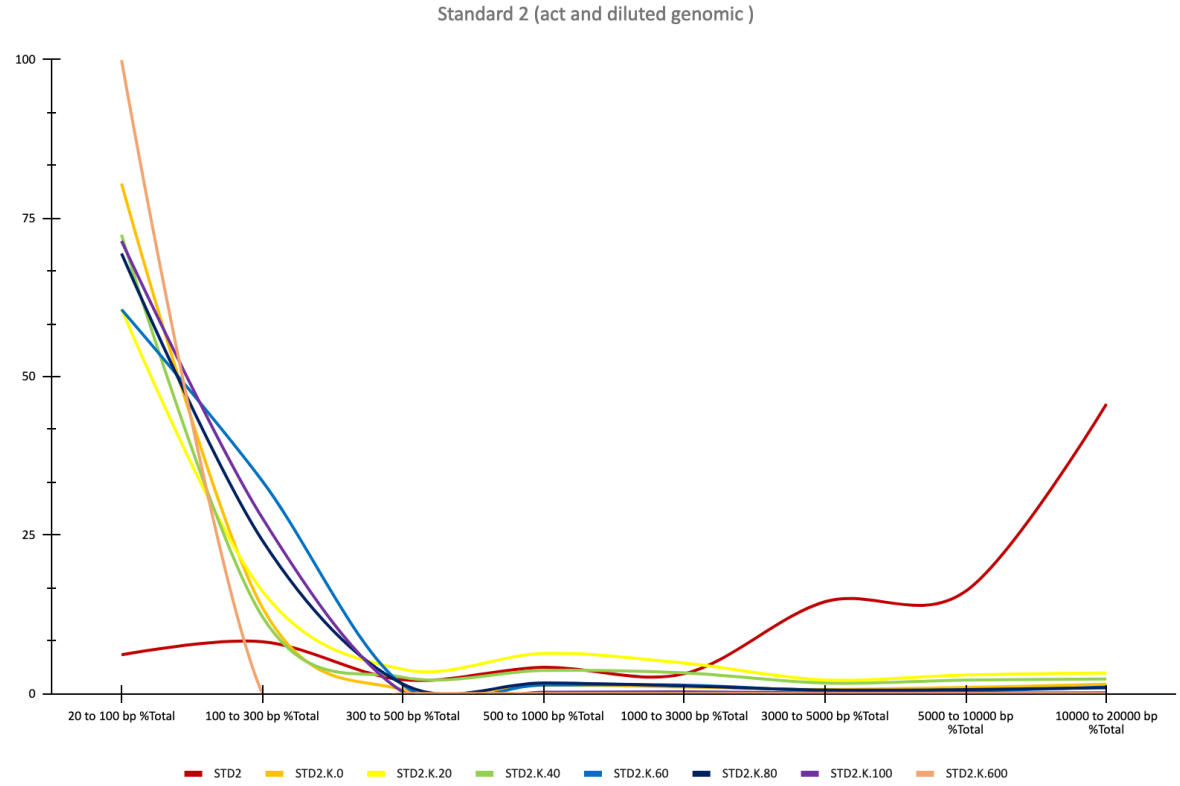
Color coding and symbols used in the following tabs: Green Cq values are indicators of target amplification. Red cells labeled N/A indicate failed production of targeted amplicons. Yellow cells highlight sequences likely compromised by post-mortem nucleotide damage.

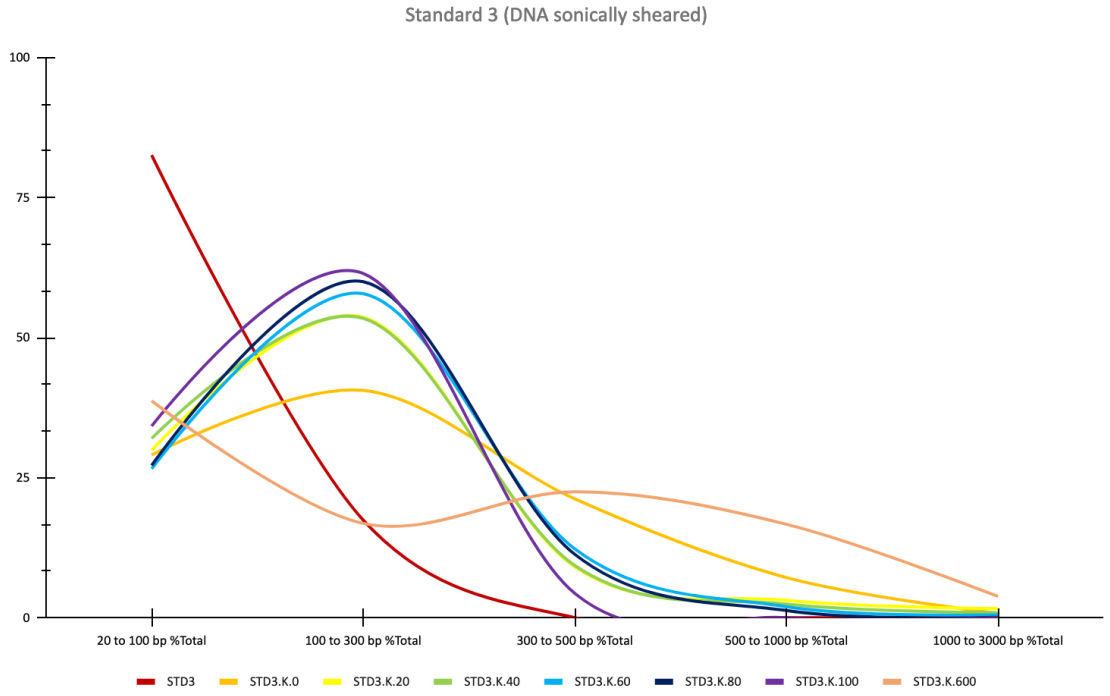
Tab B: Results of amplification of mitochondrial 12S from archaeological rockfish DNA eluates using 40-500 cycles.

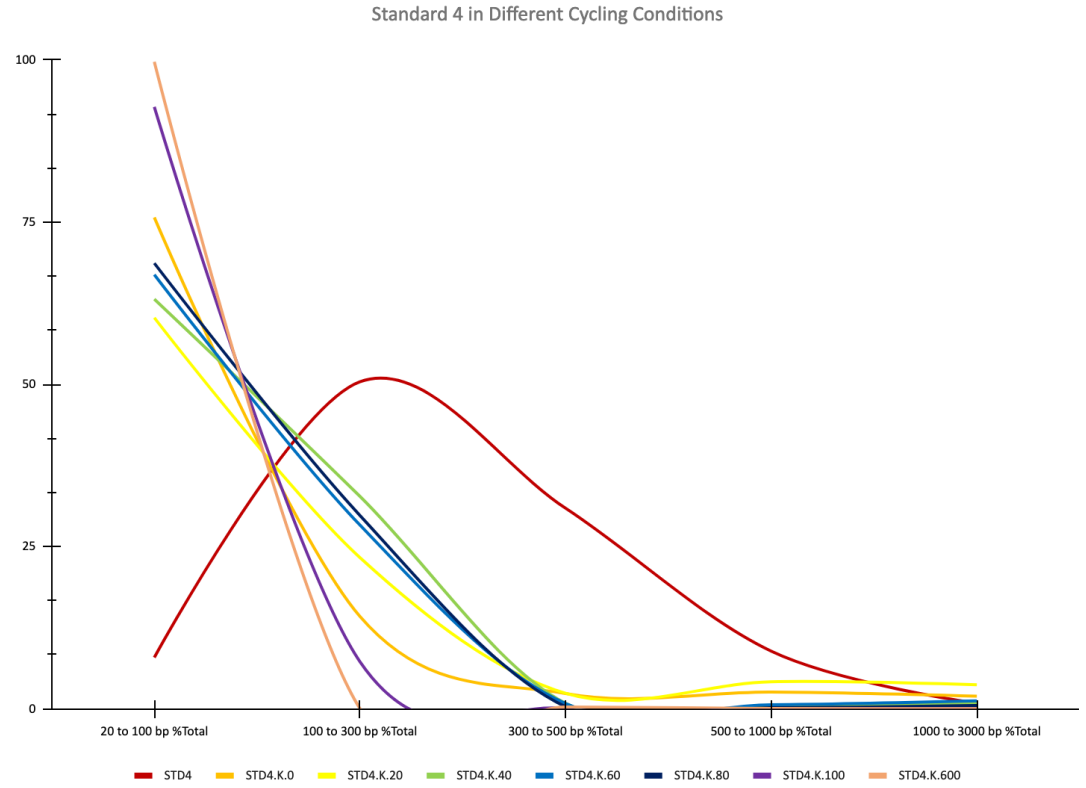
Tab C: Comparison of the Efficacy of 80 Cycle PCR Over 60 Cycle PCR of Natural State DNA, 1:10 Dilutions of that Natural State DNA, and DNA Eluates Subjected to Repeated Silica

Sub-Project 3.2. Figure 1. Visual depiction of results reported in Sub-Project 3.2. Table 1

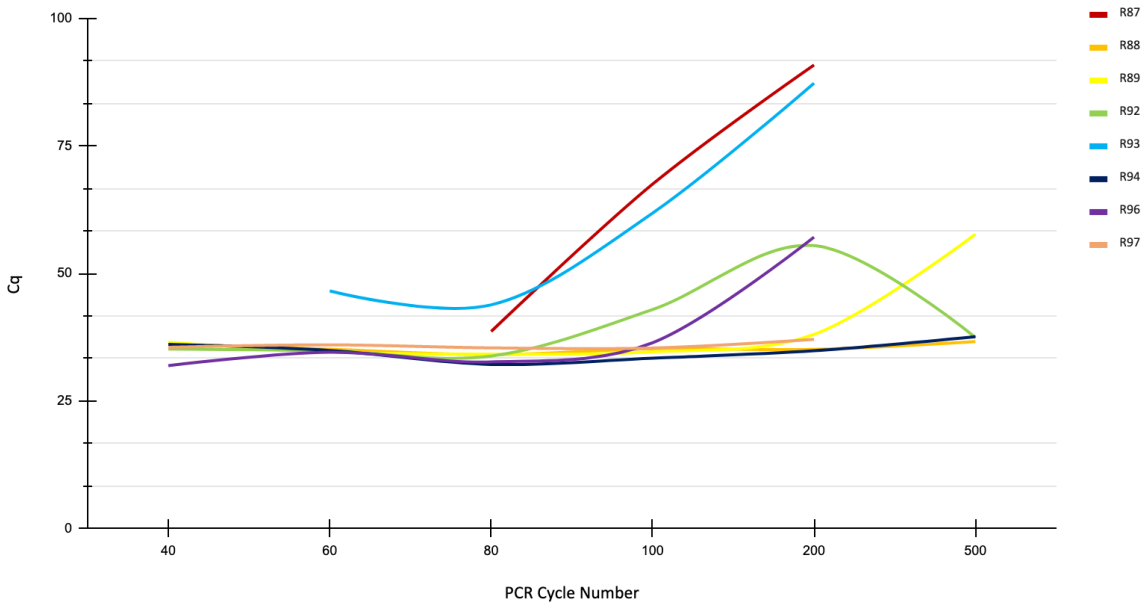




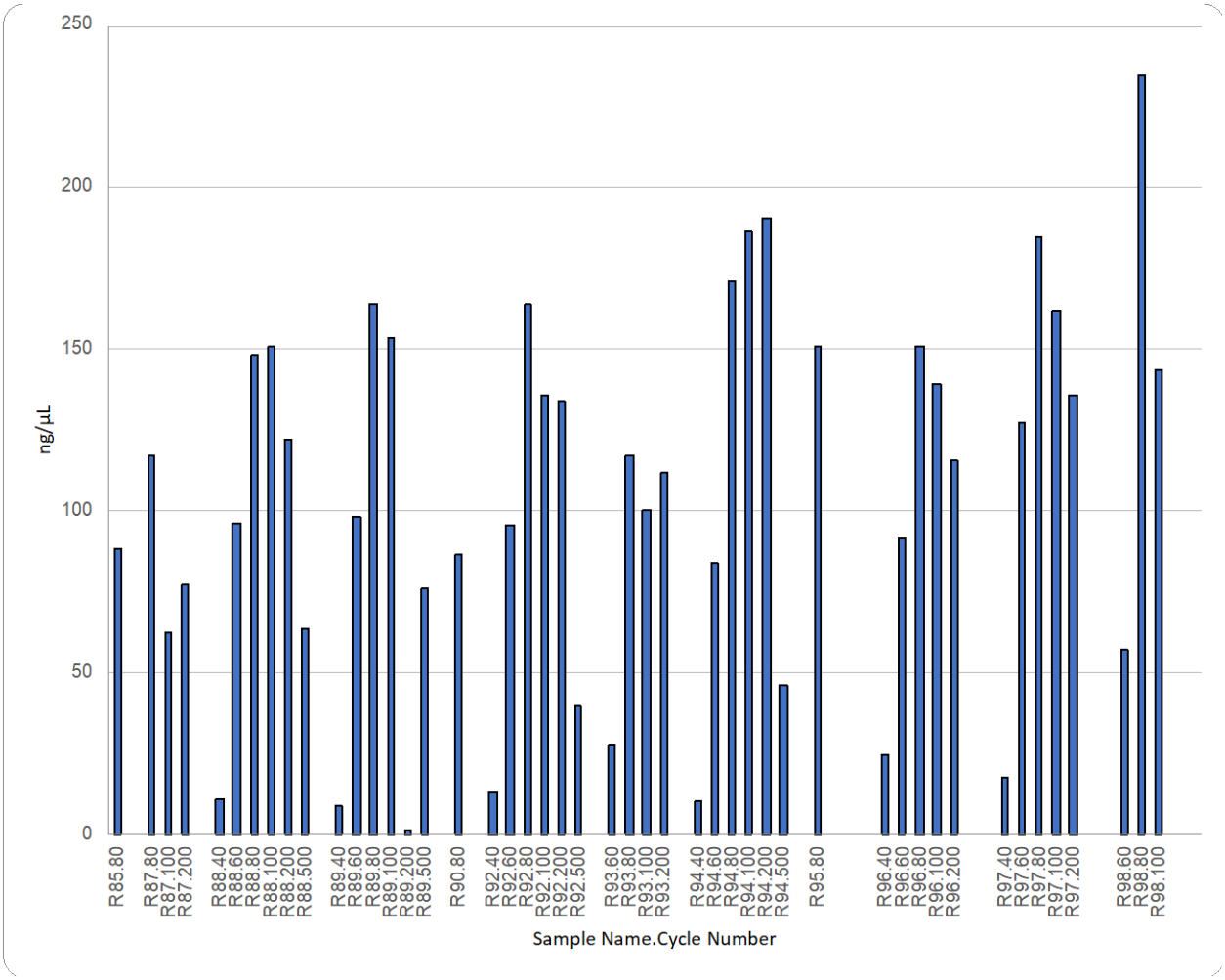




Sub-Project 3.2. Figure 2. Visual depiction of Cq values reported in Sub-Project 3.2 Table 1.



Sub-Project 3.2. Figure 3. Concentration of Targeted Amplicons by Sample Name and Cycle Number.



Sub-Project 3.3 "Influence of a Range (Especially Outside) of Standard Denaturing Temperatures on the Amplification of Aged, Degraded, and/or Low Copy Number DNA." (NOTE: proposed title for publication)

In this sub-project, we added to the observations of Orlando et al. (2011) that employed a lower than typical denaturation temperature of 80°C. The rationale is simple, in that the shorter, (typical of aged and degraded) DNA molecules will preferentially denature at lower temperatures over (presumably) more intact modern DNA contamination.

Denaturation is essential to PCR. So early, preferential denaturation of degraded DNA strands might tip the scales towards their preferential amplification over more intact (and presumably) contaminating DNA molecules. To appreciate this concept, one must consider base complementarity and its relationship to hydrogen bonding, the overall strength of which is based on the specific sequence *and* length of the molecules. In this case, shorter molecules should denature at relatively lower temperatures. Could it be possible to amplify authentic, degraded DNA molecules over those more intact? If so, this simple modification to PCR could be incredibly useful to forensic genetics approaches, as well as those of paleogenomic (i.e., ancient DNA).

Here, we subjected DNA eluates recovered from various archaeological rockfish remains to qPCR with denaturing ranging 70-100°C. Because heat treatment (see Sub-Project 2.2) and particularly heat treatment during initial denaturing/hot start (see Sub-Project 3.2) Our expectation is that DNA derived from such source materials will be amenable to lower-than-average denaturing temperatures, and if this is a useful tactic, success rates at lower temperatures should be higher compared to standard denaturing at 94°C. Moreover, it is possible that Cq values are maximized over those observed when employing lower than standard annealing temperatures.

Secondly, we created two amplicons (362 and 186 bps in length) and subsequently subjected them (and their dilutions down to $1*10^{-10}$ ng/μL) as template for qPCR with denaturing ranging 70-100°C. Our expectation is that the larger template molecules will be associated with Cq values comparably large over those observed from the shorter amplicon templates.

MATERIALS AND METHODS

Evaluating the success of amplifying the cyt b1 fragment (147 bp) from archaeological rockfish remains using denaturing temperatures ranging 70-100°C

Samples/Archaeological Context

A total of 40 specimens used in this study were selected from the fish bone assemblages recovered from a late-19th-century Chinese diaspora archaeological sites in California. See Sub-Project 3.1 for additional details regarding specimen selection and morphological identification.

The "H series" samples originated from Point Alones, a Chinese fishing village located on the shores of Monterey Bay, California that was home to up to 500 residents from the late 1850s through 1906, when the village was destroyed by fire. Excavations at the site focused on trash lenses and midden deposits associated with residential activities and the discard of fisheries byproducts (Williams, 2011).

The “M series” samples originated from Market Street Chinatown, a large, urban Chinese community in San Jose, California that was home to over 1,000 permanent residents from the 1860s through 1887, when the site was destroyed by arson fire (Laffey, 1993; Yu, 2001).

DNA Extraction and qPCR Analysis

DNA was extracted from the archaeological rockfish vertebrae and evaluated for PCR inhibitors as described under Sub-Project 3.1.

These DNA eluates (regardless of status as inhibited or for free from inhibition; Sub-Project 3.3 Supplemental Table 1) were subject to 60 cycles of qPCR of the 147 bp cyt b1 amplicon (Sub-Project Table 3.1) as follows. Reactions of 20 µL contained 1X SsoAdvanced™ Universal SYBR® Green Supermix, 0.125 mg/mL bovine serum albumin (BSA), 1.0 µM of each primer, and 1 µL of template DNA. Quantitative PCR reaction conditions were as follows: 1) 3 min denaturation at 94°C, 2) 60 cycles of 15 s denaturation holds at either 70°C, 80°C, 85°C, 90°C, 94°C or 100°C, then annealing at 56°C, and extending at 72°C (with a plate read following each cycle), and 3) a final extension at 72°C for 3 min. The qPCR reactions were followed by melt curve analysis starting at 65°C and increasing the temperature by 0.5°C, after which the plate was read. This was conducted 60 times, ultimately reaching a temperature of 95°C. Reactions were cooled to 10°C. Two PCR negatives as well as a positive DNA control of a modern rockfish‡‡‡‡‡ accompanied each batch of amplifications.

All amplicons were separated on 2% agarose gels stained with Gel-Red for visual inspection (described in Sub-Project 3.3 Supplemental Table 1 Tab A).

Evaluating the influence on the associated Cq values of amplifying a longer (362 bp) and shorter (186 bp) fragment of DNA using denaturing temperatures ranging 70-100°C

Samples and Amplicon Production

We PCR amplified two fragments of DNA (362 and 186 bp) each from an ancient turkey (*Meleagris gallopavo*) control [described in Sub-Project 3.1, one pooled from specimens dating approximately 180-1400 years ago (Kemp et al., 2017)] and a contemporary turkey DNA sample (i.e., from store-bought turkey meat). Primers for the 362 bp fragment were T15553F [note: in their original publication, Kemp et al. (2017) incorrectly labeled this primer “T15593” in their Table 3) and T15894R and primers for the 186 bp fragment were T15709F and T15984R (Kemp et al., 2017). Fifteen microliter PCR reactions contained: 1X Omni Klentaq Reaction Buffer (including a final concentration of 3.5 mM $MgCl_2$), 0.32 mM dNTPs, 0.24 µM of each primer, and 1.5 µL of standard DNA template. Following denaturing at 94˚C for 3 minutes, 60 of PCR were conducted at 94˚C for 15 s, 60˚C for 15 s, and 68˚C for 15 s. Finally, a 3-minute extension period at 68˚C was conducted prior to bringing the reactions to 10˚C.

Amplicons were separated on a 2% agarose gel to confirm amplification, purified with the Qiagen MinElute PCR Purification Kit, and quantified with a Qubit 3.0. From these PCR products, a series of volumes from full concentration and dilutions from 1 ng/µL to $1*10^{-10}$ ng/µL)

‡‡‡‡‡ Contemporary rockfish samples, used as controls in our laboratory, were graciously provided to us by Anne Pollnow of Sea Level Consulting, LLC, Sitka, Alaska

of the amplicons were prepared to subsequently serve as qPCR template molecules (i.e., eleven samples of each control).

Quantitative PCR

Quantitative PCR reactions of 20 µL contained 1X SsoAdvanced™ Universal SYBR® Green Supermix, 0.125 mg/mL bovine serum albumin (BSA), 1.0 µM of each primer, and 1 µL of template DNA (Table XX). Primers T15553F and T15894R were used to re-amplify the 362 bp fragment and T15709F and T15984R were used to re-amplify the 186 bp fragment from the full concentration amplicons and their respective series of dilutions (i.e., eleven samples of each control). Two PCR negatives accompanied each batch of amplifications. Quantitative PCR reaction conditions were as follows: 1) 3 min denaturation at 94°C, 2) 60 cycles of 15 s denaturation holds at either 80°C, 85°C, 90°C, or 94°C, then annealing at 60°C, and extending at 72°C (with a plate read following each cycle), and 3) a final extension at 72°C for 3 min. The qPCR reactions were followed by melt curve analysis starting at 65°C and increasing the temperature by 0.5°C, after which the plate was read. This was conducted 60 times, ultimately reaching a temperature of 95°C. Reactions were cooled to 10°C.

RESULTS

Evaluating the success of amplifying the cyt b1 fragment (147 bp) from archaeological rockfish remains using denaturing temperatures ranging 70-100°C

None of the 40 DNA eluates, inhibited or not, amplified with denaturing temperatures of 70˚C or 80˚C (Sub-Project 3.3. Table 1; Sub-Project 3.3 Supplemental Table 1 Tab A). Notably the positive control of Rockfish 2 (at 1.36 ng/µL) failed to amplify under these experimental denaturing conditions as well. Amplification of the archaeological rockfish DNA samples was met with similar success at 85˚C, 90˚C, 94˚C, and 100˚C. Roughly 40% of the samples amplified overall and uninhibited samples amplified at about 3X the rate in comparison to inhibited samples. All amplicons were sequenced and identified as rockfish cyt b1. Some likely post damage was observed (3/49 amplicons, 6.1%), which is nothing abnormal and correlates in no direction with denaturing temperature. Extraction negative controls and PCR controls failed to produce targeted amplicons.

Sixteen DNA eluates amplified with three or more of the experimental denaturing temperatures ranging 85˚C-100˚C, allowing us to investigate the average Cqs and their associated. Little variance was notable and Cq appears to correlate not with denaturing temperature (Sub-Project 3.3 Table 2; Sub-Project 3.3 Figure 1). Maximally, the standard deviation of sample M31 is 22.4% of its mean. This variance is clearly exaggerated by the rather high Cq (49.96) associated with amplification at 100˚C. Amplification from fourteen samples is associate with variances around 5-10% and average. The positive control behaved predictably (Cq 20.38±1.11) with denaturing at 85˚C-100˚C.

Evaluating the influence on the associated Cq values of amplifying a longer (362 bp) and shorter (186 bp) fragment of DNA using denaturing temperatures ranging 70-100°C

None of the full concentration amplicons eluates nor their dilution from 1 ng/µL to $1*10^{-10}$ ng/µL amplified with using an 80˚C denaturing temperature (Sub-Project 3.3 Supplemental Table 1 Tab B). The 362 bp amplicons produced from the ancient turkey collective sample at $1*10^{-9}$ ng/µL dilution failed using 90˚C denaturing and the $1*10^{-10}$ ng/µL dilution failed in all reactions.

The 362 bp amplicons produced from the contemporary turkey control sample at $1*10^{-8}$ ng/μL dilution failed using 90˚C denaturing, the $1*10^{-9}$ ng/μL dilution failed to amplify with either 90˚C or 94˚C denaturing, and the $1*10^{-10}$ ng/μL dilution failed in all reactions. All other reactions produced targeted amplicons. While not the most ideal qPCR reaction, as the production of off-target amplicons and primer-dimer was commonly observed (Sub-Project 3.3 Supplemental Table 1 Tab B), the Cq values across the denaturing 85˚C-94˚C are associated with little variance (Sub-Project 3.3 Table 3 and 4; Sub-Project 3.3 Figure 1). PCR negatives produced no amplicons.

CONCLUSION

In this sub-project, we followed the lead of Orlando et al. (2011) to evaluate the efficacy of PCRs, ones targeting 147 bp of the rockfish cytochrome b gene, conducted with lower than standard (94˚C -95˚C) denaturing conditions, that is at temperature and ranging 70-100˚C. We also constructed amplicons of differing sizes (362 bp and 186 bp) and observed their respective behaviors in PCR amplification using denaturing temperatures spanning 80˚C-94˚C.

PCR of these target molecules universally failed at 70˚C and 80˚C. PCR was only possible at temperature of 85˚C and higher. However, the success in amplifying archaeological rockfish was consistent across these temperatures and their associated Cq values are associated with little variance. We observed similar behaviors from amplification of the two series of amplicons dilutions. Regardless the amplicon size, success and associated Cq values revealed little variance.

In sum, this indicates first that the study of archaeological specimens (i.e., representing aged, degraded, and/or at low copy number DNA) may not universally benefit by preferential amplification at lower denaturing temperatures. This is interesting as our other experiments indicate that lower denaturing temperatures should be less damaging than PCR conducted at comparatively higher temperatures (Sub-Project 3.2; Sub-Project 2.2). Given that the archaeological specimens under scrutiny are not of great antiquity (dating to ~115-170 years old), it is possible that the successful approach of low temperature denaturing by Orlando et al. (2011) took advantage of preferential denature of ultrashort pieces of DNA [<50 bp; (Gutaker et al., 2017; de Filippo et al., 2018)] that are expected to accumulate from the time of death of an organism. However, it is notable that PCR can be routinely conducted with denaturing as low as 85˚C.

Considering our experimental design, we chose to conduct qPCR with Bio-Rad SsoAdvanced™ Universal SYBR® Green Supermix. This master mix contains polymerase that requires a hot start (recommended 2-3 min at 98˚C for genomic DNA). An insightful follow up study would be to evaluate denaturing during PCR with a non-hot start polymerase.

Sub-Project 3.3 Table 1 Results from cyt b1 amplification initiated at various denaturing temperatures. Denaturing at 70˚C and 80˚C yielded no amplicons.

	85°C	Cq	90°C	Cq	94°C	Cq	100°C	Cq
Overall	15/40 (37.5%)	35.81 ±5.18	16/40 (40%)	34.22±2.83	18/40 (45%)	36.37±5.19	14/40 (35%)	36.47±4.76
Uninhibited	11/16 (68.8%)	34.45±4.10	11/16 (68.8%)	33.06±2.21	12/16 (75%)	34.92±5.61	9/16 (56.3%)	34.08±2.21
Inhibited	4/24 16.7%)	39.54±6.61	5/24 (20.8%)	36.52±3.51	6/24 (25%)	39.03±3.19	5/24 (20.8%)	40.77±4.76

Sub-Project 3.3 Table 2. Summary of successfully amplified samples taken from Sub-Project 3.3. Supplemental Table 1.

		85℃		90℃		94℃		100℃		Average Cq and Standard Deviation
Sample	Inhibited?	Amp?	Cq	Amp?	Cq	Amp?	Cq	Amp?	Cq	
M27	Yes	✔	48.82	✔	37.66	✔	43.50	✗	N/A	43.33±5.58
M28	Yes	✔	38.49	✔	35.72	✔	37.17	✔	39.14	37.63±1.51
M31	Yes	✔	33.21	✔	32.39	✔	34.09	✔	49.96	37.41±8.39
M32	No	✔	32.65	✔	32.18	✔	32.74	✔	32.2	32.44±0.29
M33	Yes	✗	N/A	✔	37.44	✔	40.60	✔	40.06	39.37±1.69
M34	No	✔	31.53	✔	31.84	✔	31.61	✗	N/A	31.66±0.16
M35	Yes	✔	37.63	✔	39.41	✔	39.02	✗	N/A	38.69±0.94
M36	No	✔	38.60	✔	35.48	✔	37.39	✔	34.6	36.52±1.81
M37	No	✔	31.15	✔	31.28	✔	31.02	✔	32.66	31.53±0.76
M38	No	✔	34.91	✔	33.50	✔	34.20	✔	35.06	34.42±0.72
M39	No	✔	31.76	✔	33.61	✔	32.00	✔	33.97	32.84±1.12
M41	No	✔	29.68	✔	29.99	✔	30.32	✔	30.55	30.14±0.38
M42	No	✔	30.93	✔	30.76	✔	31.19	✔	33.33	31.55±1.20
M43	No	✔	41.84	✗	N/A	✗	N/A	✗	N/A	41.84*
M44	Yes	✗	N/A	✗	N/A	✗	N/A	✔	38.03	38.03*
H49	No	✗	N/A	✗	N/A	✔	49.92	✗	N/A	49.92*
H51	Yes	✗	N/A	✗	N/A	✔	39.80	✔	36.68	38.24†
H55	No	✔	39.8	✔	36.09	✔	37.40	✔	37.1	37.60±1.57
H60	No	✔	36.08	✔	35.91	✔	36.3	✔	37.29	36.40±0.62
Positive Control	N/A	✔	22	✔	19.50	✔	19.93	✔	20.09	20.38±1.11

* one measure, not an average, no standard deviation to report
†no standard deviation to report, as only two measures record

Sub-Project 3.3 Table 3. Summary of qPCR amplification of a 362 bp amplificon taken from Sub-Project 3.3. Supplemental Table 1.

		Cq			
Sample	**Concentration (ng/µL)**	**85℃**	**90℃**	**94℃**	**Average Cq and Standard Deviation**
A 1F/2R	4.47	4.07	3.81	3.45	3.78±0.31
A 1F/2R E1	1	8.19	8.43	8.2	8.27±0.14
A 1F/2R E2	$1*10^{-2}$	11.9	12.36	12.88	12.38±0.49
A 1F/2R E3	$1*10^{-3}$	17.16	17.38	18.12	17.55±0.5
A 1F/2R E4	$1*10^{-4}$	20.88	22.64	22.41	21.98±0.96
A 1F/2R E5	$1*10^{-5}$	24.02	23.88	24.47	24.12±0.31
A 1F/2R E6	$1*10^{-6}$	25.89	26.95	26.97	26.6±0.62
A 1F/2R E7	$1*10^{-7}$	24.28	24.62	25.16	24.69±0.44
A 1F/2R E8	$1*10^{-8}$	24.02	26.11	29.35	26.49
A 1F/2R E9	$1*10^{-9}$	25.42	N/A*	27.19	26.31†
A 1F/2R E10	$1*10^{-10}$	N/A*	25.29	N/A*	25.29‡
M 1F/2R	17.2	5.71	6.16	5.93	5.93±0.23
M 1F/2R E1	1	10.29	11.33	11.57	11.06±0.68
M 1F/2R E2	$1*10^{-2}$	15.03	16.6	17.3	16.31±1.16
M 1F/2R E3	$1*10^{-3}$	19.31	19.91	20.06	19.76±0.4
M 1F/2R E4	$1*10^{-4}$	24.38	24.73	24.65	24.59±0.18
M 1F/2R E5	$1*10^{-5}$	28.7	32.57	32.25	31.17±2.15
M 1F/2R E6	$1*10^{-6}$	33.49	33.23	33.34	33.35±0.13
M 1F/2R E7	$1*10^{-7}$	31.9	32.71	34.11	32.91±1.12
M 1F/2R E8	$1*10^{-8}$	35.24	40.91	31.97	36.04±4.52
M 1F/2R E9	$1*10^{-9}$	37.18	N/A*	N/A*	37.18‡
M 1F/2R E10	$1*10^{-10}$	38.49	N/A*	N/A*	38.49‡
PCR Negative		N/A*	N/A*	N/A*	

*non-applicable as amplification was not achieved
† no standard deviation, as only two measures obtained
‡ not an average, as only one measure obtained

Sub-Project 3.3 Table 4. Summary of qPCR amplification of a 186 bp amplificon taken from Sub-Project 3.3. Supplemental Table 1 Tab B.

		Cq			
Sample	**Concentration (ng/µL)**	**85℃**	**90℃**	**Sample**	**Concentration (ng/µL)**
A 2	6.04	2.43	3.82	4.16	3.47±0.92
A 2 E1	1	7.34	8.01	8.11	7.82±0.42
A 2 E2	$1*10^{-2}$	11.83	13.93	14.19	13.32±1.29
A 2 E3	$1*10^{-3}$	17.88	18.58	18.86	18.44±0.5
A 2 E4	$1*10^{-4}$	24.2	24.83	24.99	24.67±0.42
A 2 E5	$1*10^{-5}$	26.7	28.69	26.73	27.37±1.14
A 2 E6	$1*10^{-6}$	30.11	30.54	28.19	29.61±1.25
A 2 E7	$1*10^{-7}$	27.31	29.12	28.41	28.28±0.91
A 2 E8	$1*10^{-8}$	24.52	26.97	27.72	26.4±1.67
A 2 E9	$1*10^{-9}$	31.6	27.92	27.9	29.14±2.13
A 2 E10	$1*10^{-10}$	30.04	24.78	25.11	26.64±2.95
M 2	6.11	N/A*	1.19	N/A*	1.19†
M 2 E1	1	7.28	7.36	7.54	7.39±0.13
M 2 E2	$1*10^{-2}$	11.18	11.31	11.75	11.41±0.3
M 2 E3	$1*10^{-3}$	15.31	16.13	16.86	16.1±0.78
M 2 E4	$1*10^{-4}$	19.79	23.32	24.08	22.4±2.29
M 2 E5	$1*10^{-5}$	25.41	25.52	25.38	25.44±0.07
M 2 E6	$1*10^{-6}$	26.02	24.37	24.37	24.92±0.95
M 2 E7	$1*10^{-7}$	29.38	25.04	23.61	26.01±3
M 2 E8	$1*10^{-8}$	28.38	26.08	26.85	27.1±1.17
M 2 E9	$1*10^{-9}$	29.01	31.07	27.23	29.1±1.92
M 2 E10	$1*10^{-10}$	30.58	30.56	30.15	30.43±0.24
PCR Negative	N/A	N/A*	N/A*	N/A*	0±0

*non-applicable as amplification was not achieved
† not an average, as only one measure obtained

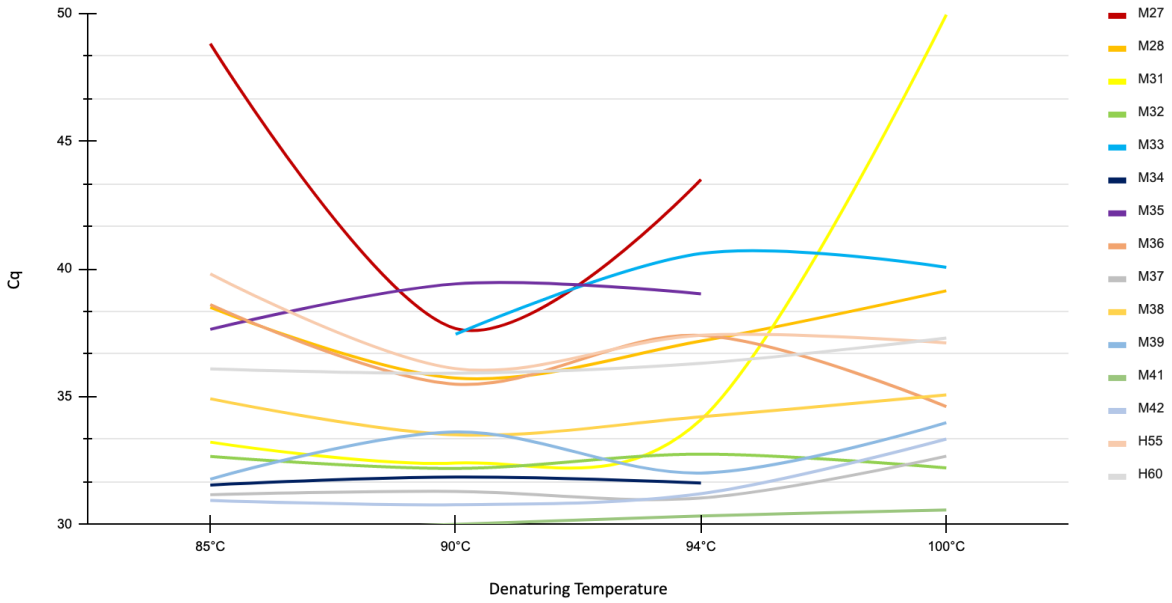
Sub-Project 3.3 Supplemental Table 1. Results from Sub-Project 3.3.

Color coding and symbols used in the following tabs. Green Cq values are indicators of target amplification. Red cells labeled N/A indicate failed production of targeted amplicons. Yellow cells highlight sequences likely compromised by post-mortem nucleotide damage.

Tab A: Results of cyt b1 rockfish amplification with denaturing from 70-100˚C.

Tab B: Cq values for qPCR amplification of standards 362 and 186 bp in length with denaturing from 70-94˚C.

Sub-Project 3.3 Figure 1. Visual depiction of Cq values reported in Sub-Project 3.3 Table 2.



Phase IV: How much contaminating DNA is routinely encountered in the laboratory and how much (and/or in what ways) does it exhibit characteristics of aged, degraded, and/or LCN DNA?

The negative controls generated during this grant will be systematically tested for the quality and quantity contaminating DNA molecules, if present. High throughput sequencing (Illumina platform) will be used to determine the source of any non-pig and non-human (e.g., bacterial DNA). These observations will allow us to address whether it is possible to discriminate between contaminating DNA routinely encountered in the laboratory against profiles generated from aged, degraded, and/or LCN DNA.

An addendum will be filed with the National Institute of Justice following completion of this phase.

Potential Impacts

The NIJ Forensic Science Technology Working Group (2016) placed a priority on addressing "Methods and/or knowledge to inform users about which processes maximize…recovery of DNA at the elution and/or extraction steps, and/or direct amplification, for best downstream DNA analysis results". In this case, the experiments outlined in Phase I will be particularly impactful.

Phase IV contributes to "Better solutions to deal with contamination, such as…nontraditional methods to monitor the presence of contamination (e.g., changes in instrument sensitivity) with minimal disruption to laboratory workflow" (NIJ Forensic Science Technology Working Group, 2016). These experiments will impact how others view the quality control measures instated in their laboratories (e.g., the way they decontaminate their laboratories and how often), and encourage the monitoring of contamination, *which is a real threat in every laboratory*.

Phases I-IV address the NIJ Forensic Science Technology Working Group (2016) demand for an "Increase in the success rate of obtaining DNA profiles from compromised (damaged) DNA evidence." This is clearly an area in which the results of all our experiments will be particularly impactful.

Implications for Criminal Justice Policy and Practice

DNA from aged, degraded and LCN sources is challenging to recover and authenticate. It is widely believed that this type of DNA will behave *differently* during routine laboratory procedures than does contaminating DNA during routine laboratory methods. However, the strength of this suggestion for use in criminal justice policy and practice is only as strong as how well we *actually* know if, and how, they differ. Our study has been specifically designed to address this, through advancing our understanding and estimation of: (1) DNA loss during extraction and purification, (2) damage accrued by DNA in the laboratory, (3) how to better PCR amplify DNA, and (4) the extent and form of contaminating DNA typically encountered in the laboratory. It is expected that our experimental results will be immediately applicable for incorporation into validation studies for improvements to the forensic DNA workflow for aged, degraded, and LCN samples and potentially for all DNA evidence to improve the likelihood of full profile amplification for forensic identification. These results will also be beneficial within the field as criminal justice policy and practice moves forward in parallel with technological advancements that permit ever increasing, fine scale observations.

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