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DNA Contamination, Degradation, Damage and Associated Microbiomes: A Comparative Analysis through Massive Parallel Sequencing and Capillary Electrophoresis

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1 Project Summary

- This project evaluated whether DNA contamination can mimic the characteristics of low copy number (LCN), aged, damaged, and degraded DNA samples. Massive Parallel/Next Generation Sequencing was used to see if specific patterns of nucleotide damage is present with surface DNA contamination that has been aged and/or exposed to varying concentrations of sodium hypochlorite (bleach) and UV.

The proposed project is divided into two phases.

- Phase 1.0: Understand the process and rate of degradation and damage of applied touch DNA contamination on human skeletal remains and evidence tape. Five time intervals (ranging from 0 days to 1 year), three bleach treatments and three UV treatments were tested. Two additional handlers (cumulative) created a scenario of minor contributors often encountered in forensic scenarios.
- Phase 2.0: Understand the utility and degradation of the skin microbiome associated with touch DNA. This included determining if unique forensic signatures can be identified and compared, especially on bone substrates that may have their own microbiome signature.

2 STATEMENT OF THE PROBLEM AND RESEARCH QUESTIONS

2.1 Phase 1.0 DNA Damage and Degradation of Touch Contamination on Bone: Implications for Capillary Electrophoresis and Massive Parallel Sequencing/Next Generation Sequencing

Massive Parallel sequencing (MPS) /Next Generation Sequencing (NGS) have the potential to retrieve increasingly more nuanced and detailed genetic profile data from both co-mingled sources and poor-quality samples. Furthermore, such technology can be cost effective by allowing samples to be analyzed in tandem rather than in separate reactions, thereby reducing not only costs, but overall workloads (Børsting and Morling 2015; Van Neste et al. 2012). However, adopting an MPS/NGS approach, especially to the study of low copy number (LCN), age, degraded, and damaged samples, does not negate the fundamental complications associated with its study, namely recovery and authentication. The latter is of the utmost importance in the forensic community, as advancements in more sensitive DNA technology has led to a proportionate increase in the detection of contaminating DNA and/or minor contributors (Taylor et al. 2017). Primary and secondary

DNA transfer can then contaminate trace evidence during collection and storage and can be detected years later (Fonneløp et al. 2016; Pickrahn et al. 2015; Pickrahn et al. 2017;Taylor et al. 2017). Underestimating the scope and extent of these problems will continue to hinder the study of DNA from such sources, regardless of what era of sequencing technology in which we currently reside. This not only potentially increases error and misidentification but is also extremely expensive- as most MPS/NGS pipelines have higher costs on the back end (i.e., the sequencing run with many pooled libraries/samples).

Extensive research, both in the fields of forensics and ancient genomics, have documented both the fragmentation and chemical modifications that co-occur in DNA that is LCN, aged, degraded, and damaged (collectively referred to as "LADD" throughout the remainder of the proposal) (Table 1) (Alaeddini et al. 2010; Ambers et al. 2014; Gilbert 2006; Hall et al. 2016; Paabo 1989). However, there are a lesser number of studies that discussed specific nucleotide damage because of deamination, or oxidation in compromised samples. For example, Fattorini and colleagues (2000) showed that reliable allele-specific probing was unsuccessful on forensic samples due to degraded DNA "artifacts" but did not further identify what these artifacts were. Instead, focus has centered on using quantitative qPCR and capillary electrophoresis (CE) methods to understand the effects of low DNA concentration and the amount of DNA degradation/damage as a result of depurination which causes DNA strand breakage, but without reference to the specific type of miscoding lesions or to any given patterning or distribution of that fragmentation [1] (Ambers et al. 2014; Gettings et al. 2015; Hanssen et al. 2017; Hughes-Stamm 2012; Onori et al. 2006). MPS/NGS studies that centered on retrieving whole mitochondrial DNA (mtDNA) as well as methods intent on DNA repair (Hall et al. 2016) are exceptions, as transition and transversion error rates due to damage were reported, (Loreille et al. 2011; Parson et al. 2015; Templeton et al. 2013) as well as the succesful repair of specific types of DNA lesions. Investigations have also studied the effects of contamination and comingling by spiking endogenous DNA with purified genomic DNA as a "contaminant", which was diluted to varying concentrations and/or mechanically/chemically modified to mimic degradation (Ambers et al. 2014; Churchill et al. 2016; Skoglund et al. 2014; Van Neste et al. 2012).

[1] The measurement and extent of degradation whether real or in silico, varied in any given study and was often measured in different ways, however the strength of florescent units [RFUs] (or peak heights) and the extent of allelic dropout and negative amplification appear to be generally acceptable methods in assessing the overall quality of a sample.

Post-mortem DNA damage*

Damage type	Mechanisms	Effects
Strand breaks	Nuclease activity Dessication, heat, chemicals etc. Direct cleavage (hydrolysis) Depurination causes a basic site (hydrolysis) Microorganism degradation	Low quantity of surviving DNA DNA fragmentation toward smaller base pair size
Miscoding Lesion via hydrolysis	Deamination causes miscoding lesions: Adenine to hypoxanthine Cytosine to uracil 5-methylcytosine to thymine Guanine to xanthine	Base misincorporation A → G C → T (or complementary strand G → A) transitions C → T (or complementary strand G → A) transitions G →A
Blocking and Miscoding lesions via oxidation	Base modifications 5-OH-5-methylhydantoin (blocking) 5-OH-5-hydantoin (blocking) 8-oxoguanosine (miscoding G→T	No amplification; jumping PCR, base misincorporation
Crosslinks	DNA to DNA crosslinks via alkylation DNA to protein crosslinks (I.e., Maillard products	No amplification

*Modified from Fulton, T. L. (2012). Setting up an ancient DNA laboratory. Ancient DNA: Methods and Protocols, 1-11.

Table 1. Overview of Types of DNA Damage Post-mortem

Additional work by Tie and Uchigasaki (2013) and McCord (2011) assessed the overall extent of DNA damage modification across the whole genome, through biotin tagging or quantifying the 8-oxoguanosine byproducts produced during degradation. This approximated total oxidative damage. However, documentation of distinct damage patterns, preferentially occurring parts of the genome, specifically the beginnings and end of the molecules, were not evaluated. Binladen et al. (2006) saw no discernable difference in damaged between nuDNA and mtDNA, while Allentoft et al. (2012) saw a two-fold increase in degradation of nuDNA vs mtDNA.

Observations from ancient metagenomic datasets have provided new insight about the amount of oxidative damage found at the beginnings and ends of ancient molecules of various small fragment lengths-but generally less than 100bp. Thus, there is a pattern to this category of DNA damage (as a result from depurination at points of strand breakage) that is predictable but may or may not be time dependent (Dabney et al. 2013; Krause et al. 2010; Meyer et al. 2013; Prüfer and Meyer 2014)(Figure 1.). Bayesian modeling and simulations has also been used to predict degradation and damage patterns of haploid and diploid cells (Hanssen et al. 2017), in addition to using these "map damage" patterns to identify endogenous vs exogenous DNA (Jónsson et al. 2013; Skoglund et al. 2014).

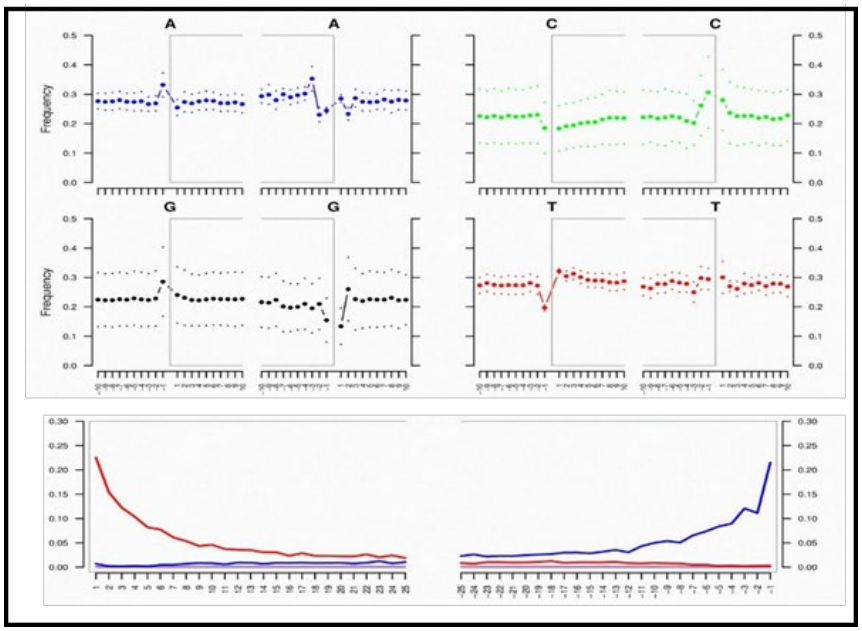

Figure 1. Typical damage pattern reported in ancient DNA MPS/NGS studies[2]

[2] This illustration summarizes damage patterns commonly observed in aDNA molecules. The image is borrowed from the mapDamage website (https:// ginolhac.github.io/mapDamage/). The four upper plots show the frequency of the nucleotide state just prior to the read, here depicted as the open grey boxes. Elevated occurrences of Gs and As occur just prior to the 5' read and Cs and Ts to the 3' (which are Gs and As on the complementary strand).
The bottom plots indicate that damaged nucleotides are accumulated at the ends of the molecule, and less so towards the center. This damage accumulates over time, but how soon it begins to accumulate has not been established.

Subjecting bones and teeth to sodium hypochlorite (NaOCl or bleach) and ultraviolet (UV) irradiation are the two most common methods used for contamination removal. Experiments aimed at evaluating methods of contamination removal, many of which conclude that it is difficult to entirely remove the contaminants (Barta et al. 2013; Kemp and Smith 2005; Shaw et al. 2008; Tamariz et al. 2006). It is most likely that failure to completely remove contamination stems from employing methods not robust enough to destroy the contaminants, as other studies have shown that complete decontamination is possible (Barta et al. 2013; Borst et al. 2004; Champlot et al. 2010; Tamariz et al. 2006). What is less understood is how these treatments may induce nucleotide lesions on contaminates, and how to recognize them in MPS sequences (Jun et al. 2012).

Garcia-Garcera et al. 2011, also cautions that human contamination co-extracted with non-human bone treated with sodium hypochlorite had depurinated base modifications like those observed from ancient endogenous human DNA. Champlot et al. 2010 also notes that bleach was not completely effective in complete degradation of short, contaminating molecules. Thus, decontamination procedures can artificially and inadvertently create specific damage patterns. Still, the rate and the extent of which exogenous contaminating DNA degrades naturally, as well as under experimental conditions is poorly understood. This could be particularly problematic if trace/touch evidence is contaminated by other trace/touch evidence, if trace/touch contaminates other matrices (i.e. bone) through direct handling, or if DNA evidence is damaged by decontamination procedures.

However, a critical assumption within the literature is that these damage patterns are exclusive to molecules that are “ancient” and therefore should not be seen regularly, or in any appreciable amount, in exogenous contaminating DNA. Thus, damage and degradation has become a form of authenticity of endogenous DNA. This is particularly problematic from a forensic standpoint, as the “time variance” between a targeted forensic sample and a non-targeted DNA contaminate can be far, far less. For example, target DNA may be of comparable age or state of decay as it’s contaminate, maybe being only days, weeks, or years apart in chronological age. This makes discernment between the two difficult. In paleogenomics, aDNA is often, hundreds to thousands of years older than any contaminating human DNA. A prediction could be made from a forensic perspective, in contrast to paleogenomic studies, that DNA “contamination” may exhibit similar damage patterns to endogenous molecules if it is of the same age or came from the same environment. However, in many cases, human DNA contamination, in the form of touch DNA, could hypothetically degrade faster due to differential exposure to the environment and variable deposition. **The reality is that there just too little information on how DNA degrades from all substrates and MPS/NGS datasets may offer key insights.**

Only very recently has it been recognized that damage patterns cannot be the sole source of authentication in MPS/NGS datasets (Eisenhofer and Weyrich 2018; Key et al. 2017).

In a comprehensive review, Key et al. (2017) outlines the possibilities, such as phylogenetics, as well as the pitfalls of in silico estimations of human DNA and microbial contamination. While some methods work relatively well (e.g., haploid data like mtDNA sequences), other methods which target autosomal markers are limited in their effectiveness to distinguish between multiple individuals (particularly female contributors).

2.1.1 Phase 1.0 (Specific Aim 1 and 2)

Utilizing MPS/NGS, we addressed the above issues by analyzing human touch DNA-from multiple donors- that has been placed on two human ribs provided by the Forensic Anthropology Center and Freeman Ranch Body Farm at Texas State University in San Marcos, Texas. This deposited “contamination”, as well as endogenous DNA from the human remains, was analyzed over the course of a year to measure the effect of time on DNA degradation. The effect of various decontamination treatments (sodium hypochlorite and UV treatments) on DNA aged at different time intervals will also be tested. For additional information, and a baseline comparison, human touch DNA from multiple donors was also extracted and analyzed from fingerprint tape and aged over the course of 1 year without treatment. This allows direct observation of damage and preservation patterns of touch DNA, identify when they begin to accrue, and whether they mimic those observed from endogenous degraded DNA from bone, as reasoned in this proposal, as well as existing literature. We further evaluated whether these “degraded and damaged contamination” molecules have a performance bias or display different forms of damage/dropout/degradation between MPS/NGS (shotgun sequencing),CE protocols, as well as with the newer FGX ForenSeq™ technology that combines polymerase chain reaction (PCR) amplicon procedures within a MPS/NGS platform.

2.2 Phase 2.0 (Specific Aim 3) DNA Damage and Degradation of Skin Microbiome placed on Bone: Implications for Capillary Electrophoresis and Massive Parallel Sequencing/Next Generation Sequencing

The use of microbiome data within forensic contexts has opened new avenues to gain even more nuanced data from crime scenes. Much of this work has focused on post-mortem changes in microbiomes post-mortem, giving insight into the decomposition process including changes to bone during various post-mortem intervals (Arenas et al. 2017; Clarke et al. 2017; Damann et al. 2015). Other exciting avenues include the potential use of skin microbiomes as, another tool to identify individuals based on their unique bacterial community profiles (Fierer et al. 2010; Hampton-Marcell et al. 2017). Experimental work on skin microbiomes from touch DNA placed on commonplace items (e.g., keyboards, etc.) suggest that personally unique microbiome profiles preserved their taxonomical structure relatively well for up to two weeks at normal building conditions (Fierer et al. 2010). However, this subfield is not without its own intrinsic challenges. Clarke et al. (2017) has argued that current forensic and comparative microbiome

datasets lack robusticity. Additionally, many of forensic MPS microbiome studies utilize universal V4 primers to amplify regions of the 16S gene. Recent research suggests this method (due to variation with the priming region) may skew microbial profiles due to preferential amplification. Additionally, it has been proposed that differential preservation and degradation of specific microbial species may also bias taxonomical profiles (Velsko et al. 2018; Ziesemer et al. 2015). Consequently, it is important to understand degradation and transitions of microbial communities through time, especially on forensically relevant time periods. With the experiments designed above, a MPS/NGS shotgun data set will be produced. This dataset then will allow us to study not only the preservation of the human DNA present but also associated skin and bone microbiomes.

3 PROJECT DESIGN AND IMPLEMENTATION

3.1 Phase 1.0 Applied Touch-DNA Degradation and Damage across Time and Decontamination

Phase 1.0 performed a comprehensive contamination/touch DNA analysis by applying DNA to two human ribs provided by the Forensic Anthropology Center and Freeman Ranch Body Farm at Texas State University in San Marcos, Texas. These samples had been exposed to taphonomic processes unknown to PIs but was visibly degraded (Figure 2.) For this phase 140 bone cross-sections (photos in Appendix A) were removed from the whole using a Dremel tool prior to handling. Fragment weight averaged 18.7mg. Touch DNA was also collected from participants using both the 3M Forensic Latent/Livescan Fingerprint Lift Pads, and Hinge Fingerprint Lifters from Sirchie®.

Figure 2. Skeletal samples used for project experiments.

Time Intervals Phase 1.1

Cross sections were cut from the rib fragments for each of the five time frames (1 week, 4 weeks, 16 weeks, 6 months, 12 months) following no treatment (Figure 3). For each additional handling experiments (n=3) and decontamination procedures (n=3) five additional bone cross sections were needed (Table 2/Figure 1). The testing of single and comingled touch DNA were tested with four different parameters (Figure 4). All had a positive control or a "time zero" to provide a baseline of initial DNA concentration and copy number (Table 2). Experiment 1) consists of bone that has not been exposed to exogenous handling, but cross-section will be aged for the five time intervals (1 week, 4 weeks, 16 weeks, 6 months, 12 months) and with each aged sample being treated to bleach and UV decontamination procedures (Table 2, Figure 4).

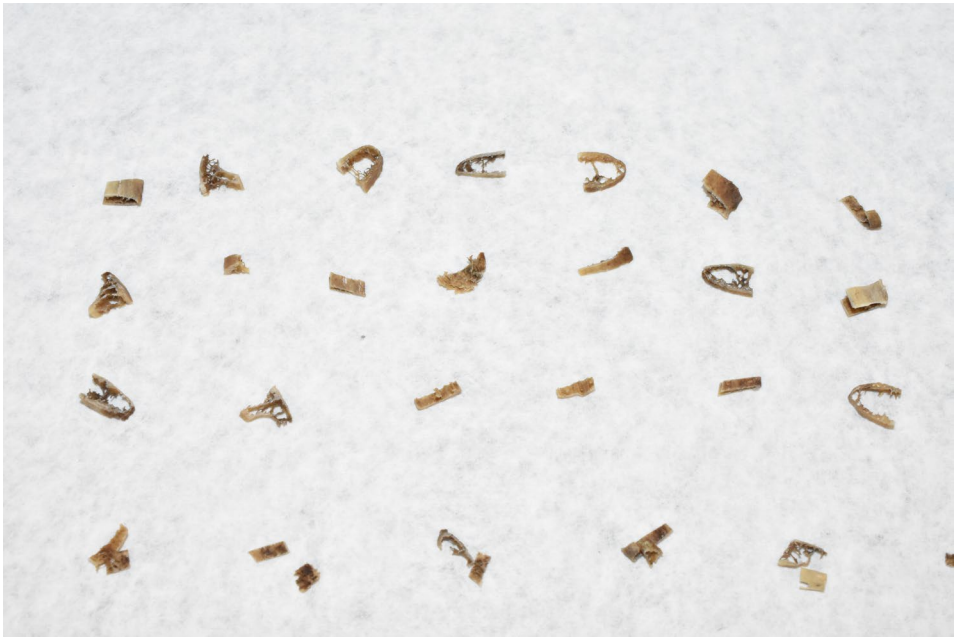

Figure 3. Example of bone sections used for experimental decontamination treatments and handling.

Table 2. Experimental treatments.

Experimental treatment	Time intervals only - no decontamination	NaOCl 0.6% (wt/vol) 15 minutes + time interval	NaOCl 3.6% (wt/vol) + time intervals	NaOCl 6% (wt/vol) 15 minutes + time intervals	UV Overhead 12 hours + time intervals	UV Crosslinker (365nm) 1 hour + time intervals	UV Crosslinker (**365nm**) 15 minutes + time intervals	Total
Untouched Cadaver Sample	5	5	5	5	5	5	5	35
Sample + Male (1) contributor	5	5	5	5	5	5	5	35
Sample + Female (2) contributor	5	5	5	5	5	5	5	35
Sample + Male (1) contributor + Female (2) contributor + Male contributor (3)	5	5	5	5	5	5	5	35
							Total	140

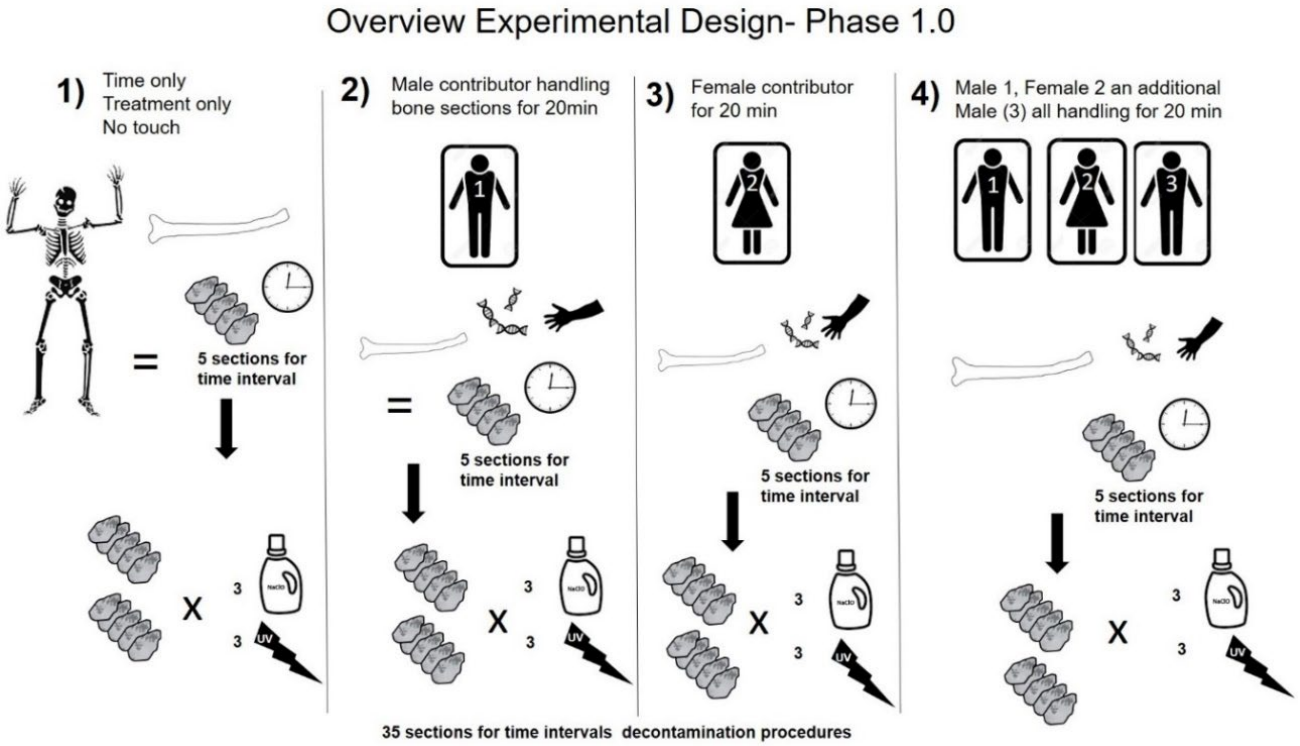

Figure 4. Visual overview of Phase 1.0 experiments

Bleach Decontamination Phase 1.3

Bone cross-section samples and accompanying controls, were submersed respectively in volumes of 0.6%, 3.6%, and 6% w/v of bleach (NaOCl) for five minutes, rinsed with dH_2O and air-dried for each respective time interval. All other non-bleach treated cross-sections were immediately place placed in sterile 1.5ml microcentrifuge tubes and stored at room temperature for the duration the samples' assigned time interval.

Ultraviolet UV Decontamination Phase 1.4

Bone cross-section samples and accompanying controls, were UV irradiated under three different conditions. The LMAMR cleanroom has overhead UVC light (λ = 254 nm) and is used routinely as a form of general lab decontamination whenever possible (Knapp et al. 2012; Kozicki 2012). However, it has been shown that DNA-crosslinking is increasingly less likely with increased distances to the source of UV (Champlot et al. 2010; Shaw et al. 2008; Tamariz et al. 2006). The extent of DNA-crosslinking induced damage on experimental touched DNA is unknown. The same is true for the endogenous bone sample. As such, exposed each surface of the contaminated/handled bone cross-sections to overhead UV light for 12 hours (for each side) for each time interval and handling procedure. Additional UV experiments subjected additional cross sections, for all time periods and handlings, to UV exposure in a crosslinker (λ = 254 nm). Samples were placed within an inch of the UV bulbs for 1 hour (per side), and 15 minutes (per side).

Touch DNA and Handling Experiments Phase 1.5

Experiments 2-4 sought to simulate contamination events through handling of human bone and allows a comparison between variable DNA deposition (both human and microbial) (e.g., different shedding rates) and its subsequent degradation and damage rates compared to bone. Experiment 2 replicated Experiment 1 but includes handling by Male #1 contributor for 15 minutes (Figure 1). Experiment 3 mimicked Experiment 2 but instead was handled by Female #2, also for 15 minutes. Finally, Experiment 4 varied by having 15 minutes of handling by all three contributors Male #1, Female #2, and Male #3.

3.2 Phase 2.0 Detection, Identification, and Degradation of Skin and Bone Microbiomes.

Phase 2.0 consists of the same samples processed Phase 1.0 (Section 3.1). Positive controls from swabs of all donors' skin and a sub-set of the deposits on the bone sections will be immediately extracted to create a microbiome baseline. Phase 2.0, post-MPS sequencing pipelines are also optimized for analyzing microbial content and operational taxonomic units.

3.3 Strategy, Methodology and Analyses for Phase 1.0 and 2.0

DNA from the bone cross-sections from the above experiments were extracted immediately after each time trial and treatment. Downstream analyses included multiple methods and platforms commonly used throughout the forensic community. MPS/NGS/HTS libraries provided a broad picture of the overall content and condition of each sample and provided information on the environmental content, such as the state of any microbiome profiles. Newer amplicon-based methods Illumina platforms, such as the MiSeq FGx, were evaluated along with CE methods such as the AmpFLSTR® Identifiler multiplex kit. Damage patterns were identified with the program Map Damage 2.0 (Ginolhac et al. 2011; Jónsson et al. 2013) which computes nucleotide misincorporation and fragmentation patterns.

3.3.1 Contamination Control

As these DNA extracts are predicted to be LADD, and the experiments are systematically trying to understand "contamination", it is incredibly important to take steps to minimize contamination, as well as successfully remove any existing contamination. LMAMR has a dedicated isO6 cleanroom laboratory and is routinely monitored for contamination[3].

3.3.2 DNA Extraction and Quality Assessment

DNA was extracted for both bone and tape samples using the Thermofisher's PrepFiler Express BTA™ Forensic DNA Extraction Kit following the protocol for each matrix type. However, the decalcification step was modified for the bone samples which included a 1 ml incubation in 0.5M EDTA for 48 hours. Bone mass ranged from 4.8=42.5 mg and averaged 18.7 mg. An extraction negative control (where no sample was added) was also included per every seven samples to monitor potential contamination from lab personnel and reagents. Positive controls (purified genomic DNA from original contaminators) were extracted using the Qiagen's DNeasy Blood & Tissue Kit. DNA concentration and distribution of fragment length of extractions was determined using Agilent Fragment Analyzer's HS Genomic Quantification kit.

[3] The LMAMR ancient DNA lab is a positively pressured clean room with hepa-filtered air. The clean room contains anterooms for storing supplies, changing into laboratory attire, and decontaminating/cutting sample. Personnel working in the ancient DNA lab wear disposable hairnets, facemasks, laboratory Tyvek suits. All equipment, reagents and consumables are dedicated for use in the ancient DNA laboratory. Bleach, ethanol, and an overhead UV system are routinely used to clean and decontaminate the ancient DNA laboratory. Personnel are restricted in their movement and are restricted from entering the cleanroom after being in a contemporary DNA laboratory.

3.3.3 Library Preparation

Blunt ended, dual-indexed DNA libraries were built using purified DNA from bone and tape extractions, positive controls (cheek swabs from handlers), an extract and library negative controls . This library preparation followed previously published protocols (Meyer & Kircher 2010, Carøe, 2018). The concentration of DNA within the libraries was then measured through qPCR (using BioRad SsoAdv Univer SYBR Master Mix and BioRad CFX Maestro qPCR machine) to determine the cycles needed for the indexing PCR. Indexing PCR reactions were performed using Illumina adapters with 8 base pair iTru DNA barcodes. This method does not utilize polymerases that repair DNA damage, thus retaining samples' original damage patterns. The resulting DNA libraries, with unique dual indexed barcodes, were then purified using Beckman Coulter magnetic SPRIselect beads. The Agilent Fragment Analyzer (High Sensitivity NGS library quantification kit) and Tapestation 4200 (Agilent D1000/D1000 High Sensitivity ScreenTape System) were used to measure quality and size distribution of purified libraries. Using a Sage Science Pippen Prep (2% agarose cassette), size specific adapter dimers and larger fragments above 500bp were removed to reduce size bias during sequencing. Larger inserts, within this range, should have a better capability to read through highly repetitive sequences (often found with STRs), whereas smaller inserts will have higher depth coverage. The goal is to have pooled libraries with both long and short inserts, which will give more accurate consensus data. Note that this had been currently untested with fragmentary/LADD DNA and there may not be recoverable fragments that are anywhere near 500 bp.

3.3.4 Metagenomic Analyses Phase 1.0 and 2.0

Downstream analyses utilized bioinformatic pipelines that: trimmed and merged demultiplexed paired end Illumina reads, mapped reads to the Green Genes 16s rRNA gene database (DeSantis et al. 2006). Using the program Bowtie 2.0. Mapped reads were clustered into OTUs using the 97% threshold in Qiime (Caporaso et al. 2010)—where downstream processing occurred. SourceTracker (Knights et al., 2011) was used to compare these profiles to databases of known sources (including skin) and estimated proportions of the sample that come from different sources.

3.3.5 FGX ForenSeq and CE Analysis of Endogenous and Touch Contamination

The FGX ForenSeq is a validated MPS/NGS system (Caratti et al. 2015; Churchill et al. 2016; Hussing et al. 2015; Jäger et al. 2017; Sharma et al. 2017) that has produced comparable results to traditional CE systems with as little as 62.5 pg of template DNA. The reaction amplifies 231 forensic loci in a single multiplexed PCR (27 autosomal STRs, 24 Y-STRs, 7 X-STRs, Amelogenin sex typing, as well as panels of identity, biogeographical, and phenotypic SNP panels). However, the system remains amplicon based and utilizes a proofreading enzyme, one that should not extend through damaged DNA bases, nicks, or lesions (Verogen, Personal communication). While FGX ForenSeq

has the advantage of reaching a high depth of coverage, it is ultimately clonal, and with LADD DNA samples, starts with a small number of molecules as in traditional PCR and CE methods. Thus, there are different expectations of success and failure with degraded/damaged contamination. In this case, modified bases would preclude amplification, regardless of concentration or strand length. The AmpFLSTR® Identifiler plus kit (which use Amplitaq Gold- a non-proofreading enzyme) was used to address concordance between this particular CE system and the FGX Forenseq platform. The studies proposed here, should also provide valuable information on the effects of using proofreading vs. non-proofreading polymerases in MPS/NGS protocols when processing LADD samples.

3.3.6 Human Subjects/Study Population

This study was approved by the Institutional Review Board at the University of Oklahoma (IRB #9993). PI Monroe obtained informed consent from participants and followed additional guidelines outlined in the approved NIJ Privacy Certificate. This study required three individuals (two male and one female) to handle human rib fragments and leave touch DNA/contamination on the surface along with additionally providing touch DNA on evidence tape, and buccal swab samples for positive controls. All samples were deidentified and coded (i.e., all reference to individual names and private information were removed from sample labels (bone material and DNA extract) all downstream analyses was also coded with no identifiers. Importantly, genomic data that could forensically identify individuals were produced, which may make the samples identifiable to a particular individual. However, as our intent is to understand the state of DNA preservation, DNA profiles were not used in our research nor will they be reported, although this data was created.

4 Results

4.1 Genomic DNA concentration and fragment length distribution from Phase 1

Total genomic concentrations and fragment size distribution was measured using Agilent Fragment analyzer, High Sensitivity Genomics Kit (Appendix B tab A-BJ). Smear analyses, using ProSize 3.0, was used to measure DNA concentrations at various size ranges (A: 30-100bp, B: 100-500bp, C:500-2000bp, D:2000-8000bp). Note that the 30bp lower range cut off was chosen to avoid crossover concentrations associated with the lower reference marker (LM) (Figure 5). Concentration of genomic DNA at various sizes varied between non-detectable-3.1628 ng/ul. Molarity ranged from 0.0000-40.1483 nmole/L. %CV (precision- coefficient of variance) are reported in Appendix B tab A. Abbreviations for sample types is as follows: U=untouched, M=male touch, F=female

touch, C=cumulative touch (all handlers), NT=no treatment, UVO=UV overhead, UVC=UV crosslinker.

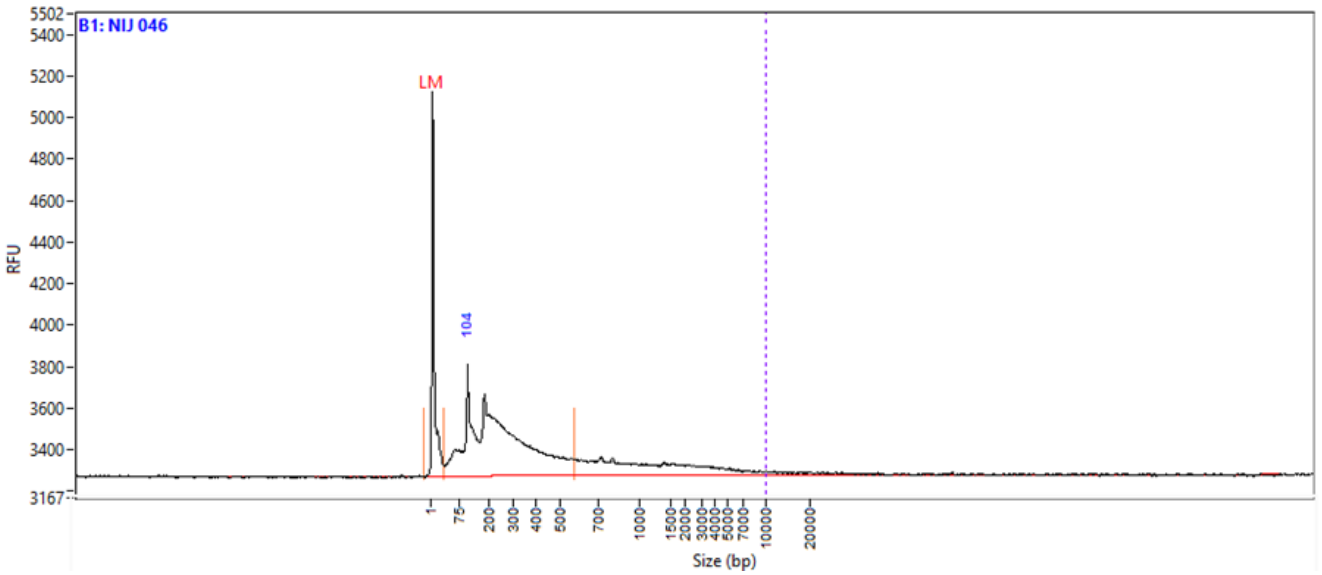

Figure 5 sample image from fragment analyzer

4.1.1 Time Trial 0 (day zero) Genomic Concentration and Fragment Distribution.

Genomic DNA concentrations varied across treatment and ranged from 0.0539-2.4499ng/ul (average 0.8514ng/ul). No treatment (NT) samples had higher yields of DNA followed by samples treated with UV. Bleached samples had the lowest genomic concentrations (Figure 6, Appendix B; Tab D). Using Pearson's correlation coefficient function in excel, there is a moderately positive correlation between DNA concentration and treatment (r= 0.6117). Most of the time trial 0 (T0) DNA fragments range between 100-500bp in size (Figure 7). When size distribution is plotted by treatment, T0 samples treated with NaClO have overall lower concentrations of DNA fragments larger than 500bp (Figure 7). The calculated correlation coefficient (r= 0.4445) between DNA concentrations and the amount of bone used in the extraction suggest little correlation between these two variables (Figure 8).

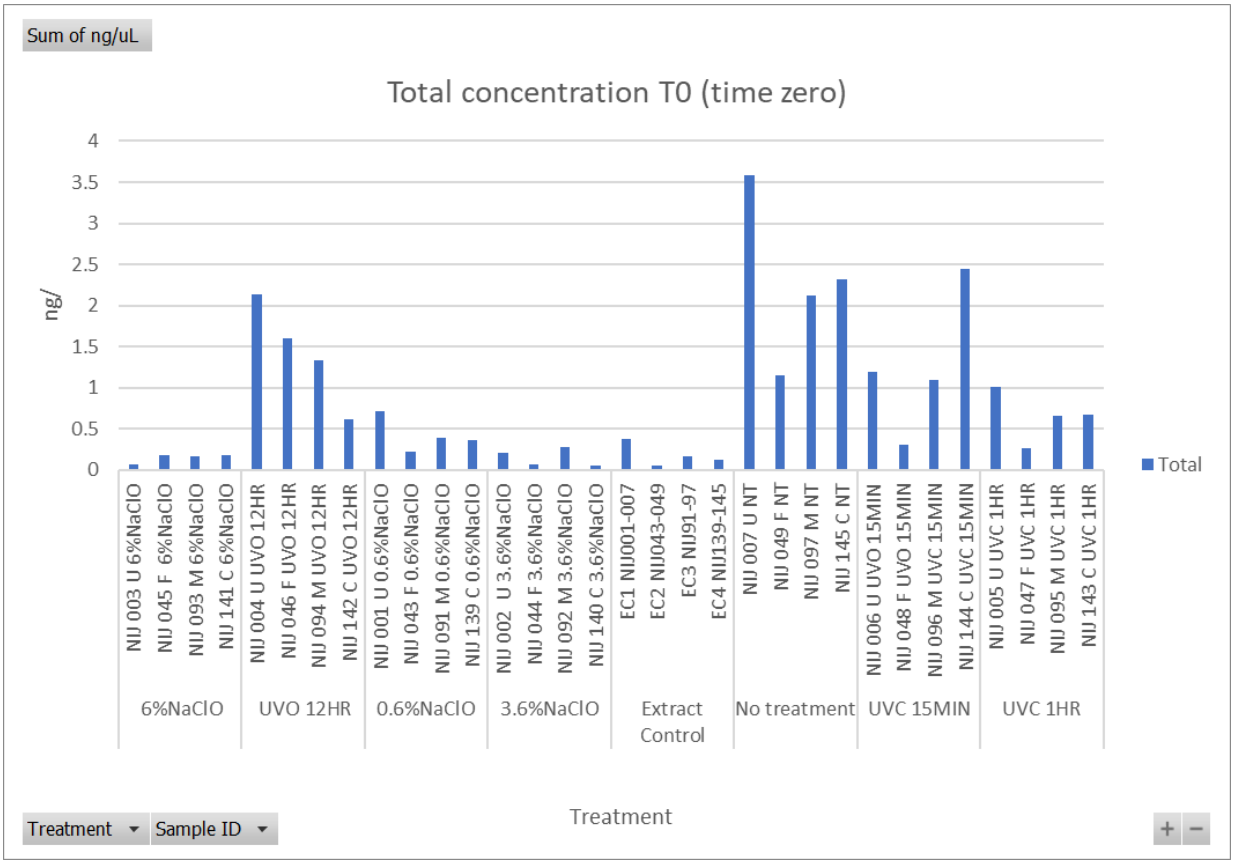

Figure 6. Time trial 0 (day zero) : genomic DNA concentrations by treatment

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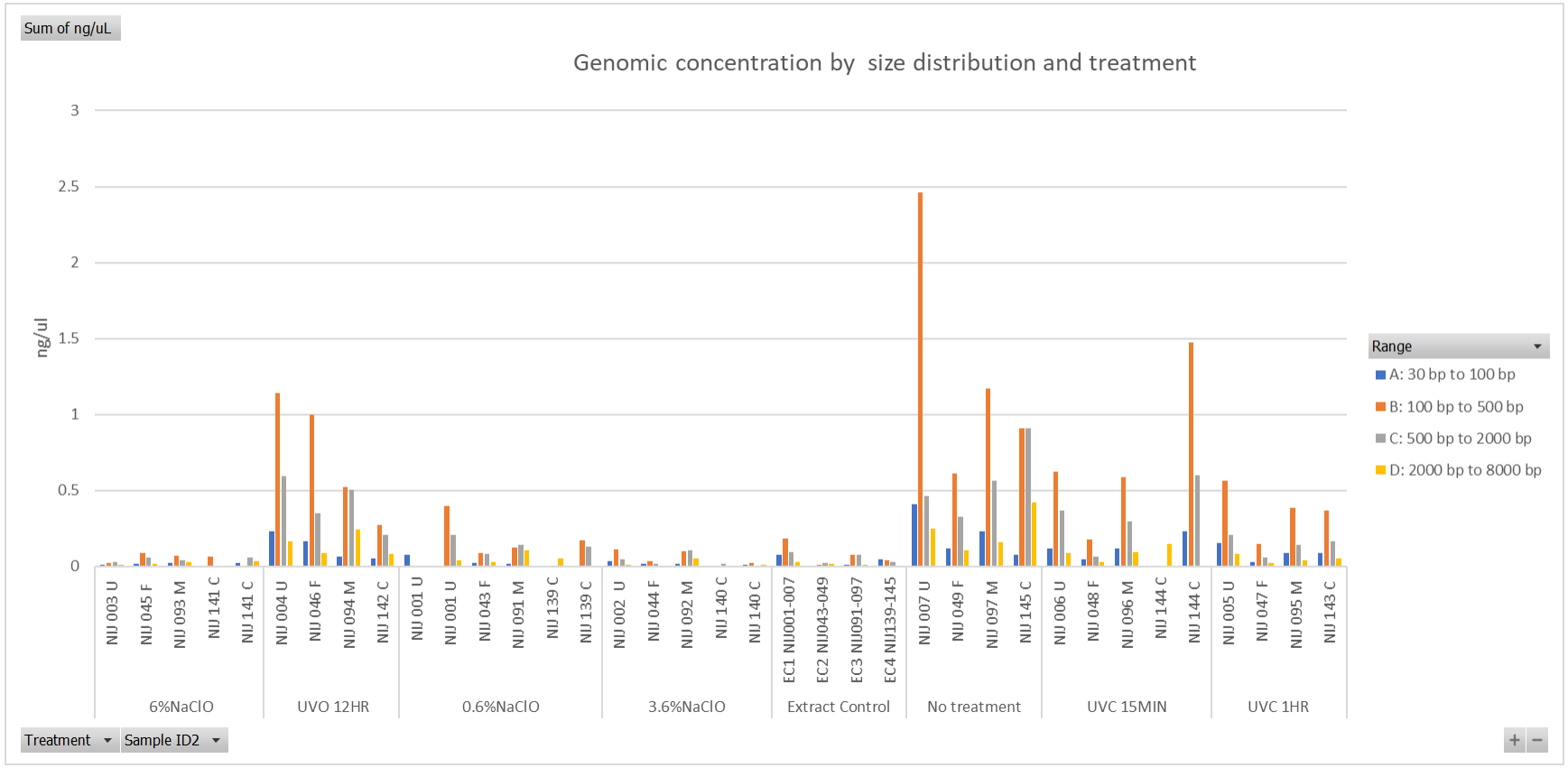


Figure 7. Time Zero samples genomic distribution plotted by treatment type.

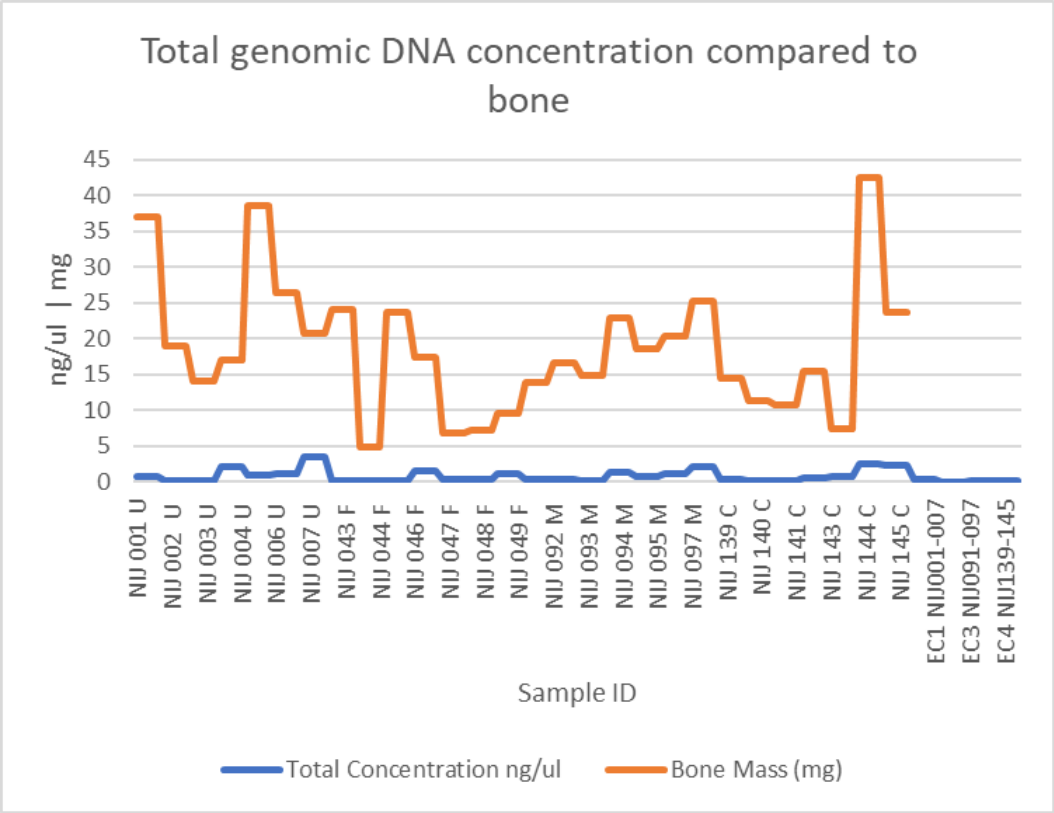


Figure 8. Time zero bone mass versus total amount of genomic DNA.

4.1.1 Time Trial 1 (4 weeks) Genomic Concentration and Fragment Distribution.

Time trial 1 (T1) DNA concentrations ranged from 0.0000- 2.9408ng/ul with an average of 0.3895ng/ul (Appendix B, Tab M). In contrast to T0, samples with no treatment and those untouched (U=untouched) tended to have lower concentrations of DNA. The highest yields were from UV treated samples. Bleached samples also had the lower genomic concentrations (Figure 9, Appendix B tab M). There was little to any correlation to treatment at 4 weeks (r= 0.1469). Most of the T1 samples had a larger proportion of DNA fragments that ranged between 100-500bp in size (Figure 10). When size distribution is plotted by treatment, T1 samples that were touch by multiple individuals (C= cumulative) had higher overall yields (Figure 10). There is a negative correlation r= -0.2545 between DNA concentrations and the amount of bone used in the extraction suggesting other factors, such as inhibitors, may be affecting DNA concentrations (Figure 11).

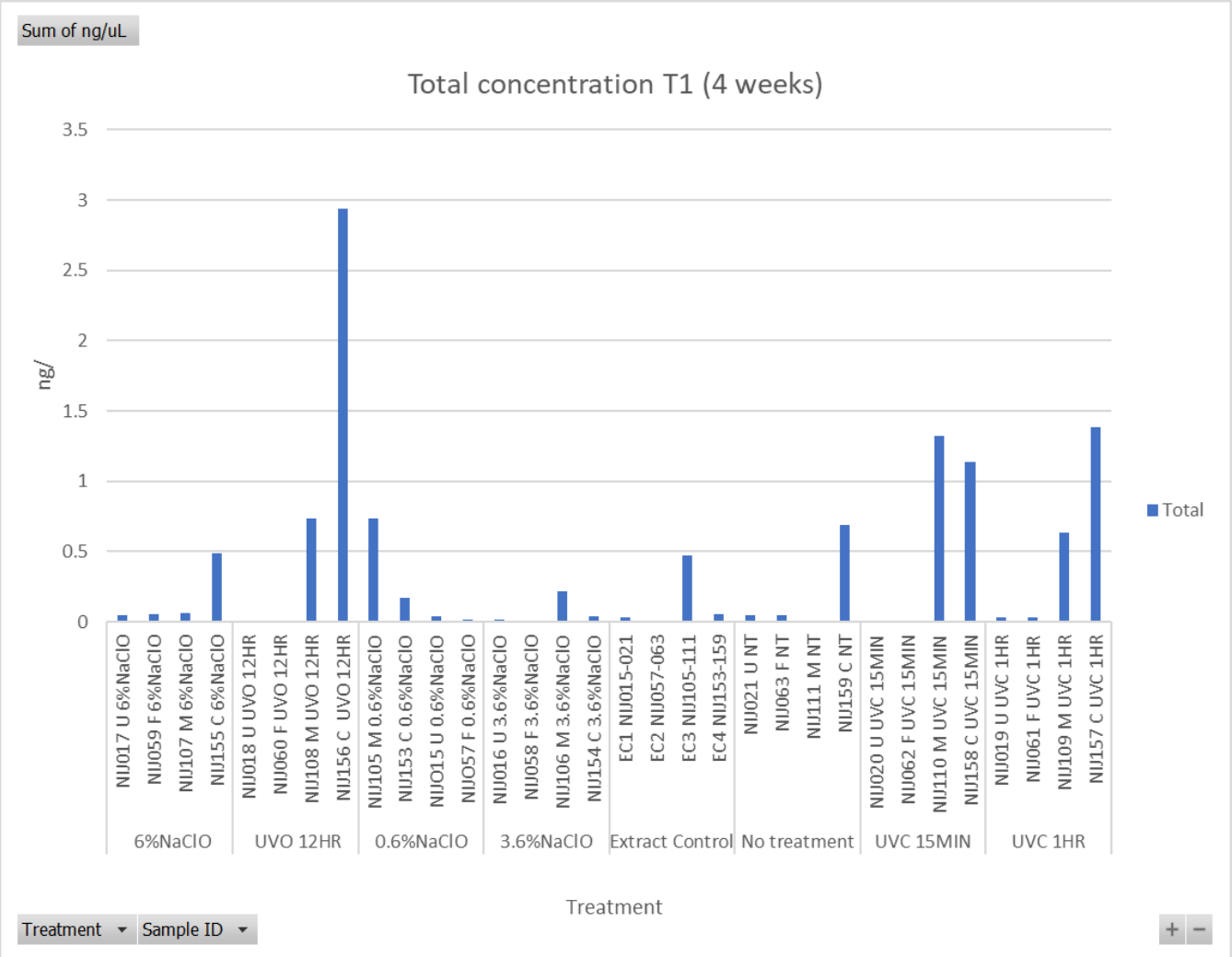


Figure 9. Time trial 1 (4 weeks): genomic DNA concentrations by treatment.

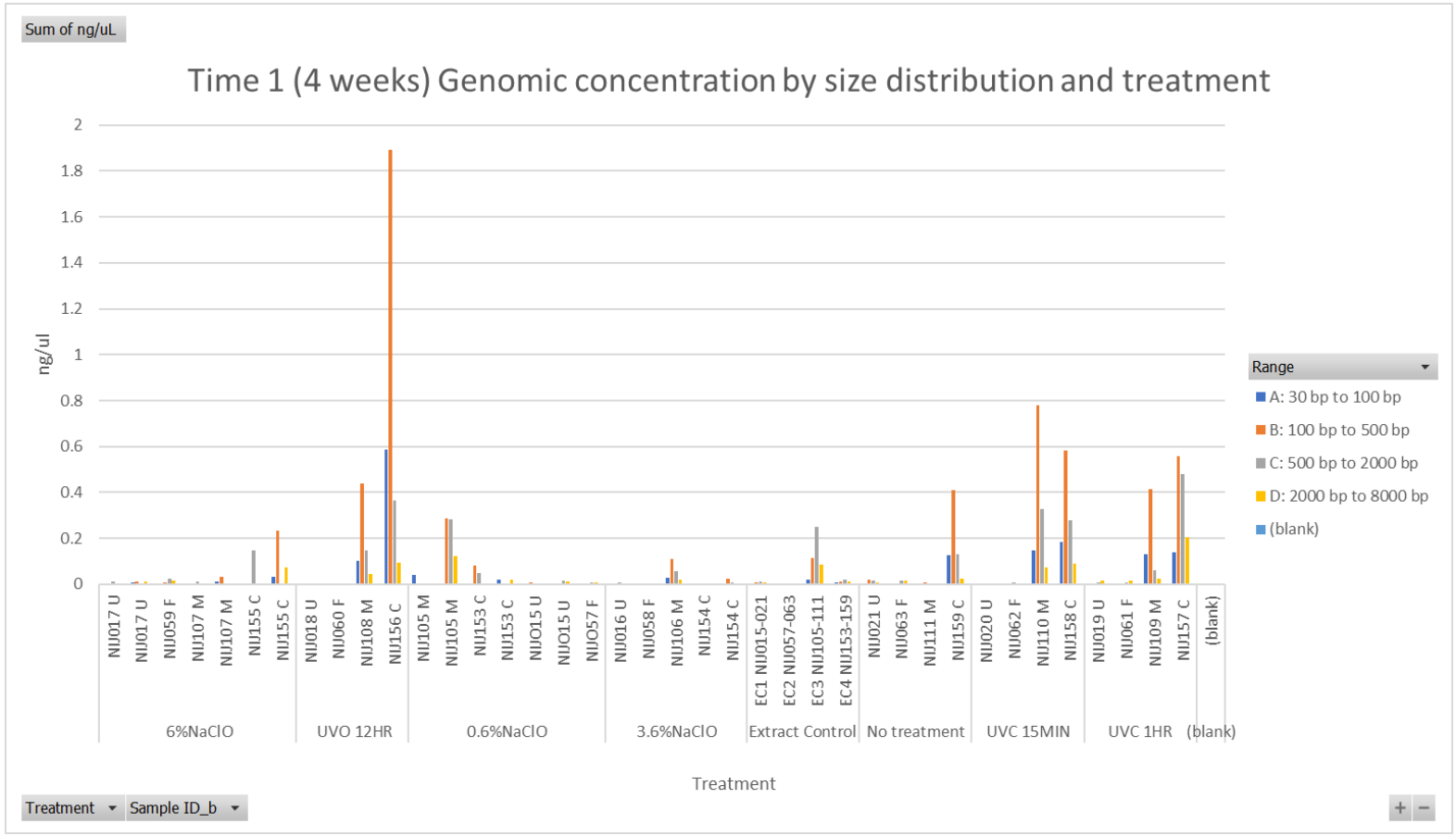


Figure 10. Time 1 (four weeks) samples: genomic distribution plotted by treatment type.

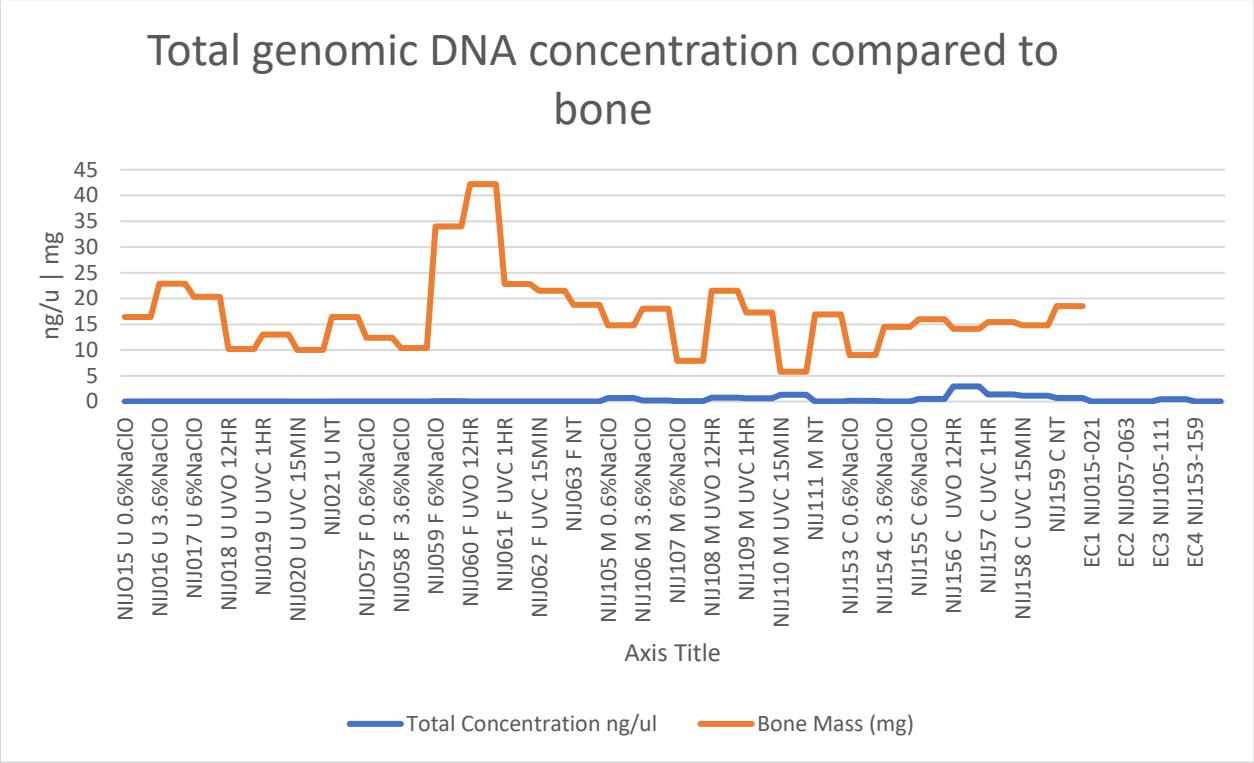


Figure 11. Time trial 1 bone mass compared to total amount of genomic DNA.

4.1.2 Time Trial 2 (16weeks/4months) Genomic Concentration and Fragment Distribution.

Time trial 2 (T2) concentrations ranged from 0.0002 to 4.9244ng/ul (Appendix B tab R). Extract controls varied from 0.0004-0.9919ng/ul and averaged 0.2526ng/ul.
No treatment and UV samples had higher yields of DNA. Compared to T0 and T1, bleached samples still have measurable amounts of DNA, with the exception 3.6% NaClO treated samples (Figure12,13, Appendix B tab R). Untouched samples had lower DNA yields. There is a small correlation to treatment at 16 weeks (r=0.3960). Like earlier time trials, most DNA fragments ranged between 100-500bp in size (Figure 13). There is a low correlation r= 0.0290between DNA concentrations and the amount of bone used in the extraction (Figure 14).

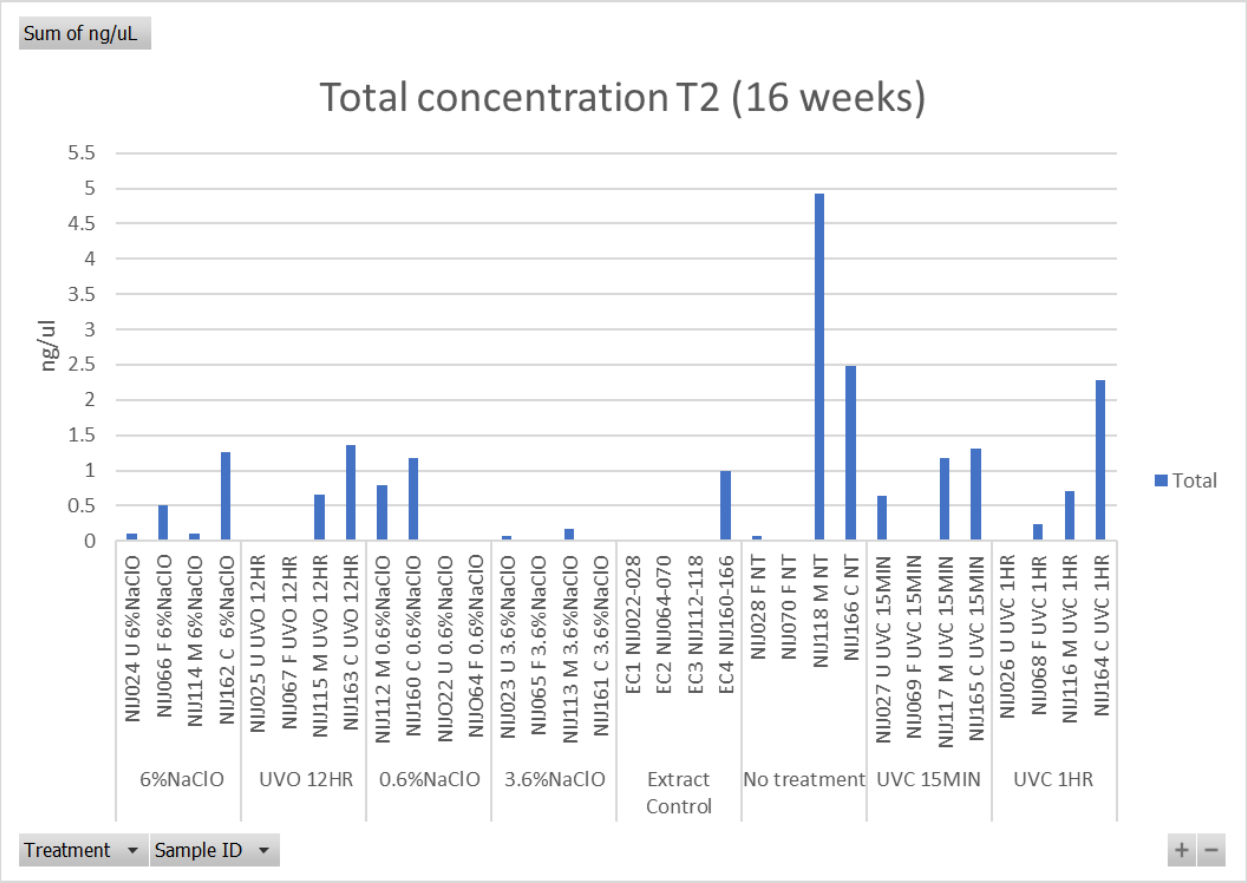


Figure 12. Time trial 2 (16 weeks): genomic DNA concentrations by treatment.

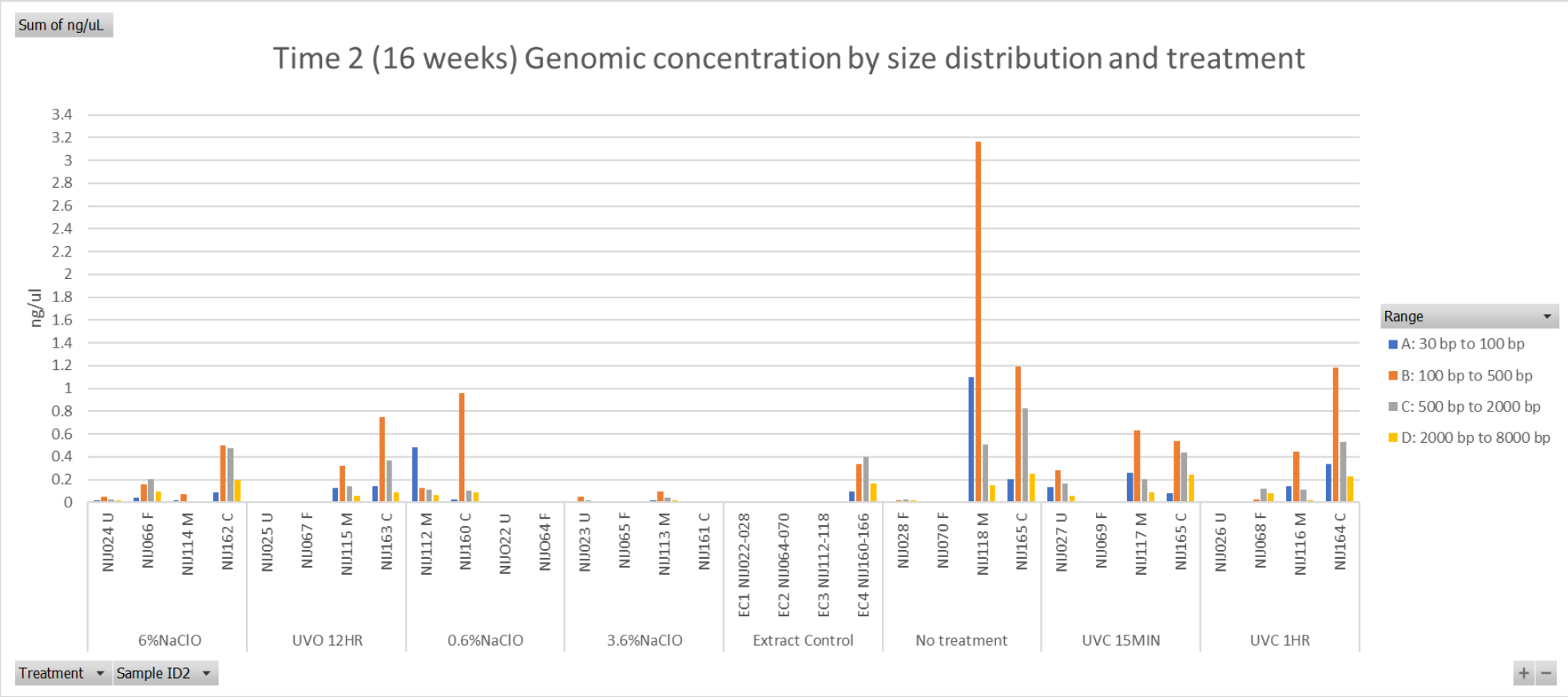


Figure 13. Time 2 (16 weeks) samples: genomic distribution plotted by treatment type.

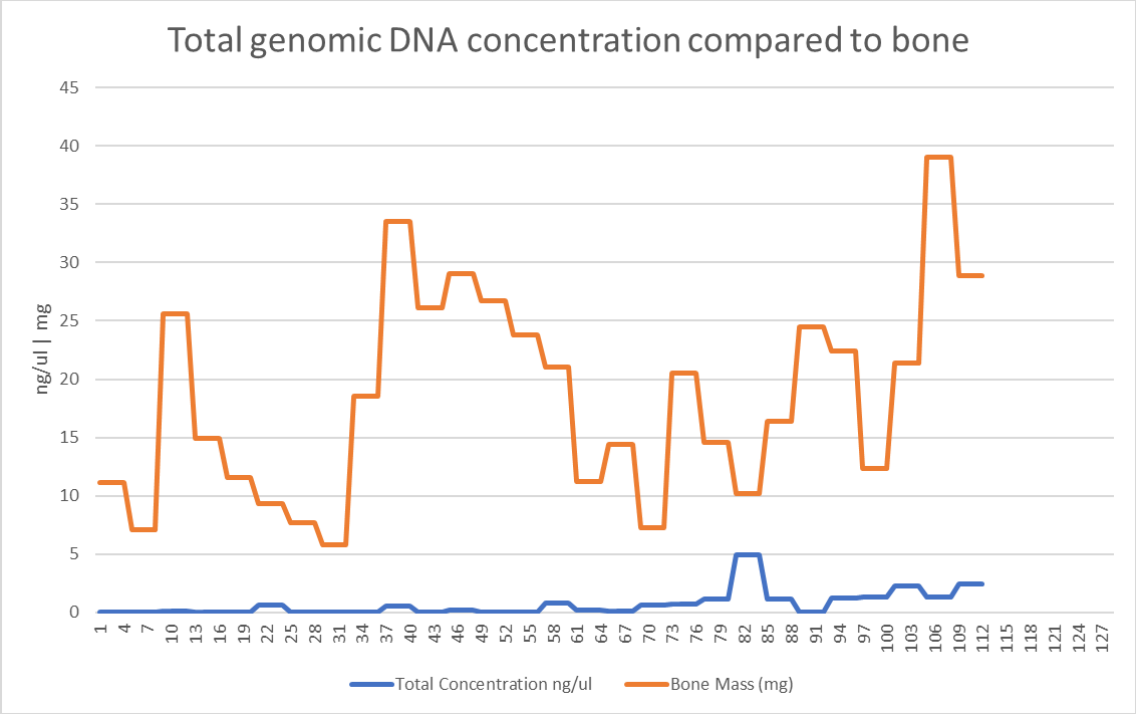


Figure 14. Time trial 2 bone mass compared to total amount of genomic DNA.

4.1.3 Time Trial 3 (6 months) Genomic Concentration and Fragment Distribution.

Across all treatments, DNA concentrations were lower for the 6-month time trial (T3) varying from 0.0026-1.4397ng/ul with an average concentration of 0.1949ng/ul (Figure 15, Appendix B tab W). Extract controls measured between 0.0112- 0.0479ng/ul and averaged 0.0296. There was little correlation between concentration and treatment (r= 0.1360). While some T3 samples had fragments sizes most concentrated between 100-500bp (NIJ122, NIJ125), lower concentration samples had a more even distribution across fragment sizes including some having more DNA fragments greater than 500bp. Most of the time trial 0 (T0) DNA fragments range between 100-500bp in size (Figure 16). There is a low correlation r= 0.1360 between DNA concentrations and the amount of bone used in the extraction (Figure 17).

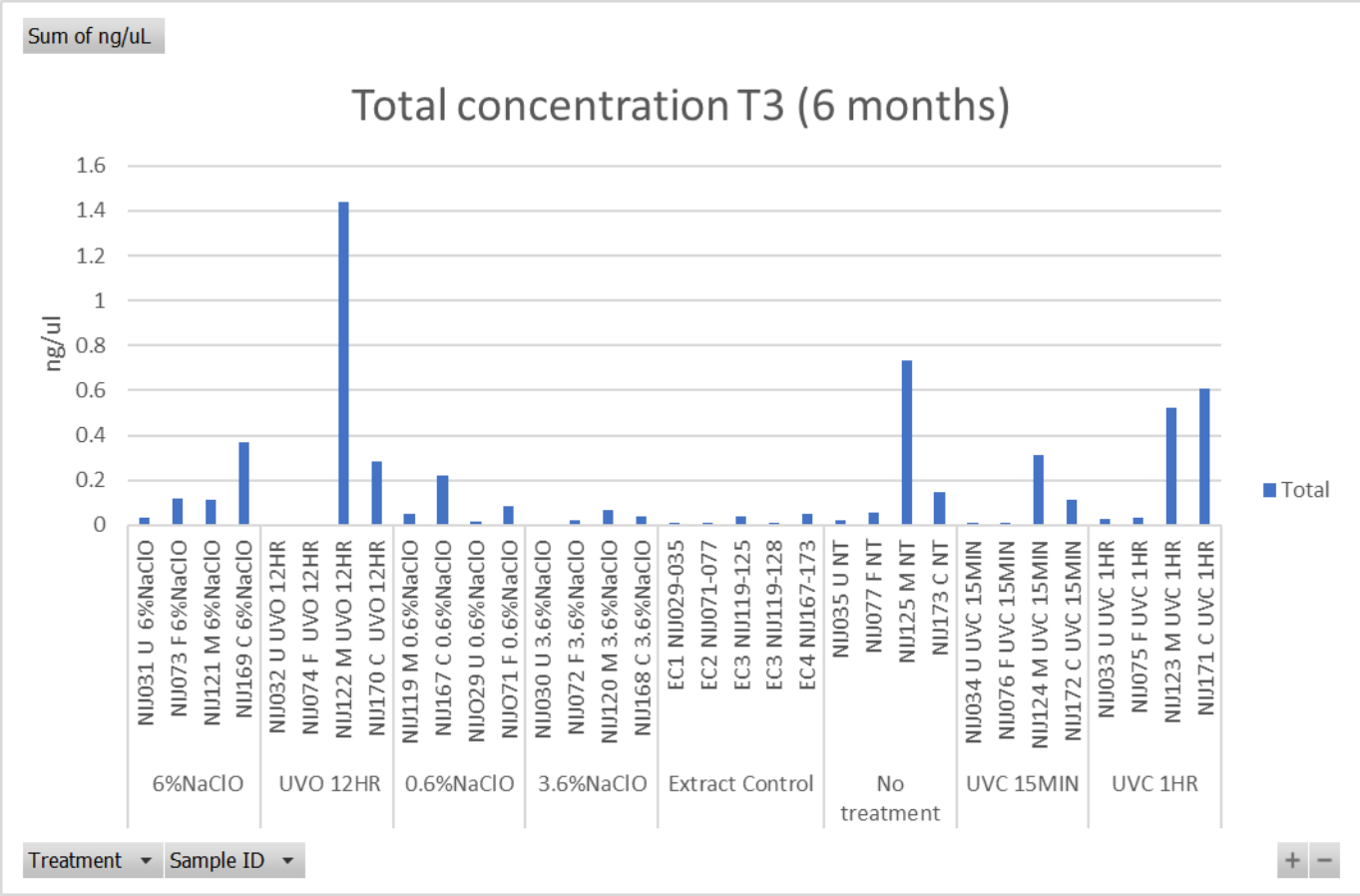


Figure 15 Time trial 3 (6 months): genomic DNA concentrations by treatment.

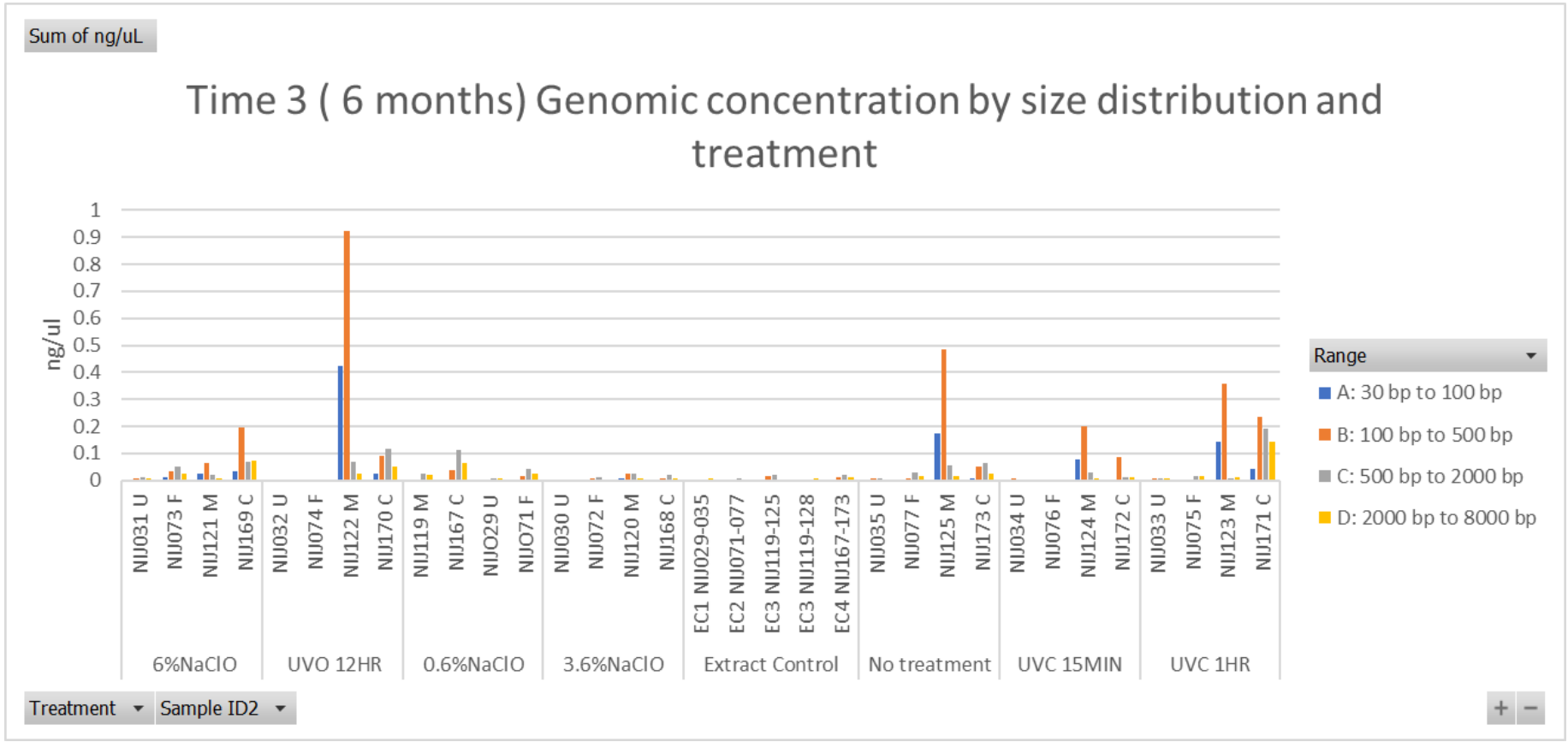


Figure 16. Time trial 3 (6 months) samples: genomic distribution plotted by treatment type

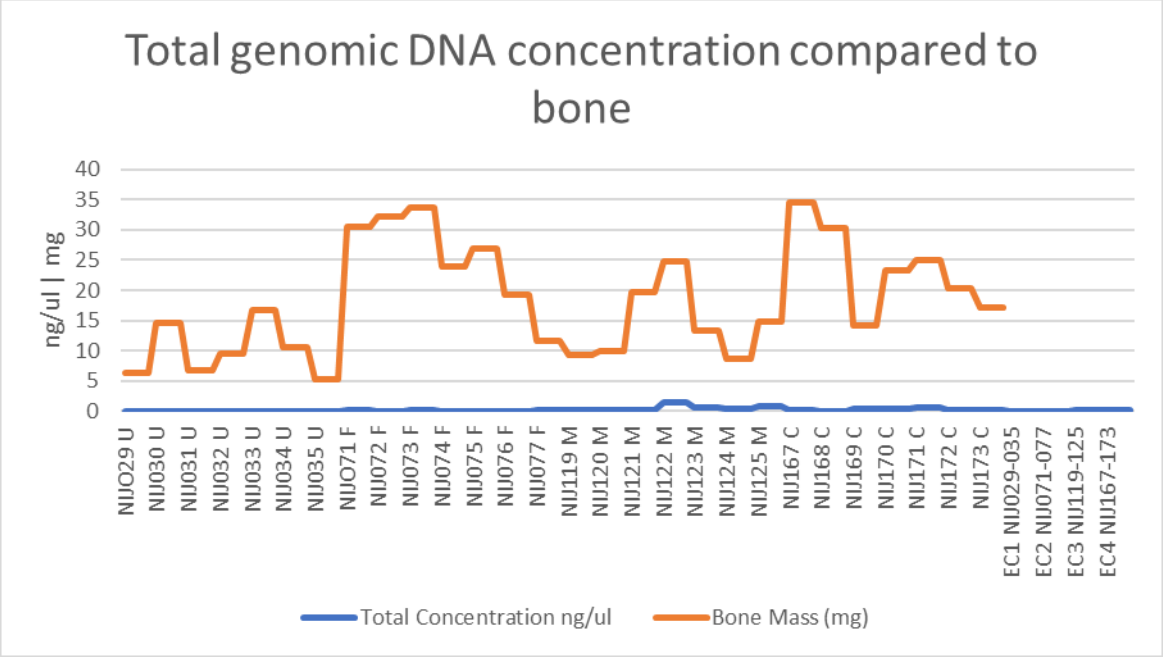


Figure 17. Time trial 3 (6 months) bone mass compared to total amount of genomic DNA.

4.1.1 Time Trial 4 (1 year) Genomic Concentration and Fragment Distribution

Genomic DNA concentrations for time trial 4 (T4) varied across treatment and ranged from 0.0006- 3.8676ng/ul (average 0.7886ng/ul). Extract controls varied from 0.0012-1.2599 (average 0.1914ng/ul)(Appendix B, tab AB). Results appear stochastic with little concordance to treatment type and with concentrations that are equal to or higher than earlier trials (Figure 18). There is a negative correlation between concentration and treatment (r= -0.1691). Many of the T4 DNA fragments range between 100-500bp in size as well as 500-2000bp (Figure 19). There is a low correlation r= 0.3450 between DNA concentrations and the amount of bone used in the extraction (Figure 20).

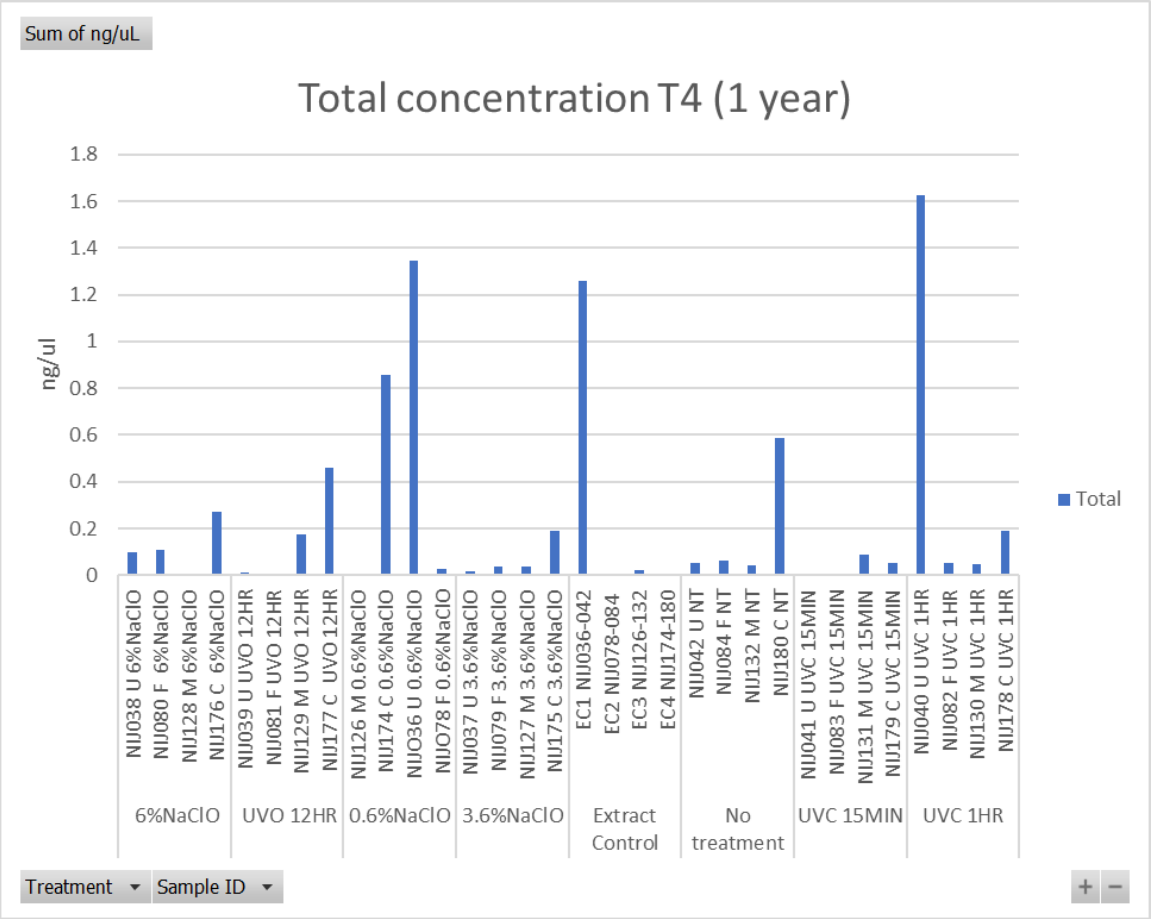


Figure 18 Time trial 4 (1 year): genomic DNA concentrations by treatment

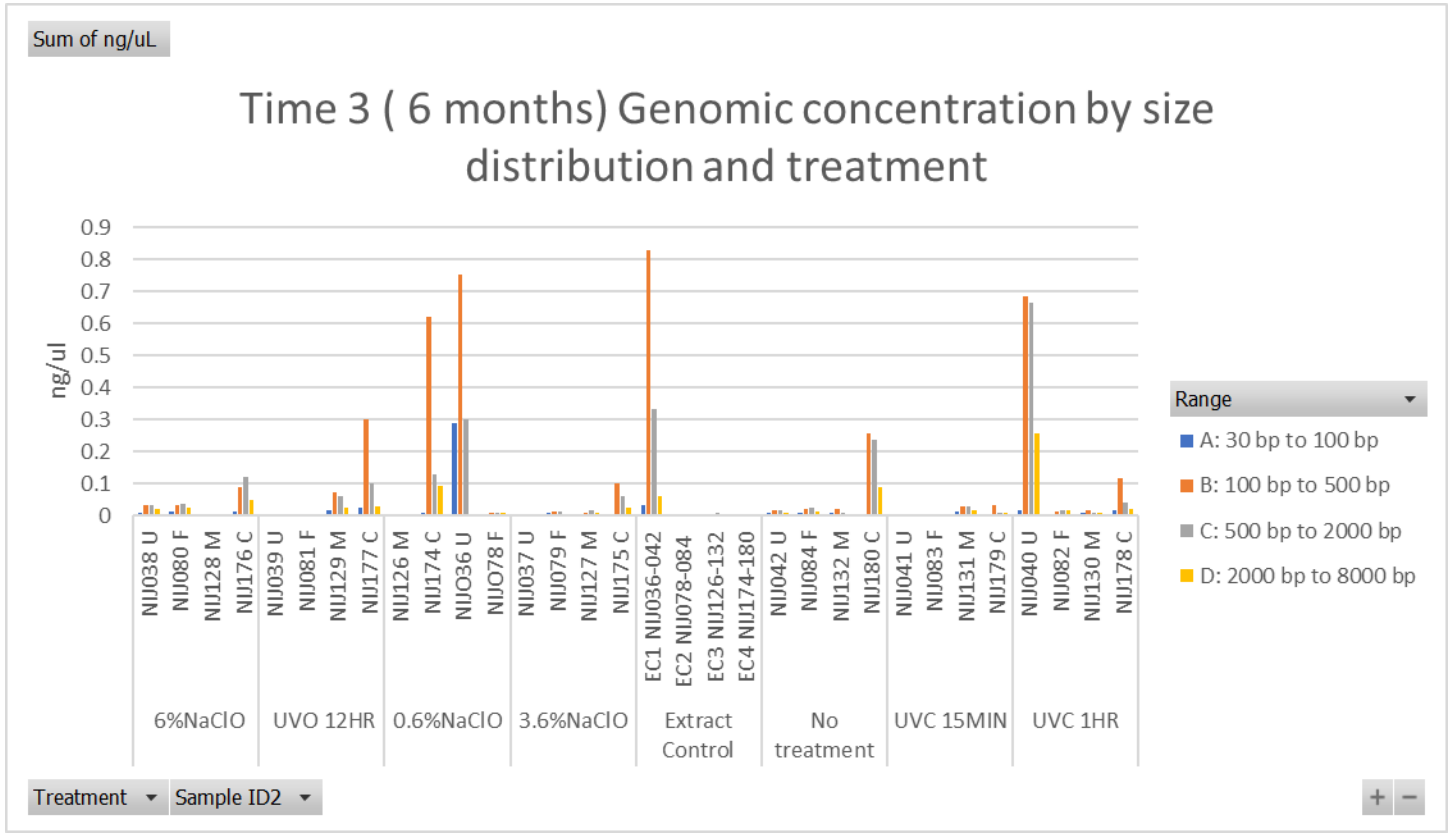


Figure 19. Time trial 4 (1 year) samples: genomic distribution plotted by treatment type.

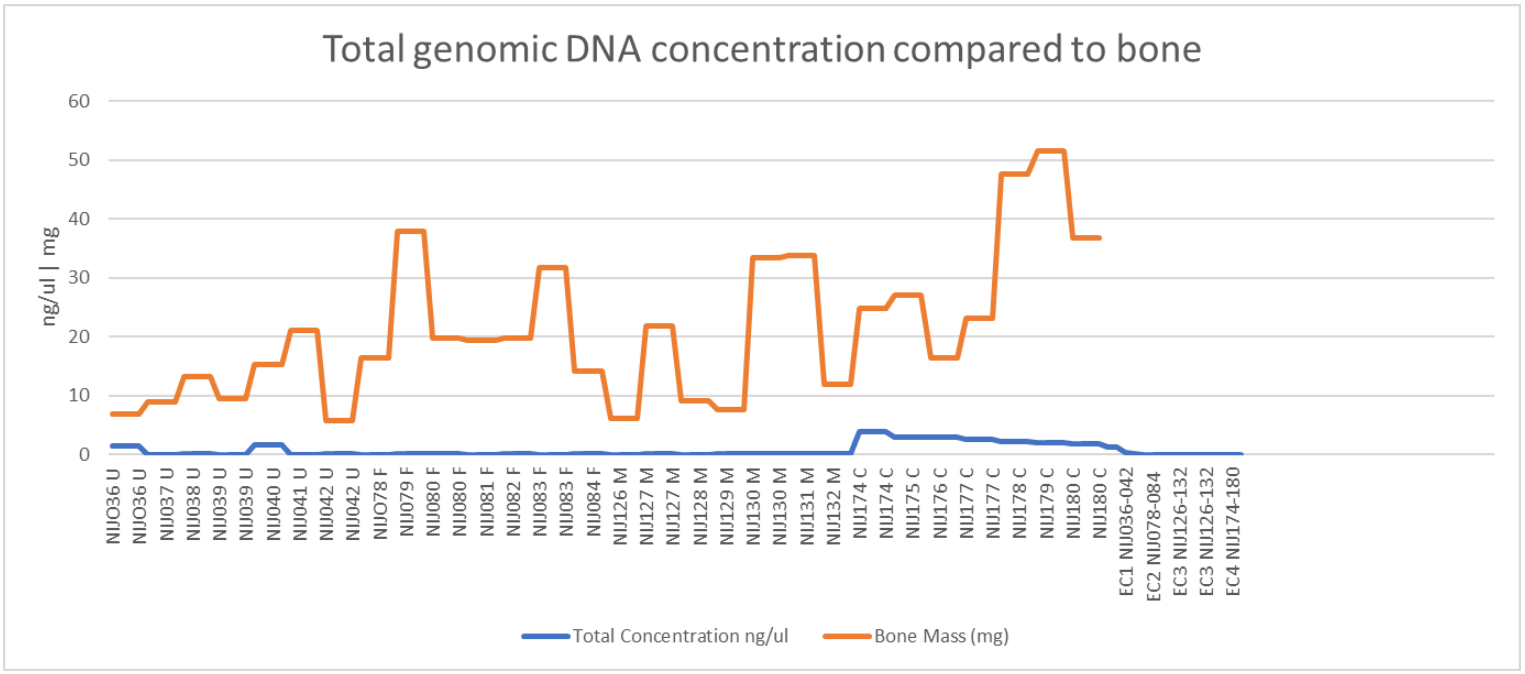
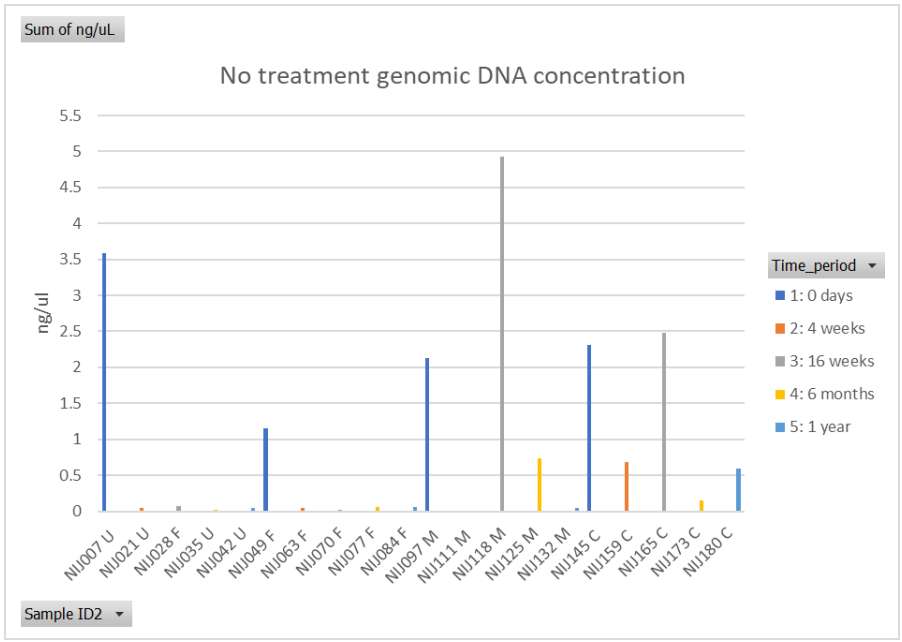


Figure 20. Time trial 4 (1 year) bone mass compared to total amount of genomic DNA.

4.1.2 Genomic DNA concentrations by treatment

Figures 21-27 display genomic concentrations by treatment over time. There is a negative correlation between concentration and no treatment (r=-0.36155), 12-hour overhead UV (r=-0.2970), and 15 min crosslinker UV (r= -0.3935) suggesting time may be a greater factor. There was a weak correlation between all bleach treatments and 1hr crosslinker UV (0.6% NaClO r= 0.266384233, 3.6% NaClO r= 0.26741203, 6% NaClO r= 0.273828409, 1hr UV r= 0.084652632).



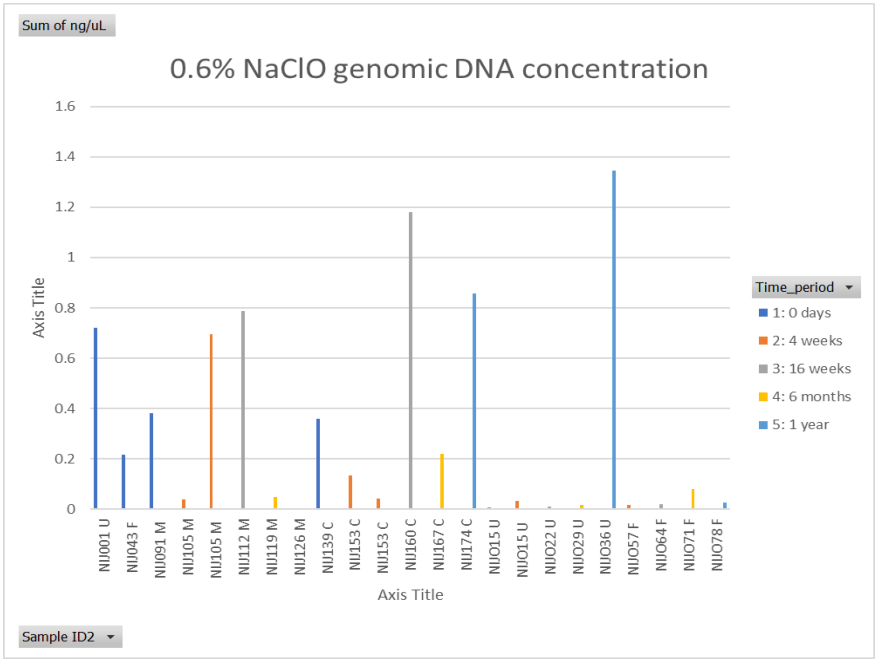
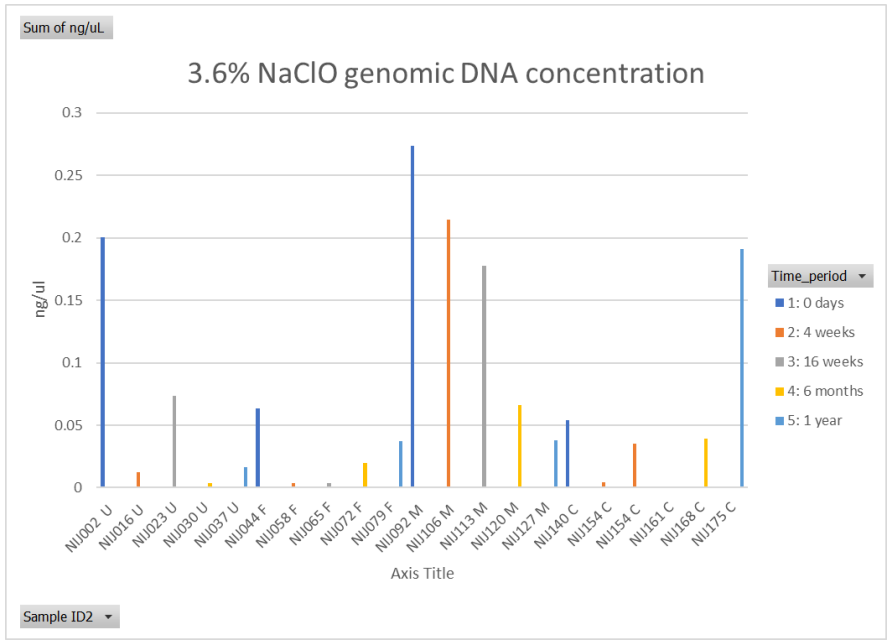


Figure 21 Genomic DNA concentrations with no treatment.

Figure 22 Genomic DNA concentrations with 0.6% NaClO treatment.



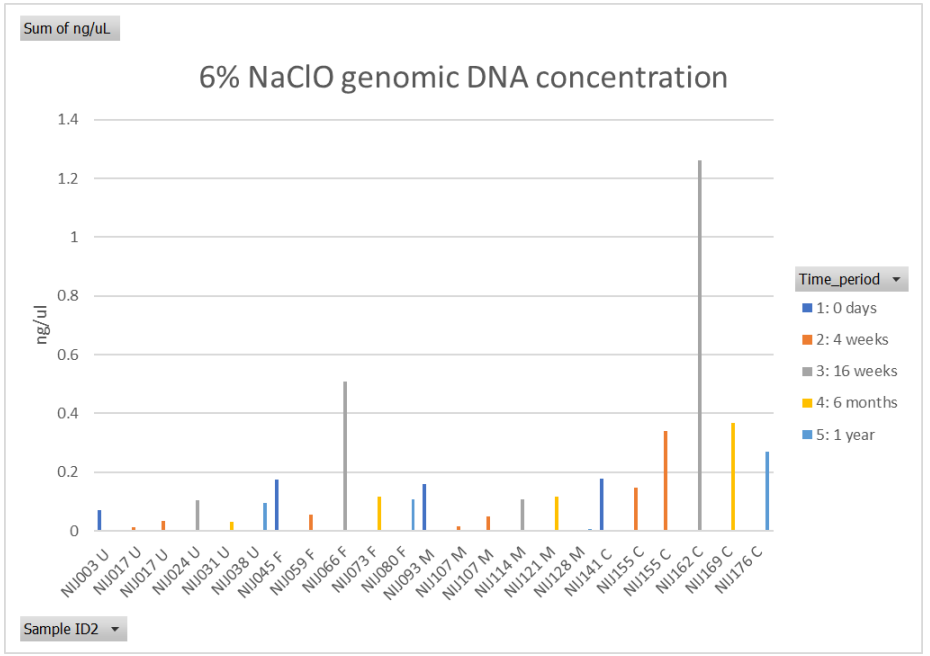
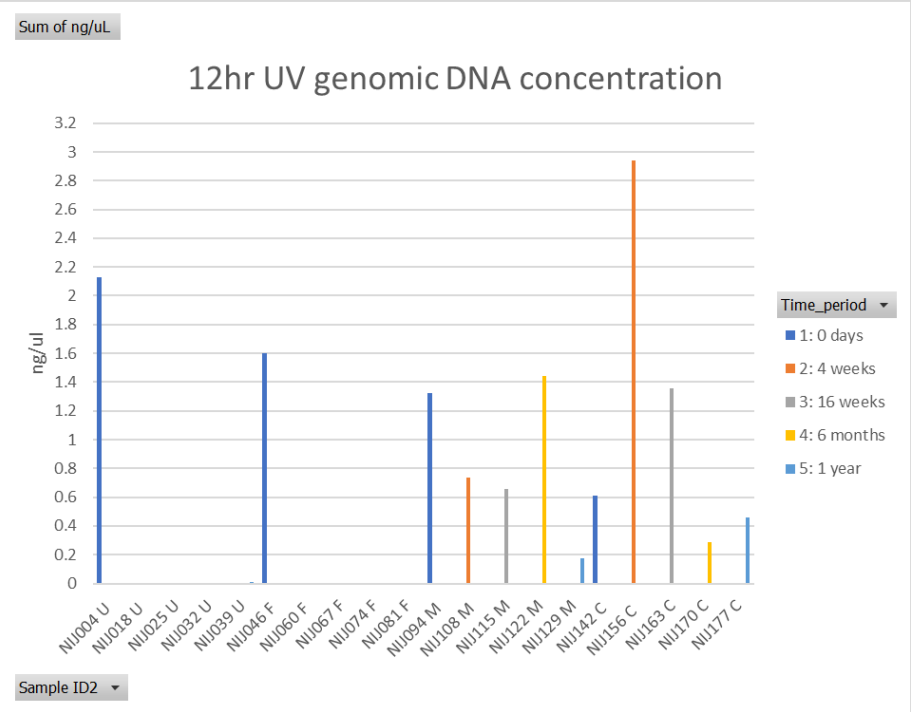


Figure 23 Genomic DNA concentrations with 3.6% NaClO2.

Figure 24 Genomic DNA concentrations with 6% NaClO2.



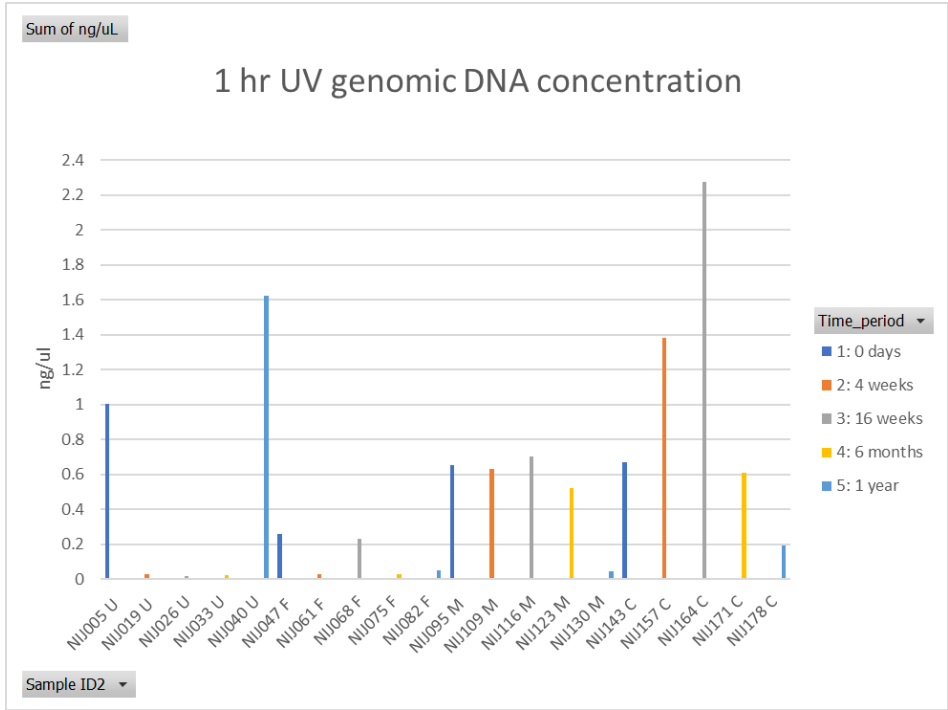


Figure 25 Genomic DNA concentrations with 12hr UV

Figure 26 Genomic DNA concentrations with 1hr UV

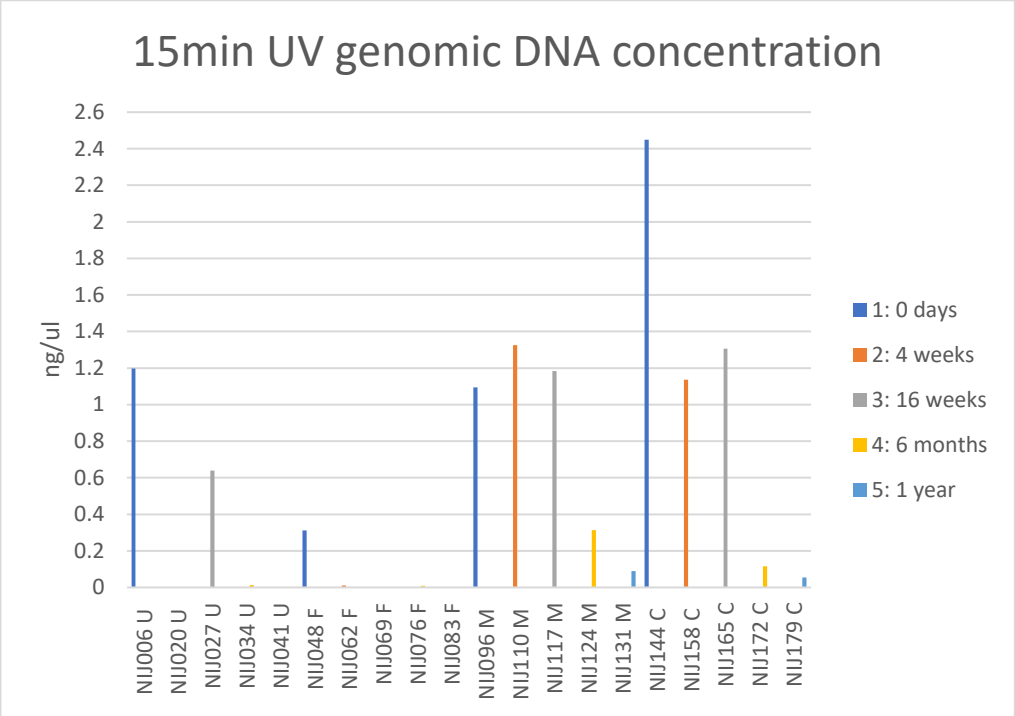
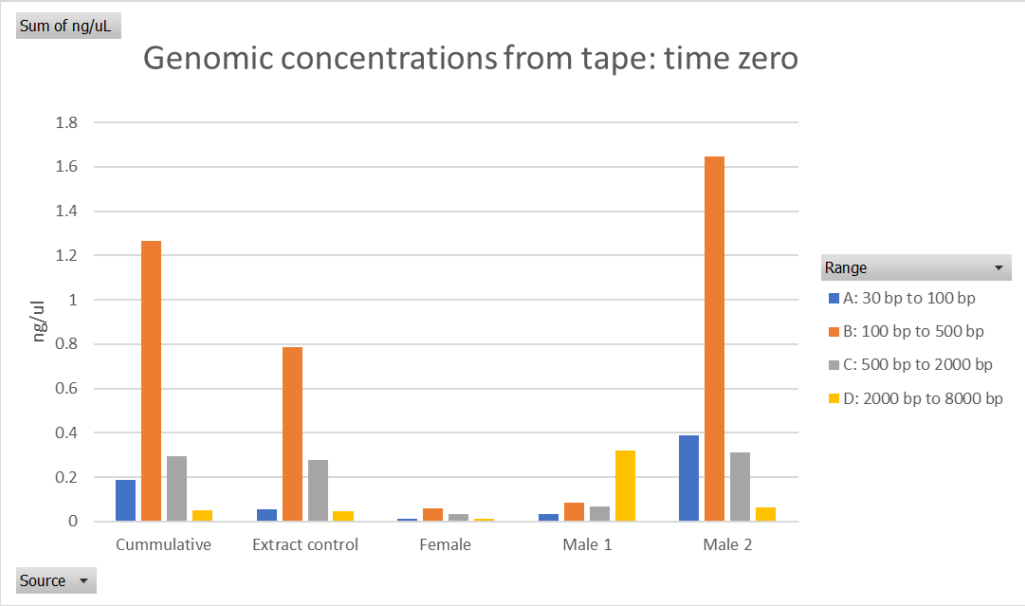


Figure 27 Genomic DNA concentrations with 15min UV

4.1.3 Genomic concentrations from tape extractions

DNA was extracted from three-hole punches from evidence tape that had been applied to the palms of one female, two male handlers. A fourth tape collected touch DNA from all handlers (cumulative). Additionally, swabs were applied to the palms of the handlers pre- and post- tape collection. Tape samples were aged across the span of a year at room temperature. No treatments were applied, and extractions were spaced across five time periods (day zero (time of collection), one week, 16 weeks, eight months, one year). Genomic concentrations Fragment analyzer results (Appendix B tabs AQ-BI, Figures 28-32) vary highly between handlers and across time suggesting varying yields may be a product of the variability/repeatability of extraction process and resulting yields or uneven distribution of touch DNA across the tape surface. Only at one year do concentrations drop across all handlers. However, the 100-500 bp fragment size range is consistently the most represent across time and handlers.



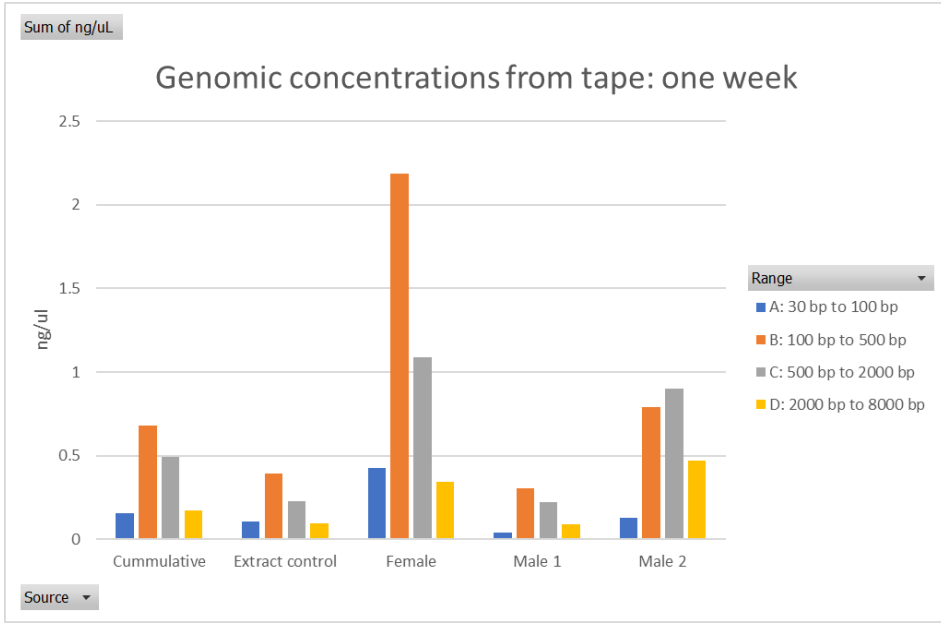


Figure 28. Genomic DNA concentration from tape extraction: time zero

Figure 29 Genomic DNA concentration from tape extraction: one week

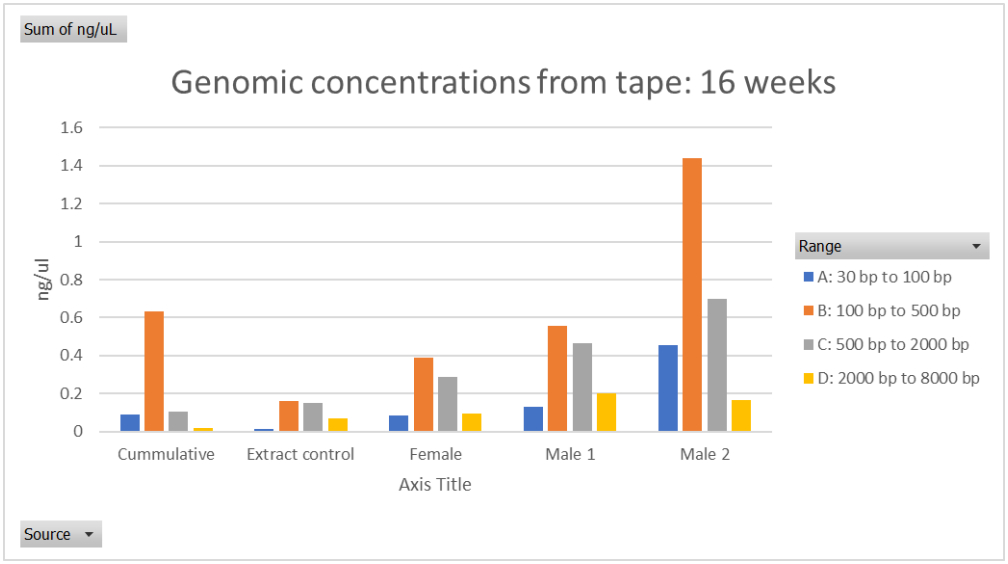


Figure 30. Genomic DNA concentration from tape extraction: 16 weeks.

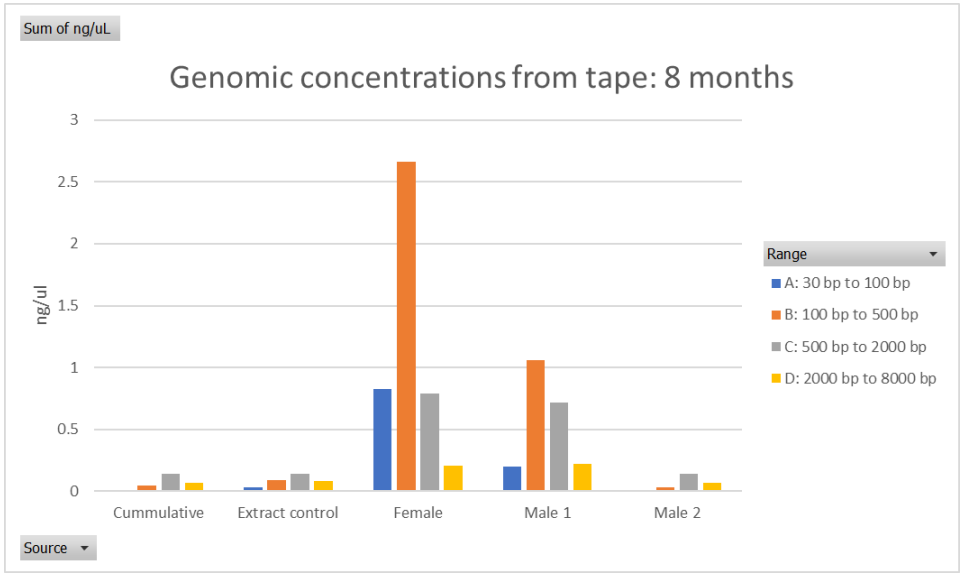


Figure 31 Genomic DNA concentration from tape extraction: 8 months.

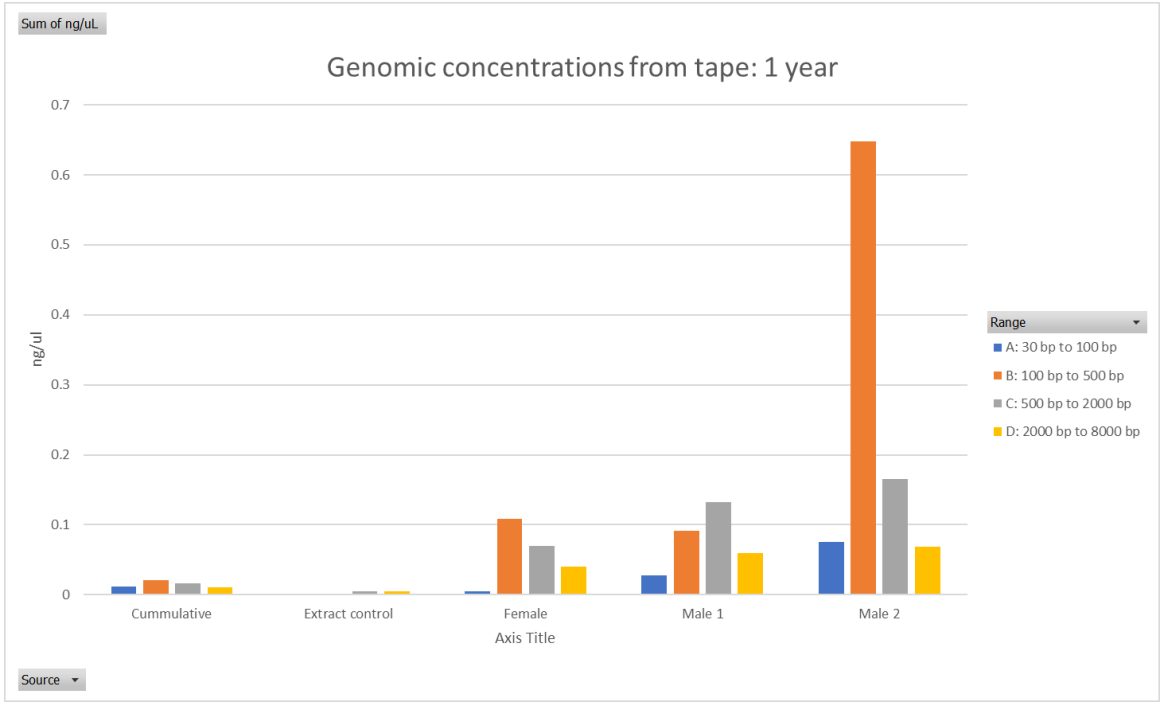


Figure 32. Genomic DNA concentration from tape extraction: one year.

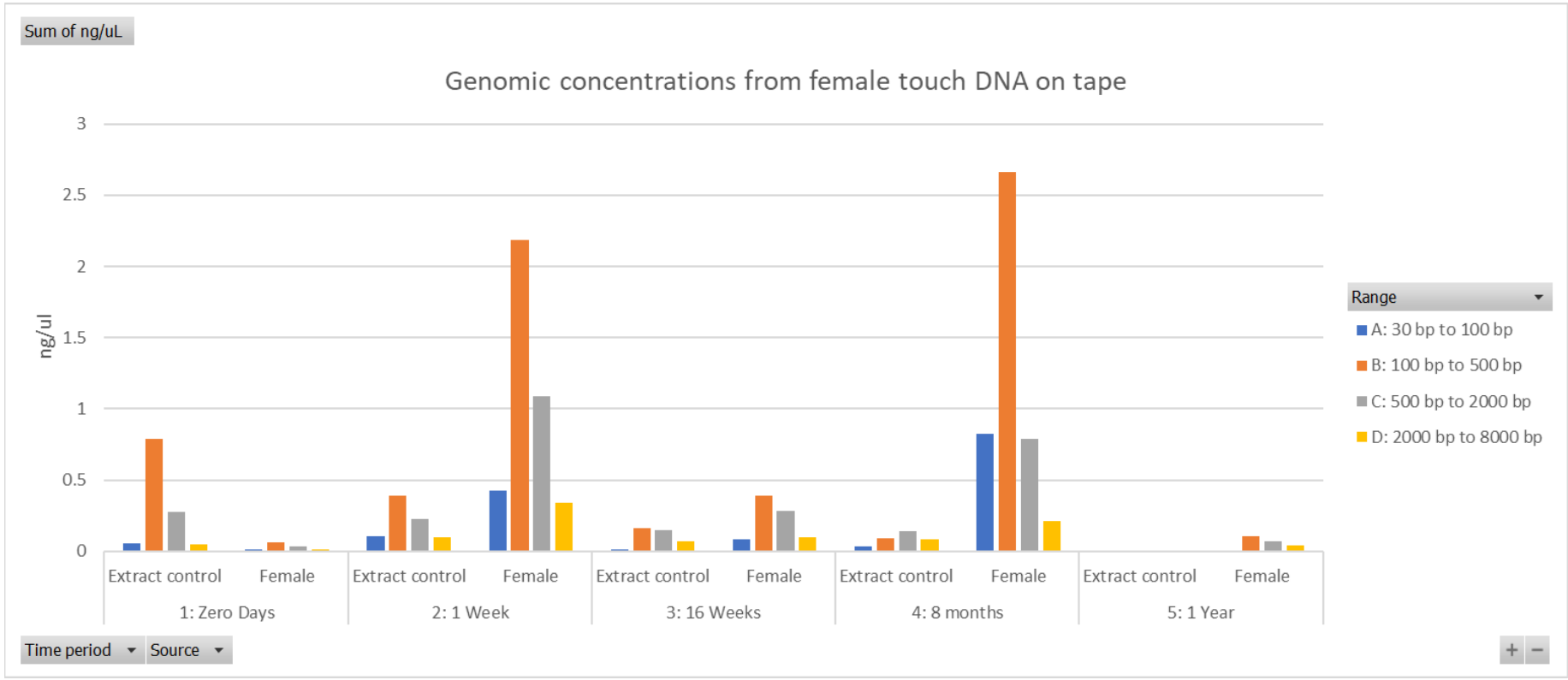


Figure 33 Genomic concentrations from female touch DNA on tape.

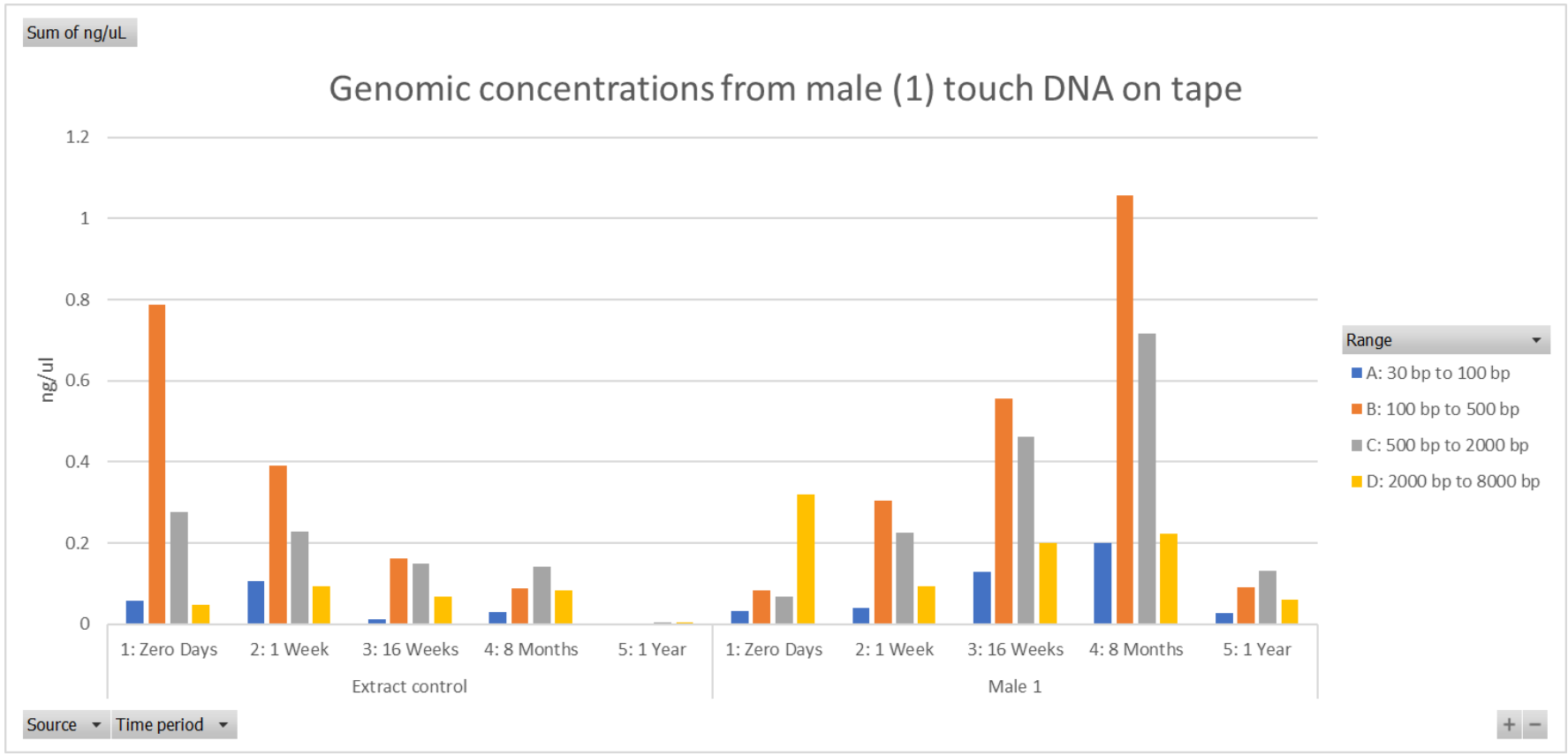


Figure 34 Genomic concentrations from male (1) touch DNA on tape.

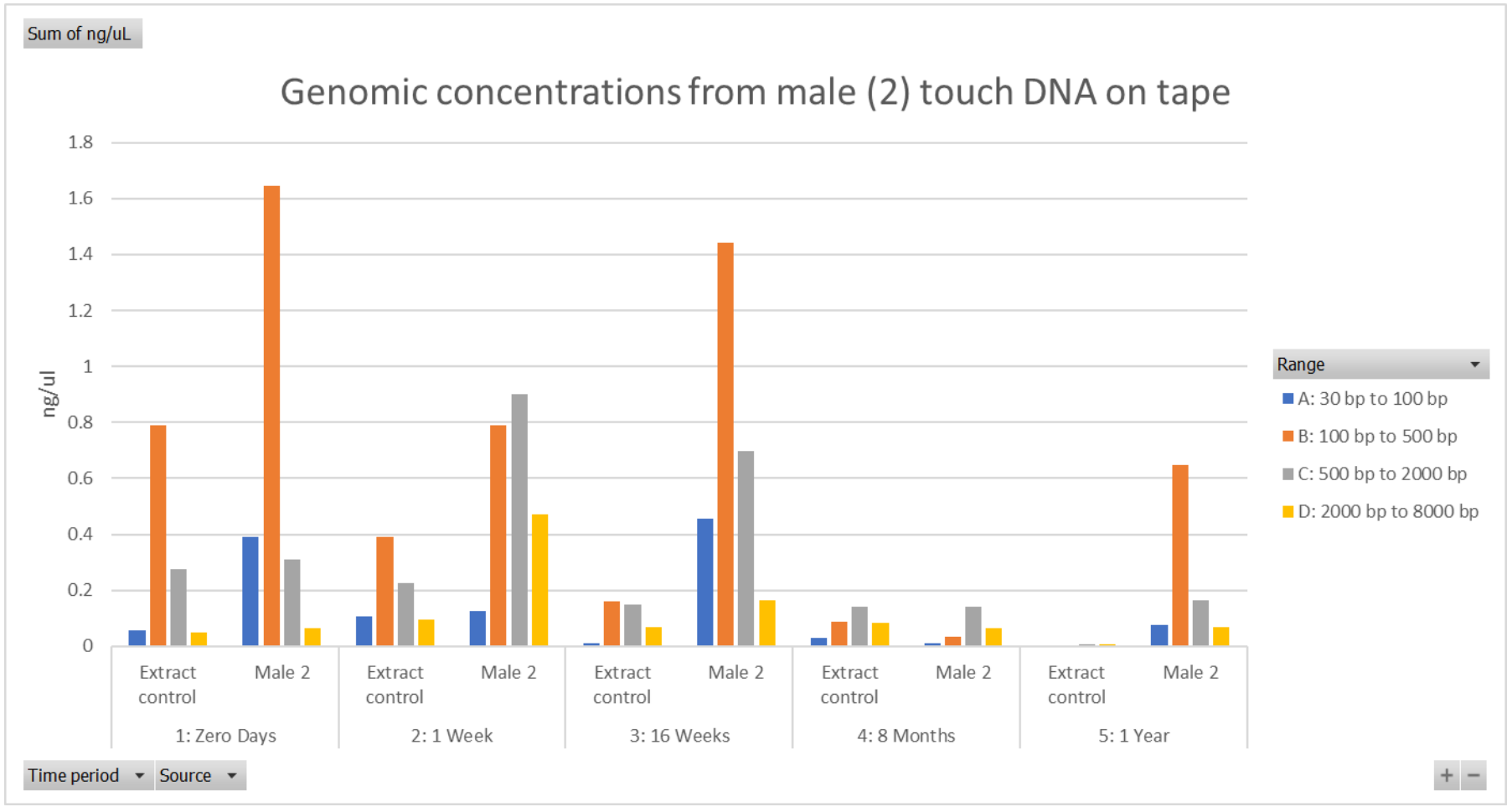


Figure 35 Genomic concentrations from male (2) touch DNA on tape.

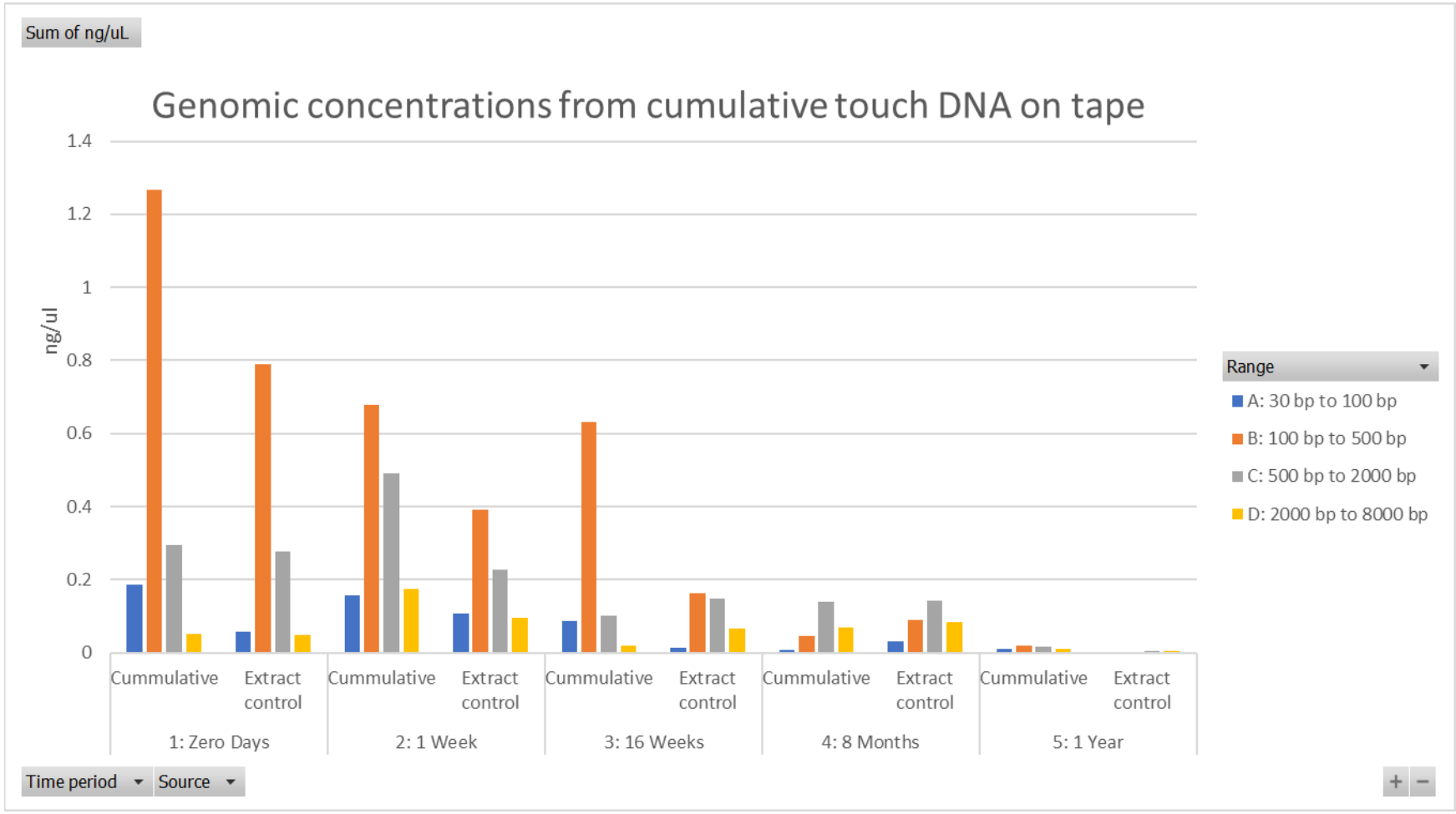


Figure 36 Genomic concentrations from cumulative touch DNA on tape

4.2 Next-generation sequencing results

Purified and pool libraries were sent for next-generation Illumina sequencing at Admera Health (Next-Seq PE150) and Oklahoma Medical Research Facility (OMRF) (Nova-Seq S4 PE100). The resulting sequence "reads" (short DNA sequences of ~200 base pair (bp) or less for ancient samples) were analyzed using a custom-designed bioinformatics pipeline. Briefly, the sequenced reads were trimmed to remove low quality or ambiguous bases/nucleotides (these are points where it is unclear, errors occur, or have too poor preservation) using fastp (Chen, Zhou, Chen, & Gu, 2018). The unique (non-clonal) attached "barcodes sequences" were then removed from each sample's dataset using Adapter Removal. These analysis-ready reads were then used for all further bioinformatics analyses including ancestry determination, sex typing, 16s microbial breakdown. In order to determine levels of endogenous DNA content, as well as potential contamination, analysis-ready sequence reads for all samples were mapped to the human rCRS mitogenome and Homo sapiens (human) genome assembly GRCh37 (hg19) from Genome Reference Consortium [GCA_000001405.1 GCF_000001405.13] using bowtie2 (Langmead & Salzberg, 2012). Mapped reads were filtered to remove low quality reads as well as duplicate reads using a program called SAMTools (Li et al., 2009). DNA damage was measured using MapDamage v2 (Jónsson, Ginolhac, Schubert, Johnson, & Orlando, 2013). Mitotyping used Haplogrep 2.0 (Weissensteiner et al., 2016).

4.2.1 Next-generation sequencing results mapped to human genome

Across all time trials, indexed and amplified libraries ranged from 0.0046- 303.1412nM with an average of (26.46nM) (Appendix C, tab A). Mapped reads to the human genome varied sample to sample and endogenous content generally ranged from 0-5% (Table 3-7). An exception is NIJ003, which had orders of magnitude higher number of sequencings reads and overall a higher human endogenous content at 21%. Since this amount of data was not targeted, the most likely scenario is error in determining library concentration or diluting/pooling of this sample. However, since endogenous content is still high (despite the sample being bleached) we cannot exclude contamination at a contributing factor for this sample. The number of sequencing reads consistently declined with time. Time periods T3 and T4 only had seven out of 28, and 13 out of 27 samples yielding any sequencing data (Appendix C, tabs A-F; Tables 3-7). Of those, most were untouched or cumulative touched samples. Interestingly, bleach treated samples had more and higher mapped reads to the human genome (endogenous content) than UV or untreated samples. While conjecture, it is possible that NaOCl, while removing the contaminating touch DNA, is also removing potential inhibitors of the DNA endogenous to the bone sample.

Table 3. Next Generation Sequencing Results for Time Zero (T0).

Sample ID	Touch Type	Treatment	Total mapped human genome reads	Mapped hgQ37 reads	hgQ37 Unique reads	% Unique	% Endogenous
NIJ001 0.6%NaClO	Untouched	0.6% Bleach	18674	10115	9278	92%	0%
NIJ002 3.6%NaClO	Untouched	3.6% Bleach	608144	320579	250627	78%	1%
NIJ003 6%NaClO	Untouched	6% Bleach	63410750	31092786	16295414	52%	21%
NIJ004 UVO12HR	Untouched	UV Overhead 12 Hours	2	2	2	100%	1%
NIJ005 UVC1HR	Untouched	UV Crosslinker 1 Hour	259566	141948	62345	44%	0%
NIJ006 UVC15MIN	Untouched	UV Crosslinker 15 mins	28757	18482	17045	92%	0%
NIJ007 NT	Untouched	No Treatment	103410	64190	57195	89%	0%
NIJ043 0.6%NaClO	Female	0.6% Bleach	159402	86275	18098	21%	0%
NIJ044 3.6%NaClO	Female	3.6% Bleach	4592	2786	2252	81%	1%
NIJ045 6%NaClO	Female	6% Bleach	408524	195090	47145	24%	1%
NIJ046 UVO12HR	Female	UV Overhead 12 Hours	44375	27815	13069	47%	0%
NIJ047 UVC1HR	Female	UV Crosslinker 1 Hour	281320	182322	108786	60%	1%
NIJ048 UVC15MIN	Female	UV Crosslinker 15 mins	35847	20777	14516	70%	0%
NIJ049 NT	Female	No Treatment	20428	13018	11409	88%	0%
NIJ091 0.6%NaClO	Male	0.6% Bleach	15392	7836	7176	92%	1%
NIJ092 3.6%NaClO	Male	3.6% Bleach	16534	7903	7108	90%	4%
NIJ093 6%NaClO	Male	6% Bleach	3942	2332	2155	92%	6%

NIJ094 UVO12HR	Male	UV Overhead 12 Hours	156107	111421	90666	81%	1%
NIJ095 UVC1HR	Male	UV Crosslinker 1 Hour	15777	9542	8048	84%	0%
NIJ096 UVC15MIN	Male	UV Crosslinker 15 mins	30746	18480	16167	87%	0%
NIJ097 NT	Male	No Treatment	37350	24750	20981	85%	0%
NIJ139 0.6%NaClO	Cumulative	0.6% Bleach	24435	17038	12312	72%	1%
NIJ140 3.6%NaClO	Cumulative	3.6% Bleach	668338	365579	166265	45%	2%
NIJ141 6%NaClO	Cumulative	6% Bleach	697446	323472	249916	77%	8%
NIJ142 UVO12HR	Cumulative	UV Overhead 12 Hours	695108	509318	231132	45%	0%
NIJ143 UVC1HR	Cumulative	UV Crosslinker 1 Hour	40398	24169	21353	88%	1%
NIJ144 UVC15MIN	Cumulative	UV Crosslinker 15 mins	889	676	542	80%	0%
NIJ145 NT	Cumulative	No Treatment	40499	32882	21979	67%	1%
NIJEC1_T0	Extract Control		2	0	0	NoData	0%
NIJEC1_T0B	Extract Control		147	109	93	85%	3%
NIJEC2_T0	Extract Control		5	4	4	100%	1%
NIJEC3_T0	Extract Control		16	10	7	70%	1%
NIJEC4_T0	Extract Control		24	16	15	94%	1%
NIJ_LN1	Library Control		0	0	0	NoData	0%
NIJ_LN2	Library Control		821	635	393	62%	2%

Table 4. Next Generation Sequencing: Human genome mapping at four weeks (T1).

Sample ID	Touch Type	Treatment	Total mapped human genome reads	Mapped hgQ37 reads	hgQ37.Unique reads	% Unique	% Endogenous
NIJ015 0.6%NaClO	Untouched	0.6% Bleach	1512034	1021595	617763	60%	0%
NIJ016 3.6%NaClO	Untouched	3.6% Bleach	7645398	5094267	1254513	25%	1%
NIJ017 6%NaClO	Untouched	6% Bleach	2210843	1277910	201691	16%	0%
NIJ018 UVO12HR	Untouched	UV Overhead 12 Hours	1565	1023	929	91%	0%
NIJ019 UVC1HR	Untouched	UV Crosslinker 1 Hour	691540	468791	130546	28%	0%
NIJ020 UVC15MIN	Untouched	UV Crosslinker 15 mins	283289	176413	6873	4%	0%
NIJ021 NT	Untouched	No Treatment	38350	22406	1751	8%	0%
NIJ057 0.6%NaClO	Female	0.6% Bleach	1109	634	533	84%	1%
NIJ058 3.6%NaClO	Female	3.6% Bleach	58	33	25	76%	0%
NIJ059 6%NaClO	Female	6% Bleach	32	24	16	67%	1%
NIJ060 UVO12HR	Female	UV Overhead 12 Hours	859496	572029	300289	52%	0%
NIJ061 UVC1HR	Female	UV Crosslinker 1 Hour	37992	24789	14488	58%	0%
NIJ062 UVC15MIN	Female	UV Crosslinker 15 mins	2113536	1355031	187822	14%	0%
NIJ063 NT	Female	No Treatment	173339	114242	65864	58%	0%
NIJ105 0.6%NaClO	Male	0.6% Bleach				No Data	

NIJ106 3.6%NaClO	Male	3.6% Bleach				No Data	
NIJ107 6%NaClO	Male	6% Bleach	13005	6739	5323	79%	2%
NIJ108 UVO12HR	Male	UV Overhead 12 Hours	27839	16999	12614	74%	0%
NIJ109 UVC1HR	Male	UV Crosslinker 1 Hour	31332	17788	12670	71%	1%
NIJ110 UVC15MIN	Male	UV Crosslinker 15 mins	15113	8363	5863	70%	0%
NIJ111 NT	Male	No Treatment	30707	19318	11894	62%	0%
NIJ153 0.6%NaClO	Cumulative	0.6% Bleach				No Data	
NIJ154 3.6%NaClO	Cumulative	3.6% Bleach				No Data	
NIJ155 6%NaClO	Cumulative	6% Bleach	474796	292923	31526	11%	0%
NIJ156 UVO12HR	Cumulative	UV Overhead 12 Hours	1198574	825673	99646	12%	0%
NIJ157 UVC1HR	Cumulative	UV Crosslinker 1 Hour	6815	4103	3427	84%	1%
NIJ158 UVC15MIN	Cumulative	UV Crosslinker 15 mins	1043	663	573	86%	0%
NIJ159 NT	Cumulative	No Treatment	0	0	0	No Data	0%
NIJEC1_4W	Extract Control		11	8	8	100%	0%
NIJEC1_4W	Extract Control		2	1	1	100%	0%
NIJEC2_4W	Extract Control		5	3	3	100%	0%
NIJEC3_4W	Extract Control		26	18	16	89%	0%
NIJEC4_4W	Extract Control		3	1	1	100%	2%
NIJ_LN3	Library Control		1	1	1	100%	4%
NIJ_LN4	Library Control		3	2	2	100%	0%

Table 5. Next Generation Sequencing: Human genome mapping at 16 weeks (T2).

Sample ID	Touch Type	Treatment	Total mapped human genome reads	Mapped hgQ37 reads	hgQ37.Unique reads	% Unique	% Endogenous
NIJ022 0.6%NaClO	Untouched	0.6% Bleach	8891	3695	3462	94%	5%
NIJ023 3.6%NaClO	Untouched	3.6% Bleach	6176	2415	2204	91%	4%
NIJ024 6%NaClO	Untouched	6% Bleach	4406	2131	1999	94%	2%
NIJ025 UVO12HR	Untouched	UV Overhead 12 Hours	220	67	66	99%	0%
NIJ026 UVC1HR	Untouched	UV Crosslinker 1 Hour	1322	613	587	96%	4%
NIJ027 UVC15MIN	Untouched	UV Crosslinker 15 mins	13	5	5	100%	0%
NIJ028 NT	Untouched	No Treatment	990	542	515	95%	0%
NIJ064 0.6%NaClO	Female	0.6% Bleach	681	255	244	96%	4%
NIJ065 3.6%NaClO	Female	3.6% Bleach	1998	870	831	96%	4%
NIJ066 6%NaClO	Female	6% Bleach	785	266	239	90%	0%
NIJ067 UVO12HR	Female	UV Overhead 12 Hours	10	3	3	100%	0%
NIJ068 UVC1HR	Female	UV Crosslinker 1 Hour	292	67	66	99%	0%
NIJ069 UVC15MIN	Female	UV Crosslinker 15 mins	152	47	47	100%	0%
NIJ070 NT	Female	No Treatment	0	0	0	No Data	0%
NIJ112 0.6%NaClO	Male	0.6% Bleach	6017	2429	2290	94%	0%
NIJ113 3.6%NaClO	Male	3.6% Bleach	23500	9853	9426	96%	3%
NIJ114 6%NaClO	Male	6% Bleach	2282	848	817	96%	0%

NIJ115 UVO12HR	Male	UV Overhead 12 Hours	6999	3058	2892	95%	0%
NIJ116 UVC1HR	Male	UV Crosslinker 1 Hour	4100	1936	1840	95%	0%
NIJ117 UVC15MIN	Male	UV Crosslinker 15 mins	4015	1642	1521	93%	0%
NIJ118 NT	Male	No Treatment	3321	1322	1220	92%	0%
NIJ160 0.6%NaClO	Cumulative	0.6% Bleach	14392	8995	7398	82%	2%
NIJ161 3.6%NaClO	Cumulative	3.6% Bleach	1583	794	694	87%	0%
NIJ162 6%NaClO	Cumulative	6% Bleach	1484	476	440	92%	0%
NIJ163 UVO12HR	Cumulative	UV Overhead 12 Hours	3752	1840	1720	93%	0%
NIJ164 UVC1HR	Cumulative	UV Crosslinker 1 Hour	1656	912	850	93%	0%
NIJ165 UVC15MIN	Cumulative	UV Crosslinker 15 mins	430	161	153	95%	0%
NIJ166 NT	Cumulative	No Treatment	4072	1287	1172	91%	0%
NIJEC1_16wk	Extract Control		11	10	8	80%	5%
NIJEC2_16wk	Extract Control		0	0	0	NoData	0%
NIJEC3_16wk	Extract Control		5	4	3	75%	0%
NIJEC4_16wk	Extract Control		0	0	0	No Data	0%
NIJ_LN5	Library Control		2	1	1	100%	4%
NIJ_LN6	Library Control		9	3	2	67%	1%

Table 6. Next Generation Sequencing: Human genome mapping at six months (T3).

Sample ID	Touch Type	Treatment	Total mapped human genome reads	Mapped hgQ37 reads	hgQ37.Unique reads	% Unique	% reads endogenous
NIJ029 0.6%NaClO	Untouched	0.6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ030 3.6%NaClO	Untouched	3.6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ031 6%NaClO	Untouched	6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ032 UVO12HR	Untouched	UV Overhead 12 Hours	No Data	No Data	No Data	No Data	No Data
NIJ033 UVC1HR	Untouched	UV Crosslinker 1 Hour	No Data	No Data	No Data	No Data	No Data
NIJ034 UVC15MIN	Untouched	UV Crosslinker 15 mins	No Data	No Data	No Data	No Data	No Data
NIJ035 NT	Untouched	No treatment	No Data	No Data	No Data	No Data	No Data
NIJ071 0.6%NaClO	Female	0.6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ072 3.6%NaClO	Female	3.6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ073 6%NaClO	Female	6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ074 UVO12HR	Female	UV Overhead 12 Hours	No Data	No Data	No Data	No Data	No Data
NIJ075 UVC1HR	Female	UV Crosslinker 1 Hour	No Data	No Data	No Data	No Data	No Data
NIJ076 UVC15MIN	Female	UV Crosslinker 15 mins	No Data	No Data	No Data	No Data	No Data
NIJ077 NT	Female	No Treatment	No Data	No Data	No Data	No Data	No Data
NIJ119 0.6%NaClO	Male	0.6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ120 3.6%NaClO	Male	3.6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ121 6%NaClO	Male	6% Bleach	No Data	No Data	No Data	No Data	No Data

NIJ122 UVO12HR	Male	UV Overhead 12 Hours	No Data	No Data	No Data	No Data	No Data
NIJ123 UVC1HR	Male	UV Crosslinker 1 Hour	No Data	No Data	No Data	No Data	No Data
NIJ124 UVC15MIN	Male	UV Crosslinker 15 mins	No Data	No Data	No Data	No Data	No Data
NIJ125 NT	Male	No Treatment	No Data	No Data	No Data	No Data	No Data
NIJ167 0.6%NaClO	Cumulative	0.6% Bleach	26598	13969	10450	75%	1%
NIJ168 3.6%NaClO	Cumulative	3.6% Bleach	611973	318189	185026	58%	2%
NIJ169 6%NaClO	Cumulative	6% Bleach	229376	109579	50039	46%	1%
NIJ170 UVO12HR	Cumulative	UV Overhead 12 Hours	7356	3857	3481	90%	0%
NIJ171 UVC1HR	Cumulative	UV Crosslinker 1 Hour	31441	17406	13565	78%	0%
NIJ172 UVC15MIN	Cumulative	UV Crosslinker 15 mins	9644	3925	3355	85%	0%
NIJ173 NT	Cumulative	No Treatment	9839	6048	4926	81%	1%
NIJEC1_6M	Extract Control		38	10	7	70%	1%
NIJEC2_6M	Extract Control		12	4	4	100%	0%
NIJEC4_6M	Extract Control		95	18	14	78%	0%
NIJEC3_6M	Extract Control		0	0	0	No Data	0%
NIJ_LN7	Library Control		5	1	1	100%	0%
NIJ_LN8	Library Control		1	0	0	No Data	0%

Table 7. Next Generation Sequencing: Human genome mapping at one year (T4).

Sample ID	Touch Type	Treatment	Total mapped human genome reads	Mapped hgQ37 reads	hgQ37.Unique reads	% Unique	% reads endogenous
NIJ036 0.6%NaClO	Untouched	0.6% Bleach	251	179	150	84%	0%
NIJ037 3.6%NaClO	Untouched	3.6% Bleach	168	119	102	86%	0%
NIJ038 6%NaClO	Untouched	6% Bleach	1876	1479	1315	89%	1%
NIJ039 UVO12HR	Untouched	UV Overhead 12 Hours	142	59	52	88%	0%
NIJ040 UVC1HR	Untouched	UV Crosslinker 1 Hour	177	108	89	82%	0%
NIJ041 UVC15MIN	Untouched	UV Crosslinker 15 mins	140	92	86	93%	0%
NIJ042 NT	Untouched	No treatment	434	236	220	93%	0%
NIJ078 0.6%NaClO	Female	0.6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ079 3.6%NaClO	Female	3.6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ080 6%NaClO	Female	6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ081 UVO12HR	Female	UV Overhead 12 Hours	No Data	No Data	No Data	No Data	No Data
NIJ082 UVC1HR	Female	UV Crosslinker 1 Hour	No Data	No Data	No Data	No Data	No Data
NIJ083 UVC15MIN	Female	UV Crosslinker 15 mins	No Data	No Data	No Data	No Data	No Data
NIJ084 NT	Female	No Treatment	No Data	No Data	No Data	No Data	No Data
NIJ126 0.6%NaClO	Male	0.6% Bleach	No Data	No Data	No Data	No Data	No Data

NIJ127 3.6%NaClO	Male	3.6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ128 6%NaClO	Male	6% Bleach	No Data	No Data	No Data	No Data	No Data
NIJ129 UVO12HR	Male	UV Overhead 12 Hours	No Data	No Data	No Data	No Data	No Data
NIJ130 UVC1HR	Male	UV Crosslinker 1 Hour	No Data	No Data	No Data	No Data	No Data
NIJ131 UVC15MIN	Male	UV Crosslinker 15 mins	No Data	No Data	No Data	No Data	No Data
NIJ132 NT	Male	No Treatment	No Data	No Data	No Data	No Data	No Data
NIJ174 0.6%NaClO	Cumulative	0.6% Bleach	40422	9427	8243	87%	0%
NIJ175 3.6%NaClO	Cumulative	3.6% Bleach	856	337	273	81%	1%
NIJ176 6%NaClO	Cumulative	6% Bleach	803	369	314	85%	1%
NIJ177 UVO12HR	Cumulative	UV Overhead 12 Hours	154	54	39	72%	0%
NIJ178 UVC1HR	Cumulative	UV Crosslinker 1 Hour	8434	499	453	91%	0%
NIJ179 UVC15MIN	Cumulative	UV Crosslinker 15 mins	22890	14248	12339	87%	0%
NIJ180 NT	Cumulative	No Treatment				No Data	
NIJEC1_1Y	Extract Control		29117	6870	901	13%	0%
NIJEC2_1Y	Extract Control		6219	37	29	78%	0%
NIJec3_1Y	Extract Control		12	5	4	80%	4%
NIJEC4_1Y	Extract Control		4027	18	17	94%	0%

4.2.2 Human DNA damage patterns

Samples' human DNA damage pattern plots are found in Appendix D. Damage frequencies are reported in Appendix C , Tabs A-F. For general reference a typical ancient sample has damage patterns around 5-10%.

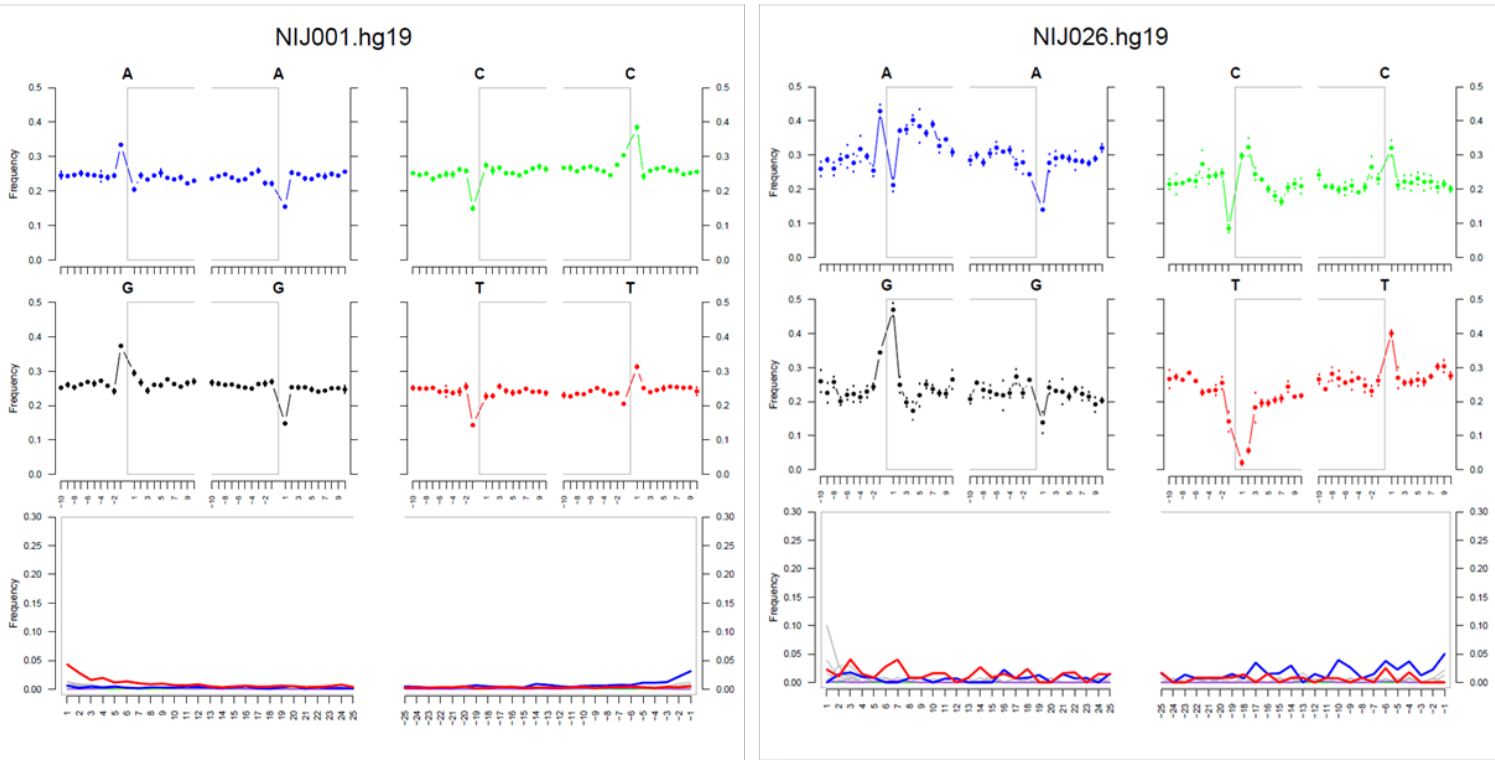


Figure 37. Map damage plots for sample NIJ001 and NIJ026

The four upper plots (Figure 37) display the base substitution frequency outside and within sequencing reads (the open grey box corresponds to the read). The bottom plots are the positions' specific substitutions from the 5" (left) and the 3" end (right). The following color codes are used in the bottom plots: red: C to T substitutions, blue: G to A substitutions, grey: all other substitutions, green: deletions relative to reference, purple: insertions relative to the reference. When damage occurs, it is preferentially at the 5' and 3' ends. Note that variance from a "smiling" pattern with higher rates at internal positions or fluctuations across the read suggests higher variance from the reference rather than damage. An example is sample NIJ026 (Figure 37).

4.2.3 Damage patterns by time trial and treatment

Damage rates were only determined if the sample retained the minimal number of reads needed to run MapDamage program. Time Trial damage pattern results (Figures 38-42, Table 3) do not suggest any pattern related to treatment or time, other than an increase in samples with insufficient sequencing reads for analysis (i.e., samples over 6 months-thus indicating low quality samples).

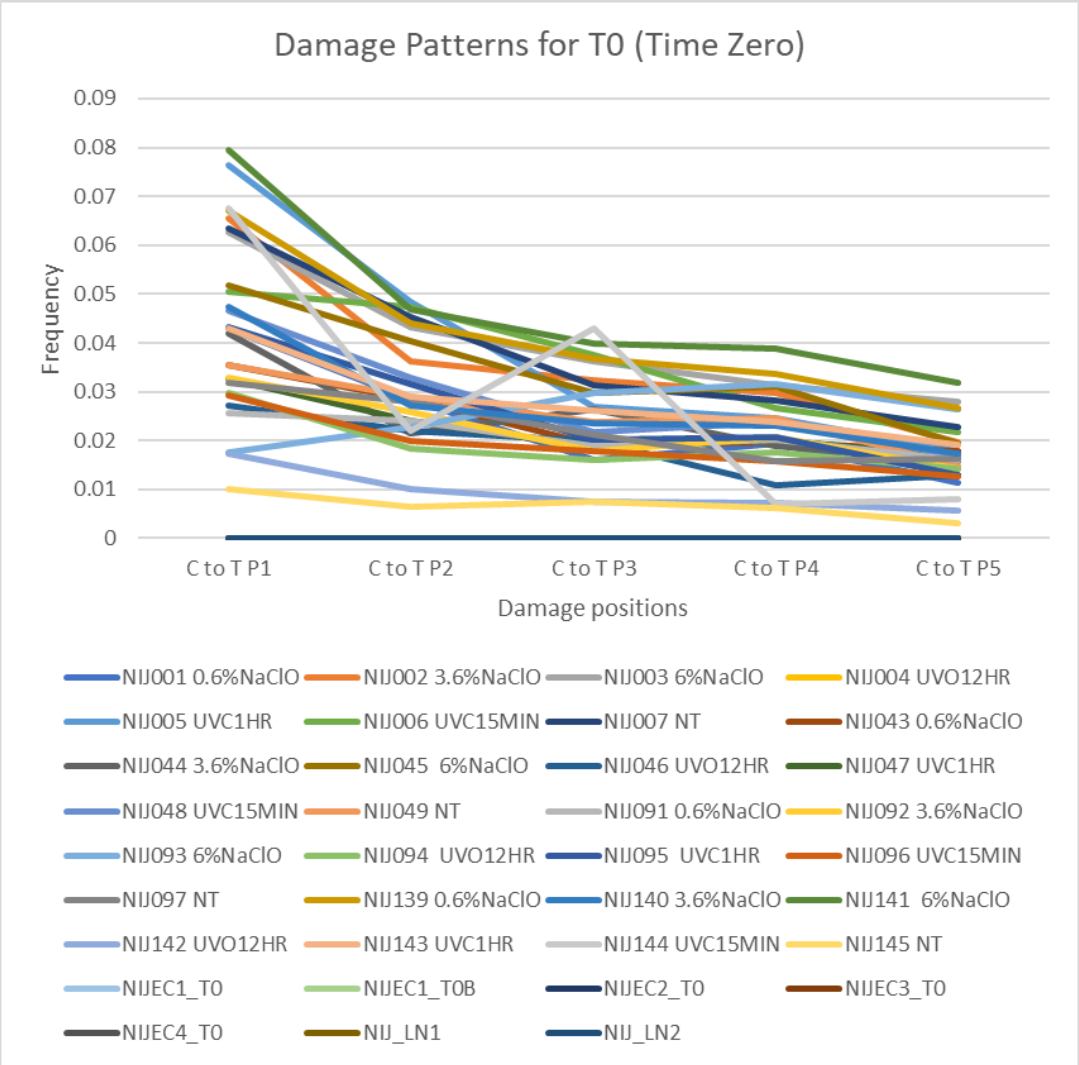


Figure 38 Damage patterns for Time Zero (T0).

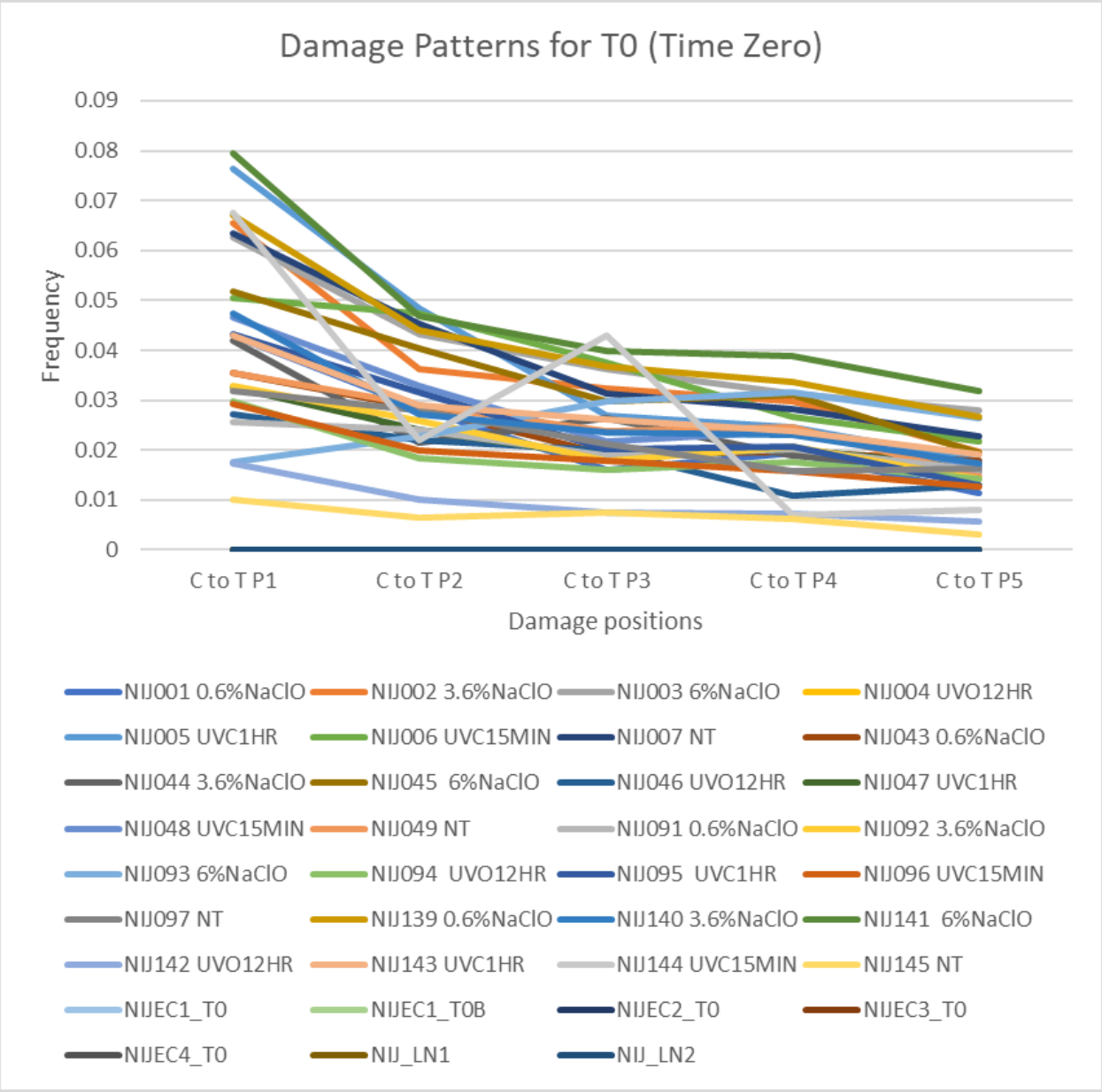


Figure 39 Damage patterns for 4 weeks (T1).

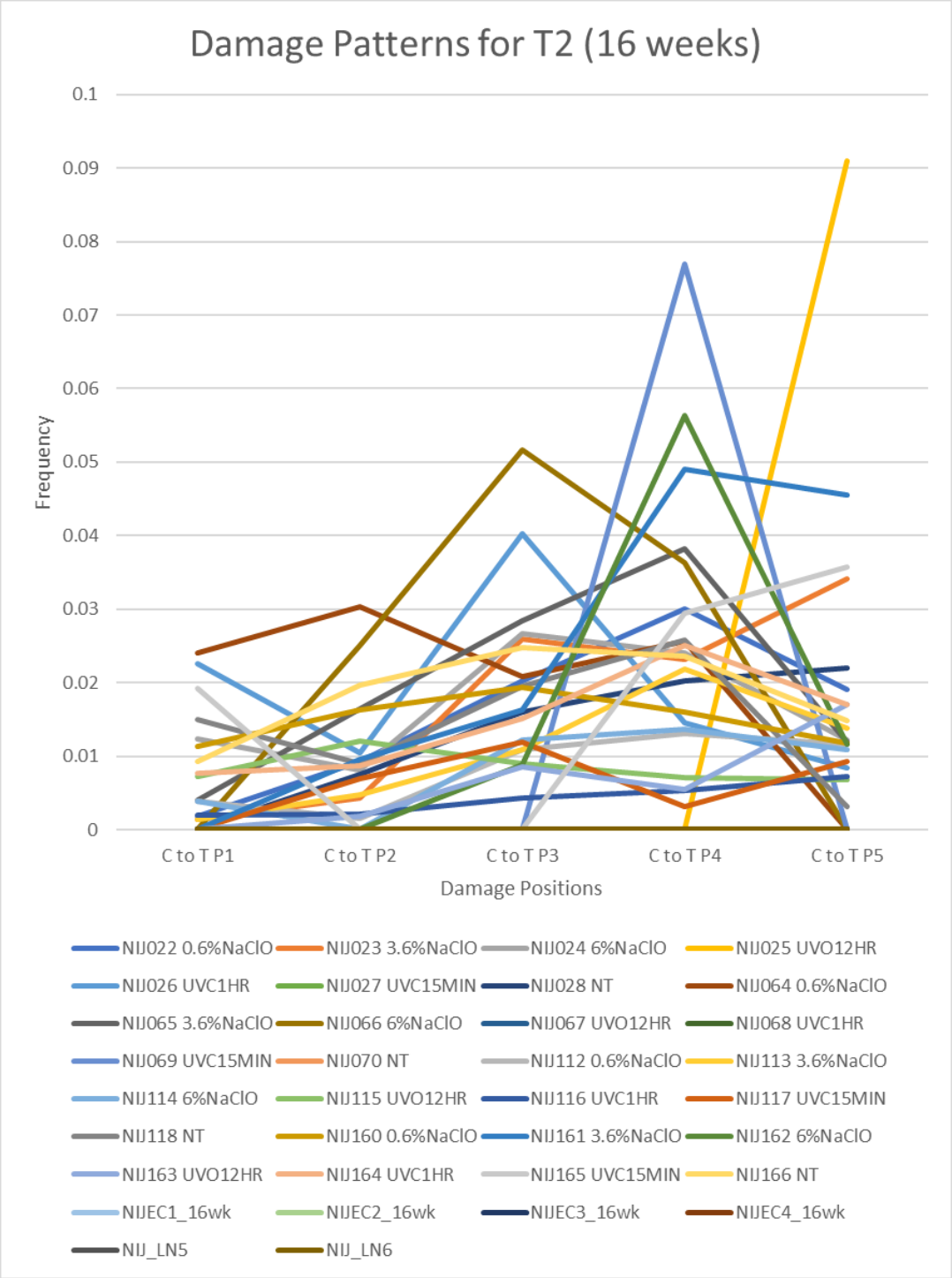


Figure 40 Damage patterns for 16 weeks (T2).

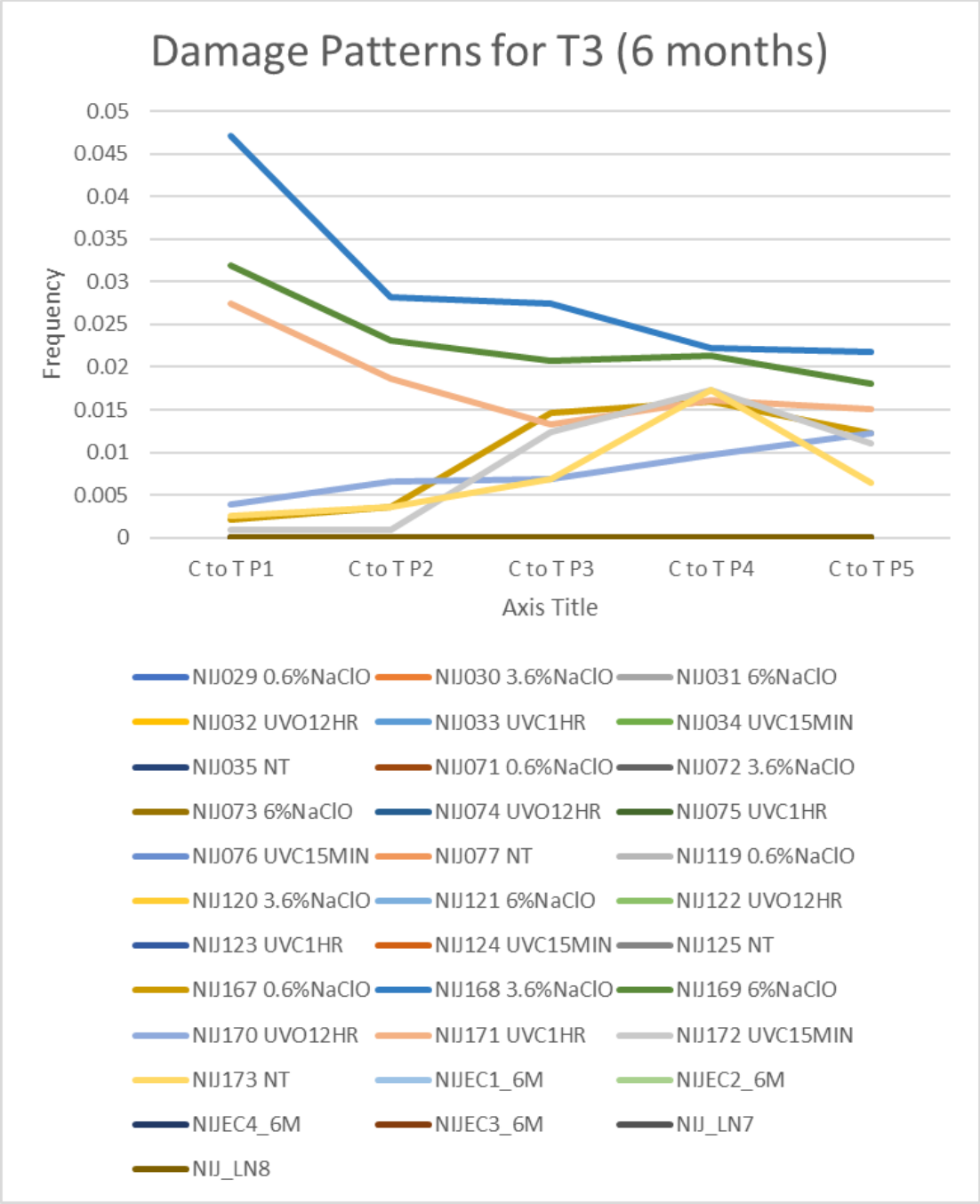


Figure 41 Damage patterns for six months (T3)

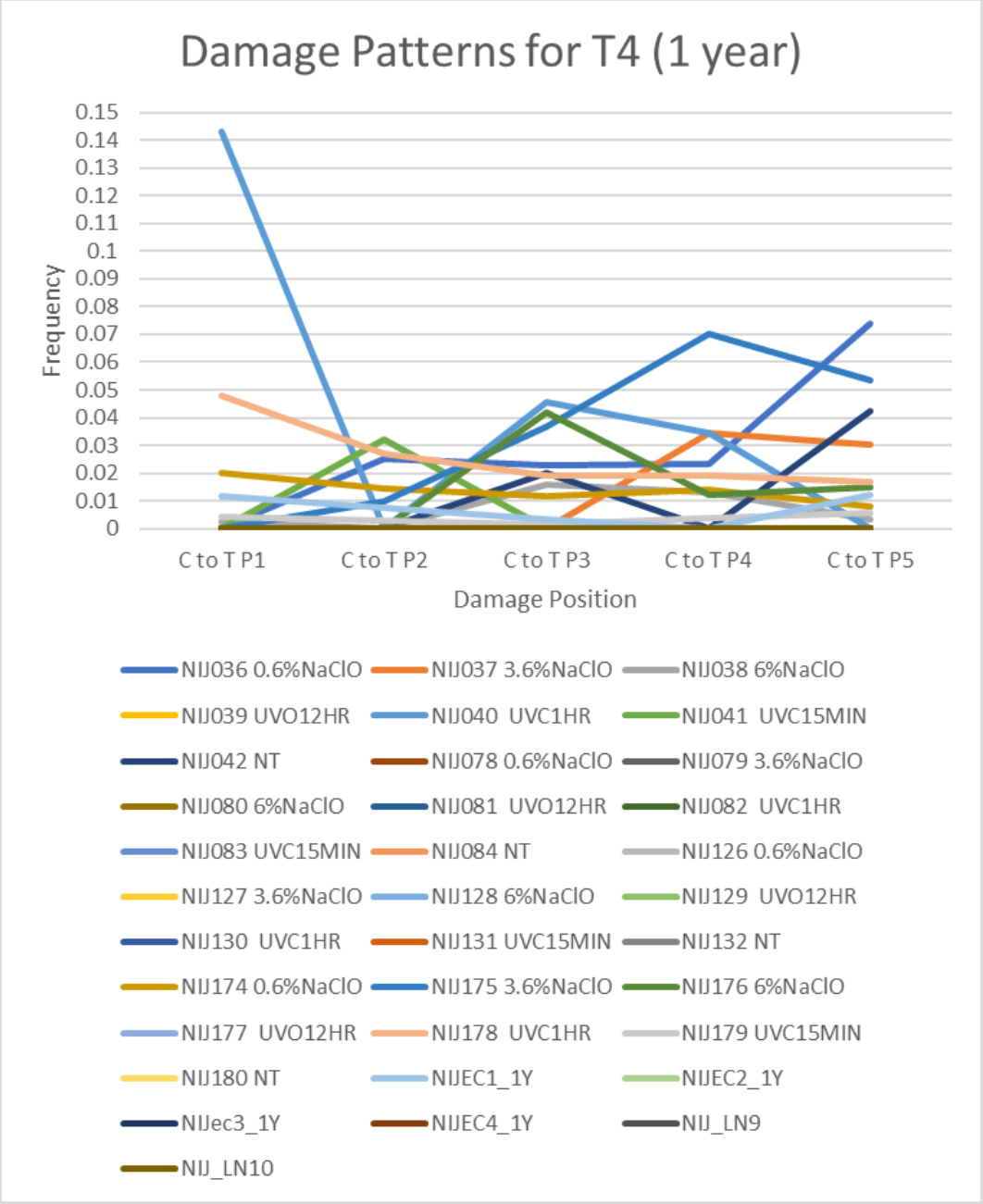


Figure 42 Damage patterns for one year (T4)

Table 8. Base substitution frequencies at first position in sequencing reads.

Shaded boxes are samples that do not have damage pattern

Sample	Time	0.6% NaClO	3.6% NaClO	6% NaClO	12HR UVO	UVC 1HR	UVC 15 MIN	No Treatment
Untouched	T0	0.043	0.065	0.063	0.000	0.000	0.000	0.000
Female	T0	0.036	0.042	0.052	0.027	0.000	0.000	0.000
Male	T0	0.026	0.033	0.018	0.030	0.000	0.000	0.000
Cumulative	T0	0.067	0.047	0.079	0.017	0.000	0.000	0.000
Untouched	T1	0.044	0.068	0.041	0.024	0.038	0.000	0.000
Female	T1	0.018	0.111	0.000	0.057	0.333	0.023	0.019
Male	T1	No data	No data	0.052	0.033	0.000	0.000	0.167
Cumulative	T1	No data	No data	0.042	0.013	0.000	0.000	0.000
Untouched	T2	0.002	0.001	0.012	0.000	0.000	0.000	0.000
Female	T2	0.024	0.004	0.000	0.000	0.000	0.000	0.000
Male	T2	0.004	0.001	0.004	0.007	0.000	0.000	0.000
Cumulative	T2	0.011	0.000	0.000	0.000	0.000	0.000	0.000
Untouched	T3	No data	No data	No data	No data	No data	No data	No data
Female	T3	No data	No data	No data	No data	No data	No data	No data
Male	T3	No data	No data	No data	No data	No data	No data	No data
Cumulative	T3	0.002	0.047	0.032	0.004	0.000	0.000	0.000
Untouched	T4	0.000	0.000	0.002	0.000	0.000	0.000	0.000
Female	T4	No data	No data	No data	No data	No data	No data	No data
Male	T4	No data	No data	No data	No data	No data	No data	No data
Cumulative	T4	0.020	0.000	0.000	0.000	0.167	0.000	No data
Average damage rate		0.023	0.032	0.026	0.014	0.036	0.002	0.013

4.2.4 Fragment length distributions

Average read length varied across time and treatment (Appendix C; Tabs A-F, Figures 43-47, Tables 4-5). Untreated samples had the smallest average read length compared to UV samples and bleach samples. The latter had the largest fragment length. It may be that smaller molecules were preferentially destroyed during this treatment. When compared to time, the T4 (one year) and T3 (6 months) samples had the largest average read length, despite having fewer overall samples with reportable sequencing data. Unhandled samples had the smallest fragment lengths compared to touched samples suggesting higher fragmentation of the bone compared to touch DNA despite time and treatment.

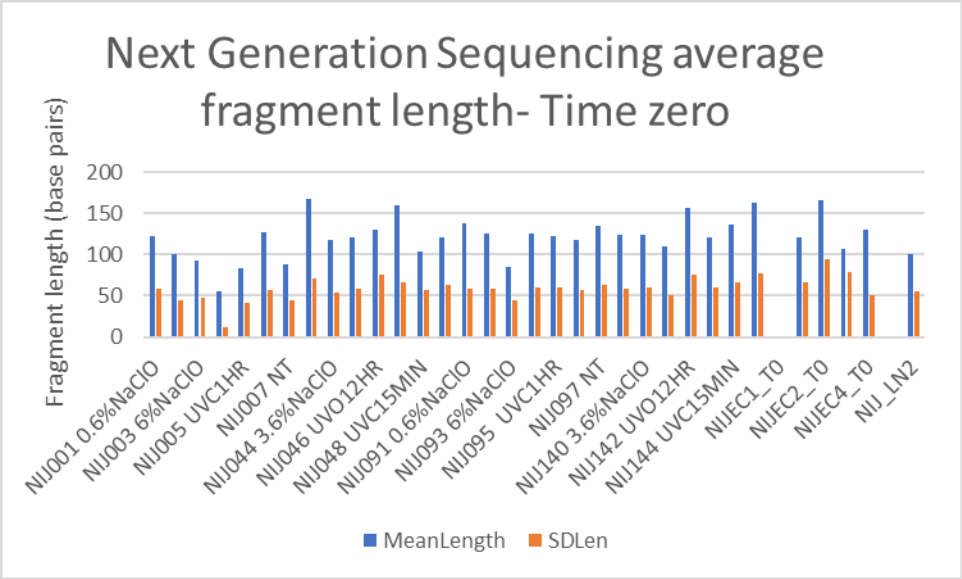


Figure 43 Average read length for time zero (T0).

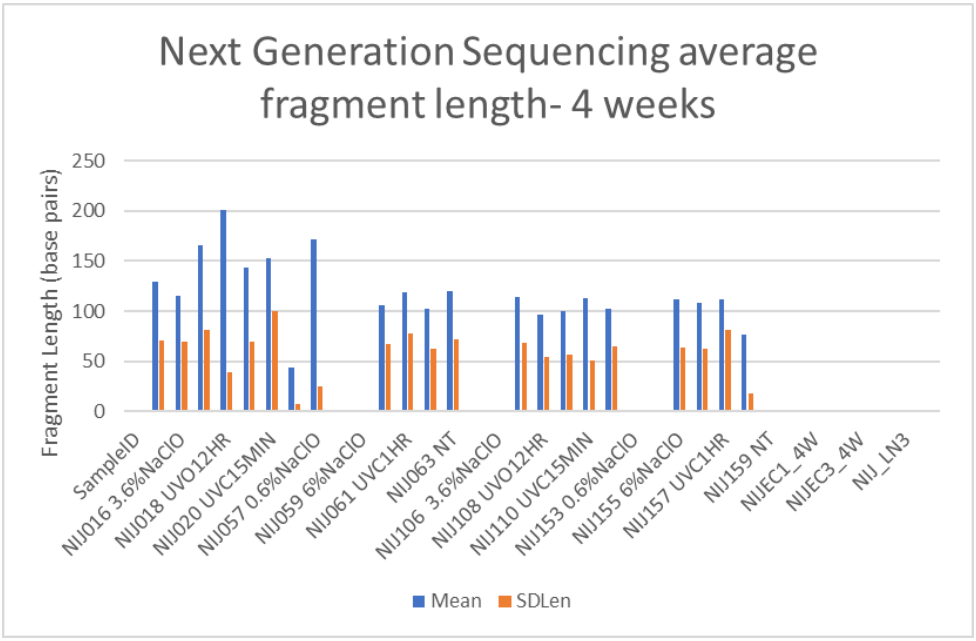


Figure 44 Average read length for 4 weeks (T1)

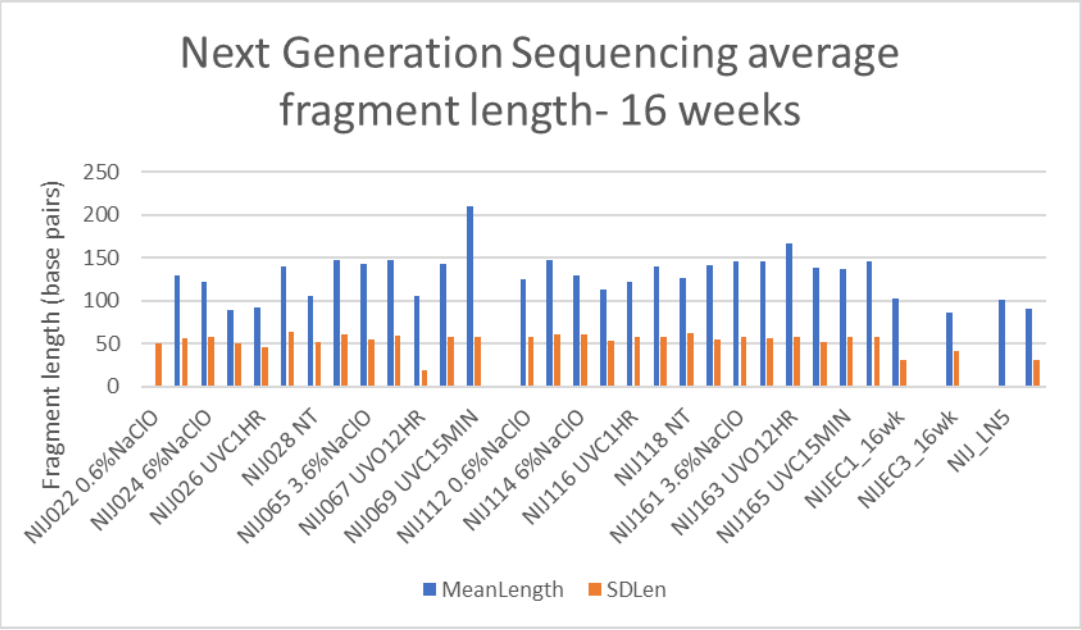


Figure 45 Average read length for 16 weeks (T2)

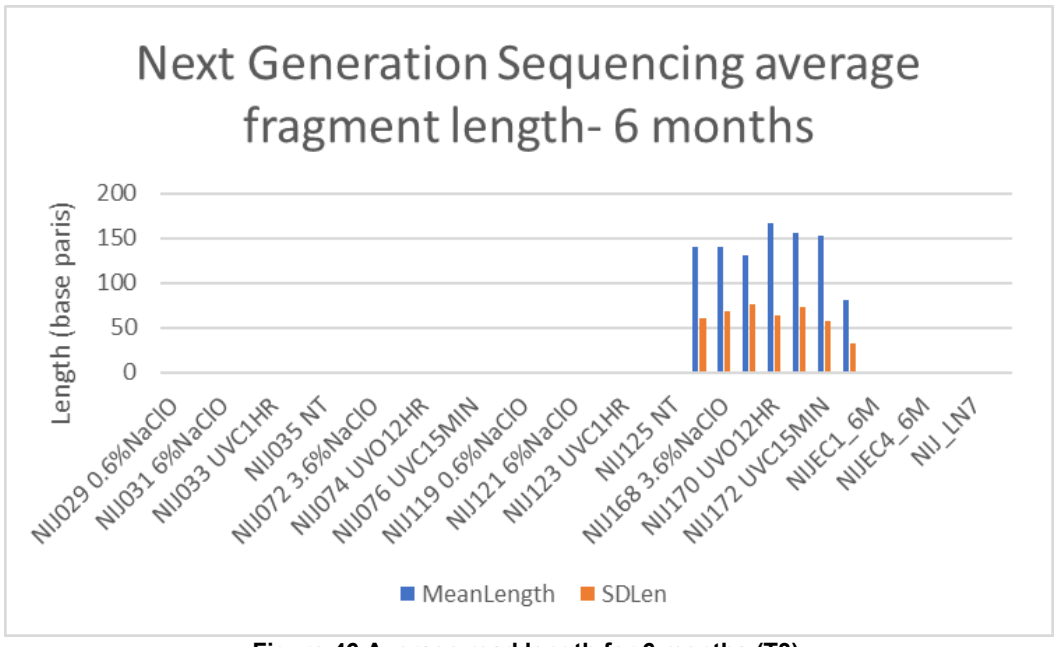


Figure 46 Average read length for 6 months (T3)

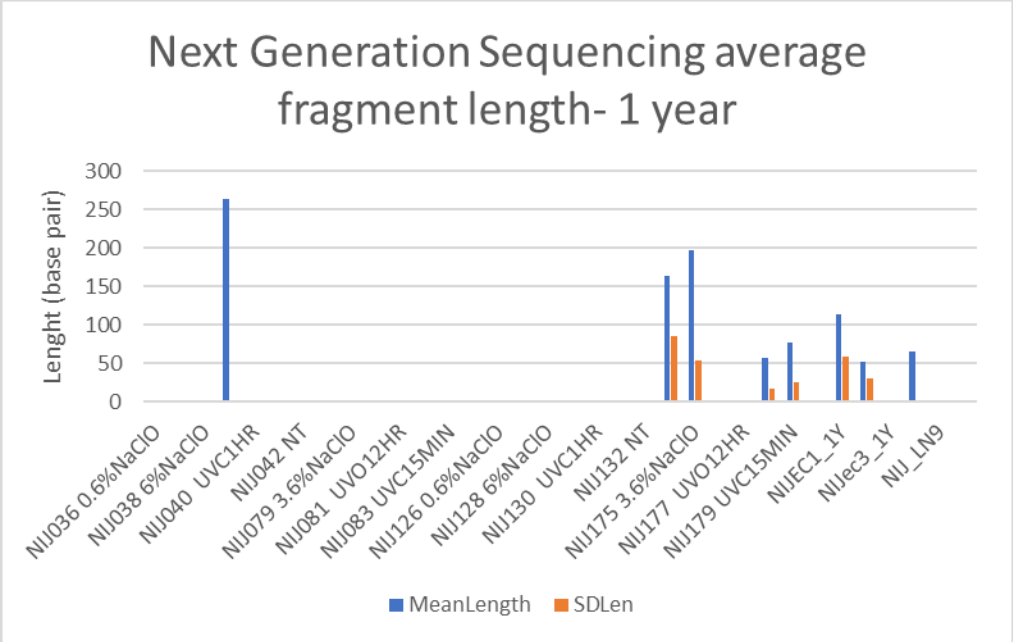


Figure 47 Average read length for 1 year (T4)

Table 9. Average read length across time trials

	Average Read Length	SD
T0 (Day Zero)	120.7317726	57.15685369
T1 (4 weeks)	119.315815	60.20891524
T2 (16 weeks)	134.237925	54.98429262
T3 (6 months)	138.0556806	61.14045709
T4 (1 year)	151.7122222	36.27761026

Table 10 Average read length across treatment.

Sample	Time	0.6% NaClO	3.6% NaClO	6% NaClO	12HR UVO	UVC 1HR	UVC 15 MIN	No Treatment	Average fragment length
Untouched	T0	121.8	93.7	107.4	no data	72.9	144.1	80.1	103.3
Untouched	T1	no data	no data	no data	264.0	no data	no data	no data	no data
Untouched	T2	no data	no data	no data	no data	no data	no data	no data	no data
Untouched	T3	121.4	142.1	112.5	113.3	112.5	86.0	77.0	109.3
Untouched	T4	no data	no data	111.9	108.2	111.8	77.0	no data	102.2
Female	T0	129.0	115.5	166.2	201.3	143.8	152.7	43.5	136.0
Female	T1	164.7	171.3	123.1	96.1	166.2	98.7	91.6	130.2
Female	T2	no data	no data	no data	no data	no data	no data	no data	no data
Female	T3	no data	no data	no data	no data	no data	no data	no data	no data
Female	T4	146.0	no data	206.0	no data	179.0	no data	139.0	167.5
Male	T0	112.0	103.0	112.3	no data	94.0	no data	70.5	98.4
Male	T1	171.6	no data	no data	106.2	118.4	102.0	120.2	123.7
Male	T2	115.3	128.0	87.6	131.8	134.4	135.8	117.1	121.4
Male	T3	no data	no data	no data	no data	no data	no data	no data	no data
Male	T4	140.3	140.1	131.0	166.2	155.0	152.4	81.4	138.1
Cumulative	T0	no data	no data	no data	no data	no data	no data	no data	no data
Cumulative	T1	no data	no data	no data	no data	no data	no data	no data	no data
Cumulative	T2	no data	no data	113.8	96.3	100.4	113.2	102.7	105.3
Cumulative	T3	113.5	124.2	118.2	129.3	92.7	242.0	191.2	144.4
Cumulative	T4	163.4	196.7	no data	no data	57.2	77.3	no data	123.6
Average fragment length		141.4	148.2	125.6	135.3	122.4	121.0	113.5	

4.2.5 Ancestry and sex typing.

The donor bone sample was previously identified as a female of European descent. The handlers were two causcasian males, and one female who was half SE Asian/half caucasian. Only 49 samples had enough data for ancestry analyses with only seven being older than four weeks. Bleach and UV treated samples were represented almost equally with no treatment samples being underrepresented. Additionally, the amount of possible genomic data available for ancestry analysis varied dramatically (Appendix C; Tab H) with fewer number of mutational sites greatly reducing discriminatory power. ADMIXTURE (Alexander & Lange, 2011) was used to estimate the relative ancestry. Samples were compared in an unsupervised analysis to 26 global reference populations (10 individuals per population) from the International Genome sample resource (IGSR) (Clarke et al., 2017) that incorporates 1000 Genomes Project data (Consortium, 2015). Additional population dataset included ancient populations from North America (Allen Ancient DNA Resource (AADR). Parameters for K (number of underlying populations) ranged from K=5 to K=6. Modeling at K=5 was selected as the most optimal level to observe continental stratification (Figure 48). Sequencing of the positive controls (i.e., the handlers) is still under way and will help clarify these results.

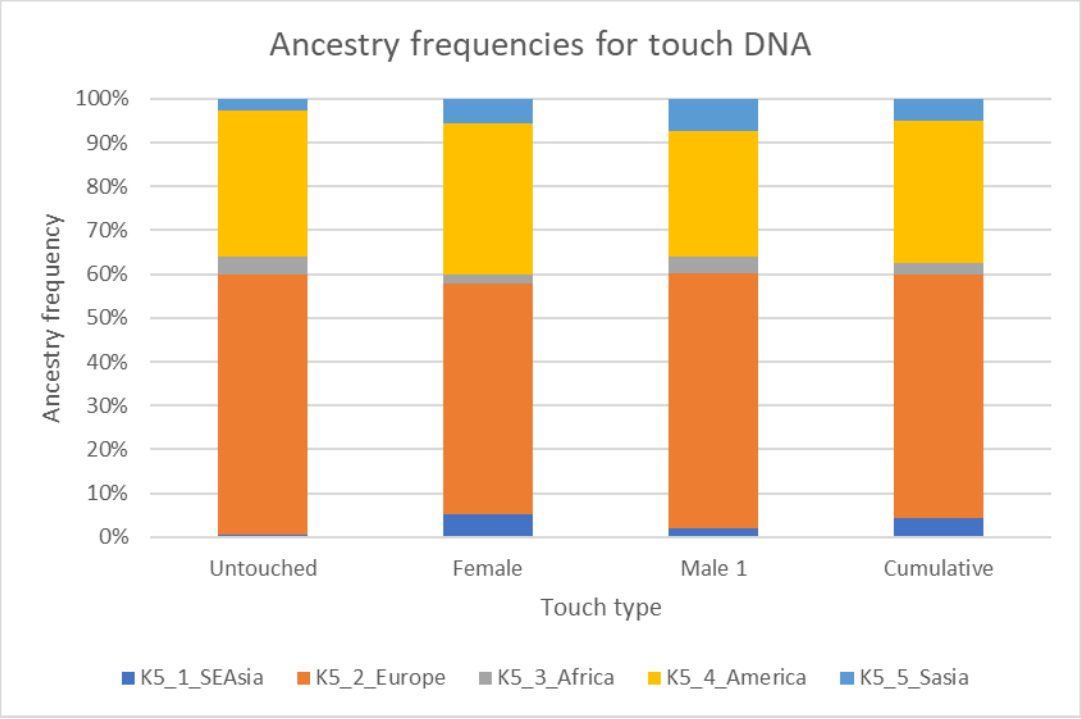


Figure 48 Ancestry frequencies.

Sex typing was determined by using a method outlined by (Skoglund, Storå, Götherström, & Jakobsson, 2013). This compares the ratio of aligned Y-chromosome sequences to the total fraction of sequence reads aligned to both the X and Y chromosome (RY) (Table 8). A male sex assignment can be made if the lower bound CI is greater than 0.077, and female is the upper CI is lower than 0.016. Only 49 samples had sufficient reads for sex assignment, with a majority of these (n=41) from less than 4 weeks (Appendix C; Tab H; Table 6). Untouched samples and female touched samples were correctly assigned as female. However, all male touched samples (including cumulative samples) were assigned female or likely female, with little Y-chromosome DNA being reported (Table 11). Due to the low number of reads it is possible that endogenous DNA from the bone sample may compete with the retrieval of touch DNA from the surface, thus creating a false sex assignment. Alternatively, there may also be low levels of contamination, as researchers for this study were female.

Table 11 Sex typing assignments

Sample	X+Y reads	Y reads	Ratio	Sex Assignment	Time
NIJ 001 U 0.6%NaClO	320	1	0.0031	XX	T0
NIJ 002 U 3.6%NaClO	9611	18	0.0019	XX	T0
NIJ 003 U 6%NaClO	737469	1294	0.0018	XX	T0
NIJ 005 U UVC 1HR	2615	12	0.0046	XX	T0
NIJ 006 U UVO 15MIN	668	1	0.0015	XX	T0
NIJ 007 U NT	2586	5	0.0019	XX	T0
NIJ 043 F 0.6%NaClO	760	2	0.0026	XX	T0
NIJ 045 F 6%NaClO	1828	3	0.0016	XX	T0
NIJ 046 F UVO 12HR	484	3	0.0062	XX	T0
NIJ 047 F UVC 1HR	4572	5	0.0011	XX	T0
NIJ 048 F UVO 15MIN	659	3	0.0046	XX	T0
NIJ 049 F NT	501	2	0.004	XX	T0
NIJ 091 M 0.6%NaClO	269	0	0	Likely XX	T0
NIJ 092 M 3.6%NaClO	287	1	0.0035	XX	T0
NIJ 094 M UVO 12HR	3578	40	0.0112	XX	T0
NIJ 095 M UVC 1HR	285	1	0.0035	XX	T0
NIJ 096 M UVC 15MIN	614	1	0.0016	XX	T0
NIJ 097 M NT	698	2	0.0029	XX	T0
NIJ 139 C 0.6%NaClO	437	1	0.0023	XX	T0
NIJ 140 C 3.6%NaClO	5941	12	0.002	XX	T0
NIJ 141 C 6%NaClO	9276	11	0.0012	XX	T0
NIJ 142 C UVO 12HR	6871	123	0.0179	XX not XY	T0

NIJ 142 C UVO 12HR. Rep	466	8	0.0172	XX not XY	T0
NIJ 143 C UVC 1HR	775	6	0.0077	XX	T0
NIJ 145 C NT	608	15	0.0247	XX not XY	T0
NIJO15 U 0.6%NaClO	27129	23	0.0008	XX	T1
NIJ016 U 3.6%NaClO	58269	65	0.0011	XX	T1
NIJ017 U 6%NaClO	7660	13	0.0017	XX	T1
NIJ019 U UVC 1HR	5546	5	0.0009	XX	T1
NIJ020 U UVC 15MIN	299	1	0.0033	XX	T1
NIJ060 F UVO 12HR	14182	26	0.0018	XX	T1
NIJ061 F UVC 1HR	621	0	0	Likely XX	T1
NIJ062 F UVC 15MIN	8600	29	0.0034	XX	T1
NIJ063 F NT	2773	3	0.0011	XX	T1
NIJ107 M 6%NaClO	225	0	0	Likely XX	T1
NIJ108 M UVO 12HR	556	1	0.0018	XX	T1
NIJ109 M UVC 1HR	611	1	0.0016	XX	T1
NIJ110 M UVC 15MIN	244	0	0	Likely XX	T1
NIJ111 M NT	485	5	0.0103	XX not XY	T1
NIJ155 C 6%NaClO	1377	2	0.0015	XX	T1
NIJ156 C UVO 12HR	3719	84	0.0226	Not Assigned	T1
NIJ113	427	2	0.0047	XX	T2
NIJ160 C 0.6%NaClO	258	0	0	Likely XX	T2
NIJ167 C 0.6%NaClO	471	1	0.0021	XX	T3
NIJ168 C 3.6%NaClO	8105	14	0.0017	XX	T3
NIJ169 C 6%NaClO	2306	8	0.0035	XX	T3
NIJ171 C UVC 1HR	572	2	0.0035	XX	T3
NIJ174 C 0.6%NaClO	262	3	0.0115	XX not XY	T4
NIJ179 C UVC 15MIN	647	2	0.0031	XX	T4

4.2.6 Mitochondrial DNA sequencing results

Only 19 samples had enough mapped mtDNA reads to be identifiable to a haplotype or to analyze DNA damage (Appendix C; Tab I, Table 12). Of these, approximately 74% were less than four weeks old. Eleven samples were bleach treated, six were UV treated and two were not treated. Map damage results are reported in (Appendix C; Tab 1, Figure 49). Read length averaged 121.78 bp with a SD 65.36bp (Figure 50). Six haplotypes were identified (Table 8) but did not match known types for female and male2 handler. Male 1 handler mtDNA type is slated for future sequencing. Haplotype 2 belongs to same haplogroup as PI Monroe but is a different lineage. Haplotype 2 also has notable damage, suggesting it may be the endogenous bone mtDNA type. However,

additional sequencing and testing will be needed to definitively conclude it was not contamination from lab personnel.

Table 12 MtDNA Next generation sequencing results

Sample ID	Time	Time Orig	Retained Merged reads	mtDNA Total reads	mtQ37.Uniq
NIJ002 U 3.6% NaClO	T0	0D	47479219	790	453
NIJ003 U 6% NaClO	T0	0D	78361845	50836	13835
NIJ007 U NT	T0	0D	17866850	184	154
NIJ015 U 0.6% NaClO	T1	4W	1.22E+08	1711	921
NIJ016 U 3.6% NaClO	T1	4W	86693230	11189	2550
NIJ017 6% NaClO	T1	4W	90720482	3541	452
NIJ019 U UVC1HR	T1	4W	70218485	1209	305
NIJ060 F UVO 12HR	T1	4W	76852485	776	388
NIJ062 F UVC 15MIN	T1	4W	1.31E+08	5365	684
NIJ063 F NT	T1	4W	45500062	333	178
NIJ094 M 6% NaClO	T0	0D	8763107	332	260
NIJ140 C 3.6% NaClO	T0	0D	6799358	799	306
NIJ141 C 6% NaClO	T0	0D	2951260	607	323
NIJ142 C UVO 12HR	T0	0D	50824040	797	313
NIJ155 C 6% NaClO	T1	4W	1.34E+08	2366	248
NIJ156 C UVO 12HR	T1	4W	1.53E+08	2104	231
NIJ168 C 3.6% NaClO	T3	6M	11428106	2417	1127
NIJ169 C 6% NaClO	T3	6M	4221903	4577	126
NIJ171 C UVC 1HR	T3	6M	3144407	239	169

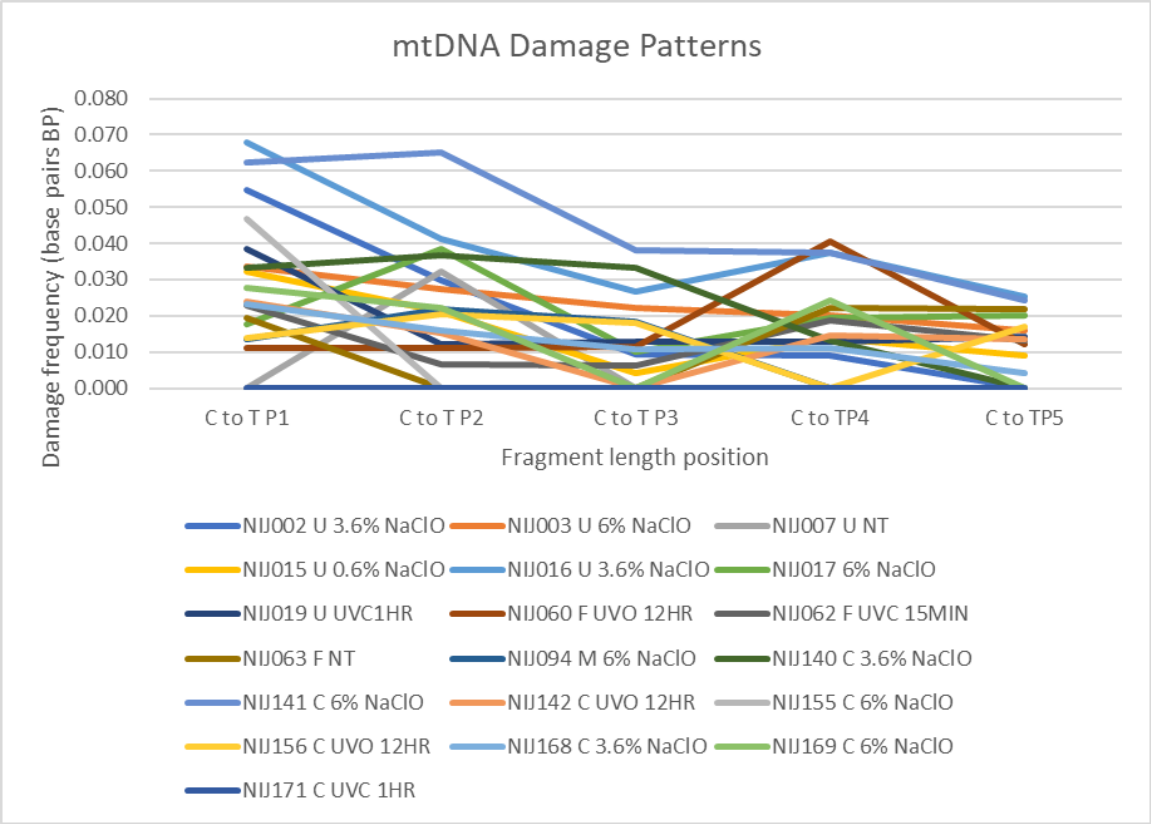


Figure 49 MtDNA map damage frequencies.

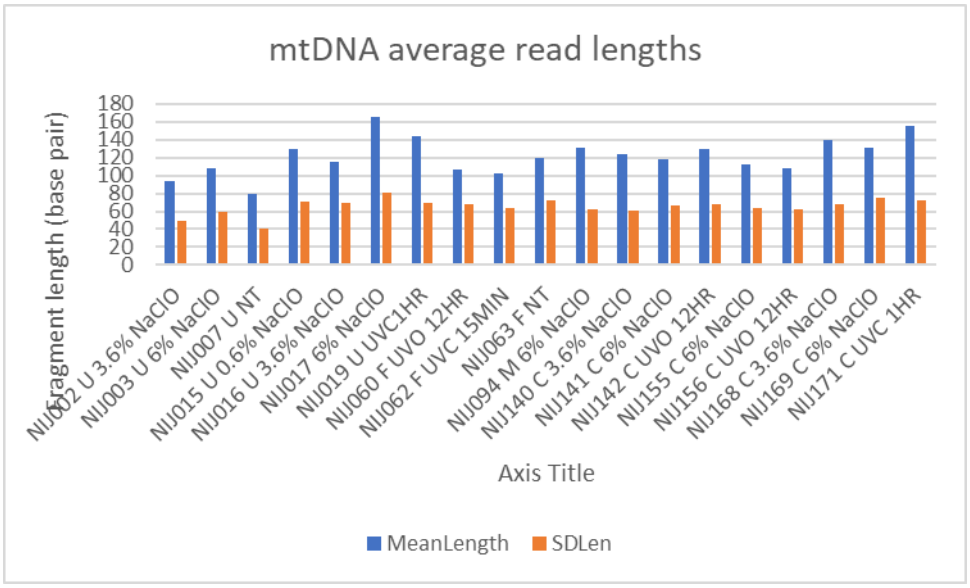


Figure 50 MtDNA average read lengths.

Table 13 MtDNA damage frequencies and haplotype ID.

Sample ID	C to T P1	C to T P2	C to T P3	C to TP4	C to TP5	Haplotype	Confidence
NIJ002 U 3.6% NaClO	0.055	0.030	0.009	0.009	0.000	hap1	0.919
NIJ003 U 6% NaClO	0.034	0.027	0.022	0.020	0.016	hap 2	0.970
NIJ007 U NT	0.000	0.032	0.000	0.000	0.000	hap 3	0.765
NIJ015 U 0.6% NaClO	0.032	0.021	0.004	0.014	0.009	hap 2	0.970
NIJ016 U 3.6% NaClO	0.068	0.041	0.027	0.037	0.025	hap 2	0.945
NIJ017 6% NaClO	0.018	0.038	0.010	0.019	0.020	hap4	1.000
NIJ019 U UVC1HR	0.038	0.012	0.013	0.013	0.014	hap2	0.864
NIJ060 F UVO 12HR	0.011	0.011	0.011	0.041	0.012	hap5	0.907
NIJ062 F UVC 15MIN	0.023	0.007	0.006	0.019	0.013	hap2	0.960
NIJ063 F NT	0.019	0.000	0.000	0.022	0.022	hap1	0.827
NIJ094 M 6% NaClO	0.014	0.022	0.019	0.000	0.000	hap2	0.683
NIJ140 C 3.6% NaClO	0.033	0.037	0.033	0.013	0.000	hap4	0.771
NIJ141 C 6% NaClO	0.063	0.065	0.038	0.038	0.024	hap1	0.746
NIJ142 C UVO 12HR	0.024	0.015	0.000	0.014	0.014	hap1	0.827
NIJ155 C 6% NaClO	0.047	0.000	0.000	0.000	0.000	hap2	0.931
NIJ156 C UVO 12HR	0.014	0.020	0.018	0.000	0.017	hap4	0.628
NIJ168 C 3.6% NaClO	0.023	0.016	0.011	0.011	0.004	hap2	0.970
NIJ169 C 6% NaClO	0.028	0.022	0.000	0.024	0.000	hap6	0.726
NIJ171 C UVC 1HR	0.000	0.000	0.000	0.000	0.000	hap5	0.787

4.2.7 Next-generation sequencing results of touch DNA from tape.

Next-generation sequencing results for tape DNA extracts (Appendix C; Tab G, Table 14) have higher overall human endogenous content (0-32% with an average of 8%) than handled bone samples, despite library quality being poorer (lower % of retained reads). Samples lack characteristic damage patterns (Appendix D) and frequency base substitutions at the 5' and 3' ends are low (Appendix C; Tab G, Appendix D, Figure 51). Fragment length is comparable to handled bone samples (Figure 52) with an average of 143.77 bp (SD=60.30 bp). There is a negative (-0.077) correlation between fragment length and time suggesting little degradation occurred over the course of a year. Fragment length may also be a product of the DNA extraction.

Table 14 Tape DNA next-generation sequencing results.

Sample ID	Human genome reads Total	human genome Q37 (quality 37)	hgQ37.Unique reads	% Unique read	% Endogenous	Library quality
NIJ205 zero	1917	1407	1025	73%	8%	Poor
NIJ206 zero	13168	7354	4792	65%	11%	Poor
NIJ207 zero	1455665	779793	296104	38%	9%	Average
NIJ208 zero	41643	21117	13333	63%	8%	Average
NIJ209EC	113	19	16	84%	0%	Poor
NIJ210 1W	608	279	201	72%	2%	Poor
NIJ211 1W	44456	26097	7987	31%	5%	Average
NIJ212 1W	132	13	10	77%	0%	Poor
NIJ213 1W	5	2	2	100%	0%	Poor
NIJ214 1W	5546	3102	2818	91%	13%	Poor
NIJ215 1W	4262	3202	689	22%	2%	Poor
NIJ216 1W	72420	40905	24628	60%	13%	Average
NIJ217 1W	17514	11600	9722	84%	22%	Average
NIJ218 1W	2086	1310	1243	95%	20%	Poor
NIJ219 1W	348783	209362	158027	75%	22%	Average
NIJ220 1W	221253	93433	68937	74%	11%	Average
NIJ221EC	185	17	15	88%	0%	Poor
NIJ222 16W	207	48	25	52%	0%	Poor
NIJ223 16W	334	191	138	72%	3%	Poor
NIJ224 16W	2481	1772	1621	91%	10%	Poor
NIJ225 16W	17	14	12	86%	3%	Poor
NIJ226 16W	241	142	101	71%	1%	Poor

NIJ227 16W	7611	6182	3966	64%	23%	Poor
NIJ228 16W	3847	3183	2241	70%	10%	Poor
NIJ229 16W	0	0	0	No Data	0%	Poor
NIJ230 16W	671	512	480	94%	7%	Poor
NIJ231 16W	17359	14251	9829	69%	32%	Average
NIJ232 16W	175987	134993	89944	67%	22%	Average
NIJ233EC	811	518	376	73%	7%	Poor
NIJ242 8M	153	54	8	15%	0%	Poor
NIJ243 8M	2506	1340	986	74%	9%	Poor
NIJ244 8M	12000	7018	4660	66%	10%	Average
NIJ245EC	107	37	23	62%	0%	Poor
NIJ246 1Y	20428	12253	7488	61%	5%	Average
NIJ247 1Y	1331	902	611	68%	5%	Poor
NIJ248 1Y	30	20	13	65%	0%	Poor
NIJ249 1Y	5112	2916	2754	94%	27%	Poor
NIJ250 1Y	1124	840	490	58%	4%	Poor
NIJ251 1Y	205	129	89	69%	1%	Poor
NIJ252 1Y	1776	1148	865	75%	10%	Poor
NIJ253 1Y	169	124	103	83%	1%	Poor
NIJ254 1Y	37	18	17	94%	1%	Poor
NIJ255 1Y	7395	4576	3182	70%	11%	Poor
NIJ256 1Y	26326	13383	9399	70%	14%	Average
NIJ257EC	42	22	21	95%	0%	Poor
NIJ_LN11	3331	28	25	89%	0%	Good

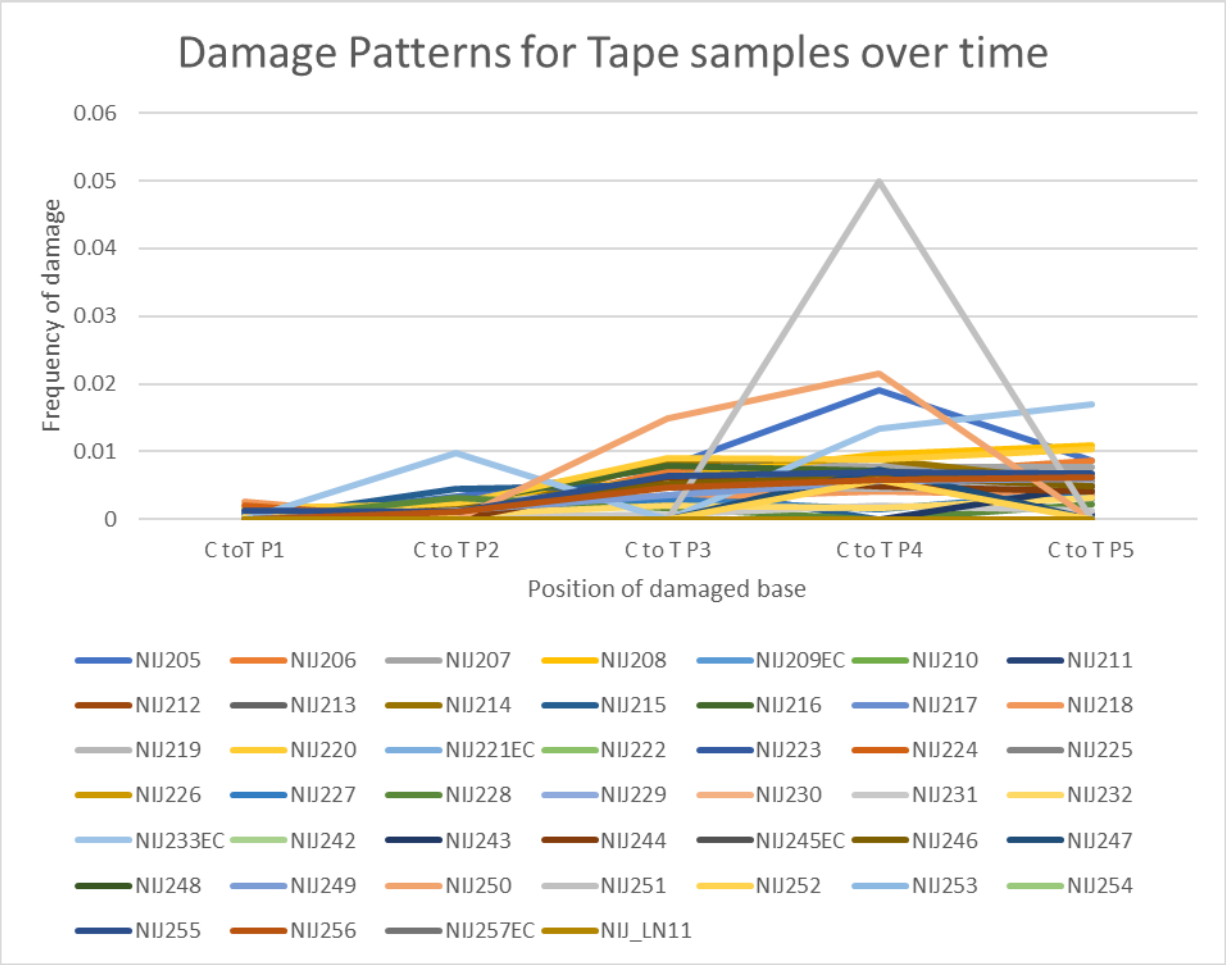


Figure 51 Tape DNA damage patterns.

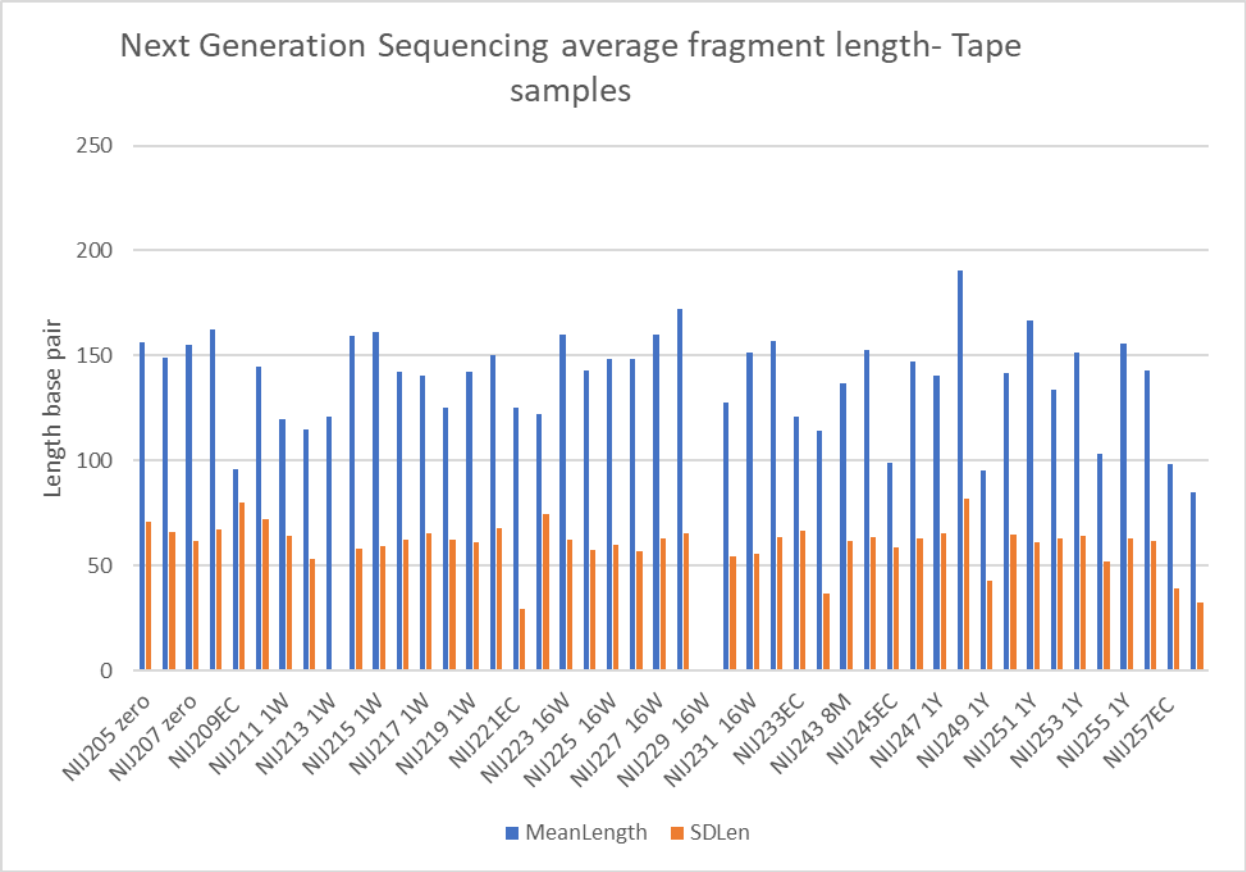


Figure 52 Tape DNA samples average read lengths

Tape ancestry results

Only 11 samples had enough aligned human reads to produce an ancestry profile. Tape samples had fewer number of comparable SNPs, versus handled bone samples, that had produced enough data for analyses (~2800 vs ~14,000). No female tape samples had enough data for analyses. Time does not appear to be a factor as samples time frames ranged from 0 days to one year. Discriminatory power is reduced as seen in the ancestry results for Male1 which had higher levels of African ancestry (Appendix C; Tab H, Table 15, Figure 53). The Ancient Americas ancestry is puzzling, while the LMAMR laboratory almost exclusively works with ancient human remains from the Americas, the samples are very degraded and often display higher levels of DNA damage and fragment lengths that are less than 100bp. Since these results are from the tape samples as well as the handled bone samples, we can conclude that the source is not the bone sample itself. Additionally, all mtDNA types found were non-Native American in origin. It is possible this dataset (all ancient samples from the Americas) have bias, errors, damage, or contamination in the reads.

Table 15 Ancestry results for Tape DNA samples.

Sample	Time	Number Reads	Number Snps	K5_1_SEAsia	K5_2_Europe	K5_3_Africa	K5_4_America	K5_5_Sasia
NIJ207 C	Time zero	296104	12103	0.172	0.446	0.000	0.283	0.099
NIJ208 C	Time zero	13333	570	0.000	0.365	0.023	0.373	0.240
NIJ219 C	1 week	158027	7536	0.045	0.617	0.005	0.317	0.015
NIJ220 C	1 week	68937	2724	0.000	0.475	0.053	0.392	0.080
NIJ231 C	1 week	9829	650	0.000	0.377	0.033	0.557	0.033
NIJ232 C	1 week	89944	5259	0.062	0.543	0.000	0.291	0.104
NIJ211 M1	1 week	7987	332	0.000	0.460	0.272	0.161	0.107
NIJ246 M1	16 weeks	7488	341	0.000	0.433	0.165	0.402	0.000
NIJ216 M2	16 weeks	24628	1043	0.000	0.692	0.000	0.308	0.000
NIJ217 M2	1 year	9722	420	0.375	0.213	0.025	0.365	0.021
NIJ256 M2	1 year	9399	350	0.000	0.540	0.000	0.237	0.222

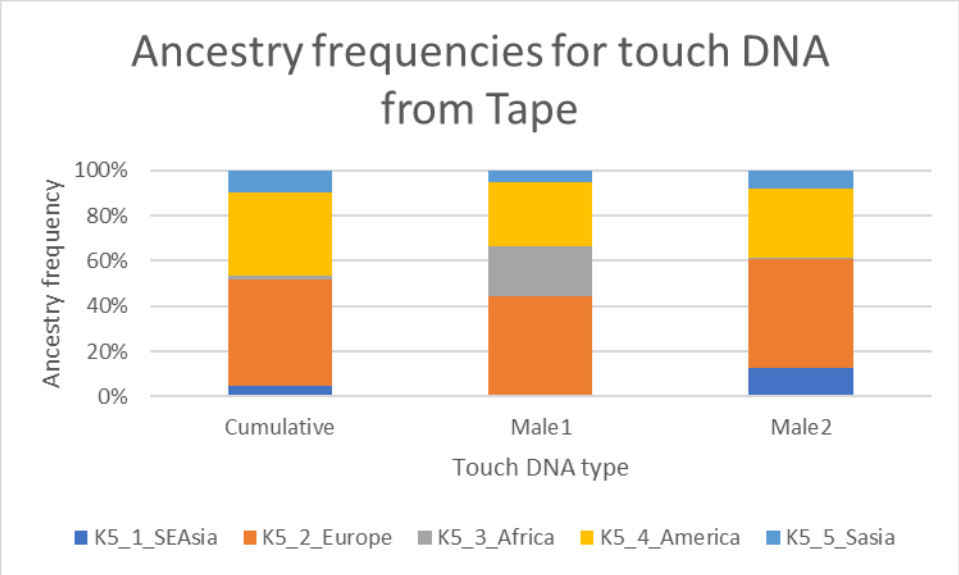


Figure 53 Ancestry results for tape DNA samples

Sex typing of tape DNA samples

Sex determination was not possible for three cumulative samples (Table 16). However, all male samples were correctly identified. Cumulative samples were identified as female in one sample and male in another.

Table 16 Sex determination for tape DNA samples

Sample	X+Y reads	Y chromosome reads	Ratio	Sex Assignment	Time
NIJ207 C	9992	212	0.0212	Not Assigned	0D
NIJ208C	300	11	0.0367	XX_not XY	0D
NIJ211 M1	206	14	0.068	XY_not XX	1W
NIJ246 M1	157	8	0.051	XY_not XX	1W
NIJ216 M2	575	35	0.0609	XY_not XX	1W
NIJ217 M2	225	18	0.08	XY_not XX	1W
NIJ219 C	4689	240	0.0512	Not Assigned	1W
NIJ220 C	1703	73	0.0429	Not Assigned	16wk
NIJ231 C	227	13	0.0573	XY_not XX	16wk
NIJ232 C	2138	116	0.0543	Not Assigned	1Y
NIJ256 M2	250	14	0.056	XY_not XX	1Y

Mitochondrial DNA results for tape samples

Three cumulative samples from three time periods had enough reads to attempt mtDNA haplotype identification (Appendix C; Tab I; Table 17). Two haplotypes were identified, one not found in the bone handled samples and one previously ID. Note that the confidence level for these assignments is rather low and should be taken with caution.

Table 17 MtDNA results for tape samples.

Sample ID	C to T P1	C to T P2	C to T P3	C to TP4	C to TP5	Haplotype	Confidence
NIJ207C T0	0.000	0.000	0.000	0.023	0.000	hap7	0.6203
NIJ219C T1	0.000	0.000	0.061	0.040	0.000	hap7	0.5
NIJ232C T2	0.000	0.000	0.000	0.000	0.000	hap6	0.6842

4.3 Phase II: Microbial DNA results

Microbial reads were retrieved by mapping to greengenes database (matching sequences from 16s gene). 16s OTU (operational taxonomic unit) clustering was done using Usearch (v11)(Schloss et al., 2009). Sourcetracker v1 analysis used human skin, gut, oral microbiomes, and soil microbiomes as potential sources (Knights et al., 2011). Closed reference was used to assign reads to OTUs. A rarefaction depth of 100 was used to filter samples. 98 samples and 1 extraction control fit this criterion.
Most of the samples contained 16s reads that were “unknown” and not associated with taxa common to oral, skin, gut, and soil microbiomes (Appendix C; Tab J, Appendix E; Figure 53-54) . Gut and oral bacteria were almost absent from the samples. There is little correlation (r= 0.029) between time and the frequency of skin microbiome signatures. Treatment of the sample also had little correlation (r= 0.132) Soil and skin microbiome signatures were detected despite bleach and UV treatments. The most common taxa identified include skin microbes: Cutibacterium, Corynebacterium, Staphylococcus; soil microbes: Bacillus, Rhizobiales (order); and common laboratory surface/reagent contaminants: Comamonadaceae (family), and Microbacterium.

4.3.1 Microbiome results from tape DNA samples.

In contrast to the handled bone samples, a majority of OTUs were associated with taxa found in skin microbiomes and were unaffected by time (Appendix C; Tab J, Appendix E, Figure 56).

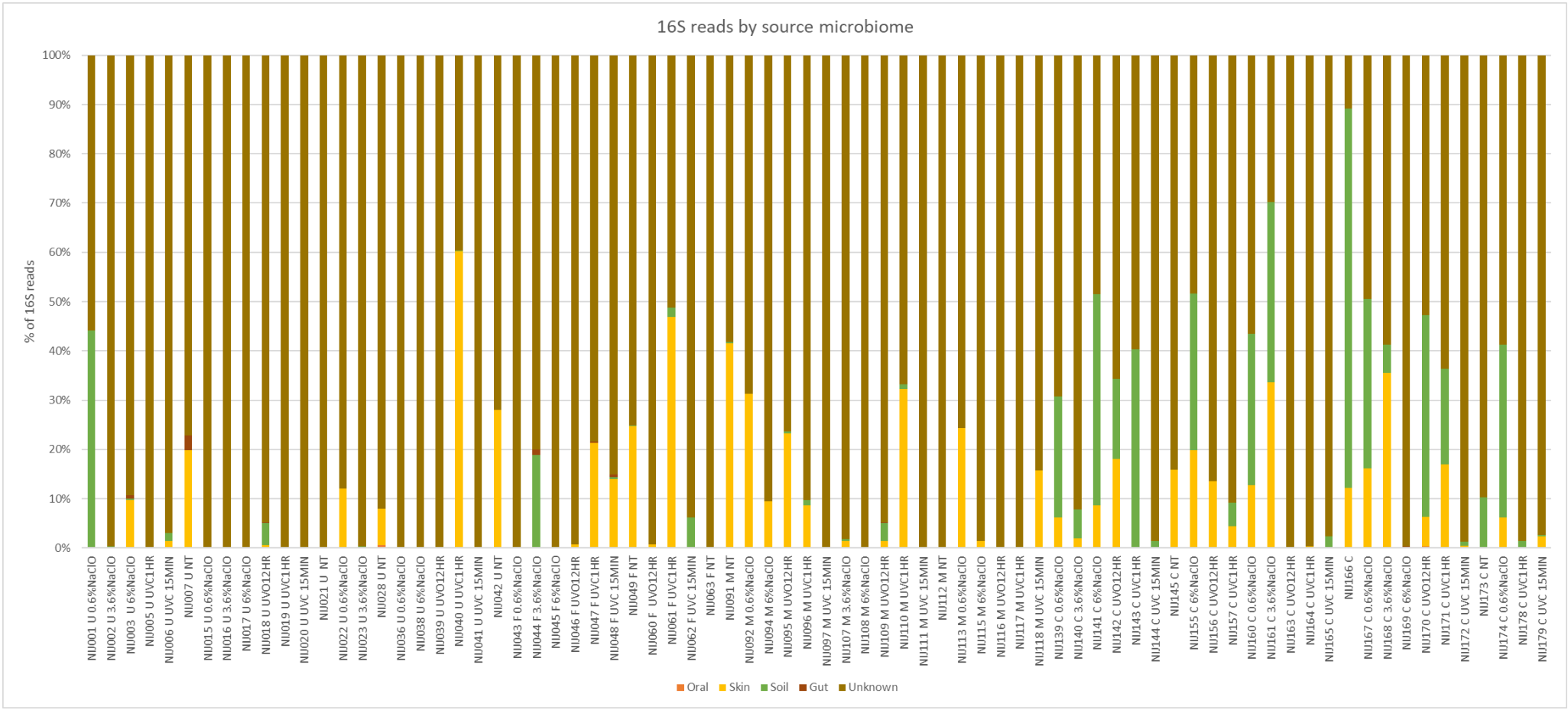


Figure 54 Sourcetracker results

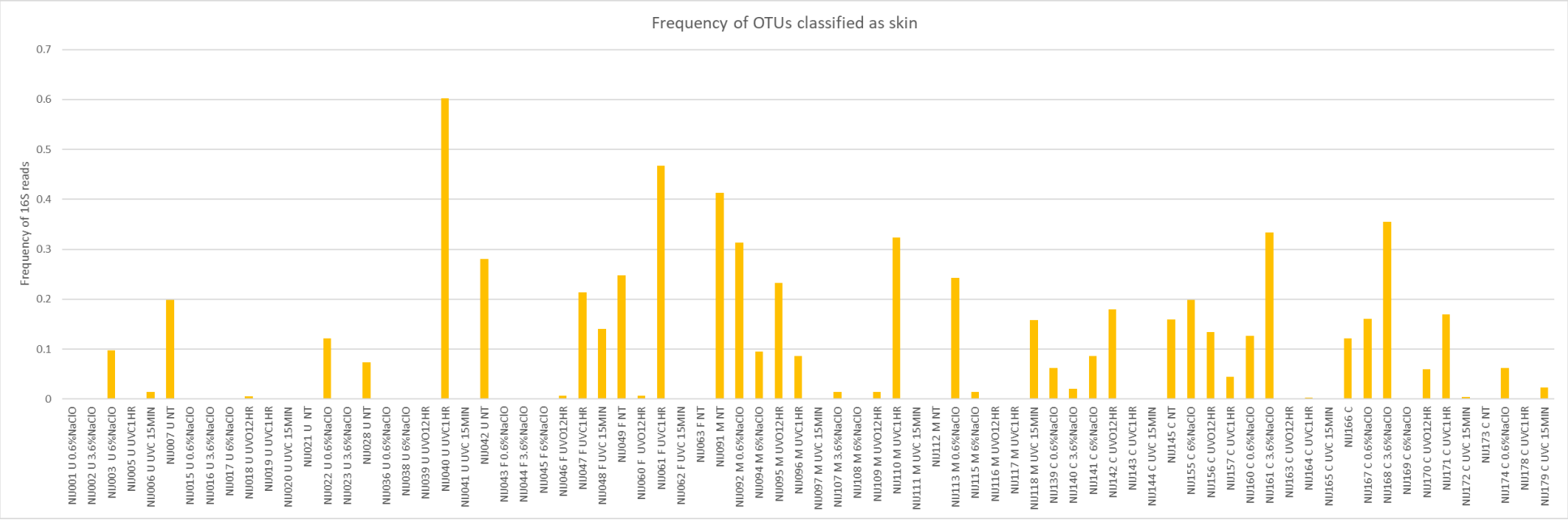


Figure 55 OTUs classified as skin.

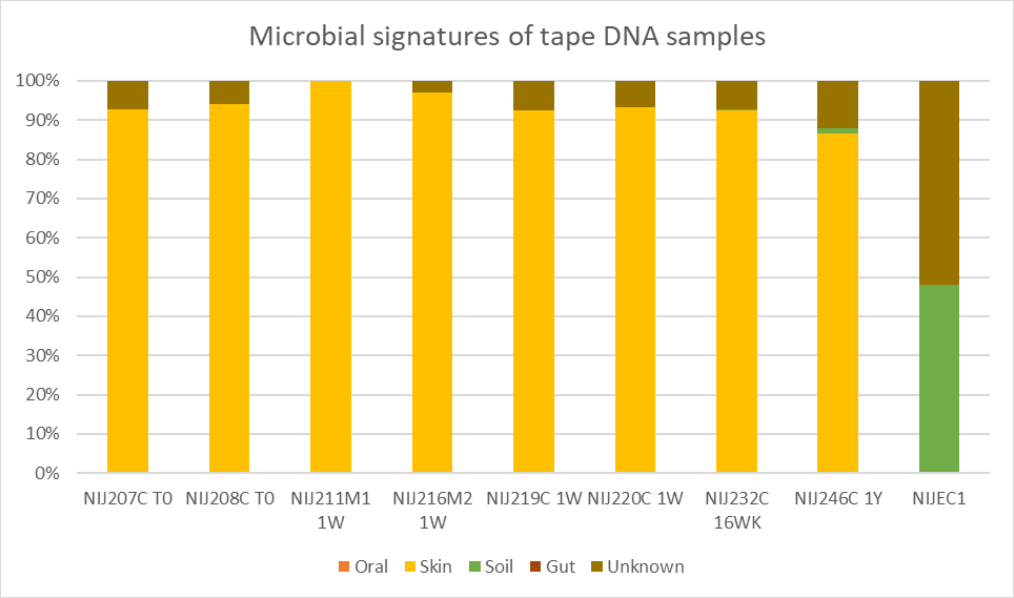


Figure 56 Microbial signatures of tape DNA samples

4.4 Capillary electrophoresis: AmpFLSTR™ Identifiler™ Plus results

Handled bone samples along with negative and positive controls (including saliva DNA extracted from handlers) were amplified using the AmpFLSTR™ Identifiler™ Plus PCR Amplification Kit and followed manufacturers guidelines. None of the bone samples amplified- with only primer dimers (less than 100bp) being present (Figure 37). The samples were not further processed for fragment analysis.

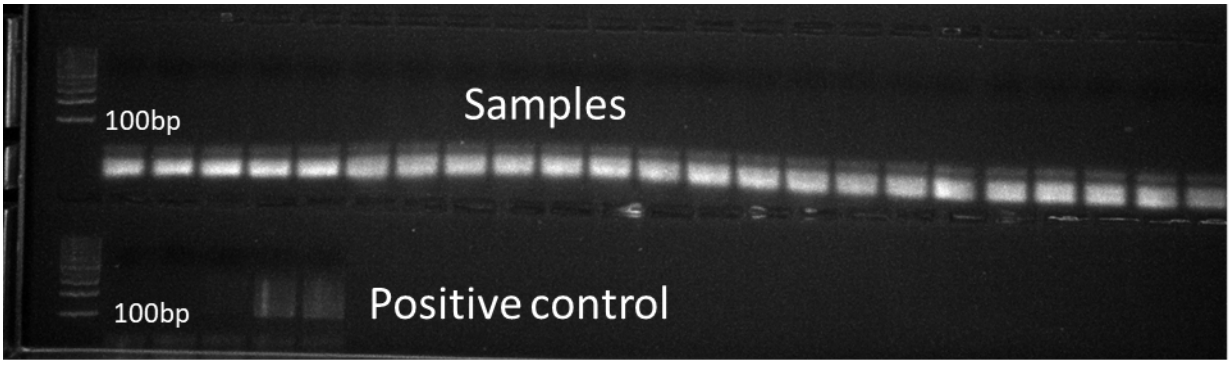


Figure 57. Example gel image from AmpFLSTR amplification

4.5 ForenSeq amplification and results

Amplicon libraries were built for handled bone samples using the Verogen DNA Signature Prep Kit and using manufacturers protocols. Prior to sequencing, PI Monroe beta tested the Verogen's mtDNA Whole DNA Kit using prehistoric bone samples and one tape sample from this project (NIJ208 cumulative). The correct mtDNA haplotypes were retrieved from all samples including the three haplotypes from NIJ208. Quantification based normalization was shown to be generally more effective for low-level samples (i.e., bone samples) compared to bead-based normalization. However, bead-based normalization proved to be highly effective and sufficient for the higher quality skin/touch DNA sample (Figure 58-59). Manual/quantification-based normalization had not been validated or attempted by Verogen for the DNA Signature prep kit at the time of this study.

4.5.1 Comparison of bead-based normalization vs quantification-based normalization.

An initial sequencing run using Verogen micro-flow cell was performed to compare the effectiveness of a bead-based versus manual (quantification-based) normalization. Manual normalization process, as recommended from Verogen, varied from more common NGS pooling methods that used molarity versus concentration. Here samples were diluted to 0.75ng/ul. Samples that were below that concentration were not diluted. Results demonstrate an improved recovery of both STRs and Identity SNPs for untouched bone samples as well as handled bone samples (female). However, there was a slight decrease in coverage for positive controls (Table 18). Since overall concentrations of Forenseq libraries were low and bead-based normalization can be associated with DNA loss it was decided to move forward with a manual normalization for subsequent sequencing. Similar to NGS results, sample NIJ003 had high recovery, but did not match PI Monroe's forensic profile.

4.5.2 Forenseq sequencing results

A majority of libraries had concentrations less than 0.75ng/ul (Appendix F, Tab A). Fragment analyses suggested high adapter dimer content and libraries lacked expected peak distributions found with positive controls (Figure 60). Sequencing using 96rxns flow cell was attempted twice using the manual-normalization method, both of which failed toward the end of the runs. Run files revealed sequencing of predominantly adapter dimers followed by a camera error that no more clusters could be sequenced. Low libraries concentrations and high adapter content were determined to be the cause of run failures. Subsequent sequencing increased the number of positive controls to prevent further failed sequencing, however this was unsuccessful. Bead-based normalization was then attempted to remove adapter dimers. NIJrun1 normalized was successful but resulted in very little data from the samples. A subsequent sequencing run using bead-based normalization was attempted but also failed due to over sequencing of adapter dimers.

Budget constraints prevented further troubleshooting, however loading fewer samples or using smaller micro flow cells may be a viable alternative, despite it large cost increase per sample.

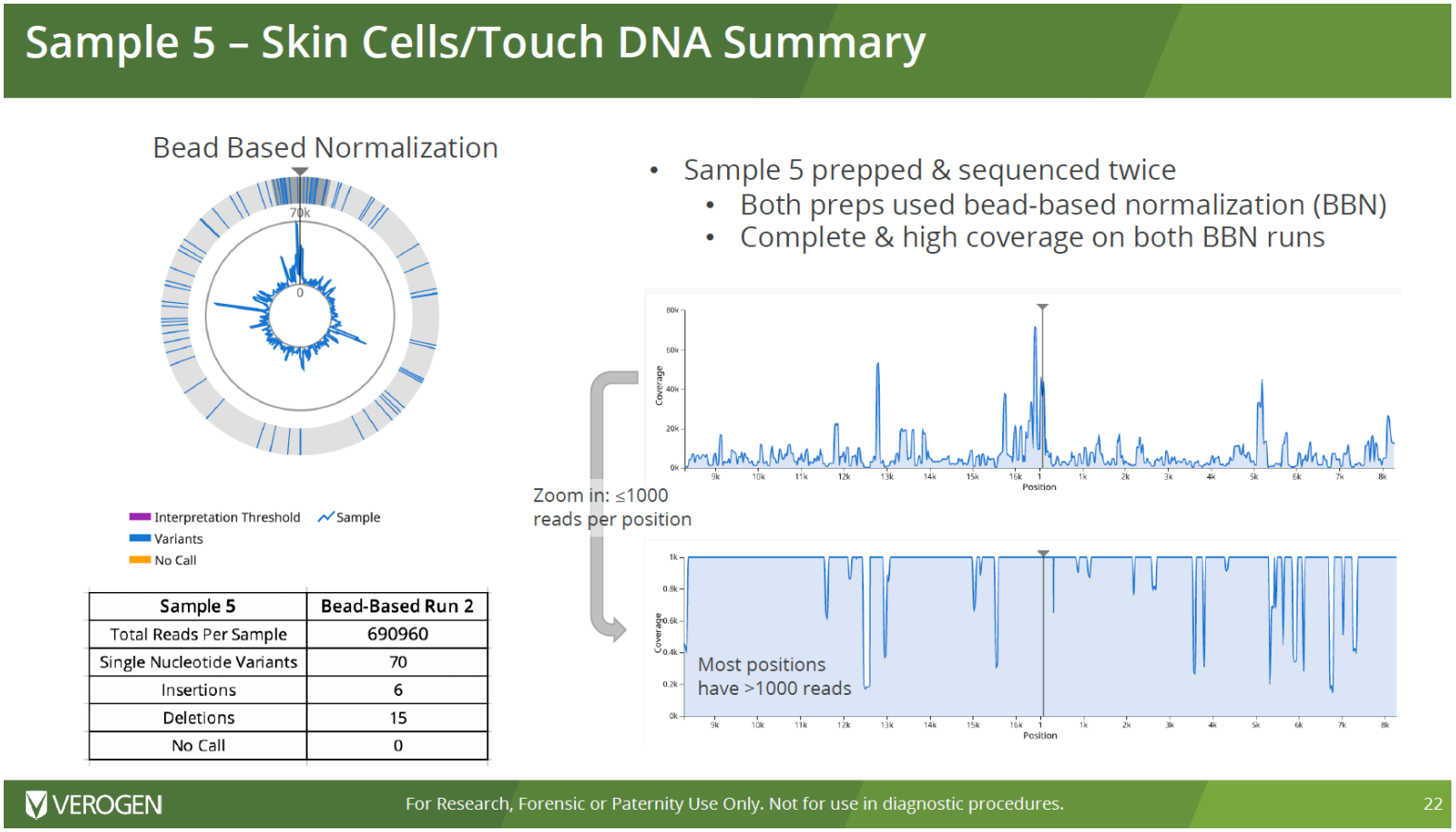
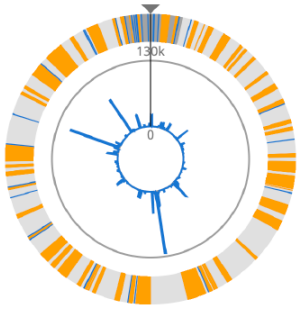


Figure 58 Verogen beta testing mtDNA results for NIJ208.

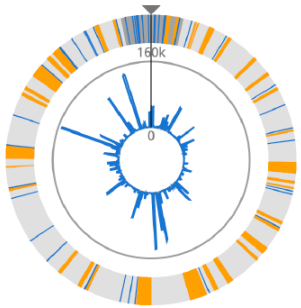
Sample 4 – Bone Variant Summary

Bead Based Normalisation



Sample 4	Bead-Based Quant	Manual Quant
Total Reads Per Sample	424239	1049540
Single Nucleotide Variants	28	42
Insertions	0	0
Deletions	3	4
No Call	4834	2545

Quant Based Normalisation



- Improved coverage using manual quantification normalization method
  - detection of more variant positions

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Figure 59 Verogen mtDNA beta testing results: bead-based normalization versus quantification based normalization.

Table 18 STR and SNP coverage of bead-based and manual quantification.

Sample ID	Touch Type	Time	Fragment Analyzer/Tape Station ng/ul	STR Coverage autosomal_bead normalization (n=35)	Identity SNP coverage_bead normalization (n=94)	Y-chromosome bead_normalization (n=24)	Manual dilution STR Coverage autosomal (n=35)	Manual dilution Identity coverage (n=94)	Manual dilution Y-chromosome (n=24)
NIJ001 0.6%NaClO	Untouched	Time Zero	0.7877	5	17	0	15	38	0
NIJ002 3.6%NaClO	Untouched	Time Zero	0.2182	14	59	0	16	44	0
NIJ003 6%NaClO	Untouched	Time Zero	0.1501	20	62	0	24	64	0
NIJ004 UVO12HR	Untouched	Time Zero	2.3008	5	13	0	4	5	0
NIJ005 UVC1HR	Untouched	Time Zero	1.1681	4	11	0	7	17	0
NIJ006 UVC15MIN	Untouched	Time Zero	1.3584	8	13	0	13	21	0
NIJ007 NT	Untouched	Time Zero	3.9627	5	11	0	6	13	0
NIJ043 0.6%NaClO	Female	Time Zero	0.3613	1	2	0	2	1	0
NIJ044 3.6%NaClO	Female	Time Zero	0.0611	0	0	0	0	0	0
NIJ045 6%NaClO	Female	Time Zero	0.1781	4	4	0	6	9	0
NIJ046 UVO12HR	Female	Time Zero	1.2089	5	2	0	4	2	0
NIJ047 UVC1HR	Female	Time Zero	0.1989	10	8	0	12	8	0
NIJ048 UVC15MIN	Female	Time Zero	0.263	0	1	0	0	1	0
NIJ049 NT	Female	Time Zero	0.9004	4	4	0	8	5	0
Female 1	Cheek swab		6.32	35	92	0	35	89	0
Male 1	Cheek swab		5.14	36	90	23	33	81	21
Male 2	Cheek swab		4.58	36	91	23	33	84	20
2800 M Positive			3.6085	33	85	23	33	79	21
2800 pos (168-173) 3.26			3.23	11	94	4	0	0	0

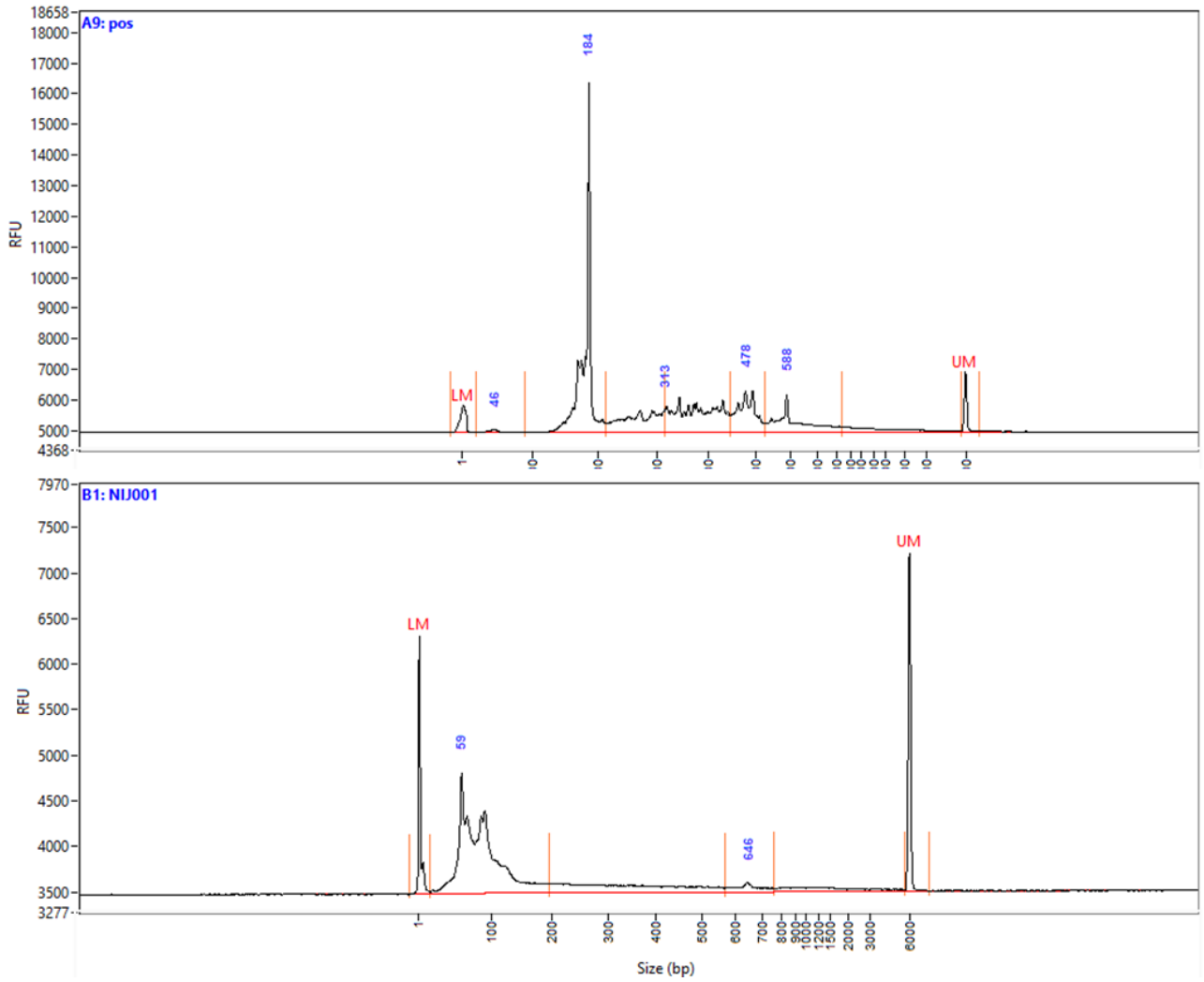

Figure 60 Fragment analyzer quantification and fragment size distribution of Forenseq libraries.

5 Conclusions

Forensic DNA researchers are confronted with interpreting data from LADD DNA from both endogenous and exogamous sources. This project was an attempt to address some of these issues by getting a fuller understanding of the extent of postmortem genetic damage as the field moves into the Massive Parallel/Next Generation Sequencing era. It is important to empirically test assumptions that contaminating touch DNA is damaged and degraded in specific ways that differ from LCN forensic samples, especially when it hinders researchers' ability to discriminate between authentic and contaminating profiles.

5.1 Phase 1

Results suggest that the quality of samples post extraction was quite low with higher-than-expected fragmentation of DNA molecules at time zero for both handled bone and tape samples. Future research should explore whether the Prepfiler BTA protocol results in DNA fragmentation and lower yields compared to paleogenomic methods.

Fragment size of genomic DNA consistently ranged between 100-500bp in size, however this was at time stochastic with 1 year sample have a higher concentration of larger sized DNA molecules over 2000bp. Genomic concentrations varied highly between handlers, treatment, and across time with no apparent pattern, suggesting varying yields may be a product of the variability/repeatability of extraction yields or uneven distribution of touch DNA. The exception is the overall lower yield of bleached samples. There was no correlation between genomic concentrations and bone mass.

The amount of endogenous human DNA ranged between 0-5% and varied greatly by sample treatment. Interestingly, bleach treated samples had more and higher mapped reads to the human genome (endogenous content) than UV or untreated samples. This contrasts with the lower amount of total genomic DNA found with bleached samples prior to library amplification and suggests bleach may in some cases improve library sequencing. The number of sequencing reads consistently declines with time with more than half the samples from 6 months and 1 year yielding no results. A majority of successfully sequenced libraries from the last two time-trials were untouched or cumulative touched samples.

Damage patterns seem unrelated to treatment or time, other than an increase in older samples with insufficient sequencing reads to perform an analysis-thus indicative of low-quality sample in general. However, some bleach treated samples have damage not on the 3' to 5' ends which suggests reduced mapping to the reference sequence or possibly internal molecule damage due to the treatment.

Average read length varied across time and treatment. Untreated samples had the smallest average read length and bleached samples having the largest fragment size. It is unclear what mechanism is causing this phenomenon as previous research has recognized that sodium hypochlorite induces lesions in contaminating DNA thus leading to smaller fragment sizes. In contrast to expectations, older samples have the largest average read lengths despite few numbers of samples successfully sequencing. Unhandled samples had the smallest fragment lengths compared to touched samples suggesting higher fragmentation of the bone compared to touch DNA despite time and treatment. Untouched samples and female touched samples were correctly assigned as female. However, all male touched samples (including cumulative samples) were assigned female or likely female, with little Y-chromosome DNA being reported. Due to the low number of reads it is possible that endogenous DNA from the bone sample may compete with the retrieval of touch DNA from the bone surface.

Sequencing results from evidence tape had markedly more pronounced endogenous DNA content than handled bone samples, despite lower levels of sequencing results and lower % of retained reads. Samples with enough data to analyze, had lower frequency base substitutions at the 5' and 3' ends. Fragment length was comparable to handled bone samples. There is negative correlation between fragment length and time suggesting little degradation occurred over the course of a year.

5.2 Capillary Electrophoresis and ForenSeq amplicon sequencing

Traditional capillary electrophoresis methods using the AmpFLSTR™ Identifiler™ Plus PCR Amplification Kit were unsuccessful. The ForenSeq DNA Signature Prep Kit was also unsuccessful for most samples due to low library concentrations and high levels of adapter dimers causing run failure. However, it does appear that manual or quantification-based normalization may be ideal for better preserved samples, which contrasts with beta testing results using the Forenseq mtDNA kit. The latter kit has fewer number of amplicons as well as a more consistent amplicon size, thus theoretically reducing the amount of potential adapter dimer (or at least allowing potential size exclusion using beads or gel-based methods like pippen prep). Future research should experiment on using smaller flow cells, as well as increased numbers of positive controls which may reduce the overall amount of dimer on any given sequencing run. If successful it may offset the large increase in per sample costs.

5.3 Phase 2

The most common taxa identified include skin microbes: Cutibacterium, Corynebacterium, Staphylococcus; soil microbes: Bacillus, Rhizobiales (order); and common laboratory surface/reagent contaminants: Comamonadaceae (family), and Microbacterium. However, a large proportion of microbial DNA sequences were classified as unknown and were not associated with oral, skin, gut, or soil microbiomes. Additional

research on bone samples could determine if there is microbiome profile unique to this substrate. There was little correlation between sample treatment and microbiome signatures, with taxa belonging to both skin and soil microbiome despite being bleach treated or UV. Tape samples varied dramatically, with high frequency of taxa belonging to skin microbiome. This pattern was consistent across the entire year. Skin microbiome signatures may not be an effective evidential tool when competing with taxa from unknown/ yet identified microbiomes.

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