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Research paper

Performance evaluation of a mitogenome capture and Illumina sequencing protocol using non-probative, case-type skeletal samples: Implications for the use of a positive control in a next-generation sequencing procedure

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ABSTRACT

Next-generation ancient DNA technologies have the potential to assist in the analysis of degraded DNA extracted from forensic specimens. Mitochondrial genome (mitogenome) sequencing, specifically, may be of benefit to samples that fail to yield forensically relevant genetic information using conventional PCR-based techniques. This report summarizes the Armed Forces Medical Examiner System's Armed Forces DNA Identification Laboratory's (AFMES-AFDIL) performance evaluation of a Next-Generation Sequencing protocol for degraded and chemically treated past accounting samples. The procedure involves hybridization capture for targeted enrichment of mitochondrial DNA, massively parallel sequencing using Illumina chemistry, and an automated bioinformatic pipeline for forensic mtDNA profile generation. A total of 22 non-probative samples and associated controls were processed in the present study, spanning a range of DNA quantity and quality. Data were generated from over 100 DNA libraries by ten DNA analysts over the course of five months.

The results show that the mitogenome sequencing procedure is reliable and robust, sensitive to low template (one ng control DNA) as well as degraded DNA, and specific to the analysis of the human mitogenome. Haplotypes were overall concordant between NGS replicates and with previously generated Sanger control region data. Due to the inherent risk for contamination when working with low-template, degraded DNA, a contamination assessment was performed. The consumables were shown to be void of human DNA contaminants and suitable for forensic use. Reagent blanks and negative controls were analyzed to determine the background signal of the procedure. This background signal was then used to set analytical and reporting thresholds, which were designated at 4.0X (limit of detection) and 10.0X (limit of quantiation) average coverage across the mitogenome, respectively. Nearly all human samples exceeded the reporting threshold, although coverage was reduced in chemically treated samples resulting in a ~58% passing rate for these poor-quality samples. A concordance assessment demonstrated the reliability of the NGS data when compared to known Sanger profiles. One case sample was shown to be mixed with a co-processed sample and two reagent blanks indicated the presence of DNA above the analytical threshold. This contamination was attributed to sequencing crosstalk from simultaneously sequenced high-quality samples to include the positive control. Overall this study demonstrated that hybridization capture and Illumina sequencing provide a viable method for mitogenome sequencing of degraded and chemically treated skeletal DNA samples, yet may require alternative measures of quality control.

1. Introduction

Next-generation sequencing (NGS) technologies have significantly expanded the ability to recover genetic information from ancient and degraded DNA samples. In particular, hybridization capture has proven useful for targeted enrichment of genomic DNA (gDNA) [1–5], as well as smaller targets such as the human exome [6] and the mitochondrial genome (mitogenome) [1,7]. Capture is amenable to DNA fragments

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that are too degraded for NGS workflows involving PCR enrichment, which are more common to forensics [8–12]. The technological advancement offered by hybridization capture and NGS therefore create the potential to obtain genetic data from forensic specimens that were heretofore too degraded for DNA analysis. In particular, next-generation mitogenome sequencing holds promise for human identification efforts and missing persons cases such as those of the Defense Personnel Accounting Agency (DPAA). Working in conjunction with DPAA, the Armed Forces Medical Examiner System's Armed Forces DNA Identification Laboratory (AFMES-AFDIL) performs all of the DNA testing to assist in the identification of missing service members from past military conflicts. The samples typically submitted by DPAA to the AFMES-AFDIL for genetic testing are aged skeletal remains that were exposed to post-traumatic (e.g., fire) and environmental insults and sometimes chemical treatment with formaldehyde or powdered hardening compounds. As a result, endogeneous DNA is often limited in quantity, degraded and damaged, and plagued with co-extracted inhibitory molecules common to ancient DNA such as microbial DNA and humic acid. Though the AFMES-AFDIL currently has a > 90% mtDNA success rate using traditional Sanger-type-sequencing (STS) methods [13,14], an NGS method for mitochondrial genome sequencing was needed for the most challenging samples that yield partial or unreproducible results from standard techniques.

The present study summarizes a performance evaluation of a mitogenome capture and Illumina sequencing procedure for non-probative case-type samples. The procedure is a low-throughput, labor-intensive process with numerous tube transfers requiring approximately ten hours of hands-on time. The laboratory workflow involves several clean-lab steps, including an initial enzymatic damage mitigation step to minimize cytosine deamination typical of aged and degraded DNA [15], followed by purification and dual-indexed Illumina library preparation. In the post-amplification laboratory, a limited-cycle PCR completes the library preparation procedure, then indexed libraries are quantified to confirm successful amplification. Libraries are subsequently enriched for the mitogenome in an overnight in-solution hybridization incubation with biotinylated RNA baits. After DNA capture, purification, and amplification of the captured product, samples are purified and quantified before being pooled for paired-end sequencing on an Illumina MiSeq. The procedure takes roughly two weeks to complete from bone preparation to mitogenome profile for a sample set of three case samples and associated controls. As summarized below, 40 non-probative, case-type samples libraries and associated controls were processed for a total of more than 100 libraries sequenced by ten DNA analysts over the course of five months. These NGS data confirm the reliability, specificity, cleanliness, and sensitivity of this novel forensic DNA testing procedure.

2. Materials and methods

2.1. Samples

A total of 22 previously reported and therefore non-probative AFMES-AFDIL case samples were selected for this study, and were categorized into four types: chemically treated, degraded, high quality, and nonhuman (Table S1). The chemically treated samples represent a set of Korean War unknowns that were treated with formaldehyde before being buried in the National Memorial Cemetery of the Pacific (NMCP) in Honolulu, Hawaii. The degraded samples were chosen from World War II (WWII), Korean War, or Vietnam War cases. Two high-quality, non-probative bone samples were included for the purposes of the mixture study. All 22 non-probative case samples were previously tested with Sanger-type sequencing (STS) technology (following methods outlined in [13,14,16]), indicating a range of mtDNA quality. The high quality samples generated > 1000-bp amplicons, the degraded and nonhuman samples generated 150–300-bp amplicons, and the chemically treated samples yielded either very small amplicons or failed to amplify. This sample quality information was utilized to assess the sensitivity of the procedure in lieu of a traditional sensitivity series with control DNAs, which lack the environmental contaminants that necessitate the capture procedure.

2.2. Contamination monitoring and controls

DNA extraction and library preparation were carried out in a pre-PCR DNA laboratory dedicated for low copy and degraded DNA sample testing. Reagent blanks (RBs) were introduced during DNA extraction and were carried through the entire downstream procedure. A non-template library negative control (NC) and a library positive control (PC) were introduced during library preparation. The inclusion of a PC is unconventional for the ancient DNA community because the PC itself presents a potential source of contamination. However, the Federal Bureau of Investigation (FBI) Quality Assurance Standards (QAS) mandates that a PC be utilized to monitor the success of a DNA testing procedure [17]. In order to adhere with the QAS for accreditation purposes, this study employed a PC but utilized the following measures to minimize its potential to contaminate associated samples: 1) only 1.0 ng of fragmented gDNA was utilized in library prep (four-fold less DNA than the manufacturer's recommendation), 2) the PC was spiked into the library pool for sequencing to control the number of reads that it consumed (see below), and 3) the control DNA exhibited a rare mitogenome profile (K562; Promega Corporation, Madison, WI) to be readily identified in the sequence data (Table S2). The K562 control DNA was enzymatically fragmented with NEBNext Fragmentase (New England Biolabs Inc. (NEB), Ipswich, MA) in a separate pre-PCR laboratory, then diluted to 0.2 ng/μL prior to being taken to the degraded sample laboratory for library preparation. Overall, a total of 27 RBs, 18 NCs and 17 PCs were processing simultaneously with the non-probative samples.

2.3. DNA extraction and USER treatment

DNA was isolated from 0.2 g–1.0 g powdered bone sample. Bone powder was first demineralized in a buffer containing 0.5 M EDTA, 1% sarkosyl and 20 mg/ml proteinase K by incubating overnight with agitation at 56 °C [18]. DNA was purified using one of three protocols: organic [18], QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) [19], or a variation of the [19] procedure that utilized the MinElute PCR Purification Kit (QIAGEN) instead of the QIAquick. As with the PC, the high quality DNA samples were enzymatically fragmented with NEBNext Fragmentase following the manufacturer's protocol in order to make the DNA suitable for library preparation. When additional bone sample was available, replicate DNA extracts were prepared for reproducibility testing (Table S3). Additionally, three mixtures were created from the remaining DNA extract volume after neat sample processing of the two high quality DNA extracts. Mixtures of 30 μL were created using a volume-based mixing scheme in ratios of 1:9, 1:1, and 9:1. To keep the input volumes consistent with neat samples, mixed samples were suspended in 70 μL volumes using Tris-EDTA (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5).

In the absence of a commercially available kit for DNA fragments averaging ~75 bp (such as those intended for the present procedure), a quantitative PCR (qPCR) was not performed. Instead, total extracted DNA (human and microbial) was quantitated to confirm the success of the DNA extraction procedure. An aliquot of each DNA extract was quantified using the Agilent 2100 Bioanalyzer using the High Sensitivity (HS) or 7500 assay (Santa Clara, CA) to confirm the success of DNA extraction. All DNA extracts were subjected to a 1-h Uracil-Specific Excision Reagent (USER) (NEB) treatment to mitigate the effects of cytosine deamination [20,21], followed by purification with the MinElute kit with elution in Tris-EDTA.