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Brief Communication

Sequencing the hypervariable regions of human mitochondrial DNA using massively parallel sequencing: Enhanced data acquisition for DNA samples encountered in forensic testing

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ABSTRACT

Mitochondrial DNA testing is a useful tool in the analysis of forensic biological evidence. In cases where nuclear DNA is damaged or limited in quantity, the higher copy number of mitochondrial genomes available in a sample can provide information about the source of a sample. Currently, Sanger-type sequencing (STS) is the primary method to develop mitochondrial DNA profiles. This method is laborious and time consuming. Massively parallel sequencing (MPS) can increase the amount of information obtained from mitochondrial DNA samples while improving turnaround time by decreasing the numbers of manipulations and more so by exploiting high throughput analyses to obtain interpretable results. In this study 18 buccal swabs, three different tissue samples from five individuals, and four bones samples from casework were sequenced at hypervariable regions I and II using STS and MPS. Sample enrichment for STS and MPS was PCR-based. Library preparation for MPS was performed using Nextera® XT DNA Sample Preparation Kit and sequencing was performed on the MiSeq™ (Illumina, Inc.). MPS yielded full concordance of base calls with STS results, and the newer methodology was able to resolve length heteroplasmy in homopolymeric regions. This study demonstrates short amplicon MPS of mitochondrial DNA is feasible, can provide information not possible with STS, and lays the groundwork for development of a whole genome sequencing strategy for degraded samples.

1. Introduction

Mitochondrial DNA (mtDNA) analysis has been applied in a number of fields [1–4]. Since mtDNA is found in higher copy number per cell than nuclear DNA, it has been an invaluable genetic marker in forensics with samples that are often limited in quantity and quality [5–7]. Sequencing a portion of the human mitochondrial genome (mtGenome), typically hypervariable regions I and II (HVI and HVII, respectively), using Sanger type sequencing (STS) and capillary electrophoresis has been validated [6,7] and prescribed under SWGDAM guidelines for forensic purposes [8]. While mtDNA sequencing can be very informative, it has not reached maximum use in forensic science due to the limitations of STS being time consuming, labor intensive, and relatively expensive. In addition, STS of mtDNA cannot readily sequence regions of amplicons downstream of length heteroplasmy homopolymers. The downstream sequence is uninterpretable due to multiple length molecules being sequenced simultaneously [9].

Massively parallel sequencing (MPS) is a high throughput technology that can rapidly generate high quality sequence from targeted areas of the human genome. The technology has reached a level of robustness such that it can be considered a viable approach to analyze challenging forensic samples. King et al. [10] have shown that a MPS system employing Nextera® XT DNA Sample Preparation Kit and the MiSeq™ platform (Illumina, Inc., San Diego, CA, USA) provides a practical protocol with long PCR and whole genome mtDNA sequencing of reference samples. To make the transition from a reference sample capacity to that of an evidence sample sequencing capacity will require that amplicons be relatively short in length. For reference samples, the initial PCR enrichment employed generated amplicons of approximately 8 kb in length. Obviously, such length amplicons are not routinely practical for the types of samples currently analyzed by mtDNA sequencing, e.g., hair shaft, bones, and teeth. Therefore, the study described herein attempted to analyze by MPS short amplicons

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of the well-defined regions HVI and HVII. Concentrating only on a short portion of the mtDNA genome does not exploit the full throughput of MPS since whole genome sequencing has been demonstrated. However, this study lays the foundation that (1) MPS can provide concordant results with STS for mtDNA analyses of the type of samples that may be encountered in a forensic laboratory; (2) demonstrates amplicons containing length heteroplasmy can be sequenced without additional laboratory effort; and (3) length heteroplasmy variants can be resolved. Based on these results, efforts are underway to develop multiplex assays containing short amplicons that span additional portions or the entire mtDNA genome.

2. Methods and materials

2.1. Samples and DNA extraction

Buccal swabs were collected from eighteen individuals. Additionally, blood was drawn by a trained phlebotomist into a lavender-top Vacutainer® tube (Becton, Dickson and Company; Franklin, NJ, USA) blood collection tube containing EDTA. Blood, buccal cells, and forcibly removed hair were collected from an additional five individuals. All samples were collected with informed consent. These samples were extracted using the Qiagen DNA Blood Mini kit (Qiagen Inc., Valencia, CA) following manufacturer's recommendations for each tissue type. Four femur bone samples, for which STS data were available, were selected from the University of North Texas Center for Human Identification (UNTCHI) missing person identification laboratory. These bone samples were selected because they displayed various issues with analysis by STS. The outer surface of each bone fragment was immersed in 50% bleach (3% NaOCl) for 15 min in a 50 mL conical tube. The bone fragments were washed 5 times with nuclease-free water, and then immersed briefly in 100% ethanol. Next, the fragments were crushed to powder using a 6750 Freezer/Mill (SPEX SamplePrep® L.L.C., Metuchen, NJ, USA), filled with liquid nitrogen, using a 10 min chill before a 5 min grind time at 15 impacts per second. Each aliquot was weighed and separated into 0.5 g aliquots. The bone powder was extracted using the Qiagen DNA Mini kit following manufacturer's recommendations for bones. The quantity of DNA was determined using the Quantifiler® Human DNA Quantification Kit on the ABI 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA) following manufacturer's recommendations. Three of the four bones were sequenced in duplicate by MPS and one bone sample (i.e., bone 7000) yielded DNA (a total yield of 1 ng) sufficient for only one analysis.

2.2. Sanger type sequencing

Initially, PCR amplification for STS was performed using primers R1 (forward primer 5′-CACCAGTCTTGTAAACCGGAGA-3′) and R2 (reverse primer 5′-CTTTGGGGTTTGGTTGGTTC-3′) to amplify positions 15,910-564 [11] at 0.3 μL each, 0.6 μL of AmpliTaq® Gold Polymerase (Life Technologies), 0.6 μL of dNTP mix (10 mM), 0.9 μL of $MgCl_2$ (25 mM), 1.5 μL of BSA, 1.5 μL of 10X PCR Buffer II (Life Technologies), 8.3 μL of nuclease-free water, and 1 μL of DNA (1 ng). The samples were amplified using the following parameters: 95 °C hold for 11 min, 36 cycles of 95 °C for 10 s, 60 °C for 45 s, and 72 °C for 1 min, a hold for 10 min at 15 °C, and a final hold at 4 °C. Amplified products were purified by adding 2 μL of ExoSAP-IT® (Affymetrix, Santa Clara, CA) to each well containing 15 μL of amplified product, and placed in a thermal cycler with cycling parameters of 37 °C for 15 min, 80 °C for 15 min, and a hold at 4 °C. The samples were cycle sequenced with primers described in Wilson et al. [7] using the BigDye® Terminator™ v3.1 cycle sequencing kit (Life Technologies) at 1 μL, 5 μL of Better-Buffer BigDye™ dilution buffer (Gel Company Inc., San Francisco, CA), 6 μL of nuclease-free water, 1.5 μL of the forward or reverse primer, and 1 μL of PCR product accounting for 8 wells per sample. The samples were placed in the thermal cycler and amplified with the following parameters: hold at 96 °C for 3 min, 25 cycles of 96 °C for 15 s, 50 °C for 10 s, and 60 °C for 3 min, and a final hold at 4 °C. Samples were purified using the BigDye® XTerminator™ kit. Here 27.5 μL of nuclease-free water, 22.5 μL of SAM™ solution, and 5 μL of BigDye® XTerminator™ were added to the amplified products and then placed in a plate vortex for 30 min at a speed setting of 7.5.

Samples were subjected immediately to electrophoresis on the Applied Biosystems 3130*xl* Genetic Analyzer using Performance Optimized Polymer (POP-4™ polymer) on a 36-cm 16 capillary array of which 8 capillaries were dedicated to sample. The sequences were analyzed using the DNA Sequencing Analysis software v5.2 (Life Technologies) and Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI).

2.3. MPS

PCR amplification for MPS was performed in four separate reactions per sample according to the Human mtDNA D-Loop Hypervariable Region Guide (Illumina http://support.illumina.com/downloads/human_mtdna_d_loop_hypervariable_region_guide_15034858.ilmn) to create four amplicons covering the following position ranges: 29–285, 172–408, 15,997–16,236, and 16,159–16,401. The quantity and quality of the PCR amplicons were determined using the Agilent High Sensitivity DNA chip on the Agilent® 2100 Bioanalyzer System (Agilent Technologies Inc., Santa Clara, CA). DNA amplicons were normalized to 0.2 ng/μL, and combined at a 1:1:1:1 ratio for a total of 20 μL (5 μL each) to allow for multiple preparations, if desired.

Libraries were prepared from the normalized PCR amplification products (1 ng total input) using the Nextera® XT DNA Sample Preparation kit according to the Human mtDNA D-Loop Hypervariable Region Guide. Size, quantity, and quality of the libraries were determined with the Agilent DNA 1000 kit on the Agilent 2100 Bioanalyzer system. During library preparation, unique indexes were added to the DNA fragments of each sample to allow for pooling and demultiplexing of the data at the analysis stage. The libraries were normalized to 2 nM and pooled for sequencing.

Three separate runs on the MiSeq™ were performed to obtain data from the 18 buccal swabs (a total of 18 barcoded samples), 15 tissue samples (a total of 15 barcoded samples), and four bone samples (a total of 7 barcoded samples). These mtDNA samples were combined with 25% PhiX control and sequenced at a concentration of 10 pM according to manufacturer's specifications with 2×151 cycles.

MPS data were analyzed using the mtDNA MiSeq Reporter (MSR) plug-in, and interpreted using the mtDNA Variant Analyzer software (Illumina, San Diego, CA). The software allows for adjustable thresholds; a default detection threshold of 0.10 (10% of reads per nucleotide position), and a default analysis threshold of 0.25 (25% of reads per nucleotide position) were used for this study. The sequence of each sample was compared individually to the revised Cambridge Reference Sequence (rCRS) [12], and sequences of samples from different tissues within a single individual were compared as well.

3. Results

Although MPS allows for high throughput beyond what was tested here, only a maximum of 18 samples were sequenced simultaneously so that exceedingly high coverage could be obtained. The

samples were grouped based on sample type and placed in three separate sequence analyses to generate high average coverage. The clustering and sequencing for each run were accomplished in 24 h with an average of 1149 K/mm^2 (±232) clusters and an average coverage of 230,003X (±174,091). Phred scores of 35 were used as a threshold of good quality data, and PhiX was used as a sequencing control.

The MPS generated mtDNA sequences from three different tissue types produced the same nucleotide calls within each of the five source individuals and also were concordant with base calls from STS sequence data (Table 1). However, MPS was able to sequence and provide interpretable data in a homopolymer C stretch in HVI of sample 400. This homopolymer stretch was unresolvable by STS due to length heteroplasmy. With STS all molecules essentially are sequenced together in one reaction. Length variants are superimposed over each other and downstream sequence is out of register and ambiguous (Fig. 1A and B). With MPS, each cluster is sequenced independently; therefore, all length variants (above threshold) can be determined. There were four length variants detected, three contained a stretch of 10, 11, or 12 Cs and one contained 4 As and 11Cs (the latter one was at low levels). The predominant length type was 3 As. Therefore, these results were considered concordant with those by STS. The high average coverage afforded by MPS (19,559X in this sample) ensured sampling of length variants sufficient to identify four different length homopolymers in this region (Table 2). At this time proportions of the length heteroplasmy cannot be provided. While the variants can be seen using Integrative Genomics Viewer (IGV [13]), the reads are substantially down sampled. Moreover, each time the data are repopulated in IGV, a different count is obtained. Software or scripts will be needed to quantify length variants. Although it would be more cost effective to barcode many different samples and sequence them simultaneously, there may be situations where a higher depth of coverage may be desired. In such cases, length variants may be resolved and facilitate tissue-to-tissue or sample-to-sample comparisons. More research is needed to determine the degree of detectable variation among heteroplasmic samples from single individuals.

Table 2
Length heteroplasmy sequences detected using MPS for sample 400.

rCRS	A	A	A	A	C	C	C	C	C	T	C	C	C	C	
type I	A	A	A	**C**	C	C	C	C	C	**C**	C	C	C		
type II	A	A	A	**C**	C	C	C	C	C	**C**	C	C	C	C	
type III	A	A	A	A	C	C	C	C	C	**C**	C	C	C	C	**C**
type IV	A	A	A	**C**	C	C	C	C	C	**C**	C	C	C	C	**C**

Table 1
Haplotypes for STS and MPS for tissue type samples.[a]

Sample	Tissue type	Method	Variants											
100		STS	73G	189R	199C	203A	204C	250C	263G	315.1C	16129A	16172C	16223T	16311C
	Blood	MPS	73G	189R	199C	203A	204C	250C	263G	315.1C	16129A	16172C	16223T	16311C
	Buccal	MPS	73G	189R	199C	203A	204C	250C	263G	315.1C	16129A	16172C	16223T	16311C
	Hair	MPS	73G	189R	199C	203A	204C	250C	263G	315.1C	16129A	16172C	16223T	16311C
200		STS	73G	150T	263G	315.1C	16192T	16311C						
	Blood	MPS	73G	150T	263G	315.1C	16192T	16311C						
	Buccal	MPS	73G	150T	263G	315.1C	16192T	16311C						
	Hair	MPS	73G	150T	263G	315.1C	16192T	16311C						
300		STS	73G	183G	263G	315.1C	16192T	16256T	16270T	16286T	16320T			
	Blood	MPS	73G	183G	263G	315.1C	16192T	16256T	16270T	16286T	16320T			
	Buccal	MPS	73G	183G	263G	315.1C	16192T	16256T	16270T	16286T	16320T			
	Hair	MPS	73G	183G	263G	315.1C	16192T	16256T	16270T	16286T	16320T			
400		STS	73G	152C	217C	263G	309.1C	315.1C	16051G	16092C	16129C	16183C	16189C	16362C
	Blood	MPS	73G	152C	217C	263G	309.1C	315.1C	16051G	16092C	16129C	16183C	16189C	16362C
	Buccal	MPS	73G	152C	217C	263G	309.1C	315.1C	16051G	16092C	16129C	16183C	16189C	16362C
	Hair	MPS	73G	152C	217C	263G	309.1C	315.1C	16051G	16092C	16129C	16183C	16189C	16362C
500		STS	73G	263G	315.1C	16129A	16223T	16294T	16362C					
	Blood	MPS	73G	263G	315.1C	16129A	16223T	16294T	16362C					
	Buccal	MPS	73G	263G	315.1C	16129A	16223T	16294T	16362C					
	Hair	MPS	73G	263G	315.1C	16129A	16223T	16294T	16362C					

[a] The haplotype data exclude the length heteroplasmy of sample 400.

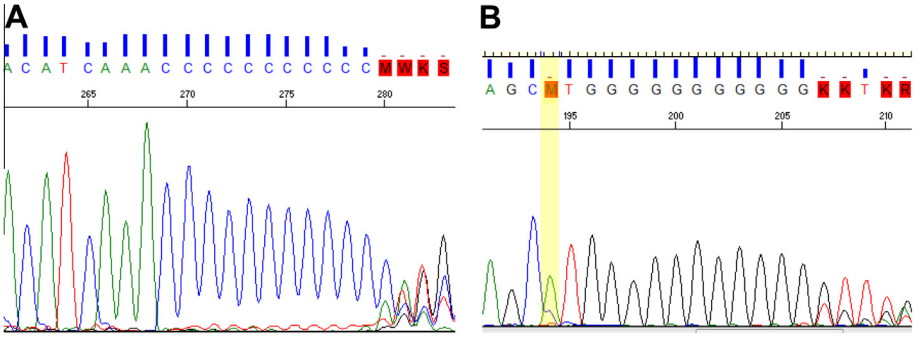

Fig. 1. STS electropherograms of homopolymer C stretch for sample 400. The left panel (A) shows the forward strand sequence. The right panel (B) shows the reverse strand sequence.

The eighteen buccal samples from various individuals yielded full target sequence for the amplified mtDNA regions (positions 16,000-400) with Sanger and MPS. Complete concordance was observed between the mtDNA types produced with STS and MPS (Table 3). Four instances of length heteroplasmy, five instances of point heteroplasmy, as well as a double deletion in one sample were observed among the 18 samples. MPS correctly identified all calls in the initial analysis with the software with no manual intervention. While the point heteroplasmy was observed with STS, the length heteroplasmy in the homopolymer C stretch was unresolvable with STS. For all four instances of length heteroplasmy, MPS was able to resolve the individual sequences.

Three of the four bone samples yielded concordant results between MPS and STS (Table 4). In addition, the replicates (i.e., DNA aliquots from the same extract) showed no considerable difference in coverage by MPS. One bone (sample 5612, Table 4) initially indicated a potential difference at position 16,093. STS showed a point heteroplasmy (i.e., Y) whereas MPS listed it only

Table 3
Haplotypes for STS and MPS for 18 buccal samples.

Sample	Sequencing method	Variants
1	STS	309.1C 315.1C 16218Y 16320T
	MPS	309.1C 315.1C 16218Y 16320T
2	STS	73G 263G 315.1C 16236T 16270T 16399G
	MPS	73G 263G 315.1C 16236T 16270T 16399G
3	STS	73G 150T 263G 315.1C 16192T 16311C
	MPS	73G 150T 263G 315.1C 16192T 16311C
4	STS	195C 214R 249- 263G 290- 291- 309.1C 315.1C 16092C 16176T 16218T 16223T 16298C 16325C 16327T
	MPS	195C 214R 249- 263G 290- 291- 309.1C 315.1C 16092C 16176T 16218T 16223T 16298C 16325C 16327T
5	STS	73G 195C 263G 315.1C 16126C 16294T 16296T 16304C
	MPS	73G 195C 263G 315.1C 16126C 16294T 16296T 16304C
6*	STS	73G 150T 263G 309.1C 309.2C 315.1C 16172C 16223T 16257A 16261T
	MPS	73G 150T 263G 309.1C 309.2C 315.1C 16172C 16223T 16257A 16261T
7*	STS	263G 309.1C 309.2C 315.1C 16129A 16183C 16189C 16355T 16356C 16362C
	MPS	263G 309.1C 309.2C 315.1C 16129A 16183C 16189C 16355T 16356C 16362C
8*	STS	257G 263G 309.1C 315.1C 16183C 16189C 16362C
	MPS	257G 263G 309.1C 315.1C 16183C 16189C 16362C
9*	STS	263G 309.1C 309.2C 315.1C 16242T 16304C
	MPS	263G 309.1C 309.2C 315.1C 16242T 16304C
10	STS	263G 309.1C 315.1C 16311C
	MPS	263G 309.1C 315.1C 16311C
11	STS	73G 263G 309.1C 315.1C 16256T 16270T 16399G
	MPS	73G 263G 309.1C 315.1C 16256T 16270T 16399G
12	STS	73G 263G 309.1C 315.1C 16126C 16294T 16296T
	MPS	73G 263G 309.1C 315.1C 16126C 16294T 16296T 16304C
13	STS	263G 309.1C 315.1C 16295T 16304C
	MPS	263G 309.1C 315.1C 16295T 16304C
14	STS	73G 146C 150T 263G 309.1C 315.1C 16126C 16292T 16294T 16296T
	MPS	73G 146C 150T 263G 309.1C 315.1C 16126C 16292T 16294T 16296T
15	STS	73G 152C 194T 263G 315.1C 16093Y 16,223T 16,362C 16,390A
	MPS	73G 152C 194T 263G 315.1C 16093Y 16223T 16362C 16390A
16	STS	73G 146C 152C 263G 315.1C 374R 16256T 16270T 16278T 16318M 16399G
	MPS	73G 146C 152C 263G 315.1C 374R 16256T 16270T 16278T 16318M 16399G
17	STS	73G 152C 199C 204C 207A 250C 263G 309.1C 315.1C 16129A 16223T 16391A
	MPS	73G 152C 199C 204C 207A 250C 263G 309.1C 315.1C 16129A 16223T 16391A
18	STS	73G 146C 154C 200G 215G 263G 310C 318C 326G 16223T
	MPS	73G 146C 154C 200G 215G 263G 310C 318C 326G 16223T

* Haplotype data excludes the length heteroplasmy of this sample.

Table 4
Haplotypes for STS and MPS for bone samples.

Sample	Sequencing method	Variants
5612	STS	73G 263G 315.1C **16093Y*** 16223T 16239T 16260T 16274A 16325C 16362C
	MPS	73G 263G 315.1C **16093C** 16223T 16239T 16260T 16274A 16325C 16362C
6997	STS	263G 315.1C 16192T 16260T
	MPS	263G 315.1C 16192T 16260T
H2	STS	64T 73G 146C 153G 155C 203A 222T 235G 263G 315.1C 16111T 16184T 16223T 16290T 16319A 16362C
	MPS	64T 73G 146C 153G 155C 203A 222T 235G 263G 315.1C 16111T 16184T 16223T 16290T 16319A 16362C
7000	STS	55C 56G 64T 73G 263G 279C 309.1C 315.1C 16126C 16223T 16325C 16362C
	MPS	55C 56G 64T 73G 263G 279C 309.1C 315.1C 16126C 16223T 16325C 16362C

* All samples had threshold set at 25%, position 16093 (bolded) for sample 5612 detected heteroplasmy at 19.1% showing all sites concordant.

Fig. 2. Point heteroplasmy determination. The top panel (A) is a STS electropherogram. The bottom panel (B) is MPS output from the MRS plug-in. Both sequencing methods detected the point heteroplasmy at position 16,093. The gray shading in panel B is an indicator for potential heteroplasmy based on the threshold that is set.

as a C (Fig. 2A). Clearly, the lower contributing T is less than 25% of the signal at position 16,093. Therefore, this "apparent" difference is due to the MPS threshold setting and not the ability to detect heteroplasmy at this position. The analysis threshold initially was set at 25%, and the MPS reads at 16,093 were 80.9% C and 19.1% T (Fig. 2B). These values were indicative of point heteroplasmy and thus all bone sample sequences were concordant between MPS and STS. It should be noted that the heteroplasmy contribution in STS data is not quantifiable, and thus no thresholds were set. In contrast, MPS data are quantifiable. At this time, there is no recommended heteroplasmy detection threshold; a full validation study would be required to set the value. In the interim, the same general heteroplasmy interpretation guidelines for STS were applied with MPS data [8].

Bone sample number 7000 was difficult to sequence using STS for the HVII region due to the presence of length heteroplasmy in the homopolymer stretch and different primers (C1-5′-CTCACGGGAGCTCTCCATGC-3′ and D1-5′-CTGTTAAAAGTGCATACCGCCA-3′ covering positions 29-429) were used to generate just the HVII amplicon. Sequencing by STS then was successful (data not shown). Only 0.25 ng of DNA was available for each PCR that would be sequenced with MPS. The sample yielded full results with the first round of testing with coverage ranging from 274 to 650,866 X and an average of 258,601 X (±211,477). The base call sequence results were concordant with those generated by STS, and the length heteroplasmy was correctly called on the first analysis. These results suggested that challenged samples can be analyzed with MPS. Given the depth of coverage, the data indicate that smaller quantities of template DNA may yield typeable results. Future studies will define the minimal amount of template DNA that can yield interpretable sequence data.

4. Discussion

MPS provided concordant mtDNA sequence results from short amplicons. In addition, this technology increased the amount of information obtained from mtDNA samples, particularly regarding resolving and quantifying length and point heteroplasmy. In this study, 18 buccal swabs, three tissue types from five individuals, and four bones from casework type samples were sequenced with STS and MPS using the Nextera® XT kit for library preparation and the MiSeq™ platform. These data support that MPS can be an attractive alternative for mtDNA sequencing, especially regarding resolution of heteroplasmic length variants, and has the potential of being an efficient and sensitive method for forensic analyses. Future work will be developing short amplicon multiplexes that span portions or the entire mtDNA genome so that greater discrimination power can be attained from degraded and low quantity samples.

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