INTERPRETATION GUIDELINES FOR MITOCHONDRIAL DNA SEQUENCING

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I. INTRODUCTION

Sequence analysis of human mitochondrial DNA (mtDNA) extracted from forensic biological specimens is becoming a routine practice, particularly when there is insufficient nuclear DNA in samples for typing (1-18). Hair shafts, bones, teeth and other samples that are severely decomposed may be characterized with mtDNA.

Mitochondria are subcellular organelles that contain an extrachromosomal genome that is separate and distinct from the nuclear genome. Human mitochondrial DNA (mtDNA) differs from nuclear DNA (Table 1) .

Table 1. Comparison of characteristics of mtDNA and nuclear DNA

mtDNA	Nuclear DNA
closed, circular molecule	linear molecule
16, 569 base pairs in size	~ 3 billion base pairs in length
~1100 base pair non coding region	a large portion of genome non-coding
maternally inherited	biparental inheritance
no recombination	recombination
50 to several thousand copies per cell	two copies per cell

Because of the different characteristics of mtDNA compared with nuclear DNA, the application of mtDNA typing and interpretation of results should not in all ways be the same as that routinely practiced for nuclear DNA typing. A higher success rate with typing mtDNA, as opposed to nuclear DNA, from old bones, severely decomposed or charred remains, or single hair shafts is expected and experienced and is due to the high copy number of mtDNA (19). Loci on the nuclear genome typically occur as only two copies per cell, mtDNA, on the other hand, is present in hundreds or thousands of copies per cell.

Excluding mutations, a mtDNA sequence is identical for all maternally related relatives (while nuclear DNA is derived from both parents) (20-22). The feature of maternal inheritance can be useful in establishing or refuting identity of putative samples by using known maternal relatives as reference to compare with the questioned mtDNA type (4,6,9,16). In general, the transmission of a mtDNA type is consistent across generations. In fact, unlike nuclear loci residing on autosomal chromosomes, relationships several generations removed may be evaluated by mtDNA typing.

The low fidelity of the mtDNA polymerase and the apparent lack of mtDNA repair mechanisms lead to a higher rate of mutation in the mitochondrial genome compared with the nuclear genome. Some regions of the mtDNA genome appear to be evolving at 5 to 10 times the rate of single copy nuclear genes (23,24). The highest degree

of variation in the mtDNA among individuals is found within the non-coding control region in hypervariable regions I and II (HVI and HVII, respectively). In fact, for the HVI and HVII regions, on average, African Americans differ from one another at approximately 14 sites; Caucasians, although less polymorphic, differ on average at 8 nucleotide positions (25).

There does not appear to be any recombination at the mtDNA. Thus, a mtDNA sequence is treated as a single locus or haplotype. The most informative single locus used for identity testing is the regions HVI and HVII. The nuclear loci used for identity testing tend to be independent from one another (26-31), because they reside on different chromosomes, or if they reside on the same chromosome, recombination destabilizes the physical linkage.

Since mtDNA is maternally inherited, the mtDNA essentially is monoclonal in an individual - only one type of mtDNA sequence should be observed per individual. However, a condition, known as heteroplasmy can occur (32-34). Heteroplasmy is defined as more than one mtDNA type being carried by an individual. A type is defined as a mtDNA sequence or haplotype. The two types in an individual usually differ at only one base. However, heteroplasmy at two or more sites is expected to occur at much lower levels. Heteroplasmy may be observed in several ways: 1) individuals may have more than one mtDNA type in a single tissue; 2) individuals may exhibit one mtDNA type in one tissue and a different type in another tissue; and/or 3) individuals may be heteroplasmic in one tissue sample and homoplasmic in another tissue sample. All three heteroplasmic scenarios were observed at site 16355 in hair samples from maternally related family members (34). Studies by Wilson, *et al* (34) and Bendall and Sykes (35) demonstrate that the degree of heteroplasmic point mutations in an individual's different hairs may vary from homoplasmy to various degrees of heteroplasmy.

Length heteroplasmy also is observed and typically manifests as variation in the number of bases residing within a homopolymeric stretch (i.e., C stretches). The most commonly occurring form of heteroplasmy (more so that point heteroplasmy) is variation in the number of C's in a homopolymeric stretch. A number of studies have documented length heteroplasmy within an individual (36-39). The mechanism suggested for generating length heteroplasmy is replication slippage (36).

II. INTERPRETATION

Although there are differences in mtDNA compared with nuclear DNA, forensic identity testing is fairly straight forward. Typically sequence concordance is assessed between reference and evidence mtDNA sequences. When heteroplasmy arises, careful analysis and direct comparisons between multiple reference samples and a questioned sample should, in most cases, alleviate interpretational differences. If the mtDNA sequences from two samples being compared demonstrate the heteroplasmy (i.e., both sequences are observed in each sample), the interpretation is cannot exclude (or concordance). If they share a common sequence (i.e., one sample is heteroplasmic and the other homoplasmic, and one of the heteroplasmic types is concordant with the homoplasmic type), then the interpretation is a failure to exclude. If both samples are deemed homoplasmic and differ slightly (i.e., typically at only one site), further investigation is warranted; if no resolution can be attained, the interpretation is inconclusive. However, when rendering an interpretation (for concordance, exclusion, or inconclusive and for assessing weight of the evidence) in any forensic comparison, one should be careful not to exceed current limitations in knowledge, mtDNA or otherwise.

When a mtDNA profile from an evidence sample and one from a known reference sample cannot be excluded as originating from the same source, it is desirable to convey some information about the rarity of the mtDNA profile. If the suspect's mtDNA type and the evidence mtDNA type are considered concordant (i.e., one cannot exclude the samples as originating from the same source), the current practice is to count the number of times a particular sequence is observed in a database(s). A confidence interval can be placed on the observation. Thus, based on the size of the database(s) and observed sampling, a range is placed on the frequency of a mtDNA haplotype (however, only an upper bound estimate usually is provided).

III. INTERPRETATIONAL GUIDELINES

Given the above considerations, interpretational guidelines to evaluate sequencing results from evidence and reference samples are necessary. The FBI Laboratory has developed and implemented such guidelines to assist the examiner. These guidelines are based on the limitations of the current state-of-the-art and knowledge regarding mtDNA genetics and technology. One should exercise caution of exceeding such bounds. For example, there is evidence for mutational hotspots residing within the mtDNA genome (e.g., site 16093) (40). Thus, there may be a desire to suggest that when there is a base difference, for example, at site 16093 between the reference and evidence sample, that the evidence is stronger support for an interpretation of a match (or concordance) than that of inconclusive or exclusion. Because the sequence of the mtDNA may affect conformational configuration of the molecule, hotspots may be mitotype specific (conformation may affect the mutation rate); thus, in specific mitotypes the site may not be a hotspot. In most cases, it actually may be correct to assume that 16093 is a hotspot; however, until the data are available, a more conservative approach should be taken (see below).

The interpretational guidelines will not apply to every possible situation encountered, but most scenarios will be addressed. Some cases may present situations which are not explicitly covered in the guidelines. However, such cases generally can be interpreted within the "spirit" of the guidelines.

1. EVALUATING AND COMPARING SEQUENCES

After proper evaluation of positive and negative controls and upon completion of gel electrophoresis and data acquisition, assess the quality of each electropherogram and the peaks. The examiner decides whether or not the electropherograms are useful for sequence analysis purposes. Those electropherograms which are not of requisite quality for comparison will not be further interpreted; if possible, the sample may be re-extracted, re-amplified, and/or re-sequenced. All sequence data determined to be of requisite quality are used for comparison purposes. However, the comparison of sequence data to the mtDNA database is limited currently to bases 16024 -16365 (HVI) and 73-340 (HVII).

The electropherograms are imported into a comparison program (currently Sequence Navigator™, PE/ABD). The heavy strand sequences are "reverse complemented" so that the bases are aligned in the light strand orientation; thus, the examiner is able to directly compare sequences (Note: generally both strands of the mtDNA amplicon are sequenced). Comparison software allows for comparison of data from separate sequencing runs on the same computer screen. Generally, designation of a nucleotide at each position of the template is made. Upon completion of sequence analysis, the examiner decides upon one of the following courses of action:

a. That all ambiguities (designated as " N") are resolved, and the sequence is to be compared to other relevant sequences in the case(s) and/or the database.

b. That ambiguous bases remain, and sufficient template exists which would allow additional sequencing runs to be performed in order to resolve these ambiguities. The examiner will decide either to re-sequence the template, or let the ambiguous base designations remain. Should the examiner decide to re-sequence the template, he or she should indicate which additional sequencing reactions should be performed for subsequent sequence analyses of the template.

c. That all relevant sequencing reactions have been attempted or re-attempted, and ambiguous bases remain. The examiner will then choose to designate the ambiguous bases as " N" s, and compare the sequence to other relevant sequence(s) and/or database(s). Where ambiguities remain, all four possibilities (i.e., A, G, C, T at that site only) should be included in the comparative search with other

sequences in the database. For example, consider the following ten nucleotide sequence where the sixth base is ambiguous:

AATGC**N**TTCC

When this sequence is searched in a reference database or compared with other relevant sequences, it should be found to be in concordance with all four of the following sequences:

AATGC**A**TTCC
AATGC**C**TTCC
AATGC**G**TTCC
AATGC**T**TTCC

Sequence concordance is therefore defined as including exact sequence matches, as well as comparisons of sequences where ambiguities are present, (provided that all unambiguous bases are the same), and a common nucleotide is present at the position(s) of ambiguity.

In those instances in which a mtDNA sequence is found to be in concordance with a mtDNA sequence from another item of evidence and the results of the sequence concordance are included in the report, each template sequence will be independently analyzed by two examiners. A sequence analysis is considered completed when both examiners have arrived at the same conclusion. If one examiner concludes that the sequence analysis is complete and all ambiguities are resolved, but the other concludes that additional sequencing runs are needed, the template will not be considered complete until the additional sequencing reactions are completed, the results analyzed, and the results from both examiners concur. If the sequences do not concur, the examiners should decide on an appropriate course of action. This course of action typically will result in situation b or c above. The examiners can request additional sequencing reactions be performed, or leave as ambiguous those bases in which they do not agree. If ambiguous bases are left in the final sequence, they will be identified as "**N**'s" and compared to other sequences in the manner discussed above.

2. COMPARISON (SEARCH) TO DATABASE AND STATISTICS

In cases where a determination is to be made as to whether the questioned sample and known sample could have originated from the same individual or maternal lineage, failure to exclude will be reported when concordance is found. Should ambiguous bases be present in either sample, a common nucleotide or length variant must be present at the position(s) of ambiguity. A search of the mtDNA database will specify both the unambiguous bases, as well as those which are not specified. Bases which are specified are recorded as A,C,G, or T. Those which are not specified (ambiguous) are designated as " N" . Alternatively, the IUPAC codes may be used (Table 2) and may be more descriptive.

Table 2. IUPAC codes for base calling when a site has more than one nucleotide (e.g., heteroplasmy).

R	A or G
Y	C or T
K	G or T
M	A or C
B	C, G, or T
D	A, G, or T

H	A, C, or T
V	A, C, or G
S	G or C
W	A or T

Those bases which are not specified will not be used for exclusionary purposes.

The statistical weight of a sequence match is presented in the following way: the sequence is searched against a population database (currently consisting of mtDNA sequences from populations of Caucasian, African, Asian, and Hispanic descent). The statement to be reported will state the number of times a particular sequence is present in the database and will include the number of entries in the database from each of the populations listed above. In some instances, a statistical calculation with a 95% confidence interval may be performed. These values should be included in the examiner's notes.

3. HETEROPLASMY

Because it is possible that heteroplasmic mtDNA sequences could be present within an individual, care should be exercised in instances where the questioned and known sequences differ slightly. In such cases, the examiner should carefully inspect the sequences obtained from the questioned and known sample(s). If possible, additional known (i.e., reference) samples, including but not limited to blood, buccal swabs, and hair, should be obtained and processed. Hair fragments from a known hair standard may be combined and processed as a single "pooled" known sample. Up to five (5) additional known samples may be used in such cases. In the instances of heteroplasmy, the chromatograms may show the presence of more than a single nucleotide at the position(s) of difference between the questioned and known samples. If the questioned and known samples differ by a single nucleotide, and no evidence of heteroplasmy is present, the comparison should be reported as **inconclusive**. In cases where more than a single nucleotide difference exists between the questioned and known sequences, careful analysis of the chromatograms at the position(s) which differ will determine the proper interpretation of the comparison.

In cases where more than one extraction is performed on a sample, failure to exclude will only be reported when complete concordance (again disregarding ambiguities) of the sequences from the multiple extracts is found. However, this requirement will not preclude the use of sequencing results from a single extract should other analyses of the same sample fail to analyze successfully. In other words, if one of two extracts from a sample is analyzed and the other is not (due to, for example, amplification failure), the results of the successful sample will be reported according to the guidelines.

Both HV I and HV II of the human mtDNA control region contain segments of sequence which can be homopolymeric. Length heteroplasmy is often observed in these regions. In HV I, the region in which this is most often noted begins at nucleotide number 16184. When a transition at position 16189 (T-C) is observed, length heteroplasmy often results. In such cases, it is difficult to unambiguously determine the exact number of cytosine residues present. For this reason, no attempt will be made to count the number of residues (for interpretation purposes), and all comparisons will assume that the same number is present.

A similar region of length heteroplasmy exists in HVII, encompassing positions 303-315. Many individuals exhibit length heteroplasmy in this region. In order to assess the presence of length heteroplasmy, careful consideration should be given to positions 309 and 310 in both light and heavy strand chromatograms. Likewise, should a transition occur in HV II at position 310, no attempt will be made to count the number of cytosine

residues in the homopolymeric region for comparison purposes. Length heteroplasmy is exhibited by "out-of-phase sequence carryover" downstream of this region. Therefore, positions after 309 in the light strand and before 309 in the heavy strand should be compared to positions before 309 in the light strand and after 309 in the heavy strand for such evidence. Again, in most cases, length heteroplasmy is not used for interpretations. However, if one determines it to be of value, a common length variant should be present in both the questioned sample and the known sample in order for a conclusion of sequence concordance to be reached. Also, the amount of length variability in the known sample should be considered in such situations.

CONCLUSION

In conclusion, MtDNA sequencing provides another useful tool for characterizing biological evidence. The guidelines described in this paper should be useful for those implementing mtDNA sequencing. With high quality practice and careful consideration when evaluating typing data (i.e., using the guidelines), confidence in the results and interpretational conclusions can be attained. As more genetic information is generated and improvements in technology occur, the guidelines should be amended to reflect such changes.

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APPENDIX

The following table lists the conclusions in such instances:

Table 3. Interpretations for homoplasmic and heteroplasmic situations.

SITUATION	**INTERPRETATION**
Identical sequences	Cannot exclude (NE)
One heteroplasmic base in both samples **at the same position.**	Cannot exclude (NE)
One heteroplasmic base in one sample, not observed in the other sample, with a common nucleotide present in both samples.	Cannot exclude (NE)
One base difference between two samples with no evidence of heteroplasmy	Inconclusive (I)
Two heteroplasmic bases at the same positions in both samples	Cannot exclude (NE)
One heteroplasmic base at the same position, one base showing heteroplasmy in one sample but not in the other, with a common nucleotide in each	Cannot exclude (NE)
One heteroplasmic base at the same position, one different base at another position with no evidence of heteroplasmy	Inconclusive (I)
Two or more base differences with no evidence of heteroplasmy	Exclusion (E)