SCIENTIFIC WORKING GROUP ON DNA ANALYSIS METHODS[1]

Supplemental Information for the SWGDAM Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories

Short Title: *MtDNA FAQs Supplement*

Approved and Effective: September 9, 2024

Scope

The Supplemental Information for the SWGDAM Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories provides supplemental information for the SWGDAM Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Laboratories (2024) in the form of frequently asked questions (FAQs). Where applicable, FAQ responses include cross-references to the specific guideline in the parent document and references to published documents, but may also use illustrative examples based on laboratory experiences. Within this document, the terms "profile" and "haplotype" are used interchangeably. The term "sequence" refers to sequence data.

[1] The Scientific Working Group on DNA Analysis (SWGDAM; see SWGDAM.org) is comprised of forensic science practitioners and other experts who represent government laboratories within the U.S and Canada, as well as intra- and international professional groups and academia. SWGDAM recommends to the FBI Director revisions to the *Quality Assurance Standards for Forensic DNA Testing Laboratories* and the *Quality Assurance Standards for DNA Databasing Laboratories (QAS)*. SWGDAM provides a forum for its members and invited guests to discuss research, technologies, techniques, and training; and conduct or recommend studies to develop, test, and validate methods for use by forensic laboratories. SWGDAM's Guidelines and Recommendations represent best practices within the discipline. The term "should" is used herein to indicate good practices identified by SWGDAM. "Shall" distinguishes mandatory elements, which may be specified in the Quality Assurance Standards for Forensic DNA Testing Laboratories and/or Quality Assurance Standards for DNA Databasing Laboratories.

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FAQ-1: What types of cases and/or samples can benefit from mtDNA testing?

Cross-reference Introduction

Mitochondrial DNA (mtDNA) testing is useful for single source biological samples with limited or severely degraded nuclear DNA. Samples such as bone, teeth, and hair may contain an insufficient amount of nuclear DNA, making them unsuitable for STR typing.

Generally, all cells, excluding red blood cells, contain two copies of nuclear DNA, one inherited from each parent. In contrast, there are hundreds or even thousands of copies of mtDNA in each cell inherited only from the mother. Due to the lack of recombination in mtDNA, the discriminatory power of a mtDNA profile is more limited compared to a STR profile.

Unidentified human remains, especially those encountered in missing persons cases, often contain degraded DNA. This presents a challenge during STR analysis, often resulting in limited STR results or STR results that are unsuitable for comparison to family reference samples. The stability and large number of mtDNA copies per cell allow for greater sensitivity than nuclear DNA analysis. Therefore, the ability to obtain a mtDNA profile is greatly increased in severely compromised samples compared to STR typing. Performing mtDNA testing in addition to STR testing on unidentified human remains increases the ability to make comparisons to associated family reference samples. For instance, a mtDNA profile developed from remains may be compared to not only first or second-degree maternal relatives but may also be compared to distant maternal relatives.

Hairs encountered in forensic casework often do not contain, or contain very minimal, root material and may not always be suitable for STR typing. Hair shafts and hairs with limited or no root material are prime candidates for mtDNA testing.

Samples with ample nuclear DNA but without direct references may also be candidates for mtDNA testing if maternal relatives are available.

FAQ-2: What type of controls are used for mtDNA testing?

Cross-reference Guideline 2.1

The use of controls is one of the most important quality measures for mtDNA testing. Controls shall include, at a minimum, a positive amplification control, a positive sequencing control (this may be the same as the positive amplification control), a negative amplification control, and a reagent blank control.

Reagent blanks and negative amplification controls are used to monitor levels of contamination and also assist in identifying at which step of the process contamination may have been introduced. Reagent blanks monitor contamination from extraction to final sequence analysis. Negative amplification controls monitor contamination from amplification to final sequence analysis. Additional negative controls may be included during sample preparation to assess contamination as needed.

Reagent blanks and negative amplification controls that contain DNA are assessed to ensure that any results for the corresponding sample(s) derive from the samples and not from contamination. If contamination in the reagent blank and/or negative amplification control does not meet the laboratory's established acceptance parameters, then the data from the associated samples cannot be used for interpretative purposes.

A single positive control of known mtDNA sequence (some examples are provided in Levin et al. 2003, Riman et al. 2017, and Cihlar et al. 2020b) may be processed starting at amplification and used throughout the process to monitor each step. Alternatively, different positive controls, such as amplification or internal sequencing controls, may be used to monitor the success of different steps of the process.

FAQ-3: How is contamination assessed?

Cross-reference Guideline 2.2

Mitochondrial DNA analysis is more susceptible to contamination compared to nuclear DNA analysis, largely due to the higher copy number per cell. A mtDNA haplotype is generally less discriminating than an autosomal STR DNA profile, and therefore identifying the source of contamination can be more difficult.

The laboratory's contamination assessment procedures should involve assessing the extent of the contamination and determining the reliability of the results from any affected sample(s). This may include the use of contamination thresholds (e.g., Wilson et al. 1995b) that are supported by the laboratory's internal validation. A local database of laboratory personnel and other relevant mtDNA haplotypes may be used to determine the source of the contamination. The laboratory should have guidelines to determine when an associated sample should be re-processed, if possible, from the most appropriate step.

FAQ-4: How is sequencing accomplished?

Cross-reference Guideline 3

Sequencing of the mtDNA genome has traditionally been accomplished through Sanger sequencing. However, many laboratories are now employing Next Generation Sequencing (NGS).

Whichever methodology is used for mtDNA sequencing, the regions of the mtDNA genome that are typically targeted for evidentiary testing are hypervariable region 1 (HVI; positions 16024-16365) and hypervariable region 2 (HVII; positions 73-340) located within the control region. Because the discrimination power of mtDNA data is dependent on the reported sequence range, it is beneficial to sequence more than HVI and HVII. With the move to NGS, sequence ranges encompassing the entire mtDNA genome are possible.

Sanger sequencing:

Sanger sequencing typically first involves PCR amplification of the mtDNA control region, either in a single amplicon or smaller, overlapping amplicons, depending on the degradation state of the template.

The sequencing step involves the addition of a single primer to a PCR-like reaction containing amplified product, deoxynucleotide triphosphates (dNTPs), buffer, polymerase, and fluorescently-labelled dideoxynucleotide triphosphates (ddNTPs). The ddNTP terminates the growing chain, creating a population of extension products that differ in length by a single nucleotide. The extension products are then separated by size using capillary electrophoresis, with the incorporated terminal labelled ddNTP in each product detected by a laser/camera sensor. Depending on the amplification strategy, multiple sequencing reactions, each with a different primer, are used to generate the composite sequence of the mtDNA for a sample. At a minimum, sequencing primers for the forward and reverse strands should be employed to reduce ambiguities in base determination.

For samples containing common homopolymeric regions such as those that may occur in the HVI Cytosine-stretch (C-stretch) region (between positions 16183-16194) and the HVII C-stretch region (between positions 302-310), the use of additional Sanger sequencing primers is recommended as these motifs can prove to be challenging.

Sequence differences in overlapping regions, as well as discrepancies in expected amplicon quantities, could indicate primer binding issues and should be interpreted with caution.

Commercial kits with primers for mtDNA amplification for Sanger sequencing are not commonly available. Suggested primers and processing strategies for Sanger sequencing are described in the literature (e.g., Wilson et al. 1995a, Wilson et al. 1995b, Gabriel et al. 2001, Edson et al. 2004, Eichmann et al. 2008, and Berger and Parson. 2009).

Next Generation Sequencing:

Next Generation Sequencing (NGS) is the simultaneous sequencing of millions of DNA molecules that are localized onto solid substrates such as particles or flow cells. Another name for NGS is Massively Parallel Sequencing (MPS). The two most prevalent NGS methods are reversible dye terminator (Illumina) and pH-mediated sequencing (Ion Torrent), both of which use sequencing by synthesis (SBS). Validated mitochondrial sequencing kits are available for both methods (Holt et al. 2021, Cihlar et al. 2020a, Brandhagen et al. 2020). Although they utilize different approaches, all NGS methods consist of four steps: library preparation, clonal amplification, sequencing, and data analysis.

Library preparation is a molecular process that incorporates adaptor sequences to the ends of the target DNA. Each adaptor contains sequencing primers and, in most cases, molecular barcodes to uniquely tag each sample. Most mitochondrial DNA library preparation methods also include a target enrichment step (i.e., PCR or probe capture). While most manufacturers provide kits that allow a user to move from raw DNA extract to sequencing, it is possible to mix and match kits throughout the process, for example, by using a different manufacturer's library preparation kit.

A single sample library or a multiplex library is then clonally amplified. The goal of clonal amplification is to separate each DNA molecule and replicate it individually. This important aspect of NGS allows for the increased detection sensitivity that is used in studies like copy number variation (CNV). Illumina technologies utilize "bridge amplification." In this process, DNA molecules hybridize to the surface of a flow cell (solid phase hybridization) termed a "lawn." An isothermic amplification is performed in which the oligonucleotides attached to the flow cell are used as primers. This causes the molecules to bend (forming bridges) as they replicate. The physical grouping of each molecule and its copies is deemed a cluster, which is then sequenced as one unit ("a read"). Another commonly utilized clonal amplification method is emulsion PCR (ePCR). In this process, DNA molecules are encapsulated into oil bubbles that act as PCR microreactors. The ideal microreactor would contain a singular DNA molecule. However, many reactors will have multiple DNA molecules (polyclonal) which cannot be interpreted and are filtered out during analysis.

Bridge amplification and SBS take place in the same flow cell to simplify the two-step process, leading most to think of this as a single sequencing step. Illumina sequencing utilizes reversible terminator technology, allowing all four dNTPs (reversible terminator-bound) to compete during incorporation. During each cycle, a base is incorporated to the growing strand which is then imaged for direct detection. The terminator and fluorophore are then chemically cleaved opening the strand for subsequent addition. This process occurs simultaneously for millions (or billions) of clusters. The sequencing read length is dependent on the number of cycles performed during SBS.

Ion Torrent sequencing utilizes a complementary metal-oxide semiconductor (CMOS) chip with a predefined number of wells. Each well can fit a singular (clonally amplified) molecule that is individually sequenced and detected. Ion Torrent sequencing flows each dNTP separately. If the growing strand incorporates that base, it will naturally release a hydrogen ion that is detected by the chip sensors. This technology is analogous to a micro pH reader. The fact that the nucleotides are not terminated means that homopolymer regions are all incorporated in the same flow and that specificity may be impacted with longer stretches.

The raw data generated during NGS requires bioinformatics data analysis methods to perform the base calling, quality trimming, filtering, and demultiplexing. These preliminary data analysis steps are performed on board the NGS instrument and result in FASTQ or BAM files. Subsequent mitochondrial DNA alignment and variant calling is performed with a secondary software package.

FAQ-5: What are the base call designations used in mtDNA analysis?

Cross-reference Guideline 3.4

In accordance with the International Union of Pure and Applied Chemistry (IUPAC), the following IUPAC codes are used in mtDNA analysis:

Adenine = A	Guanine = G
Cytosine = C	Thymine = T
Any base (A/C/T/G) = N	Deletion = '-' or 'del' or 'DEL'

Insertions are described by noting the site immediately prior to the insertion with respect to the light strand of the revised Cambridge Reference Sequence (rCRS) followed by a point and a '1' for the first inserted base, with sequential numbering for each inserted base thereafter (e.g., 315.1C). Insertions should not alter subsequent numbering of the sequence.

Deletions are described by noting the deleted site followed by either a dash '-' or 'del' or 'DEL,' which may depend on the preference of the laboratory or the requirements of the target database (e.g., 249-, 249del, or 249DEL).

FAQ-6: What is the appropriate nomenclature to describe a mixed base in a sample's sequence?

Cross-reference Guidelines 3.4 and 3.5.

At confirmed positions of more than one base, the following upper-case extended IUPAC codes should be used:

G/T = K	A/C = M
A/G = R	A/G/T = D
G/C = S	A/C/T = H
A/T = W	A/C/G = V
C/T = Y	C/T/G = B

A/C/G/T = N

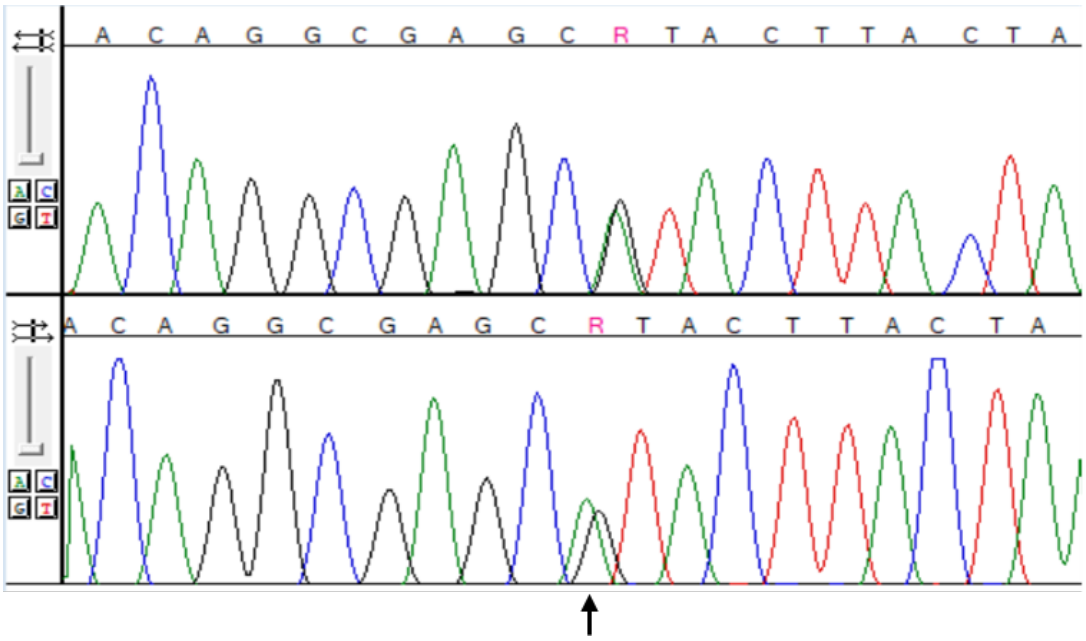
Per extended IUPAC codes, lower case letters should be used to indicate mixtures between deleted and non-deleted bases (Parson et al., 2014). For example, 249a indicates a mixture of adenine (A) and a deletion at position 249.

FAQ-7: What is heteroplasmy and how does it impact forensic analysis?

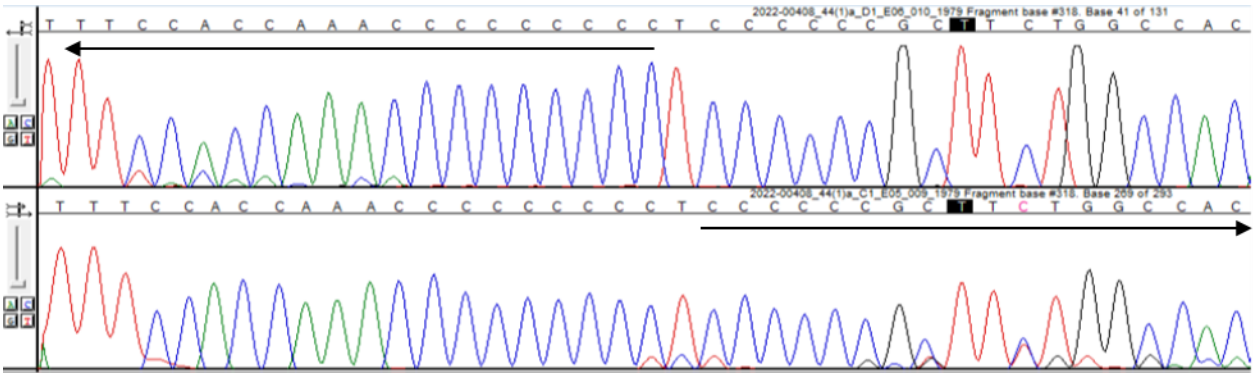
Cross-reference Guidelines 3.5 and 3.6.1

Heteroplasmy is defined as more than one mtDNA sequence present in an individual. Detectable heteroplasmy can be observed as point heteroplasmy where two DNA bases are observed at the same nucleotide position. Heteroplasmy can also be seen as length heteroplasmy caused by a variation in the number of bases in a homopolymeric stretch of bases (i.e., C-stretch). In Sanger sequencing, this is typically observed as out-of-phase downstream sequences. In order to properly report heteroplasmy, it should be observed in sequencing reactions from forward and reverse strands and above the level of background.

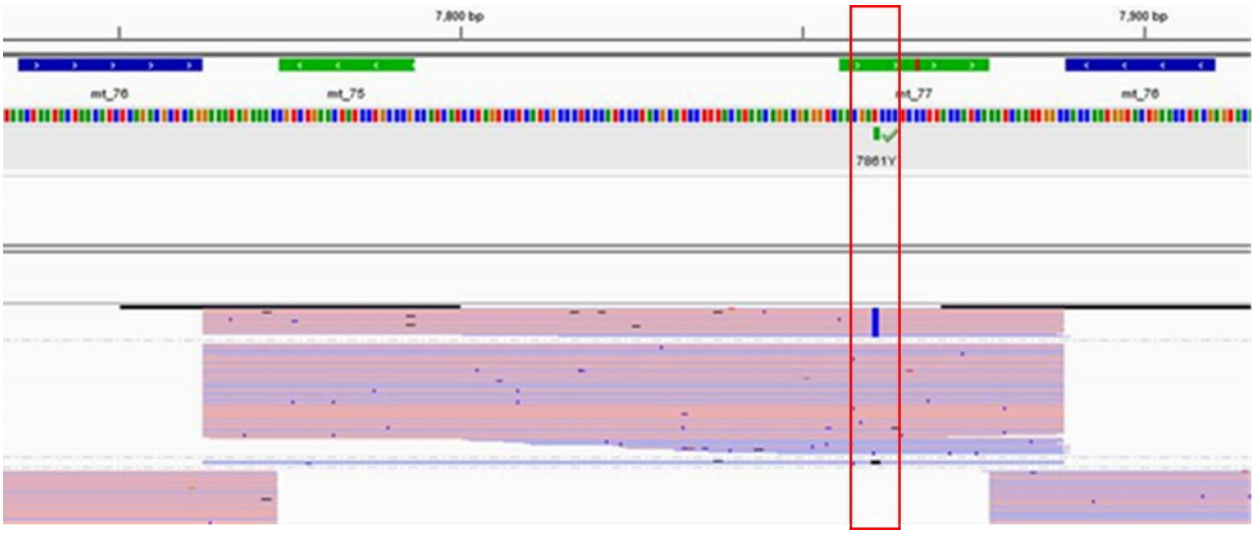
An example of point heteroplasmy observed using Sanger sequencing is shown below.



An example of length heteroplasmy observed using Sanger sequencing is shown below.



An example of point heteroplasmy observed in an NGS sequence alignment is shown below.



An example of heteroplasmy reported in an NGS variant table is shown below.

Position	Reference	Variant	Major freq...	Coverage	Covered	Quality Scores	#-Unique Start Positions	Position (e...	Reference...	Variant (edit)	Filter	Tool Comment	User Com...
152	T	T (58.7%) / C (41.1%)	58.74	503	Yes	T (37.1) / C (37.6)	T (103) / C (84)	152	T	Y			
263	A	G (100.0%)	100.00	491	Yes	G (37.4)	G (117)	263	A	G			
302.1	-	- (69.9%) / C (30.1%)	69.95	194	No	C (37.7) / - (0.0)	- (41) / C (40)				HV2 removal	Shifted, Filtered	
310.1	-	C (97.7%)	97.66	167	No	C (36.5)	C (67)				HV2 removal	Shifted, Filtered	
477	T	C (99.2%)	99.23	382	Yes	C (37.3)	C (109)	477	T	C			
750	A	G (99.7%)	99.65	1404	Yes	G (37.4)	G (138)	750	A	G			
1438	A	G (98.3%)	98.31	404	Yes	G (37.3)	G (119)	1438	A	G			
3010	G	A (99.8%)	99.81	515	Yes	A (37.2)	A (108)	3010	G	A			
3106	C	- (100.0%)	100.00	437	Yes	- (0.0)	- (116)	3107	C	:		Shifted	
4769	A	G (100.0%)	100.00	25	Yes	G (37.4)	G (20)	4769	A	G			
10646	G	A (99.7%)	99.73	367	Yes	A (37.3)	A (104)	10646	G	A			
15326	A	G (99.5%)	99.54	862	Yes	G (37.3)	G (136)	15326	A	G			
16519	T	C (99.7%)	99.68	601	Yes	C (37.4)	C (119)	16519	T	C			

Levels of heteroplasmy can vary between different tissues within the same individual, as well as between individuals within a maternal lineage (Wilson et al. 1997, Stewart et al. 2001). When the specimens under consideration differ by a laboratory-defined number of nucleotides across the region sequenced, additional samples may be run in an attempt to resolve whether the sequence difference between samples is real or due to undetected heteroplasmy.

The ability to detect point heteroplasmy depends on the sequencing methodology. The limit of detection of point heteroplasmy in Sanger sequencing is estimated to be between 10-20% (Just et al. 2015a). With the increased sensitivity of NGS, the limit of detection is estimated to be less than 5% (Just et al. 2015a). Based on Sanger sequencing studies of the control region using buccal and blood cells, approximately 6% of the population has detectable levels of point heteroplasmy (Irwin et al. 2009). Studies of the whole mtDNA genome using Sanger sequencing as well as NGS have found point heteroplasmy in roughly 25% of the individuals tested (Just et al. 2015a, Taylor et al. 2020).

In most samples, as detected by Sanger sequencing of the control region, point heteroplasmy occurs at a single site. However, observation of point heteroplasmy at two or more sites can also occur (Irwin et al. 2009, Just et al. 2105b), often with at least one heteroplasmic site at a position that has been observed to have a high incidence of heteroplasmy and has been termed a "hot spot" (e.g., 16093, 16129, 16189, and 16309 in HVI, and 73, 152, 189, 207, and 215 in HVII; Melton 2004). Caution should be used when multiple sites of point heteroplasmy are observed within a sample, particularly at positions showing known patterns of polymorphisms in mtDNA analysis. Interpretation of the sample as a mixture or DNA damage should be considered.

Length heteroplasmy is more common than point heteroplasmy, being observed in the control region of 63.6% of individuals (Just et al. 2015b) and is likely to be observed in samples with longer homopolymeric stretches (8 or more residues). Different PCR methods and sequencing methodologies may impact the display of length heteroplasmy (Sturk-Andreaggi et al. 2020). Length heteroplasmy is believed to be caused by slippage of the polymerase during replication (akin to stutter in autosomal DNA testing) in vivo and/or in vitro. Due to the higher incidence of length heteroplasmy and differences between tissues within an individual, variations in length in some homopolymeric regions (particularly the HVII C-stretch) may be ignored in database searches and for statistical purposes. In addition, some laboratories may not use length variations in these areas for comparison purposes between samples.

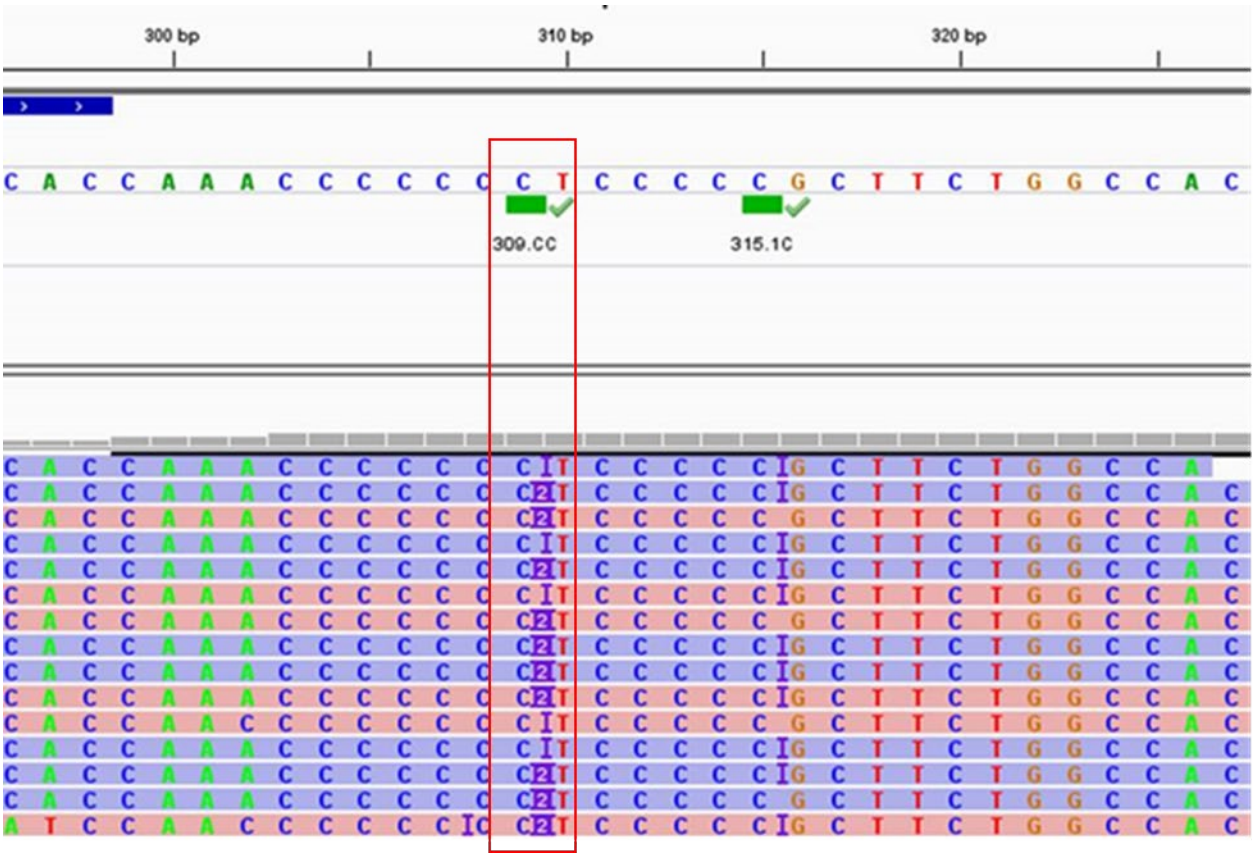
FAQ-8: How does a laboratory address length heteroplasmy?

Cross-reference Guidelines 3.5 and 3.7.

Homopolymeric tracts are prone to exhibiting length heteroplasmy, particularly in HVI between positions 16183-16194 and in HVII between positions 302-310. Homopolymeric tracts can differ in length such that sequences contain a different number of repeating bases within the same individual and/or different individuals from the same maternal lineage. A laboratory should determine if/how they will report these regions. In most cases laboratories do not report the presence of length heteroplasmy in these regions or use it for searching and comparison. By default, the CODIS and EMPOP databases ignore indels following positions 16193 and 309.

For NGS, different software programs may not necessarily align homopolymeric (and other) repeat regions uniformly or according to standard forensic practice. Thus, further review of C-stretch alignments may be warranted to ensure appropriate variant calling metrics are met.

An example of length heteroplasmy observed using NGS is shown below.



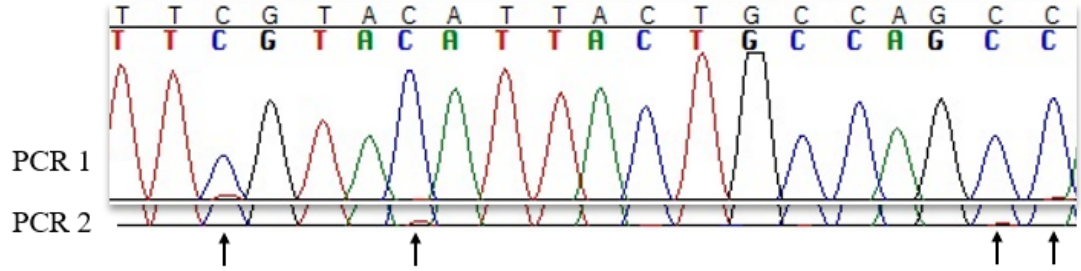
FAQ-9: How is DNA degradation addressed and what is DNA damage?

Cross-reference Guideline 3.6.1

Over time, mainly as the result of hydrolytic attack, but also through chemical and background radiological damage, DNA degrades through both fragmentation and base modification. Following (tissue) death, DNA is exposed to hydrolytic enzymes originating from bacterial activity and lysing cellular organelles. However, environmental effects are the primary source of damage (Dabney et al. 2013). Fragmentation, the most commonly encountered form of damage in a forensic setting, occurs rapidly, especially with exposure to heat and humidity. When amplifying evidentiary DNA, this is typically addressed by targeting shorter amplicons. For example, the control region may be amplified in one long (~1200 bp) amplicon when dealing with well-preserved DNA, but when dealing with degraded DNA, HVI and HVII may be

amplified using shorter overlapping amplicons. Given enough time, heat and humidity also result in the chemical modification of bases. A common example of such modification is Type II DNA damage, also known as cytosine deamination (Willerslev and Cooper 2005). In this form of damage, cytosine is deaminated to uracil, which when amplified by PCR, is observed as thymine. In this way, nucleotide positions that were 'C' in the living individual appear to be 'T' in the evidence. Often such sites are observed as 'Y' mixed bases and change position upon reamplification. In addition to DNA strand fragmentation and base changes, blocking lesions such as intrastrand crosslinks can also interfere with amplification.

An example of cytosine deamination observed as C/T mixed bases in Sanger sequencing data is shown below.



An example of cytosine deamination observed as 'T' transitions in mtDNA NGS data from the same sample is shown below.

FAQ-10: What are NUMTs/pseudogenes?

Cross-reference Guideline 3.6.1

NUMTs, or nuclear mitochondrial insertions (i.e., pseudogenes), are sequences resulting from the integration of fragments of the mitochondrial DNA genome into the nuclear genome (Marshall and Parson 2021). Because they are no longer constrained by evolutionary pressure and are free to mutate more rapidly than functional mtDNA, NUMT sequences will often differ from their ancestral mtDNA genome. NUMTs may contain binding sites for the primers used in forensic mtDNA analysis and can coamplify with the true mitochondrial genome, appearing as a mixture of sequences when analyzed. Recent insertions may appear as a mixture of individuals differing at only a few bases, while more ancient NUMTs may be characterized by many base changes even insertions and deletions and more closely resemble bacterial contamination. Reamplification of a sample using the same primers and cycling parameters will usually not remedy the situation. Instead, reamplifying the sample using different primer sets is often the best solution in a forensic setting when using traditional amplification and sequencing. Alternatively, samples can be diluted to minimize the nuclear DNA contribution. In many cases, NGS allows for the recognition and removal of NUMT amplicons.

FAQ-11: What factors should I consider when analyzing the data?

Cross-reference Guideline 3.4

An alignment of overlapping sequences is performed using software programs specifically designed for this purpose. The sequence data is then compared to the rCRS for subsequent reporting.

The following criteria should be considered when analyzing Sanger sequencing data:

- Is excessive background noise interfering with peak calls?
- Sequence should be easily legible and align with the revised rCRS. For example, peaks should not be off scale or disguised beneath elevated background. Additionally, there should be good peak morphology to allow for each peak to be distinguishable from neighboring peaks.

- Sequence information should appear to be from a single source. Although Sanger sequencing mixtures may be used for exclusionary purposes based on internal validation, no further effort should be made to deconvolute these mixtures into separate single source profiles. A mixed position can be defined as two or more base peaks occupying the same position either directly or slightly shifted in a 3' or 5' direction. The following criteria may be considered when determining if data originated from a single source vs multiple sources:
    - Are there multiple mixed positions above baseline?
    - Are there mixed positions consistent across multiple amplifications and/or extracts?
    - Are there mixed positions seen in both forward and reverse sequence data?
    - Are there mixed positions at common heteroplasmic positions, such as nucleotide position (np) 16093 or np 152?
    - Are there multiple mixed positions and if so, is there an expected number of mixed positions, i.e., exceeding the expected number of heteroplasmic positions as per the laboratory's standard operating procedures?
    - Is it possible the mixed positions are a result of NUMTs or non-human DNA?
    - Is it possible the mixed positions are a result of DNA damage, specifically cytosine deamination? This type of DNA damage will appear as minor T peaks (red) under C peaks (blue) or minor A peaks (green) under G peaks (black).

For NGS data, reads are filtered, trimmed and mapped by analysis software. Metrics including but not limited to read depth, read quality scores (Q-scores), and strand bias should be considered (Pont-Kingdon et al. 2012, Gargis et al. 2012, Rehm et al. 2013, Aziz et al. 2015, Ellard et al. 2016). Additional criteria that may be considered specifically for the designation of bases include variant frequency, variant count, and variant quality.

The following criteria should be considered when analyzing NGS data:

- The data should meet or exceed the minimum read threshold set forth by the laboratory. Areas with a reduced number of reads should be closely evaluated.

- The data should be of good quality and align with the rCRS.
- Sequence information should appear to be from a single source. The following criteria may be considered when determining if data originated from a single source vs multiple sources:
    - Are variant frequencies within laboratory established acceptance parameters?
    - Are there multiple mixed positions?
    - Are there mixed positions across extracts or lab events?
    - Are there mixed positions at common heteroplasmic positions, such as np 16093 or np 152?
    - Are the frequencies of the mixed positions similar?
    - Are the mixed positions clustered (located closely together)?
    - Are the mixed positions the result of transversions or indels?
    - Are there multiple mixed positions and if so, is there an expected number of mixed positions, i.e., exceeding the expected number of heteroplasmic positions as per the laboratory's standard operating procedures?
    - Is it possible the mixed positions are a result of NUMTs or non-human DNA?
    - Is it possible the mixed positions are a result of DNA damage, specifically cytosine deamination? This type of DNA damage will appear as a minor T variant with a major C variant or a minor A variant with a major G variant.

For NGS, laboratory guidelines for the interpretation of homopolymeric regions and regions of length heteroplasmy should be established with consideration of the analysis software employed.

FAQ-12: Are mtDNA mixtures suitable for interpretation?

Cross-reference Guideline 3.6

Due to the limited quantitative information available from Sanger sequencing, aside from contamination investigation, etc., mitochondrial DNA mixed sequences are not commonly interpreted (Melton et al. 2012). However, laboratories can perform mixture interpretation for mitochondrial DNA sequences that is supported by their internal validation. Considerations are provided in FAQ-11.

Next generation sequencing allows for more quantitative information to be obtained. Therefore, interpretation of NGS mitochondrial DNA mixed sequences for inclusions, as well as exclusions, is possible (Holland et al. 2011, Kim et al. 2015, Vohr et al. 2017, Churchill et al. 2018, Peck et al. 2018, Brandhagen et al. 2020, Cihlar et al. 2020a, Canale et al. 2021, Holt et al. 2021, Mandape et al. 2021, Dür et al. 2022).

FAQ-13: What nomenclature should I use to report the mtDNA sequence?

Cross-reference Guideline 4

Since the resulting mtDNA sequence is a long string of letters (representing the DNA bases) that can theoretically differ at any position along this sequence, a shorthand method of naming the sequences is used. The use of standardized nomenclature principles to determine the mtDNA alignment allows for the consistent representation of a sample's haplotype. However, experience has demonstrated that the same nomenclature principles have not always been employed by laboratories. In addition, for some sequences, consistent application of standardized nomenclature principles has proven difficult to achieve manually. In such situations, differences in the representation of the same sequence string could result in a false exclusion in a direct comparison or database search. Modification of search algorithms used for sequence comparisons to include string-based capability can resolve this issue.

Use of a Sequence Reference Standard:

A consensus sequence obtained from the sample is compared to the rCRS described by Andrews et al. (1999). Differences between the rCRS and the sample sequence will be recorded as polymorphisms with both the nucleotide position and the DNA base difference from the reference noted (e.g., 16089 C). This process derives the mtDNA shorthand used to record a sample's haplotype.

Applied Nomenclature (i.e., the use of Nomenclature Rules):

Historically, there have been different mtDNA nomenclature approaches to derive a sample's haplotype (Wilson et al. 2002a, Wilson et al. 2002b, Bandelt and Parson 2008, Budowle et al. 2010, Huber et al. 2018). These methods employed either a hierarchical series of rules or a phylogenetic approach. A comparison of the rule-based approach and the phylogenetic approach showed that generally both systems code the haplotypes in the same manner even though they use different strategies (Polanskey et al. 2010). However, due to the inherent differences between the approaches, the potential exists for the same sequence to be annotated differently between laboratories, particularly when mtDNA types have atypical insertions and deletions. Furthermore, there are mtDNA sequences whose base compositions (sequence strings) truly differ by only one base. Yet when these sequences are evaluated using a rule-based nomenclature, the results may yield mtDNA haplotypes which appear to differ by more than the one true base (Table I, Example 1).

Nomenclature differences like these may not be a problem with direct one-to-one comparison of samples within the same laboratory. However, it is problematic when performing forensic database searches for missing person cases using mtDNA data. In these situations, the database comparison between these samples would result in a missed association since mtDNA database searches using mtDNA sequences for missing person cases account for the possibility of only a single mutational event between generations (Table I, Example 2).

Ideally, full sequence strings would be aligned for database searches, making any subtle differences in the coded nomenclature irrelevant. However, the infrastructure for database string searches is not yet in place for forensic (Missing Person) databases in the United States. The

European DNA Profiling Group Mitochondrial DNA Population Database (EMPOP; see FAQ-16) currently performs string-based searches and provides phylogenetic nomenclature that is widely accepted by laboratories that process mtDNA casework samples. In order to maintain mtDNA nomenclature uniformity among analysts within a laboratory and across laboratories, it is recommended that all laboratories which process mtDNA casework samples utilize the phylogenetic nomenclature as indicated by the EMPOP mtDNA database.

It is important that no matter which rules are applied, efforts are made to maintain known patterns of polymorphisms in mtDNA analysis. When rules alter known patterns (i.e., established phylogenetic patterns of polymorphisms), it is possible that two mtDNA haplotypes will appear to differ at two or more sites when they actually only differ at one. For example, a rather common deletion at nucleotide position 249 is present in existing populations. When a polymorphism at position 247 is coupled with the 249 deletion, a rule-based approach would code this area as a 247DEL instead of 247A, 249DEL. By failing to maintain the phylogenetically established 249DEL, sequences coded as a 247DEL are now 2 differences away from a sequence containing only 249DEL. On the contrary, if the known pattern of 249DEL is maintained, a sequence coded as 247A, 249DEL is only one difference away from a sequence containing only a 249DEL (Table I, Example 3).

Table I – Examples of Nomenclature Issues	
Example 1: nucleotide position (np) 16024-16365, 73-340	
Historical Rule-Based Alignment	Phylogenetic Alignment
16182DEL 16183C 16193.1 C 16217C 73G 263G 309.1C 315.1C	16182C 16183C 16188T 16189C 16217C 73G 263G 309.1C 315.1C
In this example, the rule-based and phylogenetic alignment haplotypes represent the same nucleotide sequence string, yet there are 5 differences between the two haplotypes.	
Example 2: np 16024-16365, 73-340	
Historical Rule-Based Alignment	Phylogenetic Alignment

16111T 16189C 16192.1T 16223T 16233G 16290T 16319A 16331G 73G 146C 153G 235G 263G 315.1C	16111T 16189C 16191.1C 16192T 16223T 16233G 16290T 16319A 16331G 73G 146C 153G 235G 263G 315.1C
In this example, the rule-based and phylogenetic alignment haplotypes represent the same nucleotide sequence string, yet there are 3 differences between the two haplotypes.	
Example 3: np 16024-16365, 73-340	
Historical Rule-Based Alignment	Phylogenetic Alignment
16129A 16172C 16184T 16187T 16189C 16223T 16261T 16278T 16290T 16293G 16311C 16360T 73G 150T 151T 152C 182T 186A 189C 195C 247DEL 263G 297G 315.1C 316A	16129A 16172C 16184T 16187T 16189C 16223T 16261T 16278T 16290T 16293G 16311C 16360T 73G 150T 151T 152C 182T 186A 189C 195C 247A 249DEL 263G 297G 315.1C 316A
In this example, the rule-based and phylogenetic alignment haplotypes represent the same nucleotide sequence string, yet there are 3 differences between the two haplotypes.	

FAQ-14: What are the previously published (2003-2019) alignment rules?

Cross-reference Guideline 4.4

To reduce variation in reporting and provide consistent mtDNA nomenclature, EMPOP should be used to verify the alignment of a mtDNA haplotype. However, it may not always be feasible to search EMPOP (i.e., lack of EMPOP availability, limited mtDNA sequence data). In such instances, it is recommended to use the SWGDAM Nomenclature Rules in Section 4.4 in the SWGDAM Interpretation Guidelines for Mitochondrial DNA (mtDNA) Analysis for Forensic DNA Testing Laboratories (2024). Previously published rules are presented below for historical purposes. If a mtDNA haplotype was not phylogenetically aligned, the laboratory should assess if and/or when to search the mtDNA haplotype in EMPOP and/or utilize the SWGDAM Nomenclature Rules in Section 4.4 of the SWGDAM Interpretation Guidelines for Mitochondrial DNA (mtDNA) Analysis for Forensic DNA Testing Laboratories (2024).

Previous SWGDAM Nomenclature Rules (SWGDAM Interpretation Guidelines for Mitochondrial DNA (mtDNA) Analysis for Forensic DNA Testing Laboratories (approved 04/23/2019)):

Variants from the rCRS should be coded in accordance with the following nomenclature rules:

Rule 1 – Maintain known patterns of polymorphisms (i.e., known phylogenetic alignments). Most violations to known patterns of polymorphisms involve insertions and deletions. A phylogenetic alignment tool is available at https://empop.online.

Example: Maintain deletions at positions 249, 290 and/or 291 when present. See other examples in the Nomenclature Examples section.

Rule 2 – Use nomenclature with the least number of differences unless it violates known patterns of polymorphisms.

Rule 3a – Homopolymeric C-Stretches in Hypervariable Region I (HVI): C-stretches in HVI should be interpreted with a 16189C when the otherwise anchored T at position 16189 is not present. Length variations in the short A-tract preceding 16184 should be noted as transversions.

Rule 3b – Homopolymeric C-Stretches in Hypervariable Region II (HVII): C-stretches in HVII should be interpreted with a 310C when the otherwise anchored T at position 310 is not present. C-stretches should be interpreted with a 311T when the anchored T at position 310 is followed by a second T.

Rule 4 – Maintain the AC Repeat Motif in the HVIII region from np 515-525.

Rule 5 – Prefer substitutions to insertions/deletions (indels).

Rule 6 – Prefer transitions to transversions unless this is in conflict with Rule 1.

Rule 7 – Place indels contiguously when possible.

Rule 8 – Place indels on the 3' end of the light strand.

Rule 9 – The 3107 nucleotide should not be reported in sample data. As 3107 in the rCRS is simply a placeholder intended to maintain historical nomenclature (Andrews et al. 1999), differences from the rCRS (i.e., deletions) at this position are not biologically meaningful.

FAQ-15: When would a realignment be appropriate?

Cross-reference Guideline 4.7.1

Laboratories should determine on a case-by-case basis whether sequences need to be realigned. It should be noted that in most cases a sequence would not need to be realigned. Sequences aligned under previous rules may need to be updated when:

- A previously reported match going to trial may need to have statistics recalculated after realignment
- A sequence continues to be searched in CODIS (i.e., unidentified human remains and relatives of missing persons)
- Other situations such as when an analyst is performing a manual comparison and determines that a sequence may need to be realigned

FAQ-16: What is EMPOP?

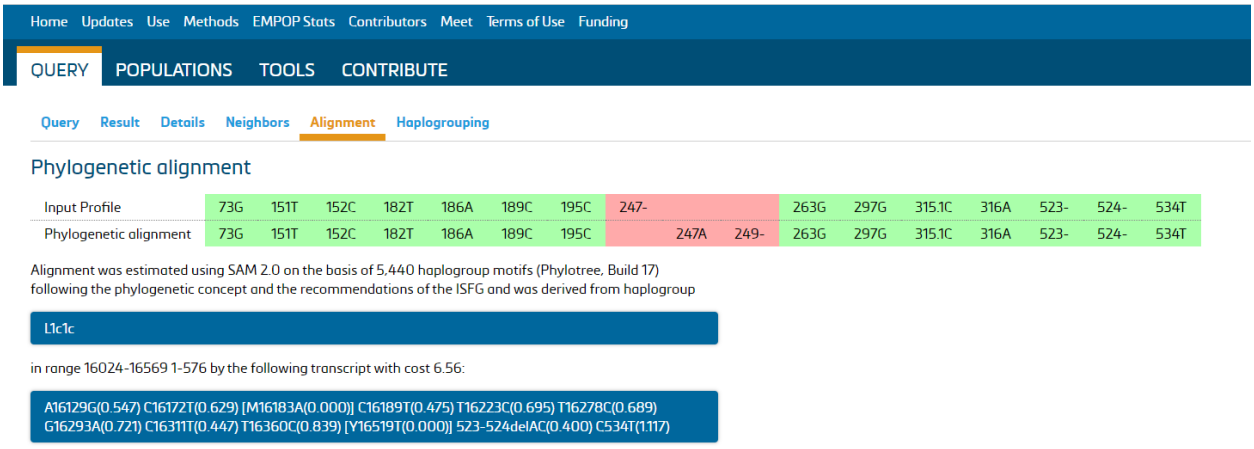
Cross-reference Guideline 4.3

Supplemental Information for the SWGDAM Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories
Approval/Effective Date: September 9, 2024

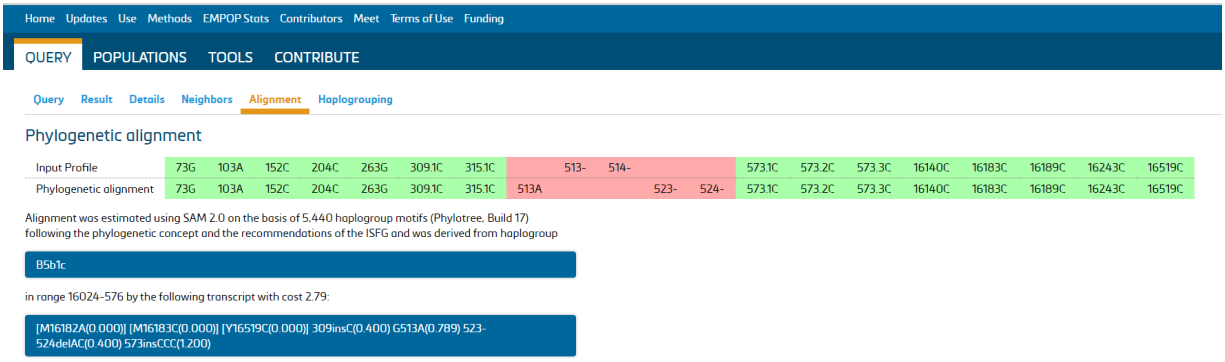
The European DNA Profiling Group Mitochondrial DNA Population Database (EMPOP) is a searchable collection of quality-controlled mtDNA haplotypes from all over the world. The haplotypes range from sequences covering only hypervariable region I to the entire mitogenome. EMPOP can be used to estimate the frequency of a mtDNA haplotype, to verify the phylogenetic alignment and to predict the haplogroup. EMPOP converts queries into alignment-free nucleotide sequence strings so that a haplotype can be found in the database regardless of its alignment. In the output, however, the haplotype is presented in the rCRS difference coded format using the phylogenetic alignment. New users are encouraged to refer to the "Directions for Use" guide available in the "Use" tab on the website (https://empop.online). SWGDAM recommends that a laboratory defines when a sequence alignment is verified phylogenetically using EMPOP.

Examples of the EMPOP phylogenetic alignment tool are provided below. In sample 1, the 247del variant is corrected to 247A and 249del based on phylogenetic alignment. In sample 2, the 513del 514del variants are corrected to 513A 523del 524del based on phylogenetic alignment.

Sample 1



Sample 2



FAQ-17: Does a laboratory need to validate EMPOP?

As per the FBI Quality Assurance Standards For Forensic DNA Testing Laboratories, new software used as a component of instrumentation, for analysis/interpretation of data, or for statistical calculations shall be subject to internal validation specific to the laboratory's intended use prior to implementation in forensic DNA analysis. The information below is meant to provide general guidance but requirements will be dictated based on how a laboratory intends to use the different functions of EMPOP.

If a laboratory intends to use EMPOP to estimate the mtDNA haplotype probability/frequency, then the laboratory must follow the FBI Quality Assurance Standards for Forensic DNA Testing Laboratories for software used for statistical calculations and perform a validation specific to the laboratory's intended use.

If a laboratory intends to use EMPOP to assist with the interpretation of mtDNA data (e.g., using the phylogenetic alignment or haplogrouping tools to assist with calling mtDNA variants), then the laboratory must follow the FBI Quality Assurance Standards for Forensic DNA Testing Laboratories for software used for the interpretation of DNA data and perform a validation specific to the laboratory's intended use. The validation may consist of testing mtDNA profiles with known phylogenetic misalignments and/or known haplogroups.

If a laboratory intends to use EMPOP in a way that does not impact the interpretation of mtDNA data (e.g., using the haplogroup tool to report a haplogroup), then at a minimum, a functional test is required.

FAQ-18: After an initial internal validation, how can a laboratory address new releases of EMPOP?

Modifications to software used as a component of instrumentation, for analysis/interpretation of data, or for statistical calculations shall be evaluated to determine if modifications result in major or minor revisions to the software and be subject to the testing required by the FBI Quality Assurance Standards for Forensic DNA Testing Laboratories. With each new release of EMPOP, a laboratory should review the extent of the updates, which includes changes to database sizes, if the changes affect the relevant populations used for statistics, and if the updates affect their procedures for searching, calculating haplotype probabilities and/or the interpretation of mtDNA data. As appropriate, necessary validations or functional tests should then be performed per the FBI Quality Assurance Standards for Forensic DNA Testing Laboratories.

If a new EMPOP release does not impact the intended use in the laboratory (e.g., no changes to the laboratory's relevant population databases, the search algorithms, sequence alignment strategy or statistical calculations), then no testing is needed; however, this evaluation must be documented.

If a new EMPOP release results in only a change to the laboratory's relevant population database, this is considered a minor revision and, at a minimum, a functional test is needed. A functional test may consist of selecting an appropriate set of mtDNA haplotypes that are searched with each new release of EMPOP. The results between the new search and previous searches can be compared.

If a new EMPOP release results in a change to the search algorithms, sequence alignment strategy, statistical calculations used by the laboratory and/or any other change determined to be a major revision by the laboratory, then a validation is needed.

FAQ-19: What is a haplogroup?

A mitochondrial haplogroup is a group of individuals who share a common maternal ancestor and are identified by their possession of a diagnostic mutation(s) in the mitochondrial genome. As mutations are accumulated, they are passed from one generation to the next and eventually may become fixed in a population sharing the common ancestor in which the mutation first occurred. Certain mutations have been identified that act as markers for the major lineage branch points in the human mitochondrial tree, and the branches are known as haplogroups. The haplogroups are identified by letters ranging from A to Z, with the major groups being L, M, N, and R (van Oven and Kayser 2008). Haplogroups have been used to identify possible human migration routes and can act as rough indicators of ancestry (Underhill and Kivisild 2007). However, mitochondrial haplogroups represent very distant ancestry and therefore may not reflect more recent ancestry. Therefore, an individual's observed phenotype may be different from that which would be expected when considering the mitochondrial haplotype alone. For this reason, mitochondrial haplogroups are not typically reported or used as investigative leads.

FAQ-20: What is string-based searching and how does it address nomenclature issues?

Cross-reference Guideline 4.1

String-based searching utilizes the string, or sequence, of bases that comprise the mtDNA haplotype. Regardless of the nomenclature rules applied, certain unusual mtDNA types that generally involve atypical insertions and deletions may be difficult to represent consistently. By converting mtDNA haplotype results to alignment-free nucleotide sequence strings, samples can be compared within forensic or population databases without the concern of interpretation differences resulting in missed associations. The publication by Röck et al. (2011; update in Huber et al. 2018 and Dür et al 2022) demonstrates that the application of a string-based search algorithm ensures that identical sequences are associated in a database query. Mitochondrial

DNA haplotypes may be searched in EMPOP as sequence strings or as a consensus profile generated from the alignment against the rCRS. Instructions for entering a mtDNA haplotype sequence string are located on the EMPOP website. CODIS/CODIS Popstats currently does not use string-based searching.

FAQ-21: If a laboratory generates whole mtDNA genome data, how does this affect database searches?

Cross-reference Guideline 6.3

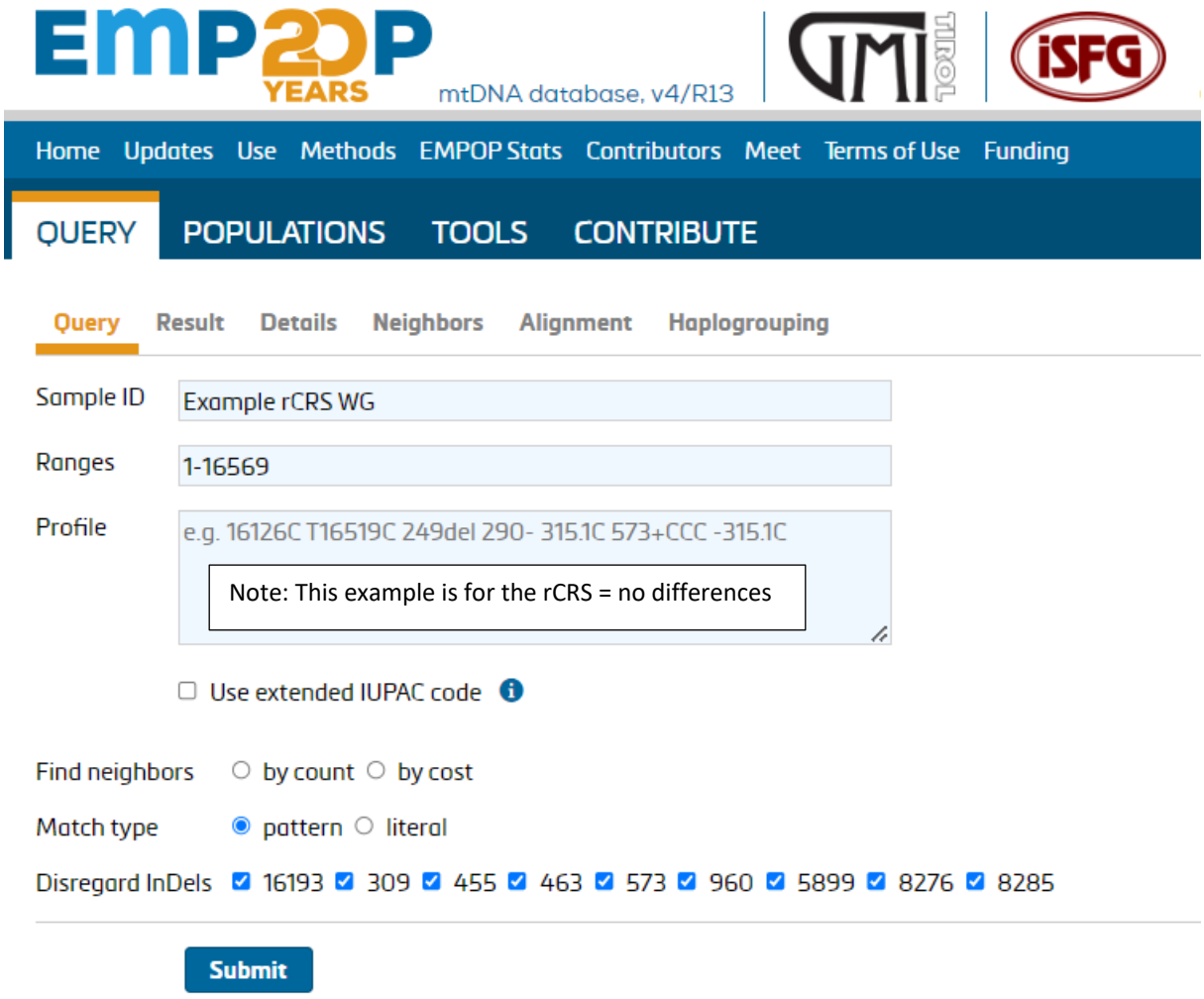
Historically, databases such as EMPOP have been built using mtDNA control region sequences, and whole mtDNA genome sequences have only relatively recently begun to be added in significant numbers. The result is that there are fewer whole mtDNA genome sequences than control region sequences in EMPOP (46,963 HVI/HVII vs 38,361 control region vs 4,289 whole genome sequence as of version 4/R13). Therefore, a more discriminating whole mtDNA genome sequence may yield a less discriminating statistic when searched in EMPOP than a less discriminating control region sequence. This is similar to the paradox observed with the transition to Y megaplexes (see Supplemental Information for the SWGDAM Interpretation Guidelines for Y-Chromosome STR Typing by Forensic DNA Laboratories, FAQ-18). This is because the 95% upper confidence interval (UCI) used to estimate population frequency is based largely upon the size of the database (See FAQ-26). One observation in a database of 10 is less meaningful than one observation in a database of 1000, and the 95% upper confidence interval reflects this by returning an artificially high frequency estimate for whole mtDNA genome data due to the relatively small database size. To address this limitation, a reduced sequence range search can be performed (see FAQ-22). Laboratories need to address this in their procedures.

FAQ-22: How is a reduced sequence range search performed using EMPOP?

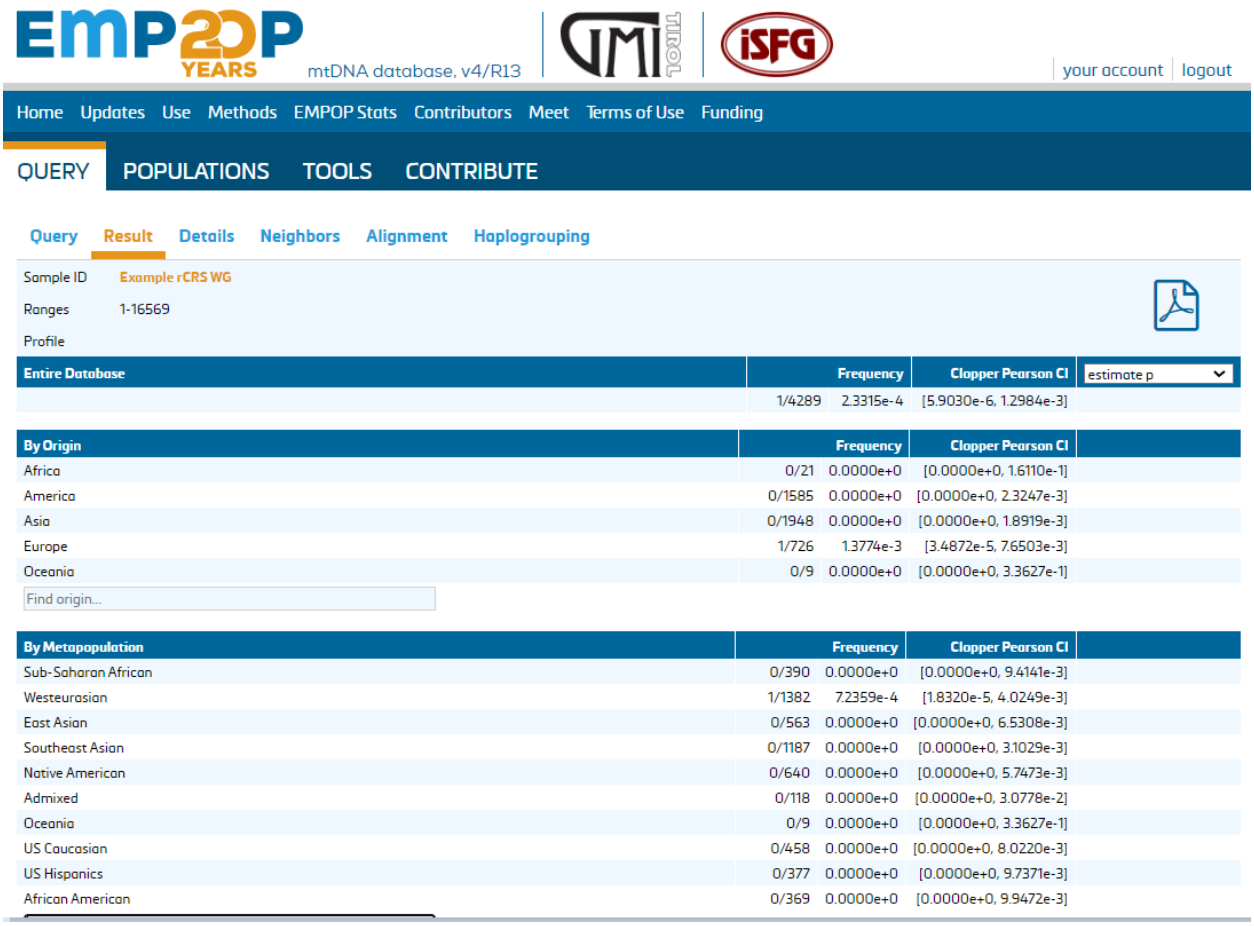
Cross-reference Guideline 6.3

There are three data fields when performing a query within EMPOP: Sample ID, Ranges, and Profile. The range selected within the mtDNA genome determines the dataset that EMPOP will use for the search. Entering a search range of 1-16569 will trigger the use of the whole mtDNA genome dataset. Alternatively, entering 16024-576 would select the Control Region dataset. EMPOP v4/R13 has 48,572 mitotypes divided into 4,289 whole mtDNA genome, 38,361 Control Region, and 46,963 HVI and HVII sequences.

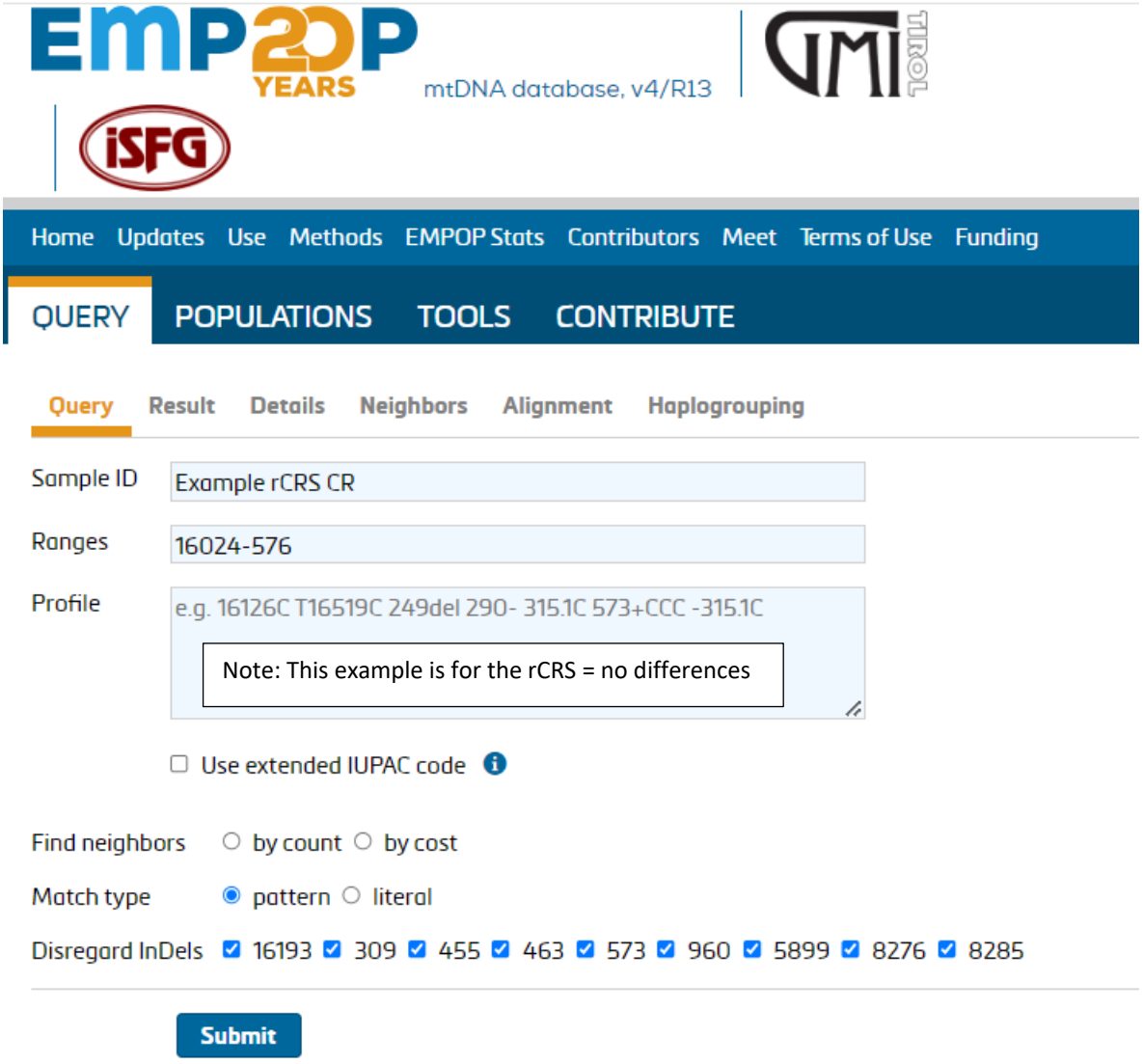
The following example demonstrates how a typical EMPOP reduced sequence range search would be performed. First, query your sample using the longest available range (typically 1-16569).



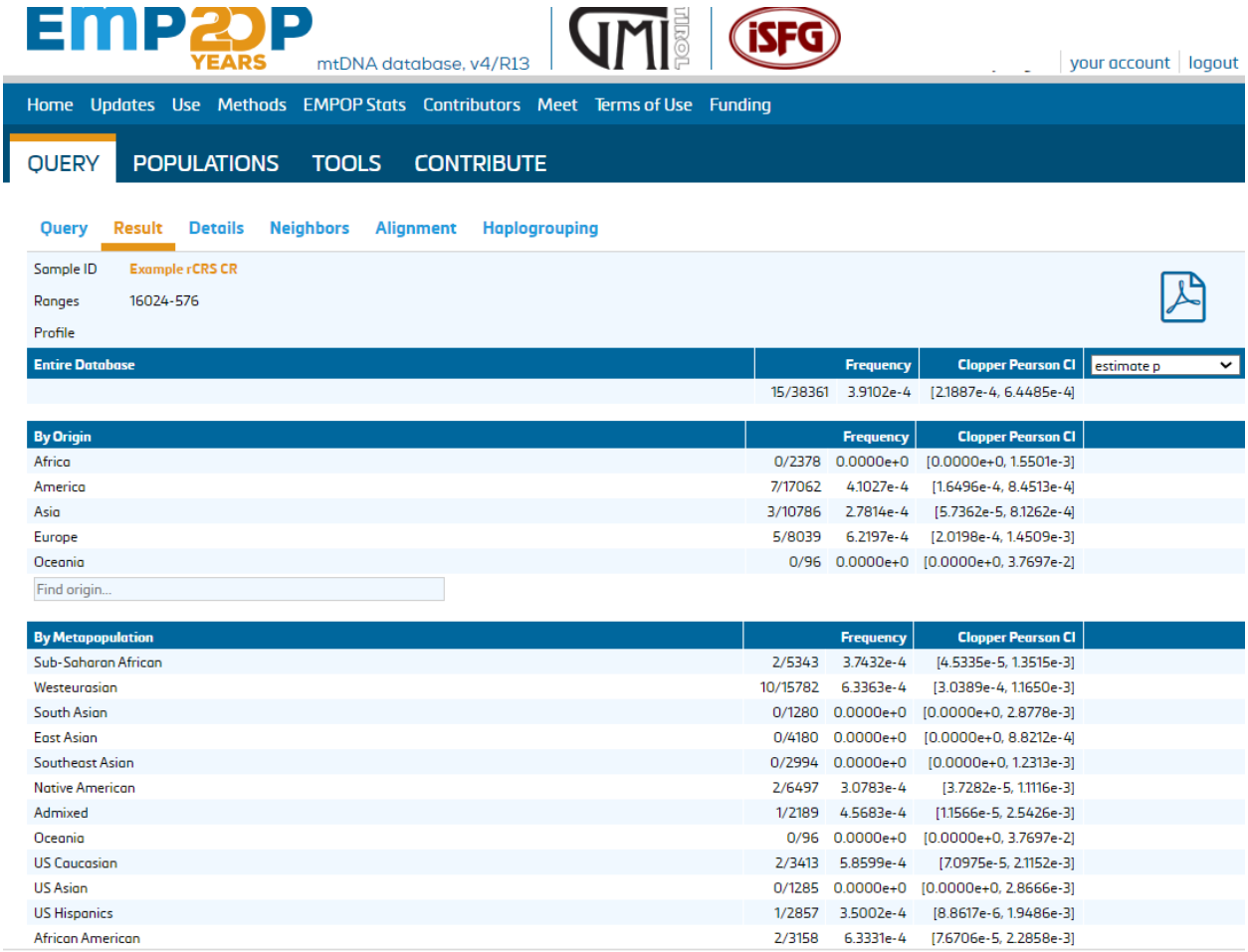
Note that the database searched has 4,289 samples, which is the number of whole mtDNA genome samples. Print the results page and note if the haplotype has been observed in the population(s) of interest. In this example, the U.S.-specific populations were added to the list, as they are the most pertinent. This haplotype has not been observed within the U.S. Caucasian population for 0/458 (95% UCI is 1/153).



Reduce the search by inputting the range for the Control Region (16024-576) and modifying the profile accordingly.



Note the database size for this search is now 38,361, which equals the number of Control Region haplotypes. The number of observations in this example increased to 2 in U.S. Caucasians for 1/1,707 (95% UCI is 1/542) and 2 in African Americans for 1/1,579 (95% UCI is 1/502). The increased number of observations is not surprising since the data range was reduced and the size of the dataset increased. The larger portion of the database considered here makes for a paradoxically more discriminating statistic using a less discriminating haplotype (shorter).



EMPOP 20 YEARS mtDNA database, v4/R13 | ISFG | your account | logout

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QUERY POPULATIONS TOOLS CONTRIBUTE

Query Result Details Neighbors Alignment Haplogrouping

Sample ID Example rCRS CR

Ranges 16024-576

Profile

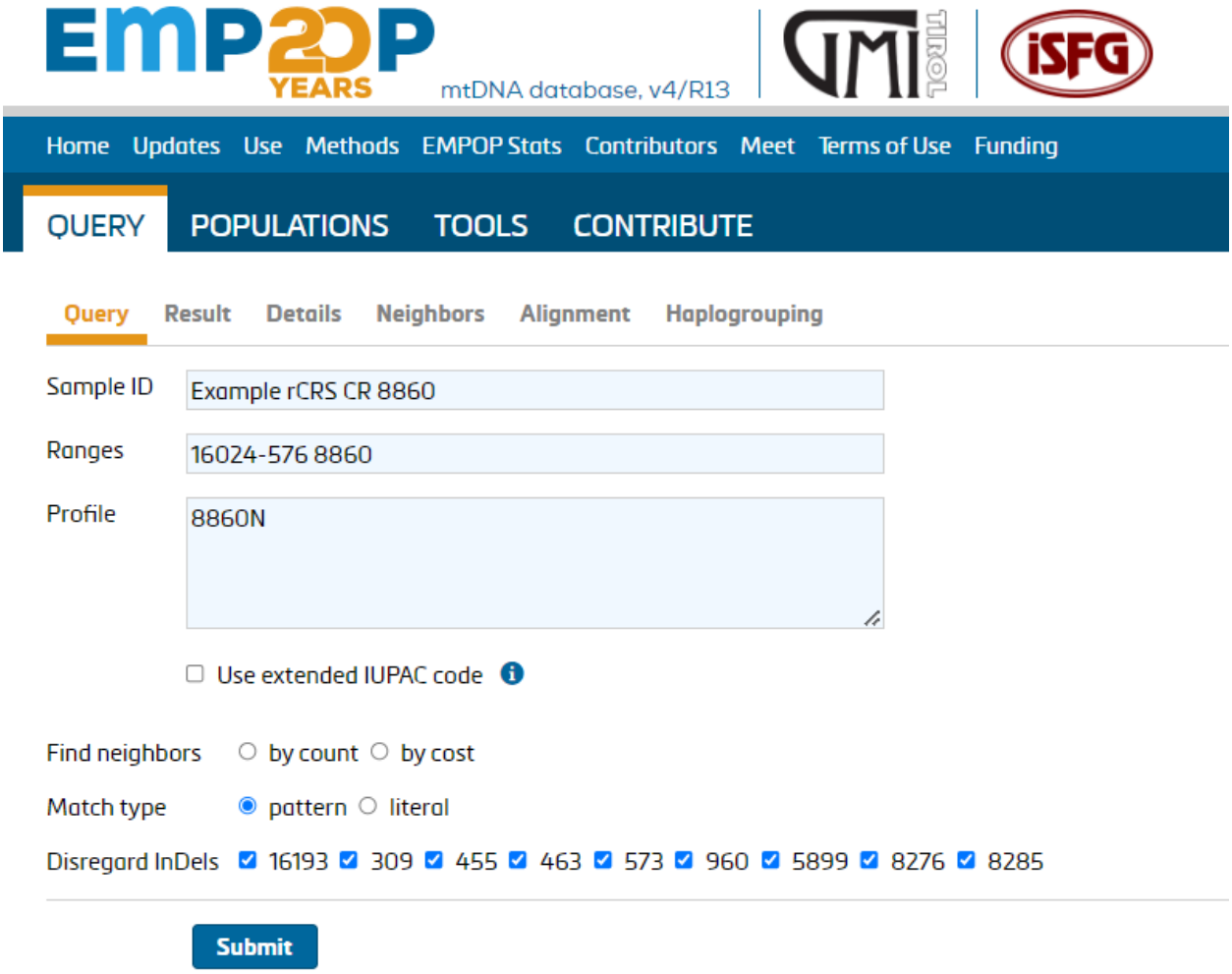
Entire Database		Frequency	Clopper Pearson CI	estimate p
	15/38361	3.9102e-4	[2.1887e-4, 6.4485e-4]	

By Origin		Frequency	Clopper Pearson CI
Africa	0/2378	0.0000e+0	[0.0000e+0, 1.5501e-3]
America	7/17062	4.1027e-4	[1.6496e-4, 8.4513e-4]
Asia	3/10786	2.7814e-4	[5.7362e-5, 8.1262e-4]
Europe	5/8039	6.2197e-4	[2.0198e-4, 1.4509e-3]
Oceania	0/96	0.0000e+0	[0.0000e+0, 3.7697e-2]

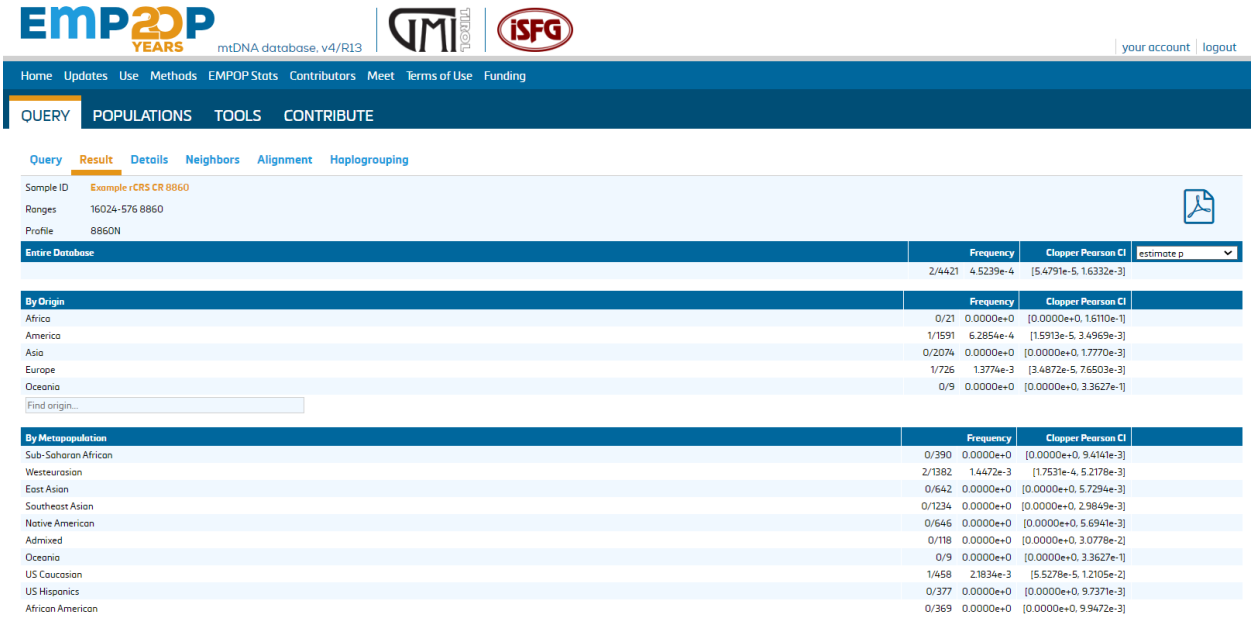
Find origin...

By Metapopulation		Frequency	Clopper Pearson CI
Sub-Saharan African	2/5343	3.7432e-4	[4.5335e-5, 1.3515e-3]
Westeurasian	10/15782	6.3363e-4	[3.0389e-4, 1.1650e-3]
South Asian	0/1280	0.0000e+0	[0.0000e+0, 2.8778e-3]
East Asian	0/4180	0.0000e+0	[0.0000e+0, 8.8212e-4]
Southeast Asian	0/2994	0.0000e+0	[0.0000e+0, 1.2313e-3]
Native American	2/6497	3.0783e-4	[3.7282e-5, 1.1116e-3]
Admixed	1/2189	4.5683e-4	[1.1566e-5, 2.5426e-3]
Oceania	0/96	0.0000e+0	[0.0000e+0, 3.7697e-2]
US Caucasian	2/3413	5.8599e-4	[7.0975e-5, 2.1152e-3]
US Asian	0/1285	0.0000e+0	[0.0000e+0, 2.8666e-3]
US Hispanics	1/2857	3.5002e-4	[8.8617e-6, 1.9486e-3]
African American	2/3158	6.3331e-4	[7.6706e-5, 2.2858e-3]

The last step is to determine if any of the Control Region observations were also part of the whole mtDNA genome dataset. If so, this allows us to subtract them from the Control Region observations as we know that when searching the whole mtDNA genome there was only one observation and therefore the others are not true to this haplotype. The final search is the Control Region plus a position in the coding region (e.g., 8860N). This will change the range to include positions beyond the Control Region and therefore search the whole mtDNA genome dataset.



This time, there was 1 observation in the U.S. Caucasian dataset. Since there were zero whole genome observations, we can subtract this one from the Control Region dataset resulting in 1 observation in 3413, or 1/3,413 (95% UCI 1/720). This method utilizes all of the information available in the database.



FAQ-23: What are the criteria for comparisons and conclusions?

Cross-reference Guideline 5.1

The laboratory must decide whether there is sufficient evidence to support an exclusion or an inclusion (cannot exclude) when comparing mtDNA sequences.

Due to heteroplasmy and mutations, it is possible to have differences between two mitochondrial DNA sequences from a common source or maternal lineage. As well, there might be limited sequence information available for comparison. Therefore, in some comparisons, there is insufficient evidence to support either an exclusion or an inclusion. In these situations, the results should be reported as inconclusive. No statistic is reported for inconclusive comparisons. Follow-up can include sequencing different sample types and/or different family reference samples and/or re-sequencing to obtain additional sequence information.

Historically, laboratories used the following criteria to make comparisons. Specific criteria should be established by each laboratory based upon validation studies.

Exclusion: If samples differ at two or more nucleotide positions (excluding length heteroplasmy), they can be excluded as coming from the same source or maternal lineage. A length variant alone cannot be used to support an interpretation of exclusion (Stewart et al. 2001).

Inconclusive: The comparison should be reported as inconclusive if samples differ at a single position only (regardless of whether they share a common length variant between positions 302-310). Length heteroplasmy alone is not a basis for an inconclusive interpretation.

Cannot Exclude (i.e. Inclusion): If samples have the same sequence or are concordant (sharing a common DNA base at every nucleotide position), they cannot be excluded as originating from the same source or maternal lineage.

FAQ-24: Do mutations or the genetic distance between relatives need to be considered when making comparisons?

Cross-reference Guideline 5.1.2

The guidelines stated above for exclusion, inconclusive, and cannot exclude may be modified by a laboratory based on validation to allow for increased mutational events in cases involving a closed population (e.g., a plane crash), or where the reference samples are from distant maternal relatives of the individual of interest (Connell 2022). The guidelines may also need to be modified when the sequences compared extend beyond the current standard ranges of HVI/HVII and the control region, as intra-individual variation has not yet been fully characterized for these regions (Strobl et al. 2019, Taylor et al. 2020, Cihlar et al. 2020a, Davidovic et al. 2020, Connell 2022).

FAQ-25: What is the counting method?

Cross-reference Guideline 6.4

When the mtDNA haplotype of a reference sample and an evidence sample cannot be excluded as potentially originating from the same source or lineage, the mtDNA haplotype is then searched in a population database to provide a statistical weight to the mtDNA association. The mtDNA frequency calculation performed is called the counting method. The counting method is used to report the prevalence of haplotypes in a sampling of a population.

Estimate of the population proportion:

$$p = x / n$$

where x is equal to the number of times the haplotype is observed in a database containing n number of haplotypes. For example, if a haplotype has been observed twice in a database of n = 2000, the frequency of that haplotype will be: 2/2000 = 0.001.

The mtDNA frequency estimate may be reported for all population groups together or as subpopulations. In addition, **an upper confidence limit (i.e., Clopper and Pearson) may be applied to the mtDNA frequency estimate obtained from the counting method.**

FAQ-26: What is the Clopper and Pearson 95% upper confidence limit?

Cross-reference Guideline 6.4.2

The Clopper and Pearson 95% upper confidence limit uses the binomial distribution for the probabilities of counts, including zero or other small numbers that are found for mtDNA **haplotypes. If the database has n haplotypes and *x* of the haplotype of interest are found, then the required upper confidence limit p_0 is the solution to the equation (see Eq. 1).**

Eq. 1

$$\sum_{k=0}^{x} \binom{n}{k} p_0^k (1-p_0)^{n-k} = \alpha$$

Here α gives the level of confidence: α = 0.05 gives a 95% confidence limit. The equation finds the value p_0 of the population proportion p for which the cumulative probability 0, 1, . . . x copies of the profile is equal to α. This equation will require a computer to solve. A special case of the result in Equation 1 is when the haplotype of interest is not seen in the database, and $x = 0$. The equation now has only one term in the sum on the left-hand side: $(1 - p_0)^n = \alpha$. The solution is illustrated in Equation 2.

Eq. 2

$$p_0 = 1 - \alpha^{1/n}$$

When applying a 95% confidence limit, this is very close to 3/n (e.g., if n = 2000 the exact 95% upper confidence limit is 0.0014967, whereas 3/2000 is 0.0015).

The upper confidence limit (Equation 1) can be estimated using the following formula[2] in various spreadsheet software packages:

=1-BETAINV(α,n-x,x+1)

Where α, x, and n are as described above. While this estimate makes use of a beta distribution, rather than the binomial distribution described for Equation 1, the resulting values are identical for all practical purposes.

[2] http://www.sigmazone.com/binomial_confidence_interval.htm

Typical Clopper and Pearson upper confidence interval p_0 values at $\alpha = 0.05$ for generic n and x values utilizing Equation 1 are provided below as examples.

x	$n = 500$	$n = 1000$	$n = 1{,}500$	$n = 2{,}000$	$n = 3000$
0	0.00597	0.00299	0.002	0.0015	0.001
1	0.00945	0.00473	0.00316	0.00237	0.00158
2	0.01254	0.00628	0.00419	0.00314	0.0021
3	0.01543	0.00774	0.00516	0.00387	0.00258
4	0.01821	0.00913	0.00609	0.00457	0.00305
5	0.02091	0.01048	0.007	0.00525	0.0035

Appropriate wording when reporting the Clopper and Pearson upper confidence limit includes "with 95% confidence this value is more conservative than the true value." Note that the use of the phrase "95% confidence" here does not imply the probability of the immediate value being more conservative is 0.95. Rather, it is an acknowledgment that in 95% of cases, the value given by this approach will be greater than the true value.

FAQ-27: Should population substructure be considered in the statistical calculations for mtDNA haplotype comparisons?

Cross-reference Guideline 6.2

Theta (θ) is a correction factor to account for substructure within a population and is most often used when calculating match probabilities of diploid autosomal markers. While it is recognized that population substructure exists for mtDNA haplotypes, using theta for haploid markers is still a topic of much debate. Haploid markers are not a primary means of identification and are most powerful when used for exclusionary purposes. Determination of an appropriate theta value is complicated by the lack of published datasets covering the various ranges that may be applied to

forensic casework. SWGDAM has not yet reached consensus on the appropriate statistical approach to estimating theta for mtDNA comparisons, and currently there are no published mtDNA theta values like those for autosomal STRs (Buckleton et al. 2016).

FAQ-28: Can the match probabilities from Y-STR, mtDNA, and/or autosomal STRs be combined into a match probability?

Cross-reference Guideline 6.5.

If there is reasonable expectation of genetic independence, match probabilities from any combination of mtDNA, Y-STR and/or autosomal STRs may be combined; however, the statistical subject matter experts have not yet reached consensus regarding the suitability of combining the likelihood ratios from lineage markers or combining an autosomal likelihood ratio with one or both lineage markers. Additional research examining such topics as independence of lineage markers and autosomal markers, the impact of combining partial profiles, and LR interpretation for combined systems is needed.

FAQ-29: Can the results of mtDNA analysis be reported as a likelihood ratio?

Cross-reference Guideline 6.4.3

Traditionally, mtDNA statistics have been reported in terms of an upper bound frequency estimate (UBFE, see FAQ 25/26). Alternatively, mtDNA statistics can be reported in terms of a likelihood ratio. Typically, this would simply be 1/UBFE. This is derived from the probability of observing the evidentiary haplotype if the person of interest is the source of the evidence (or maternally related to the source of the evidence) divided by the probability of observing the evidentiary haplotype if an unknown individual is the source of the evidence (UBFE).

FAQ-30: What are some examples of wording for reporting a frequency with an upper confidence limit?

Example when a 95% upper confidence limit is provided for each population group:

The mtDNA sequences[i] obtained from item 1 and XX are the same within the sequence range obtained in common to the samples. Therefore, XX cannot be excluded as the source of item 1.

Searching the CODIS mtDNA population database (CODIS 11.0, containing 10,629 individuals, searching positions 16024-16390, 49-408),[ii] the mtDNA sequence obtained from item 1 and XX has been observed as follows:[iii]

Population Group	Number of Observations	Number of Profiles in Population Group	Upper Bound Frequency Estimate
African-American	3	2449	0.32%
US Caucasian	1	2609	0.18%
US Hispanic	0	2576	0.12%

In addition to the population groups listed in the table, the population database search included a search of mtDNA sequences from individuals from China - unspecified region (168), China - Hong Kong (376), Japan (302), Korea (281), US Asian (645), Vietnam (187), US Native American (1036), with no observation of the queried sequence in these groups. The numbers in parentheses indicate the number of individuals in each population group.

Methods/Limitations:

The following methods and limitations apply to the results/conclusions provided in the results section(s) of this report and are referenced by number in the body of the text for clarity.

[i] DNA is extracted from each sample, and portions of the control region of the mtDNA are amplified using the polymerase chain reaction (PCR). The amplified regions are sequenced using fluorescent dye-labeled chemistry. The sequences obtained are aligned and compared to the revised Cambridge Reference Sequence (rCRS). Differences between the sample sequence and the rCRS are noted by nucleotide position and DNA base. The annotated profiles for all of the samples are then compared. Matching profiles may be searched against the CODIS mtDNA population database to provide an upper bound frequency estimate.

Mitochondrial DNA cannot be used to conclusively identify an individual because mtDNA is maternally inherited and all maternally-related individuals are expected

to have the same mtDNA profile. Also, unrelated individuals may have the same mtDNA profile within the sequenced range.

The following interpretations are possible for sequence comparisons:

CANNOT EXCLUDE: If the samples have the same sequence, or are concordant, they cannot be excluded as coming from the same source. Sequence concordance is defined as having a common DNA base at each position at which sequence data were obtained in the sample.

INCONCLUSIVE: If the samples differ at only a single nucleotide position, no conclusion can be reached as to whether they originate from the same source.

EXCLUSION: If the samples differ at two or more nucleotide positions, they are excluded as coming from the same source.

[ii] The range of sequence positions included in a database search is the one obtained in common for the samples.

[iii] The population database table(s) has been included to indicate how common or rare a sequence is expected to be in the general population. The upper bound frequency estimate is based on a 95% confidence interval and gives an estimate of the highest percentage of individuals in each population group expected to have the same profile as the referenced sample. Calculation of the upper bound frequency estimate is directly dependent upon the number of profiles in the population group; larger population group sizes will provide more refined upper bound frequency estimates. Mitochondrial DNA profiles were placed into population groups within the database based on self-identification by the donor. A searched haplotype may or may not appear in the population database or it may be observed within multiple groups in the database. Therefore, mtDNA cannot be used to identify the population group to which an individual belongs.

Additional example when a 95% upper confidence limit is provided for each population group:

The mtDNA sequences obtained from item X and Item Y are concordant[1]. Therefore, John Doe cannot be excluded as being a possible source of Item X.

[1] Concordant means that there is a common base at every position.

The following reporting format is used for results obtained from searches of pertinent Mitochondrial DNA Population Databases and the corresponding statistical calculations.

The mtDNA sequence obtained from Item X (base positions 15998-16390 and 49-402) was searched against the current SWGDAM Mitochondrial DNA Population Database resulting in the following observations for the three major U.S. population groups**:

Population Group	Observations	Database Size	Upper Bound Frequency Estimate (%)	Which equates to approximately
African American	*X*	*XXXX*	*X.XX*	*1 in XXX*
Caucasian				
Hispanic				
Additional population group(s) where sequence is observed				

Therefore, we wouldn't expect to see the mtDNA profile obtained from Item X more than once in XXX African Americans, once in XXX Caucasians or once in XXX Hispanics. (List additional populations groups as appropriate)

One of the following statements relevant to population database searches are put in the After Signature Panel of the Report Writing Template:

a) When a sequence is searched and is not observed in any population group other than the three major population groups listed.

**The above search results encompass the three major population groups. Data from searches of additional population groups is available upon request.

b) When a sequence is searched and is observed in one or more additional population group(s) in the database.

**The above search results encompass the three major population groups and all other population groups where one or more observation has occurred in the database. Data from searches of additional population groups is available upon request.

<u>Example when one 95% upper confidence limit is reported:</u>

The mtDNA haplotypes obtained from items **number** and **number** are concordant. Therefore, the individual represented by the unidentified human remains, item **number**, **<u>cannot be excluded</u>** as being a potential maternal relative of item **number**, **Reference Donor Name**.

> Utilizing the mtDNA population database currently available from the FBI Laboratory, the mtDNA haplotype obtained from item **number (16024-16365**, **73-340)** has been observed as follows: **x in 2,449** individuals of African American origin, **x in 1,959** individuals of Asian origin, **x in 2,609** individuals of Caucasian origin, **x in 2,576** individuals of Hispanic origin, and **x in 1,036** individuals of Native American origin. The mtDNA haplotype from item **number** may occur in as much as **0.xx%** of the three major U.S. populations (African American, Caucasian, and Hispanic) using a 95% confidence interval.[1]
>
> [1] MtDNA frequency data obtained via collaboration between EMPOP, AFDIL and FBI (Parson and Dör, FSIG 1 (2007): 88-92).

FAQ-31: What is an example of wording for reporting a likelihood ratio (LR)?

If a profile is entered into EMPOP and returns a result of 53 matching haplotypes out of a database of 15782 West Eurasian haplotypes, the 95% upper confidence limit would be 4.3904e-3. In a report comparing a maternal relative, this can be presented as an inverse value in the form of a likelihood ratio:

The genetic data (mtDNA) are approximately 227 times more likely to be observed if Sample XX originated from a maternal relative of the reference as opposed to if Sample XX originated from an unrelated individual from the West Eurasian population.

Nomenclature Examples

The following are examples of challenging phylogenetic alignments as described in Guideline 4.2.1 of the SWGDAM Interpretation Guidelines for Mitochondrial DNA (mtDNA) Analysis for Forensic DNA Testing Laboratories Document. The list of examples is not intended to be all inclusive.

The listed examples are organized by the range of the nucleotide positions for the observed DNA sequence, the corresponding sequence of the rCRS standard and sample for the given range and the phylogenetic alignment of the mtDNA haplotype as verified using EMPOP mtDNA database, v4/R13.

It is important to note that the phylogenetic alignment, and therefore the haplogroup assignment, could change depending on the length of the sequence searched. Practitioners should still use EMPOP to align the full sequence. **These short sequence examples are included for educational purposes only and should not be relied upon for interpretation.**

1. Range: np 16180-16196

rCRS AAAACCCCCTCC-CCATG
Sample AAAACCCCCCCCCTCATG

Phylogenetic alignment: 16189C 16191.1C 16192T

2. Range: np 16180-16196

rCRS AAAACCCCCTCC-CCATG
Sample AAAACCCCTCCCCTCATG

Phylogenetic alignment: 16188T 16189C 16191.1C 16192T

3. Range: np 16180-16196

rCRS AAAACCCCCTCCCCATG
Sample AACCCCCCTCCCCCATG

Phylogenetic alignment: 16182C 16183C 16188T 16189C

4. Range: np 16180-16196

rCRS AAAACCCCCTCCCCATG
Sample AAAACTCCCCCCC-ATG

Phylogenetic alignment: 16185T 16189C 16193DEL

5. Range: np 16180-16196

rCRS AAAACCCCCTCCCCATG
Sample AAAACCTCCCCCC-ATG

Phylogenetic alignment: 16186T 16189C 16193DEL

6. Range: np 16180-16196

rCRS AAAACCCCCTCCCC-ATG
Sample AAACACCCCCCCCCCATG

Phylogenetic alignment: 16183C 16184A 16189C 16193.1C

7. Range: np 16180-16196

rCRS AAAACCCCCTCCCCATG
Sample AAACACCCCCCCCCATG

Phylogenetic alignment:16183C 16184A 16189C

8. Range: np 16180-16196

rCRS AAAACCCCCTCCCC--ATG
Sample AAAACCCCCCTCCCCCATG

Phylogenetic alignment: 16189C 16190T 16193.1C 16193.2C

9. Range: np 16180-16196

rCRS AAAACCCCCTCCCC-ATG
Sample AAACCCCCTCCCCCCATG

Phylogenetic alignment: 16183C 16188T 16189C 16193.1C

10. Range: np 16180-16196

rCRS AAAACCCCCTCCCC--ATG
Sample AAAACCCCCCC-TCCCATG

Phylogenetic alignment: 16189C 16191DEL 16192T 16193.1C 16193.2C

11. Range: np 16180-16196

rCRS AAAACCCCCTCCCCATG
Sample AAAACCCCCCCCC-ATG

Phylogenetic alignment: 16189C 16193DEL

12. Range: np 16180-16196

rCRS AAAACCCCCTCCCC-ATG
Sample AAACCCCCCCCCCCCA-G

Phylogenetic alignment: 16183C 16189C 16193.1C 16195DEL

13. Range: np 16180-16196

rCRS AAAACCCCCTCC-CCATG
Sample AAGACCCCCCCCCTCATG

Phylogenetic alignment: 16182G 16189C 16191.1C 16192T

14. Range: np 16180-16196

rCRS AAAACCCCCTCCC-CATG
Sample AAAACCCCCCTCCCCATG

Phylogenetic alignment: 16189C 16190T 16193.1C

15. Range: np 16180-16196

rCRS AAAACCCCCTCCCCATG
Sample AAACCCCCCCCCC-CCG

Phylogenetic alignment: 16183C 16189C 16193- 16194C 16195C

16. Range: np 55-68

rCRS TATTTT-CGTCTGGG
Sample CACTTTTCGTCTGGG

Phylogenetic alignment: 55C 57C 60.1T

17. Range: np 55-68

rCRS TATTTT-CGTCTGGG
Sample TATCTTTCGTTTGGG

Phylogenetic alignment: 58C 60.1T 64T

18. Range: np 55-72

rCRS T-ATTTTCGTCT-GGGGGGT
Sample TTATT--CGTCTTTGGGGGT

Phylogenetic alignment: 55.1T 59DEL 60DEL 65.1T 66T

19. Range: 55-72

rCRS T-ATTTTCGTCTGGGGGGT

Sample TTACTCTCGTCTGGGGGGT

Phylogenetic alignment: 55.1T 57C 59C

20. Range: 55-72

rCRS TATTTT--CGTCTGGGGGGT
Sample TATCTTTTCGT--AGGGGGT

Phylogenetic alignment: 58C 60.1T 60.2T 64- 65- 66A

21. Range: 55-72

rCRS TATTTTCGTCTGGGGGGT
Sample CGTTTTCGTCTGGGGG-T

Phylogenetic alignment: 55C 56G 71DEL

22. Range: 55-72

rCRS T-ATTTTCGTCTGGGGGG-T
Sample TTATT--CGTCTGGGGGGGT

Phylogenetic alignment: 55.1T 59- 60- 71.1G

23. Range: 55-67

rCRS T-ATTTTCGTCT-GG
Sample TTATT--CGTCTTTG

Phylogenetic alignment: 55.1T 59- 60- 65.1T 66T

24. Range: np 57-70

rCRS TTTTCGTCTGGGGG
Sample TTTTCGCTCGGGGG

Phylogenetic alignment: 63C 64T 65C

25. Range: np 240-255

rCRS AACAATTGAATGTCTG
Sample AACAATTAA-TGTCTG

Phylogenetic alignment: 247A 249DEL

26. Range: np 291-295

rCRS A----------------TTTC
Sample AACATCATAACAAAAAATTTT

Phylogenetic alignment: 291.1A 291.2C 291.3A 291.4T 291.5C 291.6A 291.7T 291.8A 291.9A 291.10C 291.11A 291.12A 291.13A 291.14A 291.15A 291.16A 295T

27. Range: np 302-317

rCRS ACCCCCCCTCCCCCGC
Sample ACCCCCCCCCCCC-GC

Phylogenetic alignment: 310C 315DEL

28. Range: 302-316

rCRS ACCCCCCC--TCCCCC-G
Sample ACCCCCCTCCTCCCCCCG

Phylogenetic alignment: 309T 309.1C 309.2C 315.1C

29. Range: 303-315

rCRS CCCCCCCTCCCCC
Sample CCCCCCCCCCC--

Phylogenetic alignment: 310C 314- 315-

30. Range: 303-316

rCRS CCCCCCC-TCCCCC-G
Sample CCCCCCCCTCTCCCCG

Phylogenetic alignment: 309.1C 312T 315.1C

31. Range: 309-316

rCRS C----TCCCCC----G
Sample CCCCCTCCTCCCCCCG

Phylogenetic alignment: 309.1C 309.2C 309.3C 309.4C 313T 315.1C 315.2C 315.3C 315.4C

32. Range: 303-316

rCRS CCCCCCCTCCCCC--G
Sample CCCCCCCTTCCCCCCG

Phylogenetic alignment: 311T 315.1C 315.2C

33. Range: np 309-316

rCRS CTCCCCC---G
Sample CTCTCCCCCCG

Phylogenetic alignment: 312T 315.1C 315.2C 315.3C

34. Range: np 570-580

rCRS CCCC-ACAGTTT
Sample CCCCCCCAGTTT

Phylogenetic alignment: 573.1C 574C

35. Range: np 570-580

rCRS CCCCACAGTTT
Sample CCC-CCAGTTT

Phylogenetic alignment: 573DEL 574C

36. Range: np 8270-8292
rCRS CACCCCCTCTACCCCCTCTAGAG
Sample TACCCCCTCTA---------GAG

Phylogenetic alignment: 8270T, 8281DEL, 8282DEL, 8283DEL, 8284DEL, 8285DEL, 8286DEL,8287DEL, 8288DEL, 8289DEL

Glossary

(for use with this Supplemental Information and the SWGDAM Interpretation Guidelines for MtDNA Analysis by Forensic DNA Testing Laboratories only)

See also *Addendum to the SWGDAM Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories to Address Next Generation Sequencing (approved April 23, 2019).*

Heteroplasmy: The presence of more than one type of mtDNA genome within a cell or individual.

Indel: abbreviation for insertion/deletion used to describe a location in an alignment of two sequences where an insertion or deletion of one or more bases is required to maintain alignment.

Next Generation Sequencing or NGS: Is the simultaneous sequencing of millions of DNA molecules that are localized onto solid substrates such as particles or flow cells. NGS is also known as massively parallel sequencing, deep sequencing, high throughput sequencing, and second-generation sequencing.

NUMT (nuclear mitochondrial insertion): also known as pseudogene, this describes pieces of the mitochondrial genome that have been integrated into the nuclear genome.

Phylogenetic alignment: The process of arranging a mtDNA haplotype in an evolutionary relationship to other haplotypes.

Polyclonal: In emulsion PCR, a polyclonal reaction is one that contains two or more DNA fragments and therefore sequences as a mixed signal.

Quality scores (Q-scores): a metric that is used to indicate whether a base has been called correctly. Specifically, it is the probability that a given base has been miscalled. Mathematically, it is defined as -10log10(e), where e is the estimated probability of the base call being incorrect. Higher Q scores indicate a lower probability of base-calling error, while lower Q scores indicate a higher probability of error.

rCRS (revised Cambridge Reference Sequence): A corrected version of the first human mtDNA genome sequenced and published. See Anderson (1981) and Andrews (1999). Sequence data are aligned to the rCRS sequence and the collection of differences as compared to the rCRS constitute a sample's mtDNA haplotype.

Read depth: The number of reads that align at a given DNA location.

Strand bias: When performing paired-end sequencing, strand bias refers to any directional bias. Ideally, every nucleotide would have the same number of forward and reverse reads. While strand bias can, under certain circumstances, indicate reduced support for the affected nucleotide calls, in some assays, and in particular genomic regions, only one strand is routinely sequenced. As forward/reverse balance can be used as a quality metric, expectations for strand balance should be established during validation.

Variant count: The number of reads that contain a given variant.

Variant frequency: The percent of reads that contain a given variant.

Variant quality: Some analysis software will provide a quality score for a variant call. Some of the factors considered for this score can include frequency, strand bias, known artifact positions, read length, and number of variants within the same read.

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