

# Scientific Working Group on DNA Analysis Methods

## Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories



### SWGDAM Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories

---

---

#### Contents

Introduction.....	2
1. Evaluation of Controls.....	2
2. Sequence Analysis and Nomenclature.....	4
3. Sequence Comparison and Reporting Results.....	14
4. Weight of Evidence.....	15
5. References.....	19
6. Additional Information.....	23

---

---

The Scientific Working Group on DNA Analysis Methods, better known by its acronym of SWGDAM, is a group of scientists representing Federal, State, and Local laboratories in the United States and Canada. During meetings, which are held twice a year, Committees discuss topics of interest to the forensic DNA community and

often develop documents to provide direction and guidance for the community. Revisions to these guidelines to address next generation sequencing were approved by the SWGDAM membership on April 23, 2019.

This document provides guidelines for the interpretation of mitochondrial DNA typing results developed by either Sanger or Next Generation Sequencing (NGS) technologies and supersedes the Scientific Working Group on DNA Analysis Methods (SWGDAM) Interpretation Guidelines for Mitochondrial DNA (mtDNA) Analysis by Forensic DNA Testing Laboratories (2013).

Laboratories are encouraged to review their standard operating procedures and validation data in

light of these guidelines and to update their procedures as needed. It is anticipated that these guidelines will evolve further as future technologies emerge.

## **Introduction**

Mitochondrial DNA (mtDNA) sequence interpretation in forensic casework is an essential stage of analysis that results in the generation of mtDNA profiles typically used for comparisons, frequency estimates, and entry into pertinent databases. Since the release of the initial version of the SWGDAM Guidelines for Mitochondrial DNA (mtDNA) Nucleotide Sequence Interpretation (*Forensic Science Communications, April 2003*), newly implemented quality control methods and additional resources have become more widely available that provide assistance in the evaluation of mtDNA profiles and population databases (e.g., phylogenetic assessments). Information on these resources and methods was detailed in the previous (2013) version of this document, and is maintained here. Additionally, over the past several years, next-generation sequencing (NGS) methods have been evaluated for forensic application and been shown to be well-suited for mtDNA analysis (*Holland et al. 2011, Parson et al. 2013, King et al. 2014, Chaitanya et al. 2015, Parson et al. 2015, Seo et al. 2015, Peck et al. 2016, Marshall et al. 2017, Peck et al. 2018*). As a result, and with this document, SWGDAM is providing revised interpretation guidelines to more accurately reflect the current state of the field and to better serve forensic mtDNA practitioners. Development and implementation of written guidelines for the interpretation of analytical results for each individual laboratory still remains necessary and must be based on validation studies conducted pursuant to the FBI Quality Assurance Standards for Forensic DNA Testing Laboratories, with consideration given to the SWGDAM Validation Guidelines.

### **1. Evaluation of controls**

For data to be of requisite quality for interpretation, measures should be established to demonstrate that the testing performed as expected. The use of controls is among the most important quality measures for DNA testing. Controls shall include, at a minimum, a positive

amplification control, a positive sequencing control (if not the same as the positive amplification control), a negative amplification control, and a reagent blank control. Evaluation criteria must be established for each control and all controls shall be treated the same as, and parallel to the forensic and/or casework sample(s) being analyzed, as outlined in the FBI Quality Assurance Standards for Forensic DNA Testing Laboratories.

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. Contamination is the unintentional introduction of exogenous DNA into a DNA sample or PCR reaction. 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 must be assessed to ensure that any results for the corresponding sample(s) are legitimately from the samples and not due to contamination. If contamination in the reagent blank and/or negative amplification control is present above laboratory established acceptance parameters, then the data cannot be used for interpretative purposes.

Positive controls should be used to monitor the success of each step of the laboratory process. A single positive control, of known mtDNA sequence, may be processed starting at amplification and used throughout the process to monitor each step. Alternatively, different positive controls may be used to monitor the success of different steps of the process. In either case, the positive control should be processed concurrently with the samples being typed using the same reagents and/or instruments. The expected performance and/or sequence of the positive control(s) used for these purposes should be well-characterized, and detailed in validation or other documentation. In addition, the laboratory should have and follow procedures for evaluating the success of each step of the process.

To minimize the introduction of contamination during testing, a laboratory shall implement sample handling procedures and quality control practices designed for this purpose (see

SWGDM Contamination Prevention and Detection Guidelines for Forensic DNA

Laboratories). Methods shall be in place to monitor contamination within the laboratory. A laboratory shall verify that all control results meet the laboratory's interpretation guidelines for all reported results. In addition, a laboratory shall have and follow policies and/or procedures that are supported by validation studies for interpreting data potentially affected by contamination.

For interpretation to proceed in the event of a contaminated control, the existence of such contamination must not render the results of the corresponding sample(s) unreliable. At a minimum, a haplotype(s) obtained from a contaminant observed in a reagent blank and/or negative amplification control must not be concordant with a haplotype obtained from a corresponding sample(s). In addition to this minimum criterion, a laboratory may choose to assign a maximum threshold based on the amount of contaminant DNA observed (e.g., *Wilson et al. 1995b*). All such thresholds shall be supported by internal validation studies. Depending on the extent of the observed contamination, reanalysis may be initiated, starting with the appropriate step of the process. If reanalysis is not possible, the reliability of the results from the affected sample(s) should be critically assessed for use. Furthermore, access to the DNA profiles of laboratory personnel is helpful when attempting to trace potential sources of contamination.

## **2. Sequence Analysis and Nomenclature**

### **2.1 Sequencing Overview**

The regions of the mtDNA genome that are typically targeted for evidentiary testing are hypervariable region 1 (HV1- positions 16024-16365) and hypervariable region 2 (HV2 - positions 73-340) located within the control region. However, because the discrimination power of mtDNA data is dependent on the reported sequence range, it is beneficial to sequence more than HV1 and HV2. Regions targeted for testing should be determined by individual laboratories and be based on laboratory policy. Due to expected differences in the mtDNA quality (i.e., the state of degradation and the quantity of the mtDNA present) between evidence samples and known samples, amplification and/or sequencing strategies specific to

the sample type should be employed. Ideally, forward and reverse strands of the amplified product are sequenced to reduce ambiguities in base determination. When overlapping ranges or reads exist between separate amplifications of the same sample, these regions should be examined carefully for sequence consistency. Sequence differences in overlapping regions, as well as discrepancies in expected amplicon quantities, could indicate primer binding issues and should be interpreted with caution. It is important for each laboratory to determine sample sequence quality and read depth requirements for interpretation. In Sanger sequencing workflows, additional sequencing approaches should be implemented for samples containing common homopolymeric regions such as those that may occur in the HV1 Cytosine-stretch (C-stretch) region (between positions 16183-16194) and the HV2 C-stretch region (between positions 302-310) as these motifs can prove to be challenging. Suggested primers and processing strategies 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*).

## **2.2 Sequence Analysis**

### **2.2.1 Criteria**

A laboratory shall have and follow written guidelines for the interpretation of data that are supported through its validation. The laboratory should establish criteria to assign nucleotide base calls and to determine whether the results are of sufficient quality for interpretation purposes.

To accomplish this with Sanger data, the overall quality of the electropherogram data should be first assessed. An alignment of overlapping sequences is then performed using software programs specifically designed for this purpose. The heavy strand sequences are reverse-complemented so that the bases are aligned in the light strand orientation. Strands are compared and bases are designated. Criteria that may be considered in the designation of bases include relative fluorescence units, sequence background noise, peak resolution

and shape, and amplification and/or sequencing artifacts, for example (*Pont-Kingdon et al. 2012, Ellard et al. 2016*).

For NGS data, reads are filtered, trimmed and aligned by analysis software. The criteria used for filtering or trimming of reads should be established by individual laboratories and be supported by validation data and/or described in software literature. To determine the suitability of sequence data for analysis and interpretation, metrics including but not limited to read coverage, 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.

### 2.2.2 DNA Base Call Designations

DNA bases are designated by the nomenclature system set forth by the International Union of Pure and Applied Chemistry (IUPAC). At confirmed positions of ambiguity, the following 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

Base call designations for insertions and deletions are described in section 2.3.3.

### 2.2.3 Heteroplasmy

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, which typically is observed as overlapping sequences with a variation in the number of bases in a homopolymeric stretch of bases (i.e., C-stretch). Each laboratory should define heteroplasmy within the operational limits of the system used for sequencing and across the range of the region sequenced. When the specimens under consideration differ by a laboratory-defined number of nucleotides across the region sequenced, additional samples may be run in order to attempt to resolve the difference.

### 2.2.4 Mixtures

Sanger-based mitochondrial DNA mixed sequences are not commonly interpreted. Regardless, a laboratory employing Sanger-based sequencing shall have and follow documented procedures for mixture interpretation of Sanger-based data that are supported by its validation.

Given the quantitative nature of NGS data, it may be possible to perform interpretation on samples representing mixtures of two or more individuals (*Holland et al. 2011, Kim et al. 2015, Vohr et al. 2017, Churchill et al. 2018*). If a laboratory intends to perform mtDNA mixture interpretation with NGS, validation studies performed according to the SWGDAM Validation Guidelines, and that fully characterize the limitations of the analysis for this purpose, shall be performed. These studies should establish the laboratory's protocol for mtDNA mixture interpretation and include experiments that address criteria for distinguishing among mixture, heteroplasmy, damage and noise.

## 2.3 Sequence Nomenclature

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 commonly used. The use of standardized nomenclature principles to determine the mtDNA sequence 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 potential issue.

### 2.3.1 Use of a Sequence Reference Standard

A consensus sequence obtained from the sample is compared to the revised Cambridge Reference Sequence (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.

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

There are 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 (see the SWGDAM mtDNA Nomenclature Examples document). 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 these 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. Until string searches are possible, a nomenclature system that is easy to apply and for which readily-available tools exist is vital.

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,

**SWGDAM MtDNA Interpretation Guidelines – Approved 04/23/2019**

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		
Rule-Based	SWGDAM Guidelines	EMPOP v4 release 11
16182DEL 16183C 16193.1 C 16217C 73G 263G 309.1C 315.1C	16182C 16183C 16188T 16189C 16217C 73G 263G 309.1C 315.1C	16182C 16183C 16188T 16189C 16217C 73G 263G 309.1C 309.2C 315.1C
In this example the rule-based and SWGDAM haplotypes represent the same nucleotide sequence string, yet are coded 3 differences away from each other (ignoring the 16193.1C). When queried in EMPOP (string-based) – an exact match is found. The rule-based haplotype compared to the EMPOP haplotype results in the same 3 differences, whereas the SWGDAM haplotype compared to the EMPOP haplotype results in 0 differences (ignoring length differences at 309).		
Example 2: np 16024-16365, 73-340		
Rule-Based	SWGDAM Guidelines	EMPOP v4 release 11
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	16111T 16189C 16191.1C 16192T 16223T 16233G 16290T 16319A 16331G 73G 146C 153G 235G 263G 315.1C
In this example the rule-based and SWGDAM haplotypes represent the same nucleotide sequence string, yet are coded 3 differences away from each other. When queried in EMPOP (string-based) – an exact match is not found, but the estimated alignment ( <i>Huber et al. 2018</i> ) is consistent with the SWGDAM notation.		
Example 3: np 16024-16365, 73-340		
Rule-Based	SWGDAM Guidelines	EMPOP v4 release 11
16129A 16172C 16184T 16187T 16189C 16223T 16261T 16278T 16290T	16129A 16172C 16184T 16187T 16189C 16223T 16261T 16278T 16290T 16293G	16129A 16172C 16184T 16187T 16189C 16223T 16261T 16278T 16290T

**SWGDAM MtDNA Interpretation Guidelines – Approved 04/23/2019**

16293G 16311C 16360T 73G 150T 151T 152C 182T 186A 189C 195C 247DEL 263G 297G 315.1C 316A	16311C 16360T 73G 150T 151T 152C 182T 186A 189C 195C 247A 249DEL 263G 297G 315.1C 316A	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 SWGDAM haplotypes represent the same nucleotide sequence string, yet are 3 differences away from each other. When queried in EMPOP (string-based) – an exact match is not found, but the estimated alignment (*Huber et al. 2018*) is consistent with the SWGDAM notation.

Given the issues highlighted above, it is recommended that a blended application of rule-based and phylogenetic approaches be used. This blended approach allows for the forensic scientist to recognize and maintain known patterns of polymorphisms. For those rare sequences containing peculiar insertions and deletions, it is recommended that the forensic analyst use the EDNAP mtDNA Population Database (EMPOP) (<http://empop.org/>) to help determine a consistent mtDNA haplotype for entry into forensic databases. It is noted that these rules (noted below) do not affect previous forensic case comparisons where a forensic sample was directly compared to a submitted known reference sample. However, caution should be exercised when comparing newly interpreted samples to those interpreted using former approaches.

### 2.3.3 SWGDAM Nomenclature Rules

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 [empop.org](http://empop.org).

## SWGDAM MtDNA Interpretation Guidelines – Approved 04/23/2019

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

**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 HV1 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 HV2 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.

Insertions are described by noting the site immediately prior to the insertion with respect to the light strand of the 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’, depending on the preference of the laboratory or the requirements of the target database.

EMPOP queries are performed with alignment-free nucleotide sequence strings. In the output, however, the haplotype is presented in difference coded format using the phylogenetic alignment.

Limited sequences obtained from degraded samples may not yield enough sequential information to identify and maintain known patterns of polymorphisms when applying the nomenclature rules. With a limited sequence and the inability to apply Rule 1, the analyst should apply the remaining rules to best determine the proper nomenclature for the observed sequence. The analysts can still use EMPOP to assess the proper sequence determination. However, if the sequence information is so limited in range that the software provides multiple nomenclature options for the same string of bases, the analyst should defer to the haplotype which is consistent with the remaining rules (Rule 2 through Rule 9).

#### 2.3.4 Length Variants

Homopolymeric tracts are prone to exhibiting length heteroplasmy. Homopolymeric tracts can differ in length within the same individual and/or maternal lineage. In most cases, no attempt will be made to determine the exact number of bases in an HV1 C-stretch; however, laboratories must develop their own interpretation guidelines for HV2 length variants. A length variant alone cannot be used to support an interpretation of exclusion (*Stewart et al. 2001*).

In NGS workflows, laboratory guidelines for the interpretation of C-stretches should be established with consideration of the analysis software employed. Different software programs may not necessarily align homopolymeric (and other) repeat regions uniformly or according to standard forensic practice (see Rules 1, 7 and 8 above). Thus, further review of C-stretch alignments may be warranted to ensure appropriate variant calling metrics are met. Validation studies should characterize the performance of the analysis software and its handling of repeats to establish interpretation guidelines for these regions.

#### 2.3.5 Database Searching

Whether the developed mtDNA haplotype is being used to conduct a forensic database search for an association (e.g., missing person investigation) or a population database search to determine the haplotype frequency (see Section 4. Weight of Evidence), the search is performed on the basis of the determined 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.* (2010; update in Huber *et al.* 2018) demonstrates that the application of a string-based search algorithm ensures that identical sequences are associated in a database query. Future versions of forensic and population databases used for the comparison of mtDNA profiles should incorporate this functionality.

### **3. Sequence Comparison and Reporting Results**

Each laboratory must define conditions under which the sequence data obtained would lead to the conclusion that an individual can or cannot be eliminated as a possible source of the mtDNA

evidence in a case. This may be accomplished by an examination of the number, position, and nucleotide composition of polymorphic sites.

### **3.1 Sequence Comparisons**

Generally, the following guidelines are used for comparisons of control region data:

- 3.1.1 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.
- 3.1.2 Inconclusive: The comparison should be reported as inconclusive if samples differ at a single position only (whether or not they share a common length variant between positions 302-310).
- 3.1.3 Cannot Exclude: If samples have the same sequence, or are concordant (sharing a common DNA base at every nucleotide position), they cannot be excluded as coming from the same source or maternal lineage.

### **3.2 Additions and/or Modifications**

Laboratories should develop guidelines for the evaluation of cases that involve heteroplasmy. The guidelines stated above may need to be modified by a laboratory 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. The guidelines may also need to be modified when the sequences compared extend beyond the current standard ranges of HV1/HV2 and the control region, as intra-individual variation has not yet been fully characterized for these regions.

#### 4. Weight of Evidence

The mtDNA profile of a reference sample and an evidence sample that cannot be excluded as potentially originating from the same source should be searched in a population database in order to provide a statistical weight to a reported conclusion of “cannot exclude”. By searching an appropriate and established population database, a point estimate of the true population frequency for a haplotype can be obtained (i.e., the counting method). Scientifically, it is also valuable to provide a confidence interval for the population frequency estimate.

##### 4.1 Population Databases

Currently, the SWGDAM Mitochondrial DNA Population Database available to CODIS users is an appropriate database for assessing the relative frequency of mtDNA haplotypes within the United States using rule-based nomenclature practices. The EMPOP database is available to both public and private forensic laboratories, utilizes the applied nomenclature of Section 2.3.3 and contains representative populations for the United States. In either case, laboratories should ensure that any population database used for casework is representative of the appropriate population(s), of an appropriate size, and employs quality measures to assess the data entered into the database (e.g., limited ambiguities, minimum range of HV1 and HV2, phylogenetic assessment).

##### 4.2 Frequency Calculations

- 4.2.1 The basis for the mtDNA haplotype frequency estimation is the counting method. The application of a confidence interval accounts for database size and sampling variation.
- 4.2.2 The mtDNA haplotype sample frequency ( $p$ ) is calculated using the  $p = x/n$  formula, 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:  $2/2000 = 0.001$ .

- 4.2.3 Reporting an mtDNA haplotype sample frequency without a confidence interval is acceptable as a factual statement regarding observations in the database.
- 4.2.4 In order to provide an upper confidence limit for the frequency of the mtDNA haplotype in the population, the method described by *Clopper and Pearson (1934)* should be used. This 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

Eq. 1

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

Here  $\alpha$  gives the level of confidence:  $\alpha = 0.05$  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  $\alpha$ . 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

Eq. 2

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

and for a 95% confidence limit this is very close to  $3/n$ . (If  $n = 2000$  the exact 95% upper confidence limit is 0.0014967, whereas  $3/2000$  is 0.0015.)

4.2.4.1 The upper confidence limit (Equation 1) can be estimated using the following formula<sup>1</sup> in various spreadsheet software packages:

$$=1-\text{BETAINV}(\alpha,n-x,x+1)$$

Where  $\alpha$ ,  $x$ , 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.

4.2.5 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.

	$n = 500$	$n = 1000$	$n = 1,500$	$n = 2,000$	$n = 3000$
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
$x$ 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 interval 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.

<sup>1</sup> [http://www.sigmazone.com/binomial\\_confidence\\_interval.htm](http://www.sigmazone.com/binomial_confidence_interval.htm)

### **4.3 Population Substructure**

It is recognized that population substructure exists for mtDNA haplotypes. However, determination of an appropriate theta ( $\theta$ ) value is complicated by the variety of primer sets, covering different portions of HV1 and/or HV2, which may be applied to forensic casework. SWGDAM has not yet reached consensus on the appropriate statistical approach to estimating  $\theta$  for mtDNA comparisons.

### **4.4 Combining Statistics for mtDNA and Autosomal Results**

The frequency estimates for autosomal and mtDNA typing results obtained for a given sample may be combined. There are examples of dependencies between autosomal and mtDNA profiles, the extent of which has been demonstrated to be small (*Roby et al. 2009*).

- 4.4.1 A haplotype frequency estimation for the mtDNA profile is to be multiplied by the autosomal profile frequency as defined by National Research Council (1996) Recommendation 4.1.

### **4.5 Combining Statistics for mtDNA and Y-STR Results**

The CODIS software generates a combined likelihood ratio for autosomal, mtDNA and Y-STR results for missing person searches to rank potential candidates. *Ge et al. (2010)* and *Roby et al. (2009)* provide evidence of statistical independence between mtDNA and Y-STR profiles in U.S. and Chilean populations. Prior to reporting combined statistics for mtDNA and Y-STR results, the laboratory issuing the report should determine that each population used demonstrates independence between the mtDNA and Y-STR results. If independence cannot be determined between the mtDNA and Y-STR results for the referenced population(s), combining these systems is not recommended.

## 5. References

Andrews, R. M., Kubacka, I., Chinnery, P. F., Lightowers, R. N., Turnbull, D. M., and Howell, N. Reanalysis and revision of the Cambridge Reference Sequence for human mitochondrial DNA, *Nature Genetics* (1999) 23:147.

Aziz N, Zhao Q, Bry L, Driscoll D, Funke B, Gibson J, Grody W, Hegde M, Hoeltge G, Leonard D, Merker J, Nagarajan R, Palicki L, Robetorye R, Schrijver I, Weck K, Voelkerding K. College of American Pathologists' laboratory standards for next-generation sequencing clinical tests. *Arch Pathol Lab Med* (2015) 139:481-493.

Bandelt, H.J. and Parson, W. Consistent treatment of length variants in the human mtDNA control region: a reappraisal. *International Journal of Legal Medicine* (2008) 122:11-21.

Berger, C. and Parson W. Mini-midi-mito: Adapting the amplification and sequencing strategy of mtDNA to the degradation state of crime scene samples. *Forensic Science International: Genetics* (2009) 3:149–153.

Budowle, B., Polanskey, P., Fisher, C., Den Hartog, B., Kepler, R. and Elling, J. Automated Alignment and Nomenclature for Consistent Treatment of Polymorphisms in the Human Mitochondrial DNA Control Region. *Journal of Forensic Science* (2010) 55(5):1190-1195.

Chaitanya L, Ralf A, van Oven M, Kupiec T, Chang J, Lagacé R, Kayser M. Simultaneous whole mitochondrial genome sequencing with short overlapping amplicons suitable for degraded DNA using the Ion Torrent Personal Genome Machine. *Human Mutation* (2015) 36(12):1236-1247.

Churchill JD, Stoljarova M, King JL, Budowle B. Massively parallel sequencing-enabled mixture analysis of mitochondrial DNA samples. *International Journal of Legal Medicine* (2018) 132(5):1263-1272.

Clopper, C.J. and Pearson, E. S. The use of confidence or fiducial limits illustrated in the case of the Binomial. *Biometrika* (1934) 26:404-413.

Eichmann C, Parson W. 'Mitominis': multiplex PCR analysis of reduced size amplicons for compound sequence analysis of the entire mtDNA control region in highly degraded samples. *International Journal of Legal Medicine* (2008) 122(5):385-8.

Edson S, Ross J, Coble M, Parson T, Barritt, S. Naming the dead – confronting the realities of rapid identification of degraded skeletal remains. *Forensic Science Review* (2004)16(1):64-90

Ellard S, Charlton R, Yau S, Gokhale D, Taylor G, Wallace A, Ramsden S, Berry I. Practice guidelines for Sanger sequencing analysis and interpretation. *Association for Clinical Genetic Science* (2016)

Gabriel M, Huffine E, Ryan J, Holland M, Parsons T. Improved mtDNA sequence analysis of forensic remains using a “mini-primer set” amplification strategy. *Journal of Forensic Science* (2001) 46(2): 247-253.

Gargis, A. S., Kalman, L., Berry, M. W., Bick, D. P., Dimmock, D. P., Hambuch, T., ... Lubin, I. M. Assuring the quality of next-generation sequencing in clinical laboratory practice. *Nature Biotechnology* (2012) 30(11):1033-1036.

Ge, J., Chakraborty, R., and Budowle, B. Test of independence in contingency tables of large dimension with ordered categories and its application in population genetics. *Abstract Volume of the American Society of Human Genetics 60<sup>th</sup> Annual Meeting, Washington DC (November 2-6, 2010; Abstract # 3045/F).*

Holland MM, Mcquillan M, O’hanlon KA. Second generation sequencing allows for mtDNA mixture deconvolution and high resolution detection of heteroplasmy. *Croat Med J* (2011) 52:299-313.

Huber N, Parson W, Dür A. Next generation database search algorithm for forensic mitogenome analyses. *Forensic Sci Int: Genet* (2018) 37:204-214.

Kim H, Erlich H, Calloway C. Analysis of mixtures using next generation sequencing of mitochondrial DNA hypervariable regions. *Croat Med J* (2015) 56:208-217.

King JL, Laur BL, Movroski NM, Stoljarova M, Seo SB, Zeng X, Warshauer DH, Davis CP, Parson W, Sajantila A, Budowle B. High-quality and high-throughput massively parallel sequencing of the human mtGenome using the Illumina MiSeq. *Forensic Sci Int: Genet* (2014) 12:128-135.

Marshall C, Sturk-Andreaggi K, Daniels-Higginbotham J, Oliver RS, Barritt-Ross S, McMahon TP. Performance evaluation of a mitogenome capture and Illumina sequencing protocol using non-probative, case-type skeletal samples: Implications for the use of a positive control in a next-generation sequencing procedure. *Forensic Sci Int: Genet* (2017) 31:198-206.

Parson W, Strobl C, Huber G, Zimmerman B, Gomes S, Souto L, Fendt L, Delpont R, Langit R, Wootton S, Lagacé R, Irwin J. Evaluation of next generation mtGenome sequencing using the Ion Torrent Personal Genome Machine (PGM). *Forensic Sci Int: Genetics* (2015) 7:543-549.

Parson W, Huber G, Moreno L, Madel MB, Brandhagen MD, Nagl S, Xavier C, Eduardoff M, Callaghan TC, Irwin JA. Massively parallel sequencing of complete mtGenomes from hair shaft samples. *Forensic Sci Int: Genetics* (2015) 15:8-15.

Peck M, Brandhagen M, Marshall C, Diegoli T, Irwin J, Sturk-Andreaggi K. Concordance and reproducibility of a next generation mtGenome sequencing method for high-quality samples using the Illumina MiSeq. *Forensic Sci Int Genet* (2016) 24:103-111

Peck MA, Sturk-Andreaggi K, Thomas JT, Oliver RS, Barritt-Ross S, Marshall C. Developmental validation of a Nextera XT mitogenome Illumina MiSeq sequencing method for high-quality samples. *Forensic Sci Int Genet* (2018) 34:25-36.

Polanskey D., Den Hartog B. K., Elling J. W., Fisher C. L., Kepler R. B., Budowle B. Comparison of Mitotyper Rules and Phylogenetic-based mtDNA Nomenclature Systems. *Journal of Forensic Science* (2010) 55(5):1184-1189.

Pont-Kingdon G, Gedge F, Wooderchak-Donahue W, Schrijver I, Weck K, Kant J, Oglesbee D, Bayrak-Toydemir P, Lyon E. Design and Analytical Validation of Clinical DNA Sequencing Assays. *Arch Pathol Lab Med.* (2012) 136:41-46.

Rehm H, Bale S, Bayrak-Toydemir P, Berg J, Brown K, Deignan J, Friez M, Funke B, Hegde M, Lyon E; for the Working Group of the American College of Medical Genetics and Genomics Laboratory Quality Assurance Committee. ACMG clinical laboratory standards for next-generation sequencing. *Genet Med* (2013) 15:733-747.

Roby, R.K., Gonzalez, S.D., Phillips, N.R., Planz, J.V., Thomas, J.L., Pantoza-Astudillo, J.A., Ge, J., Morales, E.A., Eisenberg, A.J., Chakraborty, R., Bustos, and Budowle, B. Autosomal STR allele frequencies and Y-STR and mtDNA haplotypes in Chilean sample populations. *Forensic Science International Genetics, Supplemental Series* (2009) 2:533-534.

Röck, A., Irwin, J, Dür, A., Parsons, T., Parson, W. SAM: String-based sequence search algorithm for mitochondrial DNA database queries. *Forensic Science International: Genetics* (2011) 5(2);126-132.

Scientific Working Group on DNA Analysis Methods (SWGDM). Guidelines for Mitochondrial DNA (mtDNA) Nucleotide Sequence Interpretation. Forensic Science Communications (2003) [Online]. Available: <http://www.fbi.gov/about-us/lab/forensic-science-communications/fsc/april2003/swgdammitodna.htm/>

Seo SB, Zeng X, King JL, Larue BL, Assidi M, Al-Qahtani MH, Sajantila A, Budowle B. Underlying data for sequencing the mitochondrial genome with the massively parallel sequencing platform Ion Torrent™ PGM™. BMC Genomics (2015) 16(Suppl 1): S4.

Stewart, J. E. B., Fisher, C. L., Aagaard, P. J., Wilson, M. R., Isenberg, A. R., Polansky, D., Pokorak, E., DiZinno, J. A., and Budowle, B. Length variation in HV2 of the human mitochondrial DNA control region. Journal of Forensic Sciences (2001) 46:862-870.

Vohr SH, Gordon R, Eizenga JM, Erlich HA, Calloway CD, Green RE. A phylogenetic approach for haplotype analysis of sequence data from complex mitochondrial mixtures. Forensic Science International: Genetics (2017) 30:93-105.

Wilson, M. R., DiZinno, J. A., Polansky, D., Replogle, J., and Budowle, B. Validation of mitochondrial DNA sequencing for forensic casework analysis. International Journal of Legal Medicine (1995a) 108:68-74.

Wilson, M. R., Polansky, D., Butler, J., DiZinno, J. A., Replogle, J., and Budowle, B. Extraction, PCR amplification, and sequencing of mitochondrial DNA from human hair shafts. Biotechniques (1995b) 18:662-669.

Wilson, M. R., Allard, M. W., Monson, K., Miller, K. W. P., and Budowle, B. Recommendations for consistent treatment of length variants in the human mitochondrial DNA control region. Forensic Science International (2002a) 129:35-42.

Wilson, M. R., Allard, M. W., Monson, K., Miller, K. W. P., and Budowle, B. Further discussion of the consistent treatment of length variants in the human mitochondrial DNA control region, Forensic Science Communications (2002b) [Online]. Available: [www.fbi.gov/about-us/lab/forensic-science-communications/fsc/oct2002/wilson.htm](http://www.fbi.gov/about-us/lab/forensic-science-communications/fsc/oct2002/wilson.htm)

## **6. Additional Information:**

*The following articles, though not directly referenced in this document, provide relevant background information that may be helpful to laboratories performing mitochondrial DNA analysis*

Amorim A. A cautionary note on the evaluation of genetic evidence from uniparentally transmitted markers. *Forensic Science International: Genetics* (2008) 2:376–378.

Anderson, S., Bankier, A. T., Barrell, B. G., deBruijin, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. Sequence and organization of the human mitochondrial genome. *Nature* (1981) 290:457-465.

Ayadi, I., Mahfoudh-Lahiani, N., Makni, H., Ammar-Keskes, L., and Reba, A. Combining autosomal and Y-Chromosomal Short Tandem Repeat Data in paternity Testing with Male Child: Methods and Application. *Journal of Forensic Science* (2007) 52(5):1068-72.

Buckleton J.S., Krawczak, M., and Weir, B.S. The Interpretation of Lineage Markers in Forensic DNA Testing. *Forensic Science International: Genetics* (2011) 5(2):78-83.

Budowle B., Ge, J., Aranda, X. G., Planz, J. V., Eisenberg, A. J., Chakraborty, R. Texas Population substructure and Its Impact on Estimating the Rarity of Y STR Haplotypes from DNA Evidence. *Journal of Forensic Science* (2009) 54(5): 1016-21.

Budowle, B., Wilson, M. R., DiZinno, J. A., Stauffer, C., Fasano, M. A., Holland, M. M., and Monson, K. L. Mitochondrial DNA regions HV1 and HV2 population data. *Forensic Science International* (1999) 103:23-35.

Carracedo, A., Bar, W., Lincoln, P., Mayr, W., Morling, N., Olaisen, B., Schneider, P., Budowle, B., Brinkman, B., Gill, P., Holland, M., Tully, G., and Wilson, M. DNA Commission of the International Society for Forensic Genetics: Guidelines for mitochondrial DNA typing. *Forensic Science International* (2000) 110:79-85.

Gjertson, D. W., Brenner, C. H., Baur, M. P., Carracedo, A., Guidet, F., Luge, J. A., Lessig, R., Mayr, W. R., Pascali, V. L., Prinz, M., Schneider, P. M., Morling, N. ISFG: Recommendations on biostatistics in paternity testing. *Forensic Science International: Genetics* (2007) 1(3-4):223-31.

Holland, M. M. and Parsons, T. J. Mitochondrial DNA sequence analysis: Validation and use for forensic casework. *Forensic Science Review* (1999) 11:22-50.

Miller, K. W. P. and Budowle, B. A compendium of human mitochondrial DNA control region sequences: Development of an international standard forensic database. *Croatian Medical Journal* (2001) 42:315-327.

Monson, K. L., Miller, K. W. P., Wilson, M. R., DiZinno, J. A., and Budowle, B. The mtDNA population database: An integrated software and database resource. Forensic Science Communications (2002) [Online]. Available: <http://www.fbi.gov/about-us/lab/forensic-science-communications/fsc/april2002/miller1.htm>

Parson W and Dur A. EMPOP- a forensic mtDNA database. Forensic Sci Int Genet (2007) 1(2):88-92.

Sanger, F. and Coulson, A. R. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol (1975) 94(3):441-8.

Scientific Working Group on DNA Analysis Methods (SWGDM). Revised Validation Guidelines, Forensic Science Communications (2004) [Online]. Available: [http://www.fbi.gov/about-us/lab/forensic-science-communications/fsc/july2004/standards/2004\\_03\\_standards02.htm/](http://www.fbi.gov/about-us/lab/forensic-science-communications/fsc/july2004/standards/2004_03_standards02.htm/)

Tully, G., Bär, W., Brinkmann, B., Carracedo, A., Gill, P., Morling, N., Parson, W., and Schneider, P. Considerations by the European DNA profiling (EDNAP) group on the working practices, nomenclature, and interpretation of mitochondrial DNA profiles. Forensic Science International (2001) 124:83-91.

Walsh, B., Redd, A. J., and Hammer, M. F. Joint match probabilities for Y chromosomal and autosomal markers. Forensic Science International (2008) 174:234-238.

Wilson, M. R., Allard, M. W., Monson, K., Miller, K. W. P., and Budowle, B. Recommendations for consistent treatment of length variants in the human mitochondrial DNA control region. Forensic Science International (2002a) 129:35-42.

Wilson, M. R., Allard, M. W., Monson, K., Miller, K. W. P., and Budowle, B. Further discussion of the consistent treatment of length variants in the human mitochondrial DNA control region, Forensic Science Communications (2002b) [Online]. Available: [www.fbi.gov/about-us/lab/forensic-science-communications/fsc/oct2002/wilson.htm](http://www.fbi.gov/about-us/lab/forensic-science-communications/fsc/oct2002/wilson.htm).

## SWGDM MtDNA Interpretation Guidelines – Approved 04/23/2019

Document Version	Revision History
2003	Original entitled Scientific Working Group on DNA Analysis Methods (SWGDM) Guidelines for Mitochondrial DNA (mtDNA) Nucleotide Sequencing Interpretation. (Published in Forensic Science Communications in April 2003 (Vol. 5, No. 2); available at <a href="http://www.fbi.gov/about-us/lab/forensic-science-communications/fsc/april2003/index.htm/swgdammitodna.htm">http://www.fbi.gov/about-us/lab/forensic-science-communications/fsc/april2003/index.htm/swgdammitodna.htm</a> .)
July, 2013	The document was restructured and substantially revised for consistency with current best practices in mtDNA interpretation, particularly with respect to sequence nomenclature and frequency calculations. The revisions were approved by the SWGDM membership on July 18, 2013.
February, 2015	Example 3 on page 10 was revised by removing all references to 16519 and changing 16174C to 16174A (rev1). The revisions were approved by the SWGDM Executive Board on March 6, 2015.
2018	Revisions were made to the following sections to address next generation sequencing: Introduction, Sections 1, 2.1, 2.2.1, 2.2.4, Rule 9, Sections 2.3.4, 3.1.2, 4.2.4.1, and 4.2.5. The revisions were approved by the SWGDM membership on April 23, 2019.