

PROTOCOLS FOR FORENSIC MITOCHONDRIAL DNA ANALYSIS

Interpretation Guidelines for Mitochondrial DNA Massively Parallel Sequencing

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Interpretation Guidelines for Mitochondrial DNA Massively Parallel Sequencing (mitoMPS)

1 Purpose

- 1.1 Analysis of data generated by the Qiagen CLC Genomics Workbench with the AQME tool kit. The sequences are compared to the revised Cambridge Reference Sequence (rCRS) and variants, and their positions are reported.

2 Generated Data

- 2.1 The results for each sample are exported into two excel files: target file set 1, and target set 2. Each excel sheet contains three tabs: *mtDNA table*, *Coverage table*, and *Report*.

- 2.1.1 Analytical threshold coverage for target file set 1 is 600 reads and target set 2 is 100 reads. This is applied to the analysis of the following three mitochondrial regulatory regions, whose boundaries are defined below. (Note: This information can be obtained from the Excel sheet tab *Report*, line 12 and 13.)

2.1.1.1 HVII: 50 – 362

2.1.1.2 HVIII: 420 – 593

2.1.1.3 HVI: 16005 – 16384

- 2.1.1.3.1 Note: these boundaries are extended beyond the commonly defined boundaries of HVII (73-340), HVIII (438-574) and HVI (16024-16365)

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3 Guidelines for Negative Controls

- 3.1 Two negative controls are associated with each sample: the extraction negative and the amplification negative controls. The former tests for potential DNA introduced during extraction through amplification, while the latter tests for the presence of any background DNA that was introduced during the amplification, or present in the amplification reagents. Both of these controls need to be processed for library amplification and analysis.
- 3.2 Negative controls are considered **to pass** if there is no detectable DNA at or above the 600-read primary analytical threshold seen in analysis using the Qiagen CLC workbench and AQME toolkits.
- 3.3 Evaluation of negative controls
- 3.3.1 Open the primary threshold analysis file of the amplification negative control, which has a filename ending in “-primary”. If the data in the various tabs of the excel output file confirm the following, then the control passes:

Tab name:	Data reported:
mtDNA table	No data visible
Coverage table	Data is visible. Coverage for all data (column C) is below 600.
Report	Experimental run specifics are indicated on the top, including target regions, and threshold minimum coverage (cell B12) is confirmed as 600. No variants are reported. Total Differences indicated as 0. (Cell B19, if no variants reported.)

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3.3.2 Example of “Report” tab from a negative control for target file set 1:

	A	B	C
1			
2	For research purposes only. Not intended for clinical use.		
3			
4	Workbench:	CLC Genomics Workbench 20.0	
5	Plugin:	AQME Plugin 20.0	
6			
7	Date:	January 13, 2021	
8	Sample:	SWGDM NEG062419-1024	
9	Approved:	Not approved	
10			
11	Haplogroup:	N/A	
12	Minimum coverage:	600	
13	Target region:	[50..362 420..593 16005..16384]	
14			
15	Sequence Name	Comparison Range	Covered Bases
16	NC_012920	No coverage	0
17			
18	Position	Reference	Variant
19	Total Differences		0

3.3.2.1 If the results generated from the amplification negative control are not concordant with those listed, the preceding figures and table, the run fails, and the extracts may be reamplified.

3.3.3 Open the primary threshold analysis file(s) of the extraction negative control(s), with filename(s) ending in “-primary”. Compare the results to those listed in 3.3.1 and 3.3.2 to determine whether the control(s) passes.

3.3.3.1 If the results generated from an extraction negative control are not concordant with those listed in the preceding figures and table, the extraction fails, and the samples may be re-extracted.

4 Guidelines for Positive Controls

4.1 The positive control, 2800M, is included for each amplification and must produce sequence that is consistent with the known polymorphisms. The positive control serves as run control. Therefore, to be valid, every run must have a positive control that passes specification.

4.1.1 The known polymorphisms of 2800M in comparison to the rCRS are as follows: **152 C, 263 G, 315.1 C, and 477 C.**

4.1.2 Open the secondary threshold analysis file(s) of the positive control, with filename(s) ending in “-secondary”. Confirm that the minimum coverage indicated in the Report tab (cell B12) is 100 reads, covered bases is 864, and that all known polymorphisms of 2800M are present.

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	A	B	C
1			
2	For research purposes only. Not intended for clinical use.		
3			
4	Workbench:	CLC Genomics Workbench 20.0	
5	Plugin:	AQME Plugin 20.0	
6			
7	Date:	March 21, 2022	
8	Sample:	SWGDA AmpPos-03012022-090634-A1	
9	Approved:	Not approved	
10			
11	Haplogroup:	N/A	
12	Minimum coverage:		100
13	Target region:	[50..362 420..593 16005..16384]	
14			
15	Sequence Name	Comparison Range	Covered Bases
16	NC_012920	[50..362 420..593 16008..16384]	864
17			
18	Position	Reference	Variant
19		152 T	C
20		263 A	G
21		315 T	C
22		477 T	C
23	Total Differences		4

- 4.1.3 If the results developed from the positive control are concordant with those listed in 4.1.2, the positive control passes. If the positive control fails to produce the expected result, all samples associated with this control fail, and the extracts may be reamplified.

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5 Guidelines for Reporting

5.1 Base calling criteria

5.1.1 The following two interpretation criteria must be met for a sample base call to be eligible for reporting. For any given position:

5.1.1.1 The read depth must be a minimum of 100 reads AND

5.1.1.2 The read depth of the negative controls (amplification negative and extraction negative) must be less than 10% of the sample(s) at that position.

5.1.2 Positions with read depth less than or equal to 99 cannot be interpreted.

5.1.3 To identify read depth of negative control at specific positions, open target file set 2 (“-secondary” in file name) for the amplification negative control.

5.2 Sequence Nomenclature and Alignment

5.2.1 Nomenclature

5.2.1.1 Nucleotide positions are designated according to the standard one-letter code based on the nomenclature system adopted by the International Union of Pure and Applied Chemistry (IUPAC; see table below). Note that an “N” is used to denote unresolved sequence ambiguities where N can be any one of the four bases. **IUPAC codes that designate two possible bases should only be used in instances of sequence heteroplasmy.**

IUPAC code	Base designation	IUPAC code	Base designation
G	Guanine	R	A or G
A	Adenine	Y	C or T
T	Thymine	K	G or T
C	Cytosine	M	A or C
N	G, A, T or C	S	C or G
		W	A or T

5.2.2 SWGDAM Alignment rules

5.2.2.1 Sequence differences between the questioned sample and the revised Cambridge Reference Sequence (rCRS) are generated by the CLC Workbench software and exported to the *Report* tab. The differences between the sample and the rCRS are listed in order of occurrence on the mtDNA molecule.

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5.2.2.2 In most cases, the alignment of a given mtDNA sequence with that of rCRS is straightforward. However, care must be taken in the placement of insertions and deletions in reference to that of rCRS. The following rules are taken from the SWGDAM Interpretation guidelines for mitochondrial DNA analysis by forensic DNA testing laboratories (SWGDAM, 2019). They represent a blended approach of rule based and phylogenetic methods. It is recommended that the forensic analyst use the EDNAP mtDNA Population Database (EMPOP at <https://empop.online>) to help determine a consistent mtDNA haplotype.

5.2.2.3 Variants from the rCRS should be aligned to the rCRS according to the following standard nomenclature:

5.2.2.3.1 **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.online.

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

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

5.2.2.3.3 **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.

5.2.2.3.4 **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.

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

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

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

5.2.2.3.8 **Rule 7** – Place indels contiguously when possible.

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

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5.2.2.3.10 **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 (e.g., deletions) at this position are not biologically meaningful.

5.2.2.4 Insertions (INS) should be listed to the right of a particular nucleotide position. Insertions are documented by first noting the site immediately 5' to the insertion followed by a point and a "1" for the first insertion, a "2" if there is a second insertion, and so on.

5.2.2.5 Deletions (DEL) should be listed exactly where the known base in the reference sequence is missing in the sample sequence to minimize the number of differences between the questioned sample and the rCRS reference sequence. Deletions are noted by a ":" in the excel output file in the consensus sequence.

5.2.2.6 Sequence heteroplasmy (also known as point or site heteroplasmy) occurs when a single sample contains at least two mtDNA sequences that differ at one or two nucleotide positions. The appropriate one-letter IUPAC code will be used for the reporting of a given site that shows sequence heteroplasmy. This designation will be reflected in the CLC output excel files.

5.2.2.7 MtDNA **length heteroplasmy** commonly occurs in regions that contain multiple tandem C nucleotides. These regions are commonly referred to as polycytosine or C-stretch regions. Length heteroplasmy refers to a sample that has at least two types, each one differing by the total number of C nucleotides at a given C-stretch. When length heteroplasmy is present, the specific number of C nucleotides will not be considered for interpretational purposes.

5.2.2.7.1 **It will be noted if a given casework sample has length heteroplasmy in HVI.**
The number of C residues, however, in the area with HVI length heteroplasmy will not be reported. Length heteroplasmy in HVI most commonly arises when there is a substitution of a C for a T at position 16,189. The reference type in HVI is C₅TC₄. Sequences showing length heteroplasmy in HVI will be truncated to fit the C₅TC₄ format including the T to C change at position 16,189.

5.2.2.7.2 **It will be noted if a given casework sample has length heteroplasmy in HVII.**
Length variants in HVII are commonly observed in the number of C residues preceding a T residue at position 310. Only the dominant length variant in this region will be reported.

5.2.2.7.3 The reference type in the HVII C-stretch region is C₇TC₅. Severe length heteroplasmy in HVII will arise when there is a substitution of a C for a T at

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position 310. Sequences showing this type of length heteroplasmy in HVII will be truncated to fit the C₇TC₅ format including the T to C change at position 310.

5.2.2.7.4 It will be noted if a given casework sample has length heteroplasmy in HVIII. Length variants in HVIII are commonly observed in the number of AC dinucleotide repeats at positions 515-525. Only the dominant length variant in this region will be reported.

5.2.2.7.5 Length variants in HVIII can also be observed at the C-stretch at positions 568-573. Only the dominant length variant in this region will be reported.

5.3 Reporting of base calls

5.3.1 Open the secondary threshold Excel analysis file of your sample. Click on the *Report* tab and compare the profile (beginning in row 18) to the column C ("Variant") of the table in the *mtDNA table* tab, to ensure that the stated variants are concordant between the tabs. If necessary, expand the width of column C to ensure that everything in the table can be seen.

5.3.2 Position 16182, directly adjacent to a C-stretch in HVI

5.3.2.1 If a C-variant is detected and reported at 16182 in the *Report* tab, confirm it by reviewing the data in the *mtDNA table*. If the variant is recorded at ~60%, check this position in the *Coverage table* to confirm. The balance of the remaining coverage is likely an A, indicating the presence of heteroplasmy. If so, change the corresponding call in *Report* tab to an M.

5.3.3 Variants denoted by lower-case letters

5.3.3.1 A lower-case letter in the *Report* tab indicates that the major variant is the same as the reference. In the example below, the lower case c at 309.1 indicates the presence of a C insertion as a minor variant (length heteroplasmy) with the major variant being the same as the reference.

Position	Reference	Variant
309.1 :		c
309.2 :		C

5.3.4 Shifted positions

5.3.4.1 Certain positions containing mononucleotide stretches, in particular the polycytosine C-stretches (HVII and HVI) or dinucleotide AC-stretches (HVIII), are shifted for unambiguous reporting in the *Report*. These shifts are noted in the *mtDNA table* tab in

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column AE (“Tool/Comment”) as shown below.

ID	AE	AF
	Tool Comment	User Comm
	Shifted, MNV	
	Shifted, MNV	
	Shifted	

5.3.4.2 For HVII, insertions at 302 (e.g., 302.1, 302.2, etc.) are reported at 309 (e.g., 309.1, 309.2, etc). Insertions at 310 (e.g., 310.1, 310.2, etc.) are reported at 315 (e.g., 315.1, 315.2, etc).

5.3.5 For HVIII, insertions at 513 (e.g., 513.1 A and 513.2 C at the dinucleotide AC-stretch) are reported at 524 (e.g., 524.1 A and 524.2 C).

5.3.6 For HVI, insertions at 16193 are not shifted, and are reported at 16193. (e.g., 16193.1 C, 16193.2 C, etc). However, as noted in 5.2.2.7.1, sequences showing length heteroplasmy in HVI will be truncated to fit the C₅TC₄ and therefore these insertions will not be reported.

5.3.7 These naming conventions are performed in accordance with SWGDAM guidelines, with insertions and deletions presented as 3'. This can be confirmed by comparing the *mtDNA* table data in column A (“Position”) against column AA (“Position (edit)”) as shown below.

A	B	C	AA	AB	AC
Position	Reference	Variant	Position (edit)	Reference (edit)	Variant (edit)
302.1	-	C (80.6%)	309.1	-	c
302.2	-	C (22.9%)	309.2	-	C
310.1	-	C (94.7%)	315.1	-	C

5.4 Suitability for interpretation

5.4.1 A minimum read length of 90 contiguous base pairs of aligned sequence will be valid for interpretation and for generating weight assessment.

5.5 Criteria for mixture recognition

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5.5.1 More than three heteroplasmic positions in a sample are suggestive of a DNA mixture.

5.5.1.1 A heteroplasmic position will be defined as one in which a minor variant is present at 18.6% or higher. Minor variants present at less than 18.6% will not be called; only the major variants will be called at these positions.

5.5.2 If sufficient DNA was extracted, an attempt to confirm the presence of a mixture by applying autosomal STR testing may be a possibility. Consult with supervisor.

5.5.3 If the sample is deemed to be a mixture, it should be re-extracted for processing through the mitoMPS workflow. As an alternative, other samples in the same case should also be considered for testing.

5.5.4 Samples that contain three heteroplasmic positions might warrant further clarification, depending on the circumstances of the case. This is to ensure that the haplotype developed from the sample in question is not due to the presence of a mixture of DNA. Approaches may include:

- * Attempt to type the sample using nuclear DNA techniques to assess the presence of a mixture.
- * Re-run the library to assess the presence of a mixture.
- * Test an additional sample from the case.

Use caution when interpreting the data from this sample.

5.5.5 Four positions of sequence heteroplasmy would constitute a mixture of DNA.

5.6 Secondary threshold analysis

5.6.1 Sample interpretation is based on the variants detected using the 100-read secondary analysis threshold (target file set 2).

5.6.1.1 Comparison of the variants detected using the 100-read secondary analysis threshold to those detected using the 600-read primary analysis threshold may be done when troubleshooting a run or investigating samples with low coverage. If additional variants were detected using the secondary threshold beyond those that were detected using the primary threshold discuss with a supervisor for possible rework.

6 Sequence Comparisons

6.1 The positive control run with that sample must type correctly in order to report the sequence for that sample.

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- 6.2 When comparing sequences obtained from different samples, only the regions for which there is sequence information in both samples will be used for comparison purposes.
- 6.3 A specimen that yields a mixture of DNA sequences is reported as inconclusive. No comparisons and no statistical evaluation will be performed using this sample.
- 6.4 Match Criteria for Sequencing data
- 6.4.1 Conclusions of concordance, inconclusive or exclusion shall be made according to the criteria listed in the table below.

Conclusion	Criteria
Cannot exclude	When two mtDNA sequences from separate samples (e.g. from two pieces of evidence or from evidence and a maternal family reference source) are the same or are concordant (sharing a common DNA base at every nucleotide position) with each other in the regions in common (e.g. HVI or HVII, if partial, or if one sample has data in HVI, HVII, and HVIII, the other only has data in HVI and HVII), the two samples cannot be excluded as originating from the same person or from having a maternal relationship.
Inconclusive	The resulting comparison will be considered inconclusive when two mtDNA sequences from separate samples differ by one difference (whether or not they share a common length variant at positions 302-310, 515-525, or 568-573, e.g., length heteroplasmy at these positions will not be counted as a difference.) In these cases, other reference sources and/or further testing to obtain more sequence data may be helpful.
Exclusion	The resulting comparison will be considered an exclusion when two mtDNA sequences from separate samples differ by two or more differences (excluding length heteroplasmy).

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6.5 Comparison involving sequence heteroplasmy

- 6.5.1 Conclusions regarding comparisons involving sequence heteroplasmy shall be made according to the criteria listed in the table below.

Criteria	Conclusion
Two identical heteroplasmic bases are present at the same position in both samples.	Concordance. This is not a difference (eg., C/T vs. C/T).
One heteroplasmic base is present in one sample; a common base is present at the same position in the other sample.	Concordance. This is not a difference (eg., C/T vs. C; also C/T vs. T).
One heteroplasmic base is present in one sample; a different base is present at the same position in the other sample.	This is a difference (eg., C/T vs. G).

6.6 Partial results

- 6.6.1 For samples that yielded partial results, due to poor-quality- or low-quantity-template, the purified libraries may re-run as part of a smaller pool of 16 libraries, to increase data yield. Alternatively, or subsequently, samples may be reamplified using a higher quantity of template to also yield more data.

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7 APPENDIX 1 – Hypervariable Region sequence*

Hypervariable Region I (HVI)

16008 – TTTAA ACTAT TCTCT GTTCT TTCAT GGGGA AGCAG ATTTG
16048 – GGTAC CACCC AAGTA TTGAC TCACC CATCA ACAAC CGCTA
16088 – TGTAT TTCGT ACATT ACTGC CAGCC ACCAT GAATA TTGTA
16128 – CGGTA CCATA AATAC TTGAC CACCT GTAGT ACATA AAAAC
16168 – CCAAT CCACA TCAAA ACCCC CTCCC CATGC TTACA AGCAA
16208 – GTACA GCAAT CAACC CTCAA CTATC ACACA TCAAC TGCAA
16248 – CTCCA AAGCC ACCCC TCACC CACTA GGATA CCAAC AAACC
16288 – TACCC ACCCT TAACA GTACA TAGTA CATAA AGCCA TTTAC
16328 – CGTAC ATAGC ACATT ACAGT CAAAT CCCTT CTCGT CCCCC
16368 – TGGAT GACCC CCCTC AG -16384 (end)

Hypervariable Region II (HVII)

50 – TTTGG TATTT TCGTC TGGGG GGTAT GCACG CGATA GCATT
90 – GCGAG ACGCT GGAGC CGGAG CACCC TATGT CGCAG TATCT
130 – GTCTT TGATT CCTGC CTCAT CCTAT TATTT ATCGC ACCTA
170 – CGTTC AATAT TACAG GCGAA CATACT TACT AAAGT GTGTT
210 – AATTA ATTAA TGCTT GTAGG ACATA ATAAT AACAA TTGAA
250 – TGTCT GCACA GCCAC TTTCC ACACA GACAT CATAA CAAAA
290 – AATTT CCACC AAACC CCCCC TCCCC CGCTT CTGGC CACAG
330 – CACTT AAACA CATCT CTGCC AAACC CCAA AAC- 362 (end)

Hypervariable Region III (HVIII)

420 – CTTTT AACAG TCACC CCCCC ACTAA CACAT TATTT TCCCC
460 – TCCCA CTCCC AACTT ACTAA TCTCA TCAAT ACAAC CCCCC
500 – CCCAT CCTAC CCAGC ACACA CACAC CGCTG CTAAC CCCAT
540 – ACCCC GAACC AACCA AACC CAAAG ACACC CCCCC CAGTT
580 – TATGT AGCTT ACCT – 593 (end)

* regions of potential length heteroplasmy are underlined.

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