Interpretation Guidelines

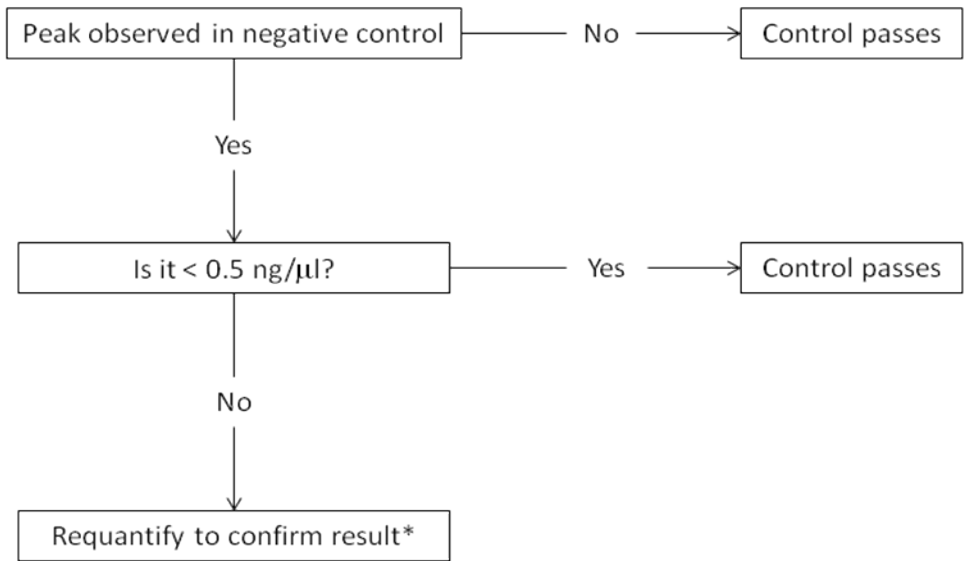
1 Guidelines for Negative controls

1.1 Negative controls are considered negative if there is no detectable DNA based on the quantitation procedure and no "readable" sequence is seen after 3130xl electrophoresis. For DNA sequencing analysis, the controls are also considered negative if sequence was obtained, but it cannot align to the reference sequence.

1.2 A "readable" sequence from a negative control run is a sequence that can be aligned to the rCRS for >90 consecutive bases with no more than 4 "N" calls within any 10 consecutive bases.

1.3 Two negative controls are associated with each sample: the extraction negative (ext neg or e neg) and the amplification negative (amp neg) 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 all sequencing primer sets.

1.4 **Agilent** Flow chart for passing, failing or retesting negative controls is as follows:



1.4.1 * If confirmed, the following actions should be taken:

1.4.1.1 For extraction negative controls

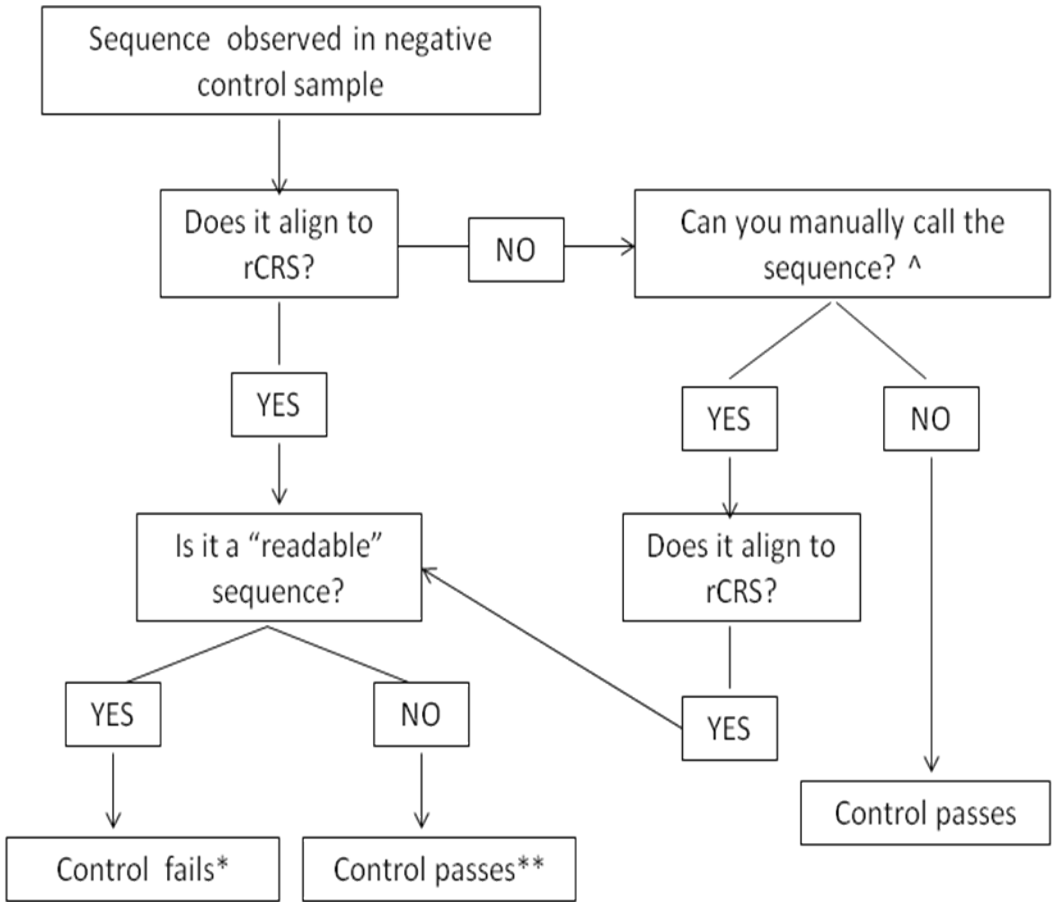
- Re-amplify to confirm presence of DNA, samples can proceed if re-amplification is clean.
- If the extraction negative control still yields a peak following re-amplification, it is preferable to re-extract if more sample is available. If sample amount is limiting, analyst may proceed with caution. The results are only valid if the sequence detected for the amplification negative control does not match any of the associated samples or any of the samples in the case.
- If the amount of DNA present in the extraction negative control exceeds 10% of any associated sample (DNA amounts determined by Agilent), that sample is invalid.

1.4.1.2 For amplification negative controls

- Re-amplify sample set.
- If sample amount is limiting, it is left to the analyst's discretion to proceed with the amplification set since this result indicates that the background DNA is limited to the amplification control tube rather than being ubiquitous in all samples. The results are only valid if the sequence detected for the amplification negative control does not match any of the associated samples or any of the samples in the case.
- If the amount of DNA present in the amplification negative control exceeds 10% of any associated sample, that sample is invalid.

Note: Any failed negative controls may be sequenced for quality control purposes.

1.5 Sequencing results flow chart for passing, failing or retesting negative controls is as follows:



1.5.1 ^ If sequence data is present for an extraction or amplification negative control, but does not have base calls assigned, an analyst should manually assign base calls to determine if the sequence data can be aligned to the rCRS and if it is a "readable" sequence.

1.5.2 * If an extraction or amplification negative control contains a "readable" sequence, the results should be confirmed by recycle-sequencing. If confirmed, then the test fails and retesting must start at the point of sample re-extraction or amplification. If the amount of original sample present is limiting, the DNA extract is limiting or the re-amplification yields the same results, then sample results can be interpreted and reported if the sequence is different from all associated samples in the case. The determined sequence for the extraction or amplification negative control must contain "readable" sequence in order to be used in sequence comparisons with case samples.

1.5.2.1 If both extraction and amplification negative control from the same test contains "readable" sequence, the extraction negative cannot be

interpreted because the amplification may have introduced a contaminant. The test fails and all samples and the extraction negative must be re-amplified and re-sequenced

1.5.3 ** If an extraction or amplification negative controls contain sequence data that can be aligned to the rCRS for <90 consecutive bases, the test passes however the results should be confirmed by recycle-sequencing. If confirmed, see a mtDNA supervisor before proceeding with further testing. The following testing can be done if further testing is deemed necessary:

- For an extraction negative control, re-amplification of the negative control in question followed by re-extraction of associated samples if necessary.
- For an amplification negative control, re-amplification of the entire amplification set.

Note: If it is necessary to re-sequence a casework sample from the cycle sequence step, a new cycle sequencing amplification negative control (CAN) must be created for this round of cycle sequencing. This negative control must yield a negative result for the results to be valid.

2 Guidelines for Positive controls

2.1 The positive control (HL60) is included for each amplification and must produce sequence that is consistent with the known polymorphisms. The positive control sample must yield results for the full read length of the associated sample contig, but at a minimum, HVI, 16024-16365, HVII, 73-340, or both. In addition, the positive control serves as the run control. Therefore, in order to be valid, every run must have a positive control that passes specification.

2.2 The known polymorphisms in comparison to the rCRS are as follows:

HVI		HVII	
16,069	T	73	G
16,193	T	150	T
16,278	T	152	C
16,362	C	263	G
		295	T
		315.1	C

2.3 If the positive control fails to produce the expected result, all samples associated with this control fail. If it is suspected that the problem is not related to the amplification but could stem from a subsequent step, the positive control and all of the samples can be retested starting either at the cycle sequencing or the 3130xl injection step.

2.4 In cases of dye interference or electrophoretic artifact, some N calls in the positive control will be allowed as follows:

2.4.1 A maximum number of 4 “N” calls within any 10 base stretch for any primer strand used to build the contig will be allowed for either HVI or HVII region provided that the calls on the complementary strand are unambiguous and not contradictory to the questioned nucleotide position(s)

2.4.2 Any positions that have ambiguous N calls on both complementary strands that cannot be resolved through retesting will result in the failing of the positive control and all of the associated sample runs.

3 A. Guidelines for Reporting - Sequencing: Reporting of Base Calls

3.1 Sequence data should be determined from both complementary strands of DNA for mtDNA regions HVI and HVII. Only under special circumstances (see 2. below) can sequence be reported for confirmed data from a single-strand.

3.1.1 All good quality data that shows concordance for both complementary DNA strands or confirmed single-strand data can be reported. A list of reported differences from the rCRS must be accompanied by the range of nucleotides of the region that was sequenced. All possible alternative alignments are not reported.

3.1.2 For sequence where an ambiguous calling situation occurs for one strand, it must be left unresolved and called an "N". No more than 4 un-editable N calls are acceptable within any 10 base stretch of strand sequence data.

3.1.3 If an "N" base call is made on one of the DNA strands (eg., due to an electrophoretic artifact), this base position can still be reported as a base in the plurality consensus sequence as long as (i) the data on both strands are not in conflict with each other, and (ii) the data generated from the complementary or confirmatory DNA strand is clean and there is no question regarding its base call.

3.1.4 A minimum read length of 90 contiguous base pairs of double-stranded or confirmed data that forms a contig will be valid for interpretation and for generating weight assessment.

3.1.5 A minimum read length of 90 consecutive bases of single-stranded data is necessary for any strand to be used to build a contig. Only under special circumstances (see 2. below) can data be reported for a read length of less than 90 bases.

3.2 Special circumstances will arise (eg., length heteroplasmy) when data from only one DNA strand can be obtained or read lengths of greater than 90 bases are not possible.

3.2.1 For samples with HVI or HVII length heteroplasmy, additional primers should be used in order to obtain as much complementary data as possible.

3.2.2 For sequence where no data is available for one of the complementary strands, this can still be reported given that the sequencing reaction that yielded the one strand of sequence data is repeated (confirmed) for this sample with the same or different primer in the same direction. All of the data from this region (eg., results from two cycle sequencing reactions) must be concordant between the two sequencing runs. Note: This type of rerun will satisfy conditions where a difference from rCRS or sequence heteroplasmy is being reported.

3.2.3 Situations will arise which result in severe length heteroplasmy (e.g. in HVII, 310 C resulting in a homopolymeric stretch of 13 C residues). Under these conditions, it will be

not be possible to sequence through this region in either the forward or reverse direction. This could result in the trimming of a strand (e.g., C1) and/or will yield runs with sequences generated from the complementary strand (e.g., D1) primer that are less than 90 bases. In these cases, the data will be acceptable at less than 90 bases. The guidelines described in b. above for run confirmations will also apply to the confirmation runs necessary in this scenario.

3.2.4 In situations when un-editable "N" base calls are made at a given sequence position for both DNA strands, then this base will be reported as "N". **Samples with 3 or more un-editable "N" calls within a 10 base pair region of the consensus sequence in either HVI or HVII are inconclusive.**

4 Criteria for Mixture Recognition

4.1 More than two heteroplasmic positions in a sample are suggestive of a DNA mixture. If possible, the sample should be re-extracted or other samples in the same case should be tested.

4.2 Samples that contain two heteroplasmic positions might warrant further testing of additional samples depending on the circumstances of the case. This is to make sure that the sample type in question is not due to a mixture.

5 Sequence Comparisons

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

5.2 If either extraction or amplification negative controls contain readable sequences, the associated case sample(s) must be compared to this data before any further sequence comparisons are made. The readable extraction or amplification negative controls must differ from all case samples by at least two bases for these case samples to be interpreted and reported (see Control Tables, section A3).

5.3 When comparing sequences obtained from samples, only the regions in common will be considered.

5.4 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.

5.5 The number of C nucleotides at the HVI polycytosine C-stretch will not be considered for interpretation purposes if length heteroplasmy is present. Likewise, the number of C residues exhibited in samples with HVII length heteroplasmy is highly variable and care must be taken when making comparisons. In order for sequence concordance to be declared, a common length variant must be observed in both samples being compared.

5.6 **Differences between samples due to the absence of an HVII common length variant are treated as one difference.**

5.7 Match Criteria for Sequencing data

Concordance	When two mtDNA sequences from separate samples (e.g. from two pieces of evidence or from evidence and a maternal family reference source) are consistent with each other in the overlapping regions, the two samples cannot be excluded as originating from the same person or from having a maternal relationship, respectively.
Inconclusive	The resulting comparison will be considered inconclusive when two mtDNA sequences from separate samples differ by one difference. In these cases other reference sources and/or further testing in order 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.

5.8 Treatment of sequence heteroplasmy

Two identical heteroplasmic bases are present at the same position in both samples.	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.	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).