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TECHNICAL NOTE

The Optimization of Input DNA for Applied Biosystems™ Precision ID mtDNA Panels

Introduction

Mitochondrial DNA (mtDNA) analysis can aid with the analysis of challenging forensic samples when nuclear DNA analysis fails due to the large number of mitochondrial copies per cell. The Precision ID mtDNA Whole Genome and Control Region Panels, combined with Ion Torrent sequencing technology, are proven to generate high-quality mitochondrial data from degraded and low-level forensic samples[1]. This technical note describes the optimization of input DNA to Precision ID mtDNA panels for best performance.

Quantification of Input DNA

Instructions on laboratory processing of the Precision ID mtDNA Whole Genome Panel and the Precision ID mtDNA Control Region Panel are detailed in the Application Guide for these panels[2]. Libraries for both panels can be prepared either manually or with the Ion Chef instrument. As noted in the Application Guide, it is recommended to quantify the DNA extract that is input to the library – both too much and too little DNA input can result in non-optimal results.

Ideally, a mitochondrial-specific quantitation method would be used to quantify the mitochondrial DNA in the sample. In the absence of a mitochondrial-specific quantitation method, the Applied Biosystems™ Quantifiler™ HP or Quantifiler™ Trio DNA Quantification Kits are recommended to quantify the sample. This quantitation will only estimate the amount of mtDNA in the sample as these kits quantify genomic DNA (gDNA).

It is recommended to use approximately 2900 copies of mtDNA per pool for these panels. If less than this amount is used, sequencing output can be lowered. If excess DNA is used, undesirable results can be obtained with sequence data that cannot be interpreted or obscures the true mtDNA haplotype of the sample. Should a mitochondrial-specific quantitation not be available, 0.1 ng of gDNA is recommended to estimate 2900 copies of mtDNA per pool. This value of 0.1 ng should be based on the result for the small autosomal quantity from either the Quantifiler HP or Trio Kit, where mtDNA can vary from sample to sample depending on the quality of the sample or the tissue type. Optimization of the target input to the reaction is recommended for all laboratories that routinely use the Precision ID mtDNA panels.

Consequences of Non-Optimal DNA Input

The amount of useable mtDNA sequence obtained from the Precision ID mtDNA panels can be lowered if excess DNA is added to library preparation. This undesirable result can be due to several factors, including the detection of nuclear mitochondrial DNA segments (NUMTs) and chimeric amplification products. NUMTs are a type of artifact read formed where the mitochondrial panel primers bind to areas of homology in the nuclear genome. If an excess of gDNA is added to the reaction, the number of NUMT reads can increase and obscure the mtDNA haplotype of the sample[3]. Chimeric reads are another type of artifact read which can form at the ligation stage of library preparation in the presence of excess input DNA. These occur when two amplicons randomly join together, resulting in an artifact read that cannot align anywhere to the reference.

Figures 1 shows a Precision ID mtDNA Whole Genome Panel sample with more than the recommended amount of input DNA. The 200-300 bp reads do not align with the reference genome and are essentially wasted throughput of the DNA sequencing run. Alignment for this run is shown in Figure 2. Figure 3 shows a sample with the same panel with an optimal amount of input DNA. The expected mtDNA reads from around 100-150 bp are still present and the artifact reads of 200-300 bp are gone.

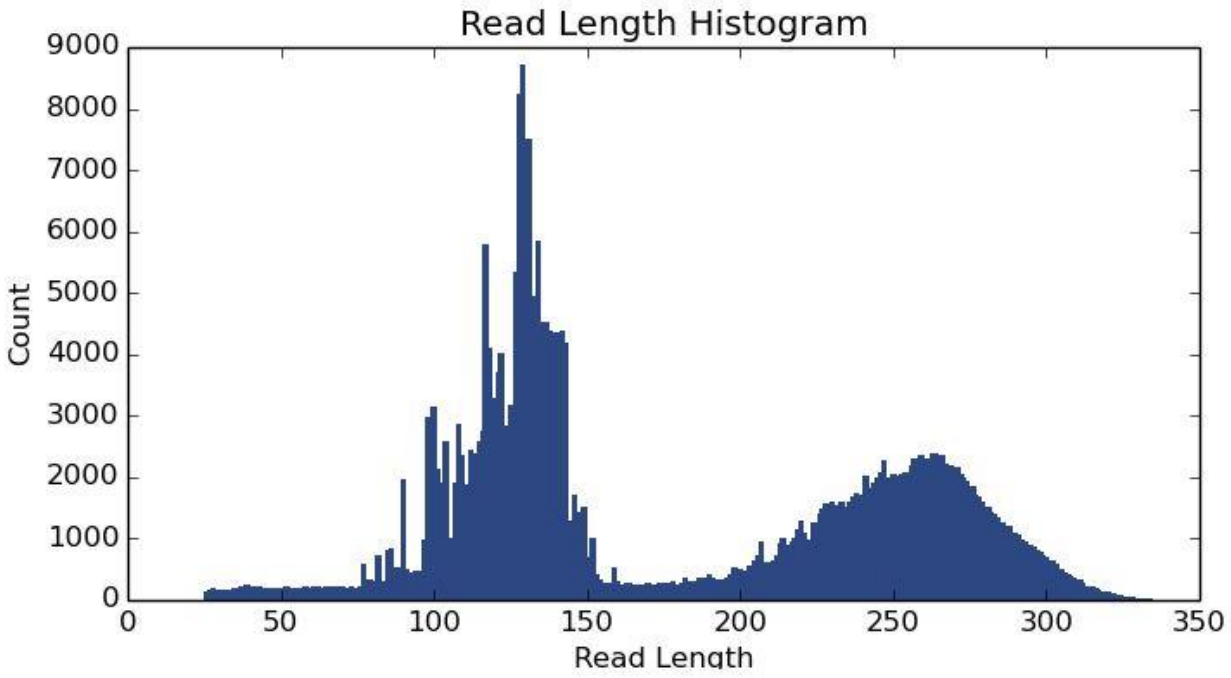

Figure 1: A read-length histogram for a sample from a Precision ID mtDNA Whole Genome Panel run. The sample contained 0.25 ng of gDNA input (more than the recommended 0.1 ng input). The jagged bars around 100 to 150bp are the expected mtDNA amplicons. The hump around 200 bp to 300 bp are reads formed due to the excess of input.

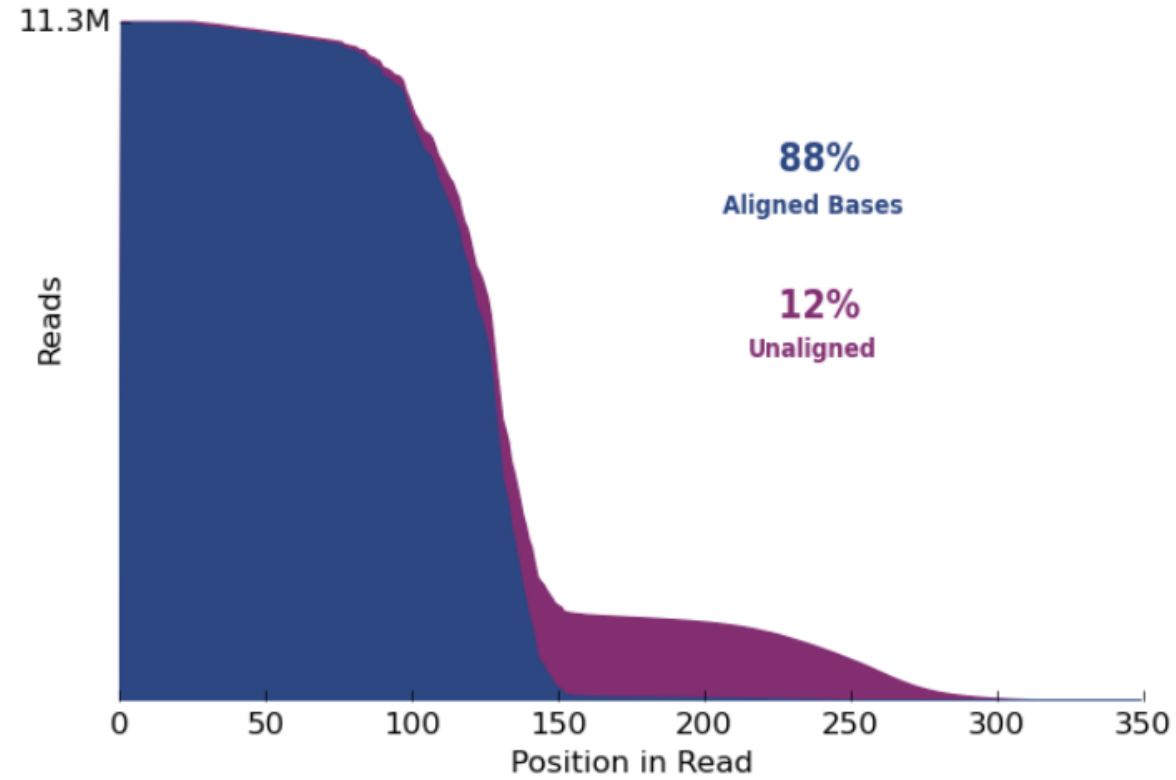

Figure 2: Alignment chart from a Precision ID mtDNA Whole Genome Panel run, which contained samples with excess DNA input. The alignment of all reads in the run is shown. Aligned reads are in blue, and unaligned reads are in purple. The large majority of reads over 150 bp are unaligned.

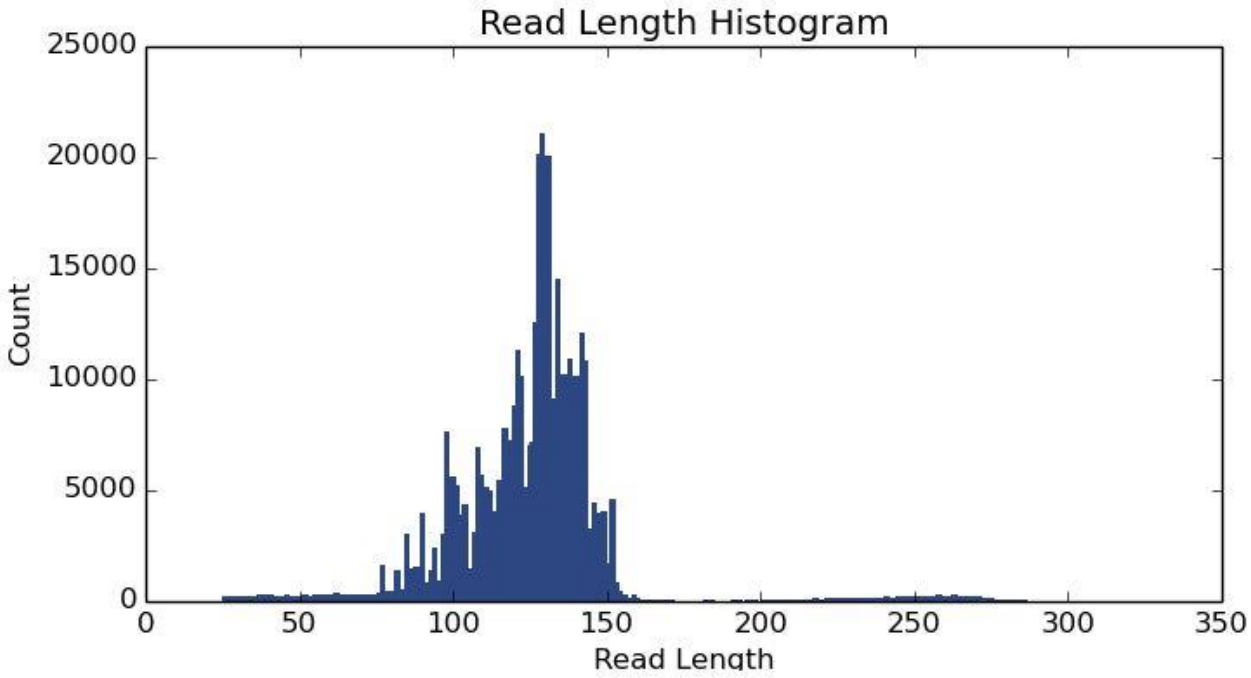

Figure 3: A read-length histogram for a sample from a Precision ID mtDNA Whole Genome Panel run. This sample contained the recommended 0.1 ng of gDNA input. The jagged bars around 100 to 150bp are the expected mtDNA amplicons. The 200-300 bp artifact reads seen in Figure 1 are not present.

If the input DNA to the Precision ID mtDNA panels is decreased, the total coverage of the assay will decline. However, valuable results can still be gained down to very low input levels, as measured by gDNA quantification. Figure 4 shows the STR CE-based result for a sample with 2.0 pg of gDNA input. Only one allele above the peak amplitude threshold of 50 RFU was observed. A complete mtDNA haplotype was achieved when the Precision ID mt DNA Whole Genome panel was used on the same sample (data not shown).

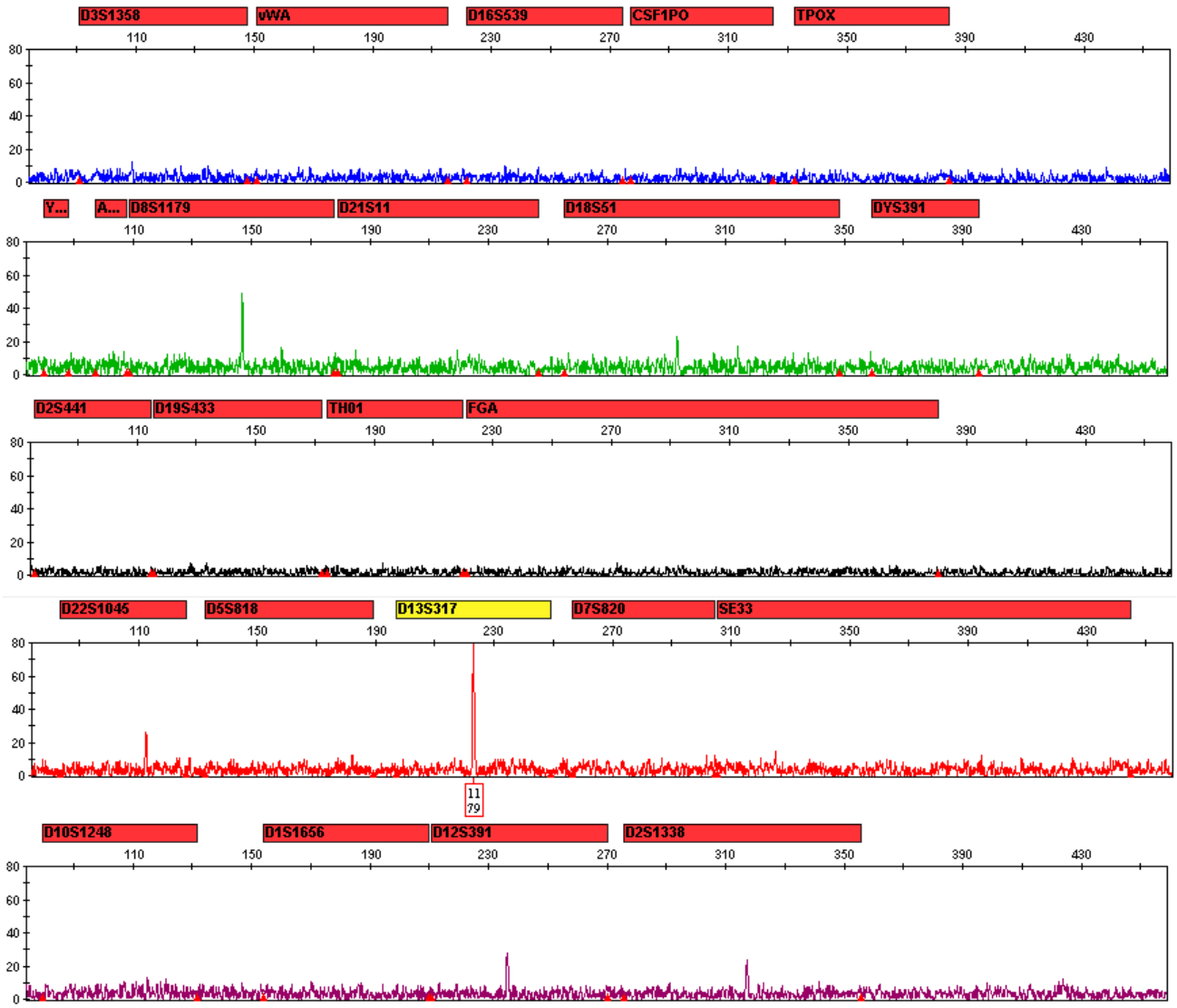

Figure 4: CE electropherogram for a DNA extract with 2.0 pg of gDNA input. The profile was generated with the GlobalFiler PCR Amplification kit and the 3500 Genetic Analyzer. A peak amplitude threshold of 50 RFU was used to analyze the data, only one peak (at D13S317) is above this threshold.

Manual vs Ion Chef Library Preparation

The Ion Chef instrument or a manual approach can be used to prepare libraries with the Precision ID mtDNA panels. Depending on the method used, considerations should be made for coverage optimization based on DNA input.

As previously mentioned, 0.1 ng of gDNA per pool is recommended for manually prepared Precision ID mtDNA libraries. More or less than this amount of DNA may be optimal depending on a laboratory's specific workflow and the sample types analyzed. Further, this recommendation is per pool, with each of the two PCR pools containing 0.1 ng of gDNA diluted in 3 µL or 6 µL of nuclease-free water for the conservative or 2-in-1 methods respectively. Therefore, a total of 0.2 ng of DNA per sample is recommended for use with the Precision ID mtDNA panels due to the two PCR pools.

If the Ion Chef is used to prepare libraries, DNA is added in a 15 µL volume to the 96-well plate supplied with the Precision ID DL8 kit. The instrument then prepares the two PCR pools with this 15 µL volume: 5 µL is used to prepare PCR pool 1, 5 µL is used to prepare PCR pool 2, and 5 µL is left unused. Therefore, to make the input for Ion Chef libraries equivalent to manually prepared libraries, 0.3 ng of input DNA should be added to the single 15 µL volume. Table 1 provides a summary.

*Table 1: Summary of DNA input mass, volume, and concentration used for Manual and Ion Chef library preparation methods with the Precision ID mtDNA panels. * These values represent the total required for the two PCR pools – half of the given value is used in each pool.*

Library Preparation Method		Total Input DNA required			DNA in each PCR pool		
		Mass (ng)	Volume (µL)	Conc. (ng / µL)	Mass (ng)	Volume (µL)	Conc. (ng / µL)
Manual	2-in-1	0.2 *	12 *	0.017	0.1	6	0.017
	Conservative	0.2 *	6 *	0.033	0.1	3	0.033
Ion Chef		0.3	15	0.020	0.1	5	0.020

The recommended concentration of DNA is similar for all three library preparation methods, with the concentration required for the Ion Chef sitting in-between the two options for manual library preparation. For especially low-level samples, with very little DNA available – possibly even returning a zero gDNA quantification – the 2-in-1 manual library method, which requires the lowest input concentration of the three methods, should be considered.

By considering these factors, a laboratory should be able to optimize DNA input and library preparation to make full use of the advantages offered by mitochondrial analysis with the Precision ID mtDNA panels.

References

1. Churchill Cihlar, Amory, Lagacé, Roth, Parson and Budowle. (2020) Developmental Validation of a MPS Workflow with a PCR-Based Short Amplicon Whole Mitochondrial Genome Panel. Genes. 11(11):1345.

2. Precision ID mtDNA Panels with the HID Ion S5™ / HID Ion GeneStudio™ S5 System Application Guide. Revision C. Available from Thermo Fisher Scientific here

3. Churchill Cihlar, Strobl, Lagacé, Muenzler, Parson and Budowle. (2020) Distinguishing mitochondrial DNA and NUMT sequences amplified with the precision ID mtDNA whole genome panel. Mitochondrion 55:122-133.

Revision History

Revision	Date	Description
A	13 June 2022	Initial publication