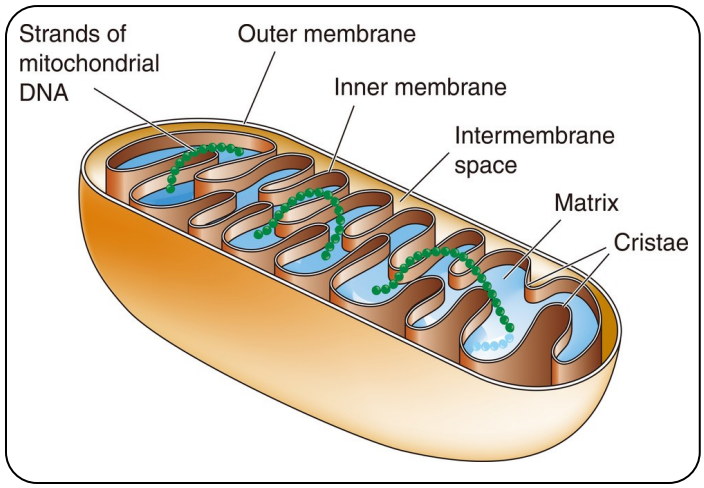
LC Sciences

TECHNICAL NOTE

Performance of the VariantPro™ Mitochondrial Panel

The VariantPro™ Mitochondrial Panel benefits from the uniformity of Relay-PCR™ technology and the specificity of Omega Primer™ technology



The mitochondrion is a double membrane-bound organelle found in most eukaryotic cells. Through respiration it functions to produce the energy of the cell and regulate cellular metabolism. The mitochondrial genome is a circular DNA molecule which is distinct from the nuclear genome. In humans, it's about 16 kb long and encodes 37 genes. Mitochondrial disorders arise as a result of dysfunction of the mitochondrial respiratory chain, and often times, these are caused by mutations of genes encoded by mitochondrial DNA (mtDNA). Because there is considerable clinical variability between mitochondrial disorders and many patients who exhibit phenotypes that overlap diseases, often diagnosis may only be confirmed by identification of a pathogenic mtDNA variant through molecular genetic testing of DNA extracted from a blood sample[1].

VariantPro™ is an innovative multiplex PCR-based targeted sequencing method that accomplishes target selection, library preparation and molecular tagging in a simple one-step workflow. The method incorporates novel Relay-PCR™ and Omega Primer™ technologies to produce amplicons of high uniformity & specificity. Designed for 100% amplicon coverage (16,569 bp) of the mitochondrial genome, the VariantPro™ Mitochondrial Panel offers cost-effective, minimal hands-on, ultra-high resolution sequence analysis for accurate detection of rare variants in mtDNA.

Assay Uniformity

Traditional multiplex PCR based capture technologies are susceptible to amplification bias due to the varying hybridization kinetics of different primer pairs. This can lead to amplicon to amplicon variations in end products due to priming efficiency variations among specific primers which would be exponentially amplified if used as amplification primers. This results in biased amplification of selected targets[2].

The VariantPro™ Mitochondrial Panel benefits from the uniformity of Relay-PCR™ technology. For Relay-PCR™, a pair of common primers is added into a mixture of multiple pairs of specific primers and mtDNA in a single tube. A single PCR run then accomplishes two functionally separated reaction phases, namely target selection and library amplification, without the need for a primer removal step. The addition of the common primers induces a profound change to multiplexing PCR by limiting the role of specific primers to only the first two thermo cycles for target replication. This enables an automatic switch to common primers in the remaining thermo cycles for library amplification.

Uniformity of amplicon coverage (enrichment uniformity) is typically measured as a percentage of reads greater than 0.2 x amplicon mean. As shown below, the VariantPro™ Mitochondrial Panel achieves > 99.1% enrichment uniformity.

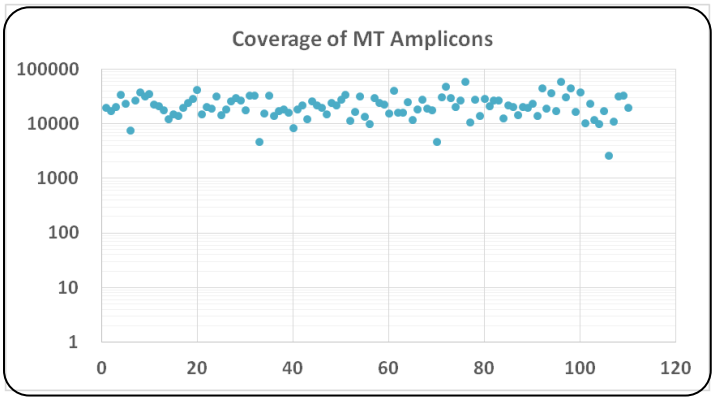


Figure 1. Average coverage distribution over the individual amplicons. The logarithmic scale of the y-axis indicates the coverage uniformity. The lowest and highest covered amplicons varied within 1.35 logs at > 2500 read depth.

Assay Sensitivity and Specificity

Traditional PCR primer design often involves trade-offs between specificity and priming stability. To address this challenge, the Omega Primers™ used in the VariantPro™ system are composed of three functional sections: a 5p

arm that anchors the primer to a DNA template, a 3p arm that checks sequence specificity and initiates polymerase extension, and a loop to provide a separation between the two arms. The use of two separate binding segments provides additional primer design freedoms to balance priming specificity and binding strength. For example: the 5p arm is designed to be relatively long so that it stays hybridized to a template even when mutations (e.g. SNP) are present in the template. The 3p arm is designed to be relatively short so that by itself it would not have any significant hybridization with the template unless 5p arm is hybridized to the template. A viable Omega Primer™ requires simultaneous bindings of both 5p arm and 3p arm, resulting in a significant increase of specificity as compared to conventional primers.

To demonstrate the sensitivity and specificity of the VariantPro™ Mitochondrial Panel and its ability to detect rare mutations, a genome type G11778 G > A (on the mitochondrial genome) genomic DNA sample was mixed with a wild type genomic DNA sample in varying ratios that ranged from 0.5% to 10%. The libraries obtained from the assay were analyzed using the Illumina NGS platform. Analysis revealed that the detected mutation frequencies matched closely to the actual mixing frequencies, indicating that the mutation detection sensitivity is better than 0.5%.

Mutation [chrMT : 11778 G>A]	
Pre-designed frequency	**Actual frequency**
0.50%	0.58%
1%	2%
2%	2%
5%	8%
10%	14%

Figure 2. The detected mutation frequencies matched closely to the actual mixing frequencies.

Assay Reproducibility

Reproducible data is an important component of a robust measurement process. One potential source of assay variability comes from errors in the sample and library preparation steps. This means that more complex assays could potentially increase the chance of errors. In the VariantPro™ assay, a single PCR run accomplishes target selection, molecular tagging and library amplification; all without a primer removal step. This results in a significantly simplified workflow which minimizes handling errors as a source of variation.

We demonstrated the robustness of the VariantPro™ Mitochondrial Panel by varying the number of PCR cycles run and the starting amount of input DNA, and then performing correlation coefficient analysis on the amplicon reads. Neither variation significantly affected the reproducibility of the sequencing results.

	15 cycles	17 cycles	19 cycles	21 cycles	23 cycles	27 cycles
15 cycles	1.000	0.992	0.983	0.951	0.978	0.932
17 cycles	0.992	1.000	0.995	0.969	0.984	0.919
19 cycles	0.983	0.995	1.000	0.965	0.980	0.910
21 cycles	0.951	0.969	0.965	1.000	0.973	0.890
23 cycles	0.978	0.984	0.980	0.973	1.000	0.944
27 cycles	0.932	0.919	0.910	0.890	0.944	1.000

Figure 3. The correlation coefficient analysis for number of PCR cycles (15 cycles to 27 cycles) indicates that the number of PCR cycles will not affect the library components.

	1.0 ng	2.5 ng	5.0 ng	7.5 ng	10.0 ng
1.0 ng	1.000	0.991	0.986	0.978	0.952
2.5 ng	0.991	1.000	0.979	0.989	0.973
5.0 ng	0.986	0.979	1.000	0.984	0.956
7.5 ng	0.978	0.989	0.984	1.000	0.984
10.0 ng	0.952	0.973	0.956	0.984	1.000

Figure 4. The correlation coefficient analysis for template gDNA (1.0 ng to 10 ng) indicates that the required template quantity is flexible.

Sequencing Degraded DNA Samples

DNA extracted from degraded tissue samples is frequently employed in medical, forensic, and anthropologic studies and degraded mtDNA are frequently encountered during the phylogenetic and population genetic analyses of ancient DNA[3]. However, there are technical challenges inherent in the assay of specimens and/or clinical samples that are of poor quality.

To demonstrate the performance of the VariantPro™ Mitochondrial Panel on degraded samples, we simulated

natural degradation of DNA by heating 250 ng of HeLa genomic DNA in the presence of dsDNA Fragmentase. The reactions were performed at 37°C for 0, 10, 20, 30, 50 min respectively. The VariantPro™ assay was performed using the degraded HeLa genomic DNA as templates and the libraries obtained were analyzed using the Illumina NGS platform. The correlation coefficient analysis indicates that the effect of DNA template degradation on VariantPro™ Mitochondrial Panel Kit results is minimal.

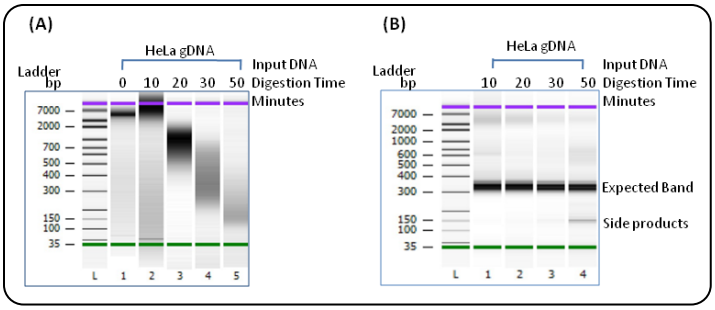


Figure 5. (A) Bioanalyzer results from degraded DNA. Obvious degradation (smearing) occurred after incubation at 37°C for 20 minutes and the average size of genomic DNA decreased with the increasing time of incubating time. (B) Bioanalyzer results from the VariantPro™ Mitochondrial Panel prepared libraries. The expected size of amplicons is approximately 330 bp. No obvious effects occurred until the average genomic DNA fragments were decreased to 200 bp (fragmentation time: 50 min).

	0 min	10 min	20 min	30 min	50 min
0 min	1.000	0.887	0.871	0.856	0.808
10 min	0.887	1.000	0.984	0.960	0.896
20 min	0.871	0.984	1.000	0.984	0.947
30 min	0.856	0.960	0.984	1.000	0.974
50 min	0.808	0.896	0.947	0.974	1.000

Figure 6. Effect of DNA degradation on VariantPro™ Mitochondrial Panel Kit. Compared to integrated genomic DNA (un-fragmented), the lowest correlation coefficient value was 0.808 when the gDNA templates were digested for 50 min.

Cost Savings

The latest Next-Gen Sequencing technologies have offered many laboratories a wide range of genomic analyses capabilities. However, sequencing costs often limit to the amount of sequencing data that can be generated and, consequently, the biological outcomes that can be achieved from an experimental design.

Despite a decline in recent years, sequencing costs remain substantial, and higher sequencing coverage requires higher costs. One of the major rationales for using targeted sequencing is to identify variants in large numbers of samples at a lower cost than using whole genome sequencing. Since researchers often seek experimental designs that generate scientific findings for the lowest sequencing cost, this inevitably means lower coverage[4]. The higher performance (coverage uniformity & on-target specificity) of the VariantPro™ system thus translates into sequencing cost savings, because less sequencing depth is required to achieve adequate coverage of all bases.

	50 x	150 x	300 x	400 x	500 x	700 x	800 x	900 x	1100 x	1500 x
50 x	1.000	0.971	0.966	0.965	0.961	0.973	0.968	0.962	0.962	0.962
150 x	0.971	1.000	0.997	0.996	0.997	0.990	0.997	0.996	0.996	0.994
300 x	0.966	0.997	1.000	0.998	0.995	0.984	0.994	0.994	0.994	0.996
400 x	0.965	0.996	0.998	1.000	0.997	0.984	0.995	0.997	0.995	0.998
500 x	0.961	0.997	0.995	0.997	1.000	0.987	0.998	0.999	0.996	0.995
700 x	0.973	0.990	0.984	0.984	0.987	1.000	0.993	0.985	0.981	0.980
800 x	0.968	0.997	0.994	0.995	0.998	0.993	1.000	0.997	0.994	0.990
900 x	0.962	0.996	0.994	0.997	0.999	0.985	0.997	1.000	0.997	0.994
1100 x	0.962	0.996	0.994	0.995	0.996	0.981	0.994	0.997	1.000	0.991
1500 x	0.962	0.994	0.996	0.998	0.995	0.980	0.990	0.994	0.991	1.000

Figure 7. The correlation coefficient analysis for sequencing depth (50X to 1500X) showed no significant coverage variations among individual amplicons.

Conclusion

Unlike current multiplex PCR targeted sequencing methods that suffer from low uniformity, sequence drop out, complicated work flow, and lengthy hands-on time, the VariantPro™ method effectively balances uniformity, specificity, coverage, and multiplexing level in an elegantly simple one step reaction via Relay-PCR™ on a regular PCR machine. This requires less hands–on work and less sequencing depth per sample. The VariantPro™ Mitochondrial Panel provides cost-effective, ultra-high resolution sequence analysis, for accurate detection of rare variants in mtDNA.

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Specifications

Description	The assay kit contains all the reagents required for capture of the entire human mitochondrial DNA genome and library preparation for next-gen sequencing
Capture Method	The VariantPro™ system incorporates novel Relay -PCR™ and Omega Primer™ technologies to form a one-step multiplex PCR based workflow.
Amplicon Coverage	Designed for 100% amplicon coverage of all regions of the mitochondrial genome (16569 bp)
Amplicon Length	Average 200 bp
Primer Pools	110 Pairs of primers in 2 primer pair pools
Input DNA required	1-5 ng human genomic DNA per reaction tube (2) – depending on the DNA quality
Assay Time	4-6 hours
Hands-on Time	5 minutes
Sequencing Platform	Illumina
Multiplexing Capability	Up to 3024 samples in one lane
Coverage Uniformity	> 99% at 0.2 x mean
Dropout Rate	0

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