

## In-Depth Screening of Polymerase Gamma 1 gene with DHPLC, SURVEYOR® Nuclease strategy and DNA sequencing

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### ABSTRACT

The maintenance of mitochondrial DNA (mtDNA) is critically dependent upon polymerase-γ (pol-gamma), encoded by the nuclear gene POLG. Mutations of POLG are a major cause of human disease. Secondary mtDNA defects characterize these disorders, with mtDNA depletion, multiple mtDNA deletions or multiple point mutations of mtDNA in clinically affected tissues. The secondary mtDNA defects cause cell and tissue-specific deficiencies of mitochondrial oxidative phosphorylation, leading to organ dysfunction and human disease. Functional genetic variants of POLG are present in up to approximately 0.5% of the general population, and pathogenic mutations have been described in most exons of the gene. Clinically, POLG mutations can present from early neonatal life to late middle age, with a spectrum of phenotypes that includes common neurological disorders such as migraine, epilepsy and Parkinsonism. Clinical samples have been analyzed with our multipronged approach in which amplicons covering DNA polymerase gamma gene coding regions with flanking intronic regions are PCR-amplified with a high-fidelity DNA polymerase and analyzed by DHPLC under fully optimized conditions for the screening. Scanning results are further confirmed with SURVEYOR Nuclease and DNA sequencing. This is an effective, in-depth approach for a clinical diagnostic assay for detection of both heterozygous and homozygous mutations seen in POLG. The accuracy and sensitivity that are characteristic of this screening methodology improve the ability to detect novel sequence variations associated with secondary mtDNA defects.

### INTRODUCTION

Pathogenic mutations of mtDNA lead to relative ATP depletion, cellular dysfunction and ultimately cell death. Until recently, primary mtDNA mutations were thought to be the major cause of mitochondrial disease in humans, particularly those presenting in adult life. Now, a newer class of autosomal mitochondrial diseases accounts for a rapidly growing clinical group causing disease through a secondary effect on mtDNA, see Figure 1.

#### Mutations in DNA polymerase γ, POLG

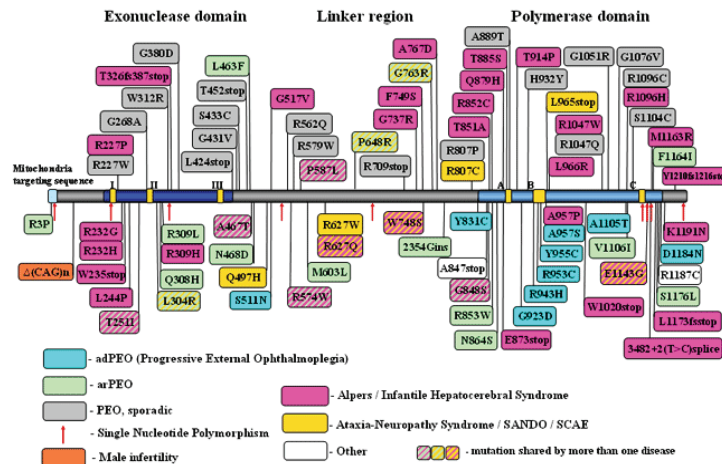


Figure 1. Human DNA Polymerase Gamma Mutation Database

Mitochondrial DNA is continuously recycled, independent of cell cycle, and this replication is achieved by a number of nuclear-encoded proteins. The only DNA polymerase present in the mammalian mitochondria is the pol-r complex. In humans, mtDNA is copied by a 195 kDa heterotrimer consisting of a catalytic subunit (p140, coded by POLG on chromosome 15q25) and two identical accessory subunits (p55, coded by POLG2 on chromosome 17q). Like other A-type polymerases, pol-r has both polymerase and 3'->5' proofreading exonuclease activity.

POLG mutations have been identified both in autosomal dominant syndromes as well as autosomal recessive syndromes. Molecular diagnostic analysis of the POLG gene has been more often done with sequencing only. Sequencing lacks the sensitivity to detect mutations that are present only at a low or high percentage. As the sensitivity of sequencing is often listed as being able to detect proportions of the minor species (heterozygous) down to 20–40%, even using the low-end value, this means that all samples with near 0% to 20%, as well as 80% to near 100%, heterozygous will be missed or interpreted as a homozygous wild type/polymorphism. Additionally, sequencing is particularly expensive and time intensive in performance and analysis.

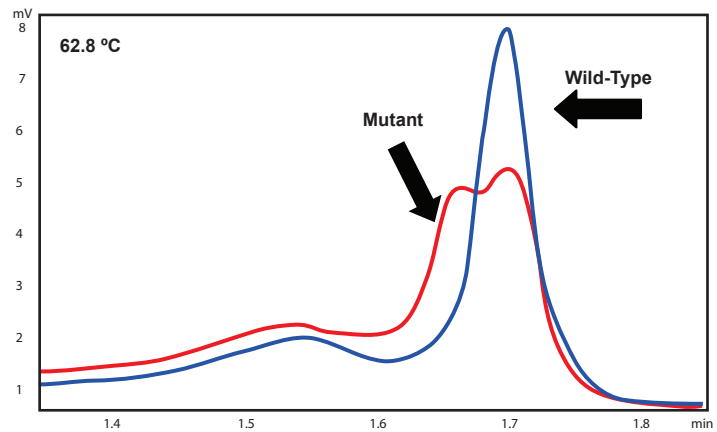
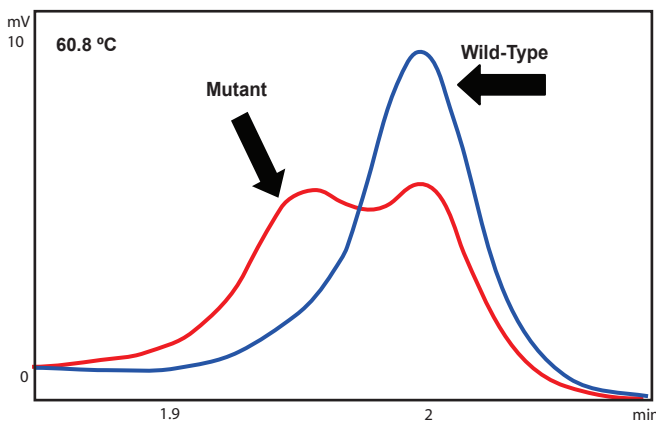
We present data from analysis of a clinical sample group with a sensitive, cost effective and less labor intensive approach. All 22 amplicons were amplified with universal PCR conditions and scanned with rapid-sensitive mutation detection with DHPLC and heteroduplex cleavage fragment analysis on the WAVE® HS System. Finally, each amplicon was analyzed with double strand sequencing.

## METHODS AND RESULTS

After PCR amplification of all amplicons, see Table 1, an aliquot was analyzed by DHPLC on the WAVE HS System. Each amplicon used specific conditions designed to scan the length of the PCR product. Thus, comparison to the wild-type showed any variation from the wild-type, see Figures 2 and 3.

**Table 1. Details of the amplicons used POLG mutational analysis.** 22 fragments were amplified from reference and clinical samples using a single PCR condition.

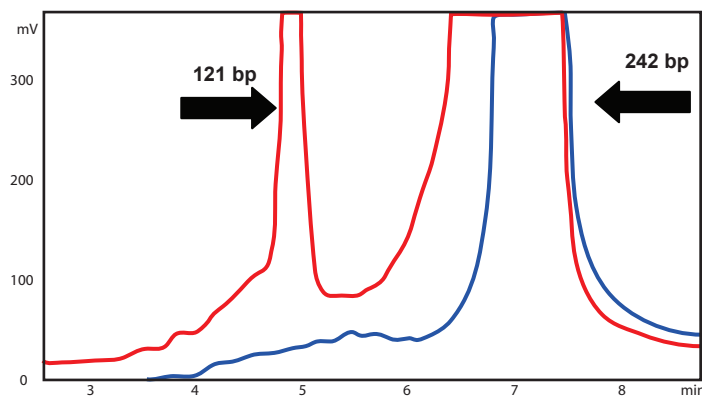
Amplicon	Size of fragment	Amplicon	Size of fragment
Amplicon 2a	348bp	Amplicon 11	201bp
Amplicon 2b	308bp	Amplicon 12	189bp
Amplicon 2c	394bp	Amplicon 13	240bp
Amplicon 3	242 bp	Amplicon 14	219bp
Amplicon 4	243bp	Amplicon 15/16	355bp
Amplicon 5	293bp	Amplicon 17	250bp
Amplicon 6	223 bp	Amplicon 18	298bp
Amplicon 7	246 bp	Amplicon 19/20	473bp
Amplicon 8	329bp	Amplicon 21	400bp
Amplicon 9	220bp	Amplicon 22	225bp
Amplicon 10	297bp	Amplicon 23	194bp



**Figures 2 and 3. POLG DHPLC analysis.** Patient samples were screened for heterozygous mutations within exon 3 of the POLG gene using PCR and subsequent analysis with the WAVE HS System under two temperature conditions. The Patient Sample R4197 clearly showed a variation from the wild-type.

Heteroduplex cleavage fragment analysis was performed with SURVEYOR Nuclease and the WAVE HS System. SURVEYOR cuts amplicons where there is a heteroplasmic variation. The presence and sizes of these fragments were viewed on the WAVE, see Figure 4.

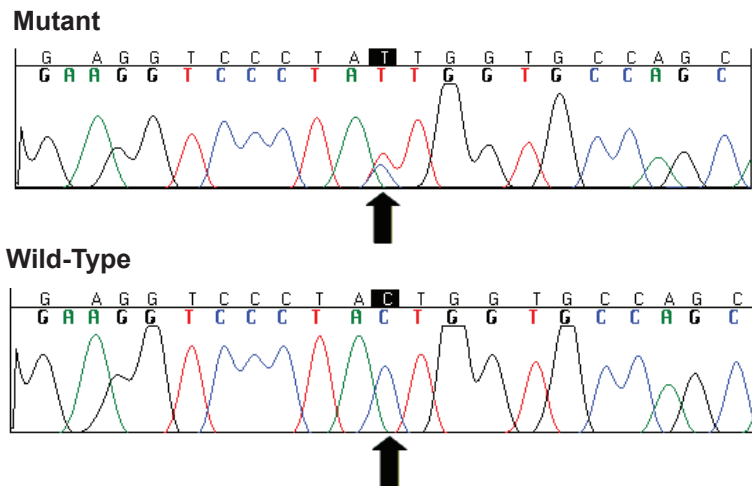
**Figure 4. POLG SURVEYOR Analysis.** Patient samples were screened for mutations following PCR by using SURVEYOR heteroduplex scanning and the WAVE HS System for detection. The Patient Sample R4197 showed a variant in the middle of the amplicon, base 121 of 242.



Finally, selected amplicons were analyzed by bi-directional, double strand sequencing on an ABI 3100 automated sequencer (Applied Biosystems). Variant identities were determined and labeled as mutational or as a common polymorphism (SNP), see Table 2.

**Table 2. Example results of somatic mutation screening of POLG patients.**

Amplicon/ Exon	Samples	DNA Base change	Amino Acid change	Freq	Type
3	R4197	C>T	Thr>Ile	0.6	Sub Mut
10	R4197	C>T	Pro>Leu	0.5	Sub Mut
11	R444	G>A	Gly>Asp	0.4	Sub Mut
12	R420	C>T	Intron	0.5	Intron SNP
13	R490	C>T	Leu>Leu	0.6	Silent SNP
17	R419 R421 R443 R446 R450 R490 R499	insGTAG	Intron	1	Ins SNPs
17	R410 R414 R416 R422	insGTAG	Intron	0.5	Ins SNPs
23	R490 R563 R585	G>T	Gln>His	1	Sub SNPs



**Figure 5. POLG Sequencing.** Patient samples that were found to be positive via DHPLC and SURVEYOR heteroduplex scanning were directly sequenced. The Patient Sample R4197 variant was shown to be the Thr>251>Ile mutation caused by aCt>aTt.

## CONCLUSIONS

- Validation of this mutation screening approach to regions that harbor the most prevalent pathogenic mutations was confirmed by the successful detection of any and all reference mutations in test samples.
- Amplicons were designed and optimized for both accuracy and efficiency in the mutation screening process.
- PCR primers and conditions were designed and optimized to facilitate the use of a single thermal cycling condition, while improving specificity and yield of all products for analysis. This heightened quality and minimized the resources required.
- DHPLC analysis conditions were optimized for mutation detection in each amplicon with use of a minimal range of different temperatures. This minimized process time.
- The POLG gene of patients and control samples were screened using this multi-pronged approach with independent screening technologies. This ensured discovery of all variants and with greater sensitivity than by sequencing alone.
- We have developed and validated an in-depth screening methodology for mutational analysis of the POLG gene for heterozygous and homozygous mutations. Low levels of heterozygous mutations of <1% are easily detected. The versatility of the WAVE HS System, coupled with SURVEYOR Nuclease and sequencing, have been successfully applied in these patient samples with POLG variations for a cost-effective and thorough analysis of somatic mutations
- This comprehensive analysis for clinical testing can be completed within 4 weeks and is very competitive in both speed and cost.

## REFERENCES

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