

# Reliable Resequencing of Human Mitochondrial Genome to Identify Mutations Linked to Mitochondrial Related Diseases

Considerable progress in understanding mitochondrial genetics has been achieved in recent years. There is mounting evidence that mitochondrial dysfunction and mitochondrial DNA (mtDNA) mutations can play a role in cancer [1, 2], diabetes [3], and aging [4]. The availability of a full-length consensus human genome sequence has helped researchers to point out mtDNA aberrations as a putative cause for complex diseases.

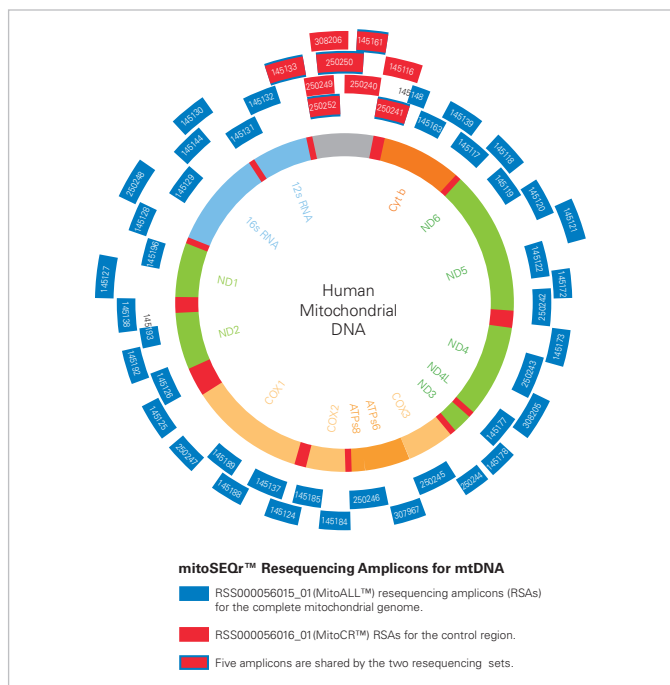
## Mitochondrial Genetics

The mitochondrial genome consists of a multicopy, circular dsDNA molecule (16.6 kb in humans), which encodes 13 essential polypeptides of the oxidative phosphorylation (OXPHOS) system and the necessary RNA machinery (2 rRNAs and 22 tRNAs) for their translation within the organelle. The

polyploid nature of the mitochondrial genome — up to several thousand copies per cell — gives rise to an important feature of mitochondrial genetics, heteroplasmy. When mtDNA are present as a mixture of two or more different mitochondrial genomes, it is present as a heteroplasmic status. If all the copies of the mtDNA are identical mtDNA status is considered homoplasmic. As a consequence, some mutations may only be present in some copies of the mitochondrial genome (heteroplasmic mutation). An easy and reliable resequencing system is essential in the study of both common and complex disorders caused by mitochondrial mutations.

## Applied Biosystems Solutions for Resequencing the Mitochondrial Genome

The mitoSEQr™ System is a PCR-based resequencing system which enables identification of sequence variations in the entire human mitochondrial genome and its control region. Overlapping regions of the mitochondrial genome are amplified with specific primer pairs tailed with universal M13 sequences at their 5' end to generate resequencing amplicons (RSAs). The RSAs are then used as templates for quick sequencing using universal M13 primers. All primer pairs are ready to use and anneal at the same temperature.



**Figure 1.** mitoSEQr™ Resequencing Amplicons for mtDNA. The mitoSEQr system is designed for detecting sequence variants in human mtDNA. Pre-designed PCR primers tailed with M13 sequences generate either 9 resequencing amplicons (RSAs) for the mtDNA control region or 46 RSAs for the entire mitochondrial genome, as indicated. The PCR primers are provided ready to use and anneal at the same temperature.

## Mitochondrial Mutations and Disease

There has been considerable interest in the possibility that mtDNA variants might cause a predisposition to several diseases. Phylogenetic studies show that human populations can be divided into several mtDNA haplogroups that are based on specific single-nucleotide polymorphisms (SNPs), reflecting mutations accumulated by a discrete lineage [5]. mtDNA inherited variants, namely the haplogroups, might be predisposing factors in common diseases such as diabetes, Alzheimer's disease [7, 9] and Parkinson's disease [8, 9], as well as play a role in longevity [4, 10]. It has also been reported that in tumors presenting mitochondrial hyperplasia (oncocyctic tumors), disruptive mutations in mtDNA-encoded complex I genes function as specific tumor markers [11–14].

## Resequencing Workflow in Action: Identifying Mitochondrial Mutations Using the mitoSEQr™ System

Dr. Giuseppe Gasparre and Dr. Elena Bonora (Genetics Department, University of Bologna, Italy) have been working for several years on a tumor characterized by a striking proliferation of mitochondria. Dr. Aurelia Santoro (Interdepartmental Center L. Galvani, University of Bologna, Italy) has been working on mtDNA, hereditary and somatic variability, in Alzheimer's disease. Their research is focused on understanding how mutations in the mitochondrial genome may be involved in high impact diseases such as cancer and age-related disorders. Here we present experimental details and two data examples for resequencing the human mitochondrial genome using the mitoSEQr™ System.

### Samples

DNA was obtained from different sources such as blood, cultured cells, frozen and paraffin-embedded tissue.

### PCR Amplification

Each reaction contained: AmpliTaq Gold® PCR Master Mix 2X, RSA primers (1.2 µM total primer concentration), 8% glycerol and 0.5 ng/µL of human genomic DNA. The reactions were amplified using the GeneAmp® PCR System 2700. Cycling conditions were: 96°C, 5 minutes; 94°C, 30 seconds, 60°C, 45 seconds, 72°C, 45 seconds amplified for 40 cycles, and post extension of 72°C, 10 minutes. For a detailed protocol, see VariantSEQr™ and mitoSEQr™ Resequencing Systems Protocol (P/N 4344468).

### Purification of PCR Product

PCR product was purified with ExoSAP-IT (USB), a mixture of exonuclease I and shrimp alkaline phosphatase used to remove unincorporated dNTPs and primers present in the PCR product. 2 µL of ExoSAP-IT was added to 10 µL of PCR product and incubated at: 37°C for 30 minutes and 80°C for 15 minutes. An aliquot of each purified PCR product was run on an agarose gel to give an estimate of the product concentration and to confirm that the PCR amplification efficiency was roughly equivalent among all samples (Figure 2). DNA from strains devoid of mtDNA (rho0 cells) shows no amplification with the mitoSEQr Resequencing System indicating that the PCR primers are specific for mtDNA (Figure 2C).

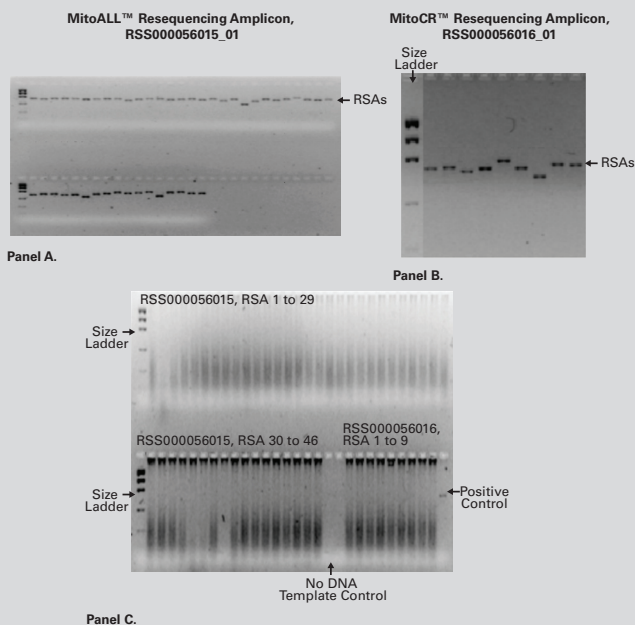
### Sequencing

The PCR product was sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit and the M13F and M13R primers. 10 µL sequencing reaction were composed of BigDye Terminator v.3.1 Ready Reaction mix; Forward or Reverse M13 primer (3.2 pmole/µL); 5X sequencing buffer and 3–10 ng (amount based on agarose gel quantification) of ExoSAP-IT purified PCR product. Cycle sequencing was performed on a GeneAmp PCR System 2700 as follows: 96°C, 1 minute; 96°C, 10 seconds, 50°C, 5 seconds, 60°C, 4 minutes for 25 cycles.

The sequenced product was purified by addition of 2.5 µL of 125 mM EDTA and 30 µL of 100% ethanol and centrifugation speed at 1600 x g for 45 min, as described in BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol (P/N 4337035). The precipitate was washed with 70% ethanol and resuspended in 10 µL of Hi-Di™ Formamide. Electrophoresis was performed on the Applied Biosystems 3730 DNA Analyzer. For further information, see VariantSEQr™ and mitoSEQr™ Resequencing Systems Protocol (P/N 4344468).

### Data Analysis with SeqScope Software v2.5

SeqScope® Software was used to perform an automated assembly of the whole mitochondrial genome from the 46 amplicons. Samples were analysed simultaneously in one project and visualised in a single window. A Project Template based on the revised Cambridge Reference Sequence (rCRS) enabled the detection of known and unknown variants ([www.mitomap.org](http://www.mitomap.org)). The project template is divided into Regions of Interest (ROIs), which comprise of all coding genes, transfer RNA (tRNA), ribosomal RNA (rRNA) sequences, and the control region (D-loop). All single ROIs are selectable from an easily accessible menu allowing the user to zoom in on the sequences of a specific region of the mtDNA. A complete list of all changes (including a description of the known variants indicating the haplogroup they define) is generated, indicating the position with respect to rCRS, the base change, the quality value of the mutation and the type, eventually indicating the amino acid substitution. Possible heteroplasmies were also listed.



**Figure 2.** Resequencing Using the mitoSEQr™ System. Amplification of total DNA with (A) 46 primer pairs, and with (B) the 9 primer pairs specific for the mitochondrial control region. (C) Amplification of total DNA isolated from rho0 cells (cells devoid of mtDNA) amplified by all the 55 specific mtDNA primers showed no amplification.



**Dr. Elena Bonora, Dr. Giuseppe Gasparre, and Dr. Aurelia Santoro.**

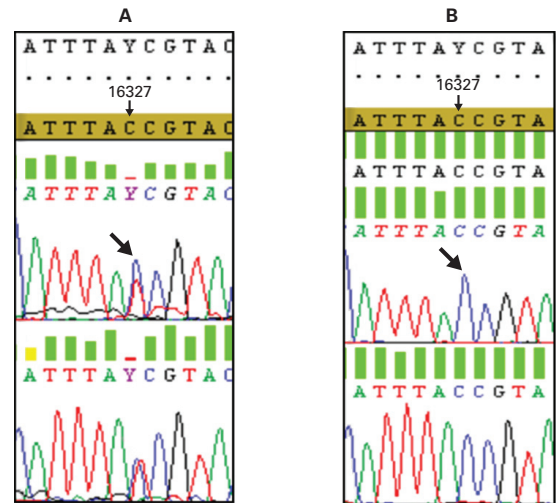
The research groups of the Interdepartmental Center L. Galvani directed by Prof. Claudio Franceschi and of the Unit of Medical Genetics, directed by Prof. Giovanni Romeo have long been working on the role of mitochondria in diseases such as cancer.

Dr. Elena Bonora (left) and Dr. Giuseppe Gasparre (middle) have been working for several years on a particular type of tumor, the oncocytoma, characterized by a striking proliferation of mitochondria. Their investigations have been focused on understanding how mutations in the mitochondrial genome (mtDNA) may be involved in the development or progression of neoplasias.

Dr. Aurelia Santoro (right) has dedicated her efforts in the past four years to the study of Alzheimer's disease, one of the most common and invalidating neurodegenerative disorders. One of the features of this complex disease is the oxidative damage in the brain likely leading to a higher rate of mtDNA mutations. Her field of research concerns the detection of mutations that may explain the pathological phenotype of the disease.

### Identifying Heteroplasmy Mutations in a patient with Alzheimers Disease (AD)

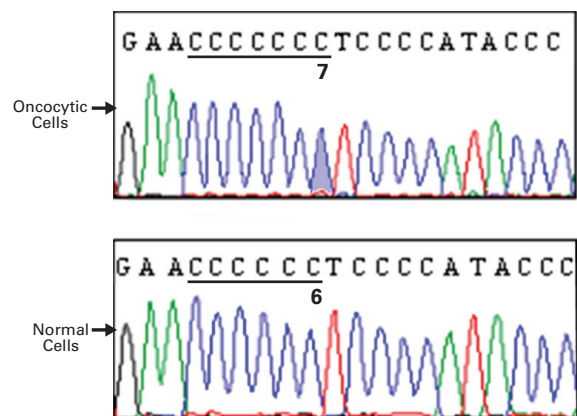
The entire sequence of mtDNA was obtained and easily compared with a reference sequence, allowing detection of variants and heteroplasmies. The somatic variability in the mtDNA sequence was evaluated in a patient with AD by analyzing the differences in mtDNA sequence between affected and non affected tissues, with particular attention to heteroplasmic positions. Figure 3 shows a heteroplasmic mutation found in the affected tissue compared to a homoplasmic one in the non affected tissue.



**Figure 3.** Detecting Heteroplasmic Mutation Using the mitoSEQr™ System. Comparison between (A) AD affected (B) and non-affected tissue from a 70 years old subject. At position 16327 a heteroplasmy is detected in the affected tissue but not in the non-affected tissue. Analysis was performed with SeqScape® software v 2.5.

### Identifying Markers of Oncocytic Tumors

Comparison of mtDNA sequence with a reference sequence identified specific disruptive mutations that cause oncocytic tumors. Figure 4 shows a homoplasmic frameshift mutation in ND1, a gene coding for one of the 45 complex I subunits of the respiratory chain, that was detected in the only existing cell model of thyroid oncocytic tumor. The same mutation along with other similar disruptive mutations in complex I was detected in vivo in biopsies from oncocytic tumors of different origins (thyroid, breast and kidney).



**Figure 4.** Detecting a Homoplasmic ND1 Insertion Using the mitoSEQr™ System. Comparison of oncocytic tumor cells and wild-type ND1 in non-oncocytic cells revealed a homoplasmic ND1 insertion.

## REFERENCES

1. Fliss MS, Usadel H, Caballero OL, Wu L, Buta MR, Eleff SM, Jen J, Sidransky D (2000) Facile detection of mitochondrial DNA mutations in tumors and bodily fluids, *Science* **287**(5460):2017–2019.
2. Kassauei K, Habbe N, Mullendore ME, Karikari CA, Maitra A, Feldmann G (2006) Mitochondrial DNA mutations in pancreatic cancer, *Int J Gastrointest Cancer* **37**(2–3):57–64.
3. Lai HC, Liu TJ, Ting CT, Yang JY, Huang L, Wallace D, Kaiser P, Wang PH (2007) Regulation of IGF-I receptor signalling in diabetic cardiac muscle: dysregulation of cytosolic and mitochondria HSP60, *Am J Physiol Endocrinol Metab* **292**(1):E292–297.
4. De Benedictis G, Rose G, Carrieri G, De Luca M, Falcone E, Passarino G, Bonafe M, Monti D, Baggio G, Bertolini S, Mari D, Mattace R, Franceschi C (1999) Mitochondrial DNA inherited variants are associated with successful aging and longevity in humans, *FASEB J* **12**:1532–1536.
5. Torroni A, Achilli A, Macaulay V, Richards M, Bandelt HJ (2006) Harvesting the fruit of the human mtDNA tree, *Trends Genet* **22**:339–345.
6. Mohlke KL, Jackson AU, Scott LJ, Peck EC, Suh YD, Chines PS, Watanabe RM, Buchanan TA, Conneely KN, Erdos MR et al (2005) Mitochondrial polymorphisms and susceptibility to type 2 diabetes-related traits in Finns, *Hum Genet* **118**(2):245–254.
7. Van der Walt JM, Dementieva YA, Martin ER, Scott WK, Nicodemus KK, Kroner CC, Welsh-Bohmer KA, Saunders AM, Roses AD, Small GW et al (2004) Analysis of European mitochondrial haplogroups with Alzheimer disease risk, *Neuroscience letters* **365**:28–32.
8. Ghezzi D, Marelli C, Achilli A, Goldwurm S, Pezzoli G, Barone P, Pellecchia MT, Stanzione P, Brusa L, Bentivoglio AR et al (2005) Mitochondrial DNA haplogroup K is associated with a lower risk of Parkinson's disease in Italians, *Eur J Hum Genet* **13**(6):748–752.
9. Santoro A, Salvioli S, Raule N, Capri M, Sevini F, Valensin S, Monti D, Bellizzi D, Passarino G, Rose G, De Benedictis G, Franceschi C (2006) Mitochondrial DNA involvement in human longevity, *Biochim. Biophys. Acta* **1757**: 1388-1399.
10. Niemi AK, Hervonen A, Hurme M, Karhunen PJ, Jylhä M, Majamaa K (2003) Mitochondrial DNA polymorphisms associated with longevity in a Finnish population, *Hum Genet* **112**(1):29–33.
11. Bonora E, Evangelisti C, Bonichon F, Tallini G, Romeo G (2006) Novel germline variants identified in the inner mitochondrial membrane transporter TIMM44 and their role in predisposition to oncocytic thyroid carcinomas, *Br J Cancer* **95**(11):1529–1536.
12. Bonora E, Porcelli AM, Gasparre G, Biondi A, Ghelli A, Carelli V, Baracca A, Tallini G, Martinuzzi A, Lenaz G, Rugolo M, Romeo G (2006) Defective oxidative phosphorylation in thyroid oncocytic carcinoma is associated with pathogenic mitochondrial DNA mutations affecting complexes I and III, *Cancer Res* **66**:6087–6096.
13. Gasparre G, Porcelli AM, Bonora E, Pennisi LF, Toller M, Iommarini L, Ghelli A, Moretti M, Betts CM, Martinelli GN (2007) Disruptive mitochondrial DNA mutations in complex I subunits are markers of oncocytic phenotype in thyroid tumors, *PNAS* **104**:9001–9006.
14. Gasparre G, Hervouet E, de Laplanche E, Demont J, Pennisi LF, Colombel M, Mège-Lechevallier F, Scoazec JY, Bonora E, Smeets R (2008) Clonal expansion of mutated mitochondrial DNA is associated with tumor formation and complex I deficiency in renal oncocyctomas, *Hum Mol Genet* **17**(7):986-995.

## ORDERING INFORMATION

Description	Quantity	Part Number
mitoALL™ Resequencing Amplicon, RSS000056015_01, for resequencing the entire mitochondrial genome with 46 RSAs	500 Reactions	4348846
mitoCR™ Resequencing Amplicon, RSS000056016_01 for resequencing the control region of the mitochondrial genome with 9 RSAs	500 Reactions	4348809
AmpliTaq Gold® PCR Master Mix, 250 Units/ 5mL	1 kit	4318739
Unlabeled Oligonucleotide (M13 Forward or M13 Reverse)	10,000 picomoles	450005
BigDye® Terminator v3.1 Cycle Sequencing Kit	100 Reactions	4337455
Applied Biosystems 3130 Genetic Analyzer	1 instrument	Inquire
Applied Biosystems 3730 DNA Analyzer	1 instrument	Inquire
SeqScape® Software v2.6, Initial License	1 license	4327091
Hi-Di™ Formamide	25mL	4311320

## RELATED PRODUCTS

VariantSEQR™ Resequencing System	For more resequencing primer sets visit <a href="http://www.appliedbiosystems.com/variantseqr">www.appliedbiosystems.com/variantseqr</a>	
BigDye® XTerminator™ Purification Kit	100 Reactions	4376486
Variant Reporter™ Software	1 Initial License	4385261

### For Research Use Only. Not for use in diagnostic procedures.

Applied Biosystems, ABI PRISM, AB (logo), GeneAmp, Big Dye and SeqScape are registered trademarks and Hi-Di, mitoSEQR, VariantSEQR, mitoALL, mitoCR, Variant Reporter, xTerminator, and methylSEQR are trademarks of Applied Biosystems Corporation or its subsidiaries in the U.S. and/or certain other countries. AmpliTaq Gold and TaqMan are registered trademarks of Roche Molecular Systems, Inc. All other trademarks are the sole property of their respective owners.

© 2008 Applied Biosystems. All rights reserved. Printed in the USA. 04/2008 Publication 132AP01-01



### Headquarters

850 Lincoln Centre Drive | Foster City, CA 94404 USA  
Phone 650.638.5800 | Toll Free 800.327.3002  
[www.appliedbiosystems.com](http://www.appliedbiosystems.com)

### International Sales

For our office locations please call the division headquarters or refer to our Web site at [www.appliedbiosystems.com/about/offices.cfm](http://www.appliedbiosystems.com/about/offices.cfm)