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## Detection of mitochondrial DNA (mtDNA) mutations

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### Abstract

The maternally inherited mitochondrial DNA (mtDNA) is a circular 16,569-bp double stranded DNA that encodes 37 genes, twenty-four of which (2 rRNA and 22 tRNA) are necessary for transcription and translation of 13 polypeptides that are all subunits of respiratory chain. Pathogenic mutations of mtDNA cause respiratory chain dysfunction, and are the underlying defect in an ever-increasing number of mtDNA-related encephalomyopathies with distinct phenotypes. In this chapter, we present an overview of mtDNA mutations and describe the molecular techniques currently employed in our laboratory to detect two types of mtDNA mutations: Single-large scale rearrangements and point mutations.

### Keywords

Mitochondria; mtDNA; mutations; Real-time PCR; Next generation Sequencing

## I. Introduction

The human mitochondrial genome is a 16,569-bp (base pair) circle of double stranded DNA. It contains 37 genes, which specify 2 ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs), and 13 polypeptides (Fig. 1). All 13 polypeptides encoded by mtDNA are components of the respiratory chain/oxidative phosphorylation system and include 7 subunits of complex **I** [NADH-coenzyme **Q** (CoQ) oxidoreductase], 1 subunit of complex **III** (CoQ cytochrome *c* oxidoreductase), 3 subunits of complex **IV** (cytochrome *c* oxidase), and two subunits of complex **V** (ATP synthase).

Each of these complexes also contains subunits encoded by nuclear genes, which are imported from the cytoplasm, assembled into holoenzymes with the mtDNA-encoded subunits, and embedded in the mitochondrial inner membrane. Complex II (succinate dehydrogenase-CoQ oxidoreductase) is encoded entirely by nuclear genes.

As mitochondria have a very dynamic multimembrane organization that facilitates the organelle's distribution throughout the cell as a bioenergetic network, mtDNA is maintained in macromolecular assemblies called nucleoids at discrete sites throughout the mitochondrial

network. These nucleoids are protein-DNA assemblies, typically consisting of one or two copies of mtDNA and associated proteins (Kukat *et al.* 2011; Brown *et al.* 2011), that ensure the distribution and effective transmission of mtDNA (Bonekamp and Larsson, 2018).

There are several distinctive features of mtDNA that are relevant to the understanding of mtDNA-related disease. (1) At fertilization all mitochondria are contributed by oocytes; thus mtDNA is inherited only from the mother. Therefore, most mtDNA point mutations are maternally inherited: a woman carrying an mtDNA point mutation will transmit it to all her children, males as well as females, but only the daughters will transmit it to their progeny. However, while mtDNA itself is transmitted by the mother, disorders of mtDNA are not always inherited maternally. For example, large-scale single deletions in mtDNA are often sporadic and are not maternally inherited. Similarly mtDNA depletion and multiple deletion errors are often the result of mutation in nuclear genes that affect the integrity or quantity of mtDNA. As such, they are transmitted as autosomal recessive or autosomal dominant traits. (2) In contrast to nuclear genes, each consisting of one maternal and one paternal allele, there are hundreds or even thousands of copies of mtDNA in every cell. Thus, when there is a deleterious mutation, both normal and mutated mtDNAs may coexist within a patient's tissues, a condition known as heteroplasmy. A critical number of mutant mtDNAs must be present before tissue dysfunction and clinical signs become apparent, the so-called threshold effect. Tissues with high requirement for oxidative metabolism, such as muscle, heart, eye, and brain have relatively low thresholds and are particularly vulnerable to mtDNA mutations. (3) At cell division, the proportion of mutant mtDNA in daughter cells can shift, a phenomenon termed or known as mitotic segregation. If and when the pathogenic threshold for a particular tissue is exceeded, the phenotype can change. Thus in a patient who is heteroplasmic for a pathogenic mutation, the clinical phenotype can change over the course of time.

## II. MtDNA Mutations

Beginning in 1988, an ever-increasing number of mitochondrial diseases with distinct clinical phenotypes have been associated with mutations in mtDNA (reviewed in Schon, 2000), almost all of which result in neurological or neuro-muscular disorders. These errors fall into two major classes: (1) large-scale rearrangements of mtDNA, and (2) point mutations in mtDNA. Large-scale single deletion of mtDNA are associated with three major clinical conditions: Kearns-Sayre syndrome (KSS), progressive external ophthalmoplegia (PEO), and Pearson syndrome (PS). Although dozens of different deletions have been described, each patient harbors only a single type of deletion and the number of deleted genome varies in different patients and in different tissues. Most patients reported with deletions in mtDNA have been sporadic; mothers of affected individuals and children of affected women are clinically unaffected and, when tested, have no detectable deletions (Graff *et al.*, 2000; Larsson *et al.*, 1992; Zeviani *et al.*, 1990). This suggested that deletion arose *de novo* early in embryogenesis or ovum. However, there have been several reports of deletions that appear to have been transmitted maternally (Bernes *et al.*, 1993; Boles *et al.*, 1997; Shanske *et al.*, 2002). Thus, one needs to be cautious when counseling.

Regarding mtDNA point mutations, as of to date, more than 200 pathogenic mtDNA point mutations have been documented and new ones are still being described (<http://www.mitomap.org>). These point mutations are located in all regions of the genome. About two-thirds of them are located in tRNA genes. The rest are located in the polypeptide-coding genes, with a few in rRNA genes. Some of these mutations have been reported in only one or a few families, while others appear to be more common.). The first point mutation in a tRNA gene to be reported was an A-to-G transition at nt-8344 (A8344G) in patients with MERRF (Shoffner *et al.*, 1990). This mutation is present in >90% of patients with MERRF, but other mutations have also been reported (Servidei, 2003). The A-to-G transition at nucleotide (nt) position 3243 (A3243G) is the most frequently encountered mtDNA point mutation and it is associated with a spectrum of clinical presentations. It was originally described in patients with MELAS (Goto *et al.*, 1990), but was subsequently observed in maternal relatives of patients with MELAS who were often oligosymptomatic, presenting with diabetes, short stature, migraine headaches, hearing loss, or other features, either alone or in various combinations (DiMauro and Schon, 2001). The more common mutations in protein-coding genes are associated with two disorders: LHON (Leber hereditary optic neuropathy) and NARP/MILS (neuropathy, ataxia, and retinitis pigmentosa/maternally inherited Leigh syndrome). The G-to-A transition at nt-11,778 in patients with LHON, a disorder characterized by acute or subacute onset of bilateral visual loss in young adults, was the first point mutation in mtDNA to be reported (Wallace *et al.*, 1988). LHON is associated with three primary mutations in genes encoding subunits of complex I of the respiratory chain—G3460A, G11778A, and T14484C (Servidei, 2003)—and these mutations are usually homoplasmic. A T-to-G mutation at nt-8993 (T8993G) in the mtDNA gene encoding ATPase 6 was initially described in patients with a maternally inherited multisystem disorder characterized by NARP (Holt *et al.*, 1990). A family was subsequently reported in which an adult family member had the NARP syndrome and three children had MILS (Tatuch *et al.*, 1992). It is now well established that, for this particular mutation, there is a good correlation between the percentage of mutant genomes (i.e., degree of heteroplasmy) and the severity of the clinical phenotype. Patients with low levels of mutation are unaffected or have only mild symptoms, those with intermediate levels present with NARP, whereas those with very high levels (more than 95%) of mutation are associated with MILS (White *et al.*, 1999). In addition to the T-to-G mutation, patients with a T-to-C substitution at the same position (T8993C) have been reported; they have milder clinical manifestations (DiMauro and Bonilla, 2003). Some patients with exercise intolerance often harbor mutations in protein-coding genes causing defects in complexes I, III, or IV (Andreu *et al.*, 1999; DiMauro and Schon, 2001). These patients are almost always sporadic, with no evidence of maternal transmission. Additionally, in most cases, these mutations are present only in muscle and are not detectable in blood or fibroblasts. It is believed that these mutations are somatic, that is, they are spontaneous events that occurred in muscle and do not affect germ line cells. Finally, pathogenic mutations in rRNA genes are rare. The most common one is in the 12S rRNA gene (A1555G) and has been reported in families with aminoglycoside induced deafness or nonsyndromic hearing loss (Prezant *et al.*, 1993).

### III. Detecting mtDNA Large-Scale Single Deletion by Southern Blot analysis

Traditionally, single or multiple deletions in mtDNA are detected by Southern blot analysis and are best demonstrated by testing DNA isolated from muscle, since in most patients deletions are not detectable in DNA isolated from blood. To perform Southern blot analysis, total DNA is isolated, digested with restriction enzymes, separated on an agarose gel, transferred to a membrane (nitrocellulose or nylon), and incubated with a labeled (radioactive or non-radioactive) mtDNA probe that will hybridize only to the mtDNA. The bands are detected by autoradiography (when radiolabeled probe is employed) or by chemiluminescence imaging (when non-radioactive probe is employed). The fragments and their size(s) indicate whether only the normal-sized mtDNA (16.6 kb) is present or whether there is also a population of a smaller mtDNA, indicating a deletion. A detailed protocol for this methodology has previously been published in this book series (Schon *et al.*, 2002; Naini and Shanske, 2007).

### IV. Detecting mtDNA Large-Scale Single Deletions by Fluorescence In Situ Hybridization

#### a. Overview/rationale

Fluorescence *in situ* hybridization (FISH) provides a sequence-specific method to visualize competing wildtype (WT) mtDNA and large-scale deletions (-mtDNAs) within the heteroplasmic cell. As a complementary method to traditional Southern blotting and emergent next generation sequencing methods for quantitating the levels of competing mtDNA species in a heteroplasmic cell population, two-color FISH is an adaptation of the method of Margineantu *et al.* (2000) using a combined set of fluorophore-labeled mtDNA-specific probes to reveal the distribution of competing mtDNAs through fluorescence microscopy (Gilkerson *et al.* 2008). In heteroplasmic cells or tissue carrying WT and -mtDNAs, probes can be designed to hybridize both within the deleted mtDNA region and to a sequence common to both mtDNAs. By labeling the two probes with different fluorophores, WT mtDNAs will hybridize with both probes, while the -mtDNA will hybridize with only the common probe (Santra *et al.* 2004). While DNA-binding dyes (such as DAPI) and immunolabeling for mtDNA-associated proteins such as TFAM can effectively track mtDNA nucleoids within the cell, the FISH method described here allows the sequence-specific visualization of WT and -mtDNAs within heteroplasmic cells.

#### b. Materials/equipment/reagents

**Probe preparation**—MtDNA forward and reverse primer sets for common and deletion-specific mtDNA regions.

Taq polymerase

10x PCR buffer

dNTPs

Invitrogen Molecular Probes ARES DNA labeling kits in two varieties (e.g. AlexaFluor 488 (green, ThermoFisher A21665) and AlexaFluor594 (red, ThermoFisher A21669)).

DNase

Salmon sperm DNA

Human COT-1 DNA

Molecular biology-grade ethanol

### **Cell preparation and hybridization—Cultured human cells**

Glass coverslips

6-well cultured cell dishes

Plastic transfer pipets (5")

PBS

Heating block

## **c. Protocol**

### **Probe preparation**

- i.** Amplify desired mtDNA sequences from total isolated cellular DNA. To ensure specificity, probe sequences are typically ~1.5 kb in length. Check a small amount via agarose gel electrophoresis to ensure the correct size of amplicon is generated. PCR reactions in 50  $\mu$ L volume from 100 ng of template DNA may be pooled to provide sufficient yield for probe labeling.
- ii.** To prepare probe DNA for incorporation of aminoallyl dUTP and subsequent fluorescent labeling, prepare 1  $\mu$ g DNA nick translation reactions, per manufacturer's instructions. For optimal subsequent labeling of probe DNA, PCR amplicons should be nicked (but not degraded), yielding a smear ~100–200 bp when visualized by agarose gel electrophoresis. Each new aliquot of DNase will require empirical determination of optimal concentration for use.
- iii.** Fluorescent labeling of amine-modified DNA allows the covalent linking of the aminoallyl dUTP (incorporated into the probe in step 2 above) with the reactive fluorophore. Reactions are set up per manufacturer's instructions, followed by column purification and ethanol precipitation. Spectrophotometric readings at 260 nm and the fluorophore excitation maximum allow determination of the specific labeling of the probe. Optimal probe labeling results in 1 dye molecule per 12–25 bases. Resuspend probe at 10 ng/ $\mu$ L stock following ethanol precipitation with 3  $\mu$ g salmon sperm DNA and COT-1 DNA. Store probe stock at –80 degrees C.

### Cell preparation and hybridization

1. Grow cultured cells on glass coverslips in appropriate culture media until ~50% confluency. Wash cells twice with PBS, 2 min. each. Fix cells in 4% paraformaldehyde in PBS for 30 min. at room temperature. Wash twice with PBS, 2 min. each.
2. Dehydrate cells through 2 min. in 70%, 90%, 100% ethanol, followed by rehydration through 2 min. incubation in 90% ethanol, 70% ethanol, and PBS.
3. Treat coverslips with RNase (0.1 mg/mL in PBS), 1 hr. 37 degrees. If desired, a small volume of RNase (50–100 mLs) can be added to each coverslip followed by addition of a second coverslip to minimize volume. Wash twice with PBS, 2 min. per wash.
4. Prehybridize coverslips in 2xSSC at 37 degrees, 30 min., followed by serial dehydration in 70%, 90%, and 100% ethanol, 2 min. each incubation. After 100% ethanol, air dry coverslip.
5. Denature coverslip by incubation in 70% formamide in 2xSSC at 72 degrees C for 5 min. Use of a watch glass placed on a heating plate allows optimal heating of solution for denaturation. Dehydrate with ice-cold 70% ethanol (5 min.), 90% ethanol (1 min.), and 100% ethanol. Keep coverslip in 100% ethanol until probe is ready.
6. Dilute probe DNA to 2 ng/ $\mu$ L in hybridization solution (50% formamide, 10% dextran sulfates, 2xSSC). Denature 72 degrees 5 min. Snap cool and spin down.
7. Add probe to coverslip, ~15  $\mu$ L per coverslip. Place another coverslip to create a sandwich, avoiding air bubbles. Seal with rubber cement. Hybridize overnight at 37 degrees in a humidified chamber (wet filter paper in a sealed petri dish).
8. Wash with 0.4xSSC +0.3% Tween-20, 72 degrees 5 min. Wash with 2xSSC +0.1% Tween-20 room temperature, 1 min. If desired, incubate with DAPI to visualize cell nuclei, 5 min. Wash 2  $\times$  2 min. with 2xSSC.
9. Mount coverslip with 50% glycerol in PBS or other mounting medium.

**Visualization and analysis**—Cells may be visualized with any microscopy capable of visualizing the fluorophores utilized for hybridization. Typically, confocal microscopy provides high resolution images that eliminate out-of-focus cellular fluorescence. Within individual cells, mtDNA will be apparent as punctate foci, consistent with their assembly in nucleoids (Margineantu *et al.* 2002, Gilkerson *et al.* 2008). As nucleoids are typically ~100 nm in diameter, a 100x objective (or 60x with optical zoom) is optimal to visualize mtDNAs at nucleoid-level resolution. Using two-color FISH, WT mtDNAs will hybridize with both red and green probes, while  $\Delta$ -mtDNAs will hybridize with only one probe. Notably, WT mtDNAs will display both red and green probes with partial (but not complete) colocalization, an effect presumably due to the packaging of mtDNA into nucleoids. If desired, colocalization can be effectively quantitated using NIH ImageJ (Gilkerson *et al.*



2008). The images and colocalization data can be correlated with Southern blot and PCR data to analyze mtDNA heteroplasmic dynamics.

## V. Detecting mtDNA Large-Scale Single Deletion by Next Generation Sequencing

Although Southern blot analysis is regarded as a gold standard technique for the detection of mitochondrial DNA rearrangements, the method suffers from several limitations. (1) It requires a relatively large amount of DNA (in  $\mu\text{g}$  range), (2) is labor intensive and time consuming (takes 2 to 3 days), and (3) does not give an accurate information as to the level of the heteroplasmy of the deleted DNA or the site of the breakpoints.

The introduction of massively parallel sequencing technology (next generation sequencing) and its application to the analysis of the whole mitochondrial genome, has overcome all the limitations associated with the mtDNA Southern blot analysis. The large amount of sequencing data generated by this methodology enables an accurate detection and quantification of not only mtDNA point mutations, but also of single-large scale deletions or multiple-deletions with their precise breakpoints. As an example, detecting a large mtDNA deletion by next generation sequencing in a patient harboring the mitochondrial DNA “common deletion” with 96% heteroplasmy is shown in Fig. 2. Detailed protocol of this methodology is described in the next section of this chapter.

## VI. Detecting mtDNA Point Mutations by PCR/RFLP Analysis

Known point mutations in mtDNA are screened for by amplifying an appropriate fragment of mtDNA by PCR, digesting with a diagnostic restriction enzyme and analyzing the fragment sizes on a gel. This involves the following steps: (1) oligonucleotide primers are designed to allow amplification of mtDNA encompassing the mutated site, (2) appropriate mtDNA fragments are amplified by PCR amplification in the presence of  $^{32}\text{P}$  dATP, (3) PCR products are digested with a restriction enzyme that will cleave normal versus mutated sequences differentially (i.e., restriction fragment length polymorphism or RFLP), (4) digestion products are electrophoresed through nondenaturing polyacrylamide gels, and (5) fragment sizes are detected by autoradiography. The primer sequences, amplification conditions, and detailed RFLP analyses to detect common point mutations of mtDNA can be found in the previous edition of this book series (Schon *et al.*, 2002; Naini and Shanske, 2007).

## VII. Detecting Mitochondrial DNA Point Mutations and Rearrangements by Next Generation Sequencing (NGS)

### 1. Principle

The massively parallel sequencing of the entire length of mitochondrial genome by Next-Generation Sequencing (NGS) methodology, which examines every nucleotide of the genome with very high coverage ( $>10,000$ ) offers a powerful tool with unparalleled sensitivity for the detection of mtDNA mutations (point mutations as well as



rearrangements). This methodology not only identifies mtDNA mutations, but also determines, with high accuracy their level of heteroplasmy (Cui *et al.*, 2013; Zhang *et al.*, 2012) which is important in the diagnosis of patients with mtDNA-related disorders. A few different versions of this technology have been published in recent years to sequence complete mitochondrial genome. They differ in the required initial step of mtDNA enrichment, which amplifies mitochondrial genome prior to sequencing. For example, in the method published by Palcuict *et al.*, 2016, one pair of primers amplifies complete sequence of mtDNA, whereas in the method published by Huang, 2011, two pairs of overlapping primers were used to amplify complete mtDNA sequence. Although mtDNA enrichment with one pair of primers is less labor intensive, it may however, increase the risk of primer dropouts and heteroplasmy miscalculation in the presence of mtDNA polymorphisms.

The method described here is an adaptation of the Illumina MiSeq protocol for sequencing of the human mitochondrial genome (Document # 15037958) and the method published by Huang, 2011. This method was validated and has been in use in our laboratory since 2013. In this assay, for amplification of mtDNA before sequencing, four sets of primer pairs are used. The first three primer pairs amplify the entire length of mtDNA in three overlapping fragments of 3968, 5513, and 7814 base pairs. The fourth primer, which amplifies the entire sequence of mitochondrial genome, is used for the detection of mtDNA large-scale deletions that could potentially interfere with the binding sites of the first three primer pairs. Following PCR amplifications, all fragments are purified and their sizes are verified on agarose gel electrophoresis. If no deletion is detected as verified by the size of fragment four, the three amplified segments are sequenced together. The three amplicons are quantitated and pooled before library preparation and subsequent sequencing on an Illumina MiSeq. Data analysis and reads alignment to the revised Cambridge Reference Sequence (rCRS) of human mtDNA are performed using NextGENe software (Softgenetics Inc.).

## 2. Reagents and Supplies

### 2.1. Long-Range PCR

1. 2.5 mM dNTP Mix (16  $\mu$ l per sample).
2. 10X LA PCR Buffer II (10  $\mu$ l per sample).
3. 96-well 0.3 ml PCR plate.
4. 200  $\mu$ l PCR tubes.
5. Microseal 'B' Adhesive Seal (Bio-Rad, cat no. MSB-1001).
6. Nuclease-free water (45  $\mu$ l per sample + volume to dilute primers).
7. Purified Human DNA (50 ng/  $\mu$ L).
8. TaKaRa LA TaqDNA Polymerase (1 $\mu$ l per sample).
9. Custom made PCR primers: Four sets of primers are custom synthesized. and purified by Integrated DNA Technologies Inc. The sequence and their positions on mtDNA sequence are shown in Table 1.

## 2.2. Gel Electrophoresis

1. E-Gel (2%) with Ethidium bromide (Thermo Fisher Scientific).
2. E-Gel PowerBase (Thermo Fisher Scientific).
3. E-Imager (Thermo Fisher Scientific).
4. 10X BlueJuice Gel Loading Buffer - Invitrogen, Cat. 10816–015.
5. DNA Molecular Weight Marker II - Roche, Cat. 10236250001.

## 2.3. DNA Purification

1. Agencourt AMPure XP 60 ml kit – Beckman Coulter, Cat. A63881.
2. DNA LoBind Tube 1.5 mL - Eppendorf, Part # 022431021.
3. Ethanol 200 proof (absolute) for molecular biology (500 ml) – Thermo Fisher Scientific, cat. BP2818500.
4. Nuclease-Free Water - Life Technologies, Cat. AM9937.
5. Magnetic stand 96 – Ambion, Cat. AM10027.
6. Heat Block (Thermo Fisher Scientific).

## 2.4. DNA Quantification and Library Preparation

1. Lens paper, 4X6 in. – VW R, Cat. 52846–001.
2. Microseal ‘B’ adhesive seals – Bio-Rad, Cat. MSB-1001.
3. MicroTube (6×16mm), AFA fiber with snap-cap – Covaris, Cat. 520045.
4. MiSeq Reagent Kit v2 (300 cycle) – Illumina, Cat. MS-102–2002.
5. NaOH - Fisher Scientific, Cat. SS255–1.
6. Pipette tips (with filters).
7. Qubit assay tubes – Life Technologies, Cat. Q32856.
8. Qubit dsDNA BR assay kit – Life Technologies, Cat. Q32850.
9. TruSeq Nano DNA Sample Prep Kit v2 – Set A or B – Illumina, Cat. FC-122–2001 or FC-122–2002.
10. Twin.Tec 96-well PCR plates – Eppendorf, Cat. 951020303.
11. Wizard® Genomic DNA purification kit - Promega, Cat. A1120.

## 2.5. Equipment

1. Centrifuge 5804 with rotor A-2-DW P – Eppendorf, Cat. 022628203.
2. Microcentrifuge 5424 with rotor FA-45–24-11 – Eppendorf, Cat. 022620401.
3. Nanodrop 2000 – Thermo Scientific.
4. Qubit 2.0 Fluorometer – Life Technologies, Cat. Q32866.

5. S2 Sonication System – Covaris, Cat. S2.
6. SP Vortex Mixer – Baxter Scientific Products, Cat. S8223–1.
7. Thermal Cycler 2720 – Applied Biosystems, Cat. 4359659.
8. MiSeq instrument from Illumina.
9. Dell T7500 workstation with two 6 core Intel E5645 Processors (2.4GHz) and 50Gb
10. DDR3 Ram using the NextGENe software from Softgenetics running on Windows 7 64 bit version.

### 3. Specimens

1. Blood: anti-coagulated, preferably with citrate or EDTA and less than a week old. Dilute extracted DNA with water to the concentration of 50 ng/  $\mu$ L.
2. Urine specimen, minimum 20 mL of fresh urine is required. Urine specimens should be shipped at room temperature to the laboratory within 24 hours after collection. Dilute extracted DNA with water to the concentration of 50 ng/  $\mu$ L.
3. Muscle biopsy specimens (>50mg) should be kept frozen from the time of biopsy to arrival to the lab. (Dilute extracted DNA with water to the concentration of 10 ng/  $\mu$ L.

### 4. METHOD

#### A. Long-Range PCR (For 16 specimens, 9 hours)

1. Dilute each PCR primer to 5  $\mu$ M with nuclease free water.
2. For each PCR primer (Mito1-F+Mito1-R; Mito2-F+Mito2-R; Mito3-F+Mito3-R;Mito4-F+Mito4-R), prepare the following PCR master mix in the 200  $\mu$ L PCR tube labeled with the appropriate primer pair number as outlined in the table 2.
3. Set up PCR on a plate on ice to prevent possible primer degradation from the polymerase activity.
4. Add 2  $\mu$ L purified human DNA to each plate well (add 2  $\mu$ L water to the blank well).
5. Add 48  $\mu$ L of master mix from step 2 to each well, then pipette to mix and then seal the plate securely.
6. Place the plate on the pre-heated thermal cycler (to 94°C). Close the lid and run the following program: 98°C for 1 min  $\rightarrow$  35 cycles of (98°C for 10 sec  $\rightarrow$  72°C for 8:15 min)  $\rightarrow$  72°C for 10 min  $\rightarrow$  4°C HOLD indefinitely. **At this stage, samples can be stored at –20 °C indefinitely.**
7. Run 1  $\mu$ L of each reaction product on a 1% E-Gel for 40 minutes.

8. If optimum amplification is obtained (intense bands with correct sizes: Mito1: 3968bp, Mito2: 5513bp, Mito3: 7814bp, and Mito4: 16484, proceed to the next step.
9. If size of amplicon 4 is as expected, proceed with quantification and pooling of PCR product 1, 2 and 3. Only these fragments will be sequenced.
10. If amplicons 1, 2 and 3 are of the proper size, but amplicon 4 is not, in addition to sequencing amplicons 1, 2 and 3, amplicon 4 has to be sequenced as a separate specimen, in order to identify heteroplasmic deletions that result in loss of primer binding sites 1F-R, 2F-R, or 3F-R in a subset of the mitochondrial DNA.

#### **B. Amplicon Purification (20 min)**

1. Gently shake the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled. Add AMPure XP beads (1.6 X reaction volume) to each sample based.
2. Mix reagent and PCR reaction thoroughly by pipette mixing 10 times. Let the mixed samples incubate for 5 minutes at room temperature for maximum recovery.
3. Place the reaction plate on the magnetic stand for 2 minutes to separate beads from the solution.
4. Aspirate the cleared solution from the reaction plate and discard.
5. Dispense 200  $\mu$ L of 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature. Aspirate the ethanol and discard. Repeat this step once for a total of two washes.
6. Off the magnet plate, add 40  $\mu$ L of Resuspension Buffer to each well of the reaction plate and pipette mix 10 times. Let the samples incubate for 2 minutes.
7. Place the reaction plate onto the magnetic stand for 5 minutes to separate beads from the solution.
8. Transfer the eluent to a new plate.

#### **C. Quantification and Pooling (55min)**

1. Measure the concentration of each purified amplicon (1–3 and 4 if large deletion is suspected to be present) on Qbit fluorimeter and record concentrations.
2. For pooling three amplicons, in a spreadsheet, calculate the molar concentration and required volume of each amplicon to pool for fragmentation.
3. Store the pooled mtDNA (containing three equimolar purified amplicons) at  $-20^{\circ}$ C. It is now ready for library preparation.

**D. Library Preparation (5 hours)**—For library preparation, amplification, validation and normalization, we use TruSeq Nano DNA Library preparation kit (Illumina part # 15041877) according to manufacturer's recommended protocol.

### **E. Denaturing and Diluting Libraries and phiX Control for Sequencing on MiSeq Platform (40 min)**

1. Denature and dilute DNA: In a microfuge tube, combine equal volume (5  $\mu$ L) of 4 nM library pool and 0.2 N NaOH to create a 2 nM dilution of the library pool.
2. Vortex briefly to mix, and then centrifuge at 280 xg for one minute.
3. Incubate for 5 min at room temperature to denature the DNA into single strands.
4. After 5 min incubation, add 990  $\mu$ L of pre-chilled HT1 to the tube containing denatured DNA. This results in a 20 pM denatured library in 1N NaOH.
5. Place the denatured DNA on ice until you are ready to make the final dilution.
6. Dilute denatured DNA to 12.5 pM by combining 625  $\mu$ L of DNA solution and 375  $\mu$ L of pre-chilled HT1 buffer. Vortex mix, spin and keep on ice.
7. Prepare PhiX control:
  - a. dilute the PhiX library with Elution Buffer to 4 nM:
  - b. Denature phiX control by combining 5  $\mu$ L of each 4 nM PhiX library and freshly diluted 0.2 N NaOH in a microcentrifuge tube.
8. Vortex briefly to mix, then centrifuge at 280xg for one minute.
9. Incubate for five minutes at room temperature to denature the PhiX library into single stands.
10. After the 5 min incubation, add the following volume of pre-chilled HT1 buffer to the tube containing denatured PhiX library to result in a 20 pM PhiX library:
  1. Denatured PhiX library (10  $\mu$ L)
  2. Pre-chilled HT1 (990  $\mu$ L)
11. Dilute an aliquot of the 20 pM PhiX library to 12.5 pM as follows:
  1. 20 pM denatured PhiX library (187.5  $\mu$ L)
  2. Pre-chilled HT1 (112.5  $\mu$ L)
  3. Vortex and spin the tube.
12. Mix sample library and PhiX control:
  1. Combine the following volumes of denatured PhiX control library and your denatured sample library to result in a 20 % volume ratio:
  2. Denatured sample library (800  $\mu$ L)
  3. Denatured PhiX control (200  $\mu$ L)
13. Set the combined sample library and PhiX control aside on ice until you are ready to load it onto the MiSeq reagent cartridge.

## F. Performing the run on a MiSeq (26 hours)

1. Pierce the foil seal over the reservoir labeled Load Samples.
2. Pipette 600  $\mu$ L of your sample libraries into the Load Samples reservoir.
3. Proceed to the run setup steps using the MiSeq control software interface.
4. Create a sample sheet and add your sample information by creating a blank row and entering the Sample ID and index ID (MID 1–12) for each sample.
5. Click Finish and choose the directory where it will be saved.

## G. Starting the run

1. Review “Analysis Workflow”, and “Read Length”. These parameters are specified in the sample sheet.
2. Review the folder locations in the lower-left corner. If you need to change any folder locations, select Change Folders. When you have completed the changes, select Save and then Next.
3. Select Next. The Pre-Run Check screen opens.
4. The system performs a check of all run components, disk space, and network connections before you start the run. If any items do not pass the pre-run check, a message appears on the screen with instructions to correct the error. When all items successfully pass the pre-run check, you are ready to start your run.

**H. Run evaluation**—Sequencing analysis Viewer (SAV) application installed on MiSeq sequencer gives all run information. Two main measures determine whether the run is acceptable or needs to be repeated. 1) Greater than 70% of reads should have Q-score equal or greater 30 (Q  $\geq$  30), and 2) the total yield should be greater than 16Mb per sample.

## I. RESULTS ANALYSIS AND INTERPRETATION (2 hours)

Data is transferred to the workstation where it can be analyzed by NextGENe software version 2.3 run on 64-bit workstation and Windows 7 operating system. The following steps are taken to generate SNP reports:

1. Format conversion: using “Conversion” tool provided by NextGENe software to convert FASTQ format to FASTA format.
2. Data Analysis:  
In Application page, three functions are specified as:  
Instrument Type – Illumina.  
Application Type - SNP/Indel.  
Discovery Step- Sequence Alignment.  
In Load data page: Reference file “NC\_012920\_1.gb” is used.

In Alignment page: set the mutant rate cut off 10% with the minimum coverage of 10. The other settings are set as default.

3. Results: After the software finishes the analysis, the NextGENe viewer window will open automatically
4. The results will be outputted as text file and vcf file. To generate mutation report, go to Toolbar, click on the transcript report icon and select “Show Transcript Report below Sequences”. The mutation table will display.
5. For the detection of large deletions use sequences obtained from amplification with PCR primer pairs 1–3 as well as PCR primer Pair 4. For determination of the exact coordinates of the missing genomic segment, run the analysis with the “Detect Structural Variations” tab checked. During such analysis reads that match over 0.1% of their length or greater than 15 nucleotides to two noncontiguous segments of the reference genome are stored in so called “Pseudo- Paired-End” sequence files. To identify the breakpoints, run NextGENe again using the sequences in the PseudoPairedEnd files as input files. The chimeric sequences are displayed in the NextGENe viewer in relation to the reference genome sequence making identification of the breakpoint possible. To do this, position the blue cross on the genomic region where the coverage drops dramatically; the sequences corresponding to this region will terminate at a single nucleotide. Repeat this on the other breakpoint on the right.

## J. Troubleshooting

**1. Input DNA quantification**—Correct quantification of genomic DNA is essential. The ultimate success or failure of a library preparation strongly depends on using an accurately quantified amount of input DNA. Illumina recommends using fluorometric based methods for quantification including Qubit or PicoGreen to provide accurate quantification for dsDNA. Small volume pipetting can be a source of potential error in protocols that require generation of standard curves, such as PicoGreen assays or qPCR, or those that require small but precise volumes, such as the Agilent BioAnalyzer. If small volumes are unavoidable, then make sure that all pipettes used are precise and are correctly calibrated.

**2. Master-mix reagent handling**—Minimize freeze-thaw cycles. If you do not intend to consume the reagents in one use, dispense the reagent into aliquots after the initial thaw and refreeze the aliquots in order to avoid excessive freeze- thaw cycles. Add reagents in the order indicated in the TruSeq DNA Sample Preparation Guide. Care must be taken while adding the A-Tailing Mix (ATL) and Ligation Mix (LIG) due to the viscosity of the reagents.

**3. Fragmentation**—Problem: Incomplete shearing caused by too much DNA input.

Solution: Adhere to recommended starting amounts (1µg).

Problem: Increased size range.

Solution: Covaris problem, check Covaris with DNA of known quality (like Lambda).



#### 4. Library quality problems

1. Presence of adapter dimers.

Potential causes: Improper AMPure bead ratios, inefficient Ligation (too much input DNA, too little input DNA).

2. Additional peaks in Bioanalyzer trace.

Cause: SPRI bead carryover

Solution: Use a strong magnet for bead separation, pipette carefully during elution to avoid disturbing bead pellet

#### 5. MiSeq run setup issues—Error: Initialization Failed.

Action: The option to re-initialize appears on the screen. Select Re-initialize.

Error: Fluidics check failed.

Action: Select Pump. When fluids have been pumped, select Restart Check.

Error: Disk space low.

Action: If disk space is low, a message appears indicating how much disk space is required. Use the Manage Files feature to clear the required space from the instrument computer.

Error: Network disconnected.

Action: Make sure the network cable is plugged into the instrument. If the network connection is not resolved; reboot the instrument by turning off the power switch. Wait at least 60 seconds, and then turn the instrument on using the power switch.

**5. Poor quality MiSeq sequencing results**—The performance characteristics of the sequencing are assessed in each run using Illumina provided controls. The controls are useful for identifying the specific mode of failure. The end repair control, A-Tailing control, and Ligase control reagents are used as controls for the enzymatic activities of the End Repair Mix, A- Tailing Mix, and DNA Ligase Mix, respectively. Each reagent contains double stranded DNA fragments designed to report the success or failure of a specific enzymatic activity used in the library preparation process. If a control's sequence appears in the final sequencing data, it indicates that its corresponding step was successful. If it does not, or if it appears in substantially diminished numbers, it indicates the step failed. Illumina also provides a PhiX control library which is spiked in to the prepared sample library at a low concentration in order to help evaluate any clustering issues on the sequencing instrument.

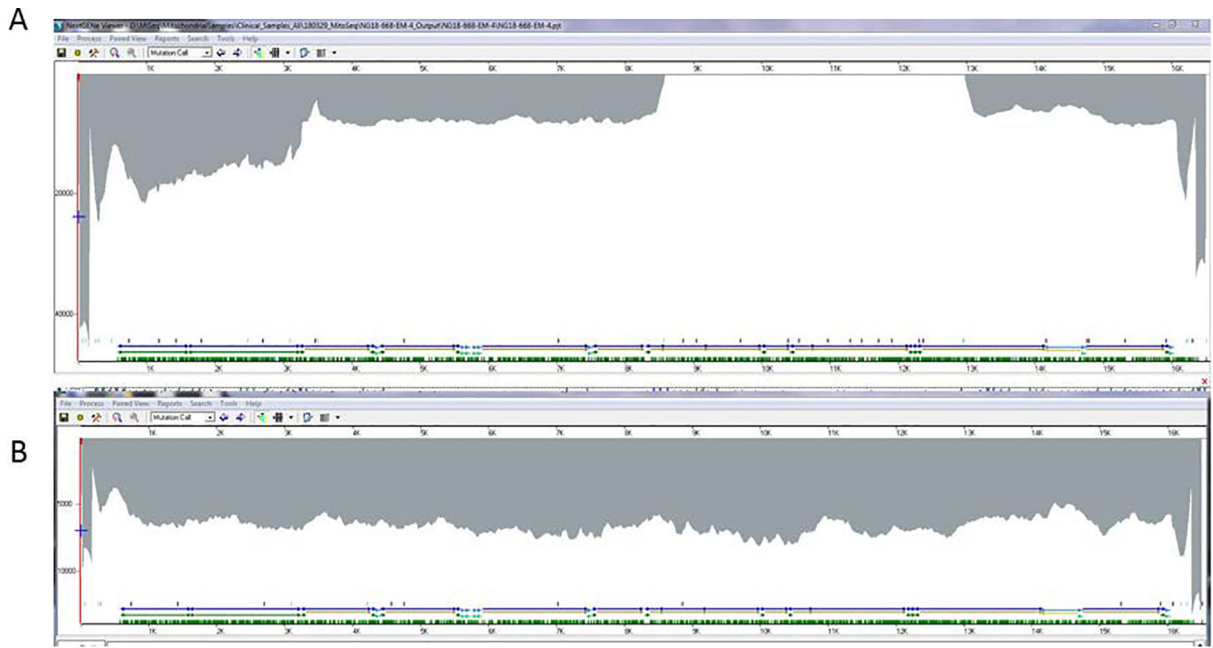
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**Fig. 2.** Detection of a large-scale (~5kb) deletion by NGS in a patient harboring the mtDNA “common deletion” with 96% heteroplasmy (panel A patient, panel B control). The nucleotide positions (*X*-axis) are according to *r*CRS reference mitochondrial genome. Y-axis represents the coverage of each detected nucleotide.

**Table 1.**

Primers sequences and their positions on mtDNA sequence.

Primer	Sequence	No. of nt	Position on mtDNA
MTI-F1	5' acatagcacattacagtc aaatcccttctctgccc 3'	35	16331–16365
MTI-R1	5' tgagattgittgggctactgctcgcagtgcc 3'	30	3729–3700
MTII-F2	5' tactcaatcctctgatcagggtgagcatcaaactc	35	3646–3680
MTII-R2	5' gcgtggattaaggcgacagcgatttctaggatagt	35	9158–9124
MTIII-F3	5' tcatttttattgccacaactaacctcctcggact 3'	35	8753–8787
MTIII-R3	5' cgtgatgtcttattaaggggaacgtgtgggctat	35	16566–16532
MTIV-F4	5' tctataccctattaaccactcaggagact 3'	31	10–41
MTIV-R4	5' gatacagttcactttagctacccccaagtgtt 3'	32	16494–16462

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**Table2**

Composition of Long-Range PCR Master Mix for one primer Pair

Stock	Volume/Rx	Final Concentration in 50 $\mu$ L PCR reaction
Nuclease Free Water	22.5 $\mu$ L	
2.5 mM dNTPs (from TaKaRa Kit)	5.0 $\mu$ L	250 $\mu$ M
10X LA PCR buffer ( $Mg^{2+}$ )(from TaKaRa Kit)	5 $\mu$ L	1x (2.5 mM $Mg^{2+}$ )
LA Taq enzyme (from TaKaRa Kit)	0.5 $\mu$ L	1.25 U/25 $\mu$ L
10 $\mu$ M Forward primer	5 $\mu$ L	1.0 $\mu$ M
10 $\mu$ M Reverse primer	5 $\mu$ L	1.0 $\mu$ M
Nuclease free water	5 $\mu$ L	
Total Volume	48.0 $\mu$ L	

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