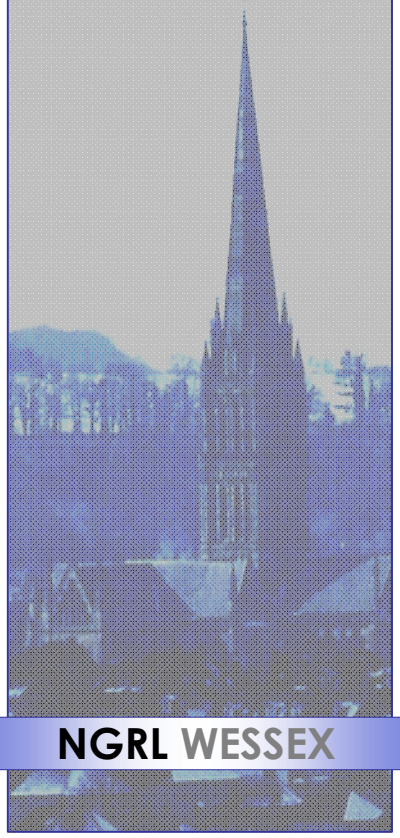
National Genetics Reference Laboratory (Wessex)

NHS

Technology Assessment Report



NGRL WESSEX

Detection and estimation of heteroplasmy for mitochondrial mutations using NanoChip® and Pyrosequencing™ technology

June 2005

Title	Detection and estimation of heteroplasmy for mitochondrial mutations using NanoChip® and Pyrosequencing™ technology
NGRL Ref	NGRLW_HTA_1.0
Publication Date	June 2005
Document Purpose	Dissemination of information about use of Pyrosequencing for quantitative SNP analysis
Target Audience	Laboratories performing mitochondrial mutation detection or quantitative SNP analysis
NGRL Funded by	

Contributors

Name	Organisation
Helen White	NGRL (Wessex)
Victoria Durston	NGRL (Wessex)
Anneke Seller	Oxford Medical Genetics Laboratory
Carl Fratter	Oxford Medical Genetics Laboratory

Peer Review and Approval

This document has been subject to peer review. In addition, Nanogen and Biotage have had the opportunity to comment on the content of this report.

Conflicting Interest Statement

The authors declare that they have no conflicting financial interests

How to obtain copies of NGRL (Wessex) reports

An electronic version of this report can be downloaded free of charge from the NGRL website (http://www.ngrl.co.uk/Wessex/downloads)

or by contacting

National Genetics Reference Laboratory (Wessex)
Salisbury District Hospital
Odstock Road
Salisbury
SP2 8BJ
UK

E mail: ncpc@soton.ac.uk
Tel: 01722 429016
Fax: 01722 338095

Table of Contents

1. SUMMARY 1
2. INTRODUCTION 1
3. MATERIALS AND METHODS 2
   3.1 DNA Samples and PCR RFLP Analysis 2
   3.2 Pyrosequencing™ assays 2
      3.2.1 PCR amplification and clean-up 5
      3.2.2 Pyrosequencing reactions and data analysis 5
      3.2.3 Threshold Detection of A3243G mutation 5
   3.3 NanoChip® Molecular Biology Workstation Assays 5
      3.3.1 PCR amplification and desalting 8
      3.3.2 Ratio reference controls. 8
      3.3.3 Cartridge loading 8
      3.3.4 Reporter hybridisation and detection. 8
      3.3.5 Data analysis. 9
4. RESULTS 9
   4.1 Overview 9
   4.2 Pyrosequencing Assays 9
      4.2.1 Sensitivity, specificity and reproducibility 9
      4.2.2 Threshold Detection of A3243G mutation 11
   4.3 Nanogen Assays 11
   4.4 Costings and Speed of Analysis. 13
5. DISCUSSION 15
6. REFERENCES 17

1. SUMMARY

Background
Disease causing mutations in mitochondrial DNA are typically heteroplasmic and therefore interpretation of genetic tests for mitochondrial disorders is problematic. The reliable measurement of heteroplasmy in different tissues may help identify individuals who are at risk of developing specific complications and allow improved prognostic advice for patients and family members. We have evaluated the NanoChip® Molecular Biology Workstation and Pyrosequencing™ technology for the detection and estimation of heteroplasmy for six mitochondrial point mutations associated with the following diseases: Lebers Hereditary Optical Neuropathy (LHON), G3460A, G11778A & T14484C; Mitochondrial Encephalopathy with Lactic Acidosis and Stroke-like episodes (MELAS), A3243G; Myoclonus Epilepsy with Ragged Red Fibres (MERRF), A8344G and Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa (NARP)/Leighs: T8993G/C.

Methods and Results
Results obtained from the Nanogen and Pyrosequencing assays for 50 patients with presumptive mitochondrial disease were compared to those obtained using the commonly used diagnostic technique of PCR and restriction enzyme digestion. The NanoChip® Molecular Biology Workstation provided accurate genotyping for the six mitochondrial assays but had limitations in determining the level of heteroplasmy for some mutations. The Pyrosequencing assays provided accurate genotyping and quantitative determination of mutational load with a sensitivity and specificity of 100%. Using Pyrosequencing the MELAS A3243G mutation was detected reliably at a level of 1% heteroplasmy.

Conclusions
Whilst both systems can be used for detection and quantification of mitochondrial mutations, Pyrosequencing offered a number of advantages in terms of accuracy, speed and cost. We conclude therefore that Pyrosequencing is a robust, effective and efficient means of detecting and quantifying mitochondrial mutations in a clinical diagnostic laboratory setting.

2. INTRODUCTION

Mitochondrial diseases are a clinically heterogeneous group of disorders that occur as a result of mutations of nuclear or mitochondrial DNA (mtDNA), leading to dysfunction of the mitochondrial respiratory chain (DiMauro *et al.*, 1998). Nuclear DNA defects are inherited in an autosomal dominant or recessive manner and generally present in childhood. However, the transmission of mtDNA is maternal and affected individuals generally present late in childhood or as adults. MtDNA deletions usually occur *de novo* and cause sporadic disease with no significant risk to other family members but mtDNA point mutations and duplications can be transmitted.

Disease causing mutations in mtDNA, unlike neutral polymorphic nucleotides (Lagerström-Fermér *et al.*, 2001), are typically heteroplasmic with normal and mutant sequences co-existing in the same cell (Wallace, 1999). This is analogous to the heterozygous state in Mendelian genetics but because each cell may contain thousands of copies of the mitochondrial genome the level of heteroplasmy can vary from 1 to 99%. Furthermore, the level of heteroplasmy can vary between cells and tissues (Macmillan *et al.*, 1993). Hence, a female harbouring a mtDNA mutation may transmit a variable amount of mutated mtDNA to her offspring which can potentially result in considerable clinical variability amongst siblings within the same family. Pre- and post-natal genetic testing and interpretation for mitochondrial disorders is therefore problematic. Although there is evidence to show that there is a correlation between the level of heteroplasmy and mitochondrial respiratory function *in vivo* it has been more difficult to demonstrate an association between level of heteroplasmy and clinical phenotype. It seems likely that a minimum critical number of mutated mtDNA molecules must be present before clinical symptoms appear and that the pathogenic threshold will be lower in tissues that are dependant on oxidative metabolism. The reliable measurement of heteroplasmy of various mutations in different tissues may help identify individuals who are at risk of developing specific complications and allow improved prognostic advice for patients and family members.

For mutations such as the MELAS mutation (A3243G) it is accepted that higher levels of mutated mtDNA are detected in muscle rather than rapidly dividing tissue (e.g. blood). However, most diagnostic laboratories receive samples that can be obtained in a non-invasive manner such as blood, hair and buccal swabs which may contain a lower level of mutated mtDNA. Therefore, diagnostic techniques must be capable of reliably detecting low levels of heteroplasmy in such samples. A

number of established techniques have been used to genotype and quantitate the level of heteroplasmy for a variety of mitochondrial mutations; denaturing gradient gel electrophoresis (Tully *et al.*, 2000), single-stranded conformational polymorphism (Mashima *et al.*, 1995; Tanno *et al.*, 1995), real time fluorescent PCR (Szuhai *et al.*, 2001; Bai and Wong, 2004; He *et al.*, 2002), temporal temperature gradient gel electrophoresis (Boles *et al.*, 2001), Invader technology (Mashima *et al.*, 2004), DHPLC (Conley *et al.*, 2003), solid-phase minisequencing (Juvonen *et al.*, 1994; Suomalainen and Syvanen, 2000) and PCR RFLP (*e*.g. Holt *et al.*, 1990). At present, the most commonly used diagnostic technique used in the UK is PCR RFLP (either radioactive or fluorescent). Here we describe the use of Pyrosequencing™ and the Nanogen® Molecular Biology Workstation to genotype and estimate the level of heteroplasmy for six mitochondrial point mutations associated with the following diseases: Lebers Hereditary Optical Neuropathy (LHON), G3460A, G11778A & T14484C; Mitochondrial Encephalopathy with Lactic Acidosis and Stroke-like episodes (MELAS), A3243G; Myoclonus Epilepsy with Ragged Red Fibres (MERRF), A8344G and Neurogenic muscle weakness, Ataxia and Retinitis Pigmentosa (NARP/Leighs): T8993G/C.

3. MATERIALS AND METHODS

3.1 DNA Samples and PCR RFLP Analysis

Total genomic DNA samples from 50 patients (25 males, 25 females; 45 extracted from peripheral blood, 5 extracted from muscle) with presumptive mitochondrial disease were initially analysed by the commonly used diagnostic method of PCR (fluorescent or non-fluorescent) followed by restriction enzyme digestion using the PCR primers listed in table 1. Amplicons were generated in a 20µl reaction volume with 5pmol of forward and reverse PCR primers, 0.2mM dNTPs (Promega), 1X Hotstar Buffer with $MgCl_2$ (QIAGEN), 1U Hotstar Taq (QIAGEN) using 10ng genomic DNA. PCR conditions for all reactions were 94°C for 12 min; 30 cycles with denaturation at 94°C for 30s, annealing at 58°C for 30s and elongation at 72°C for 30s; 1 cycle at 72°C for 10 min; and a final hold at 4°C. Thermocycling was performed using a PTC-0225 DNA Engine Tetrad (MJ Research). 5µl PCR product was digested with 2U of the appropriate restriction enzyme according to manufacturer's instructions.

The LHON mutations G3460A, G11778A and T14484C were analysed by non–fluorescent PCR and restriction digestion with *AcyI* (G3460A causes site loss), *MaeIII* (G11778A causes site gain) and *BanI* (primer mismatch creates site for T14484C). The MELAS A3243G, MERRF A8344G and NARP/Leighs T8993G/C were analysed by fluorescent PCR. To prevent heteroduplex formation and consequent variability in restriction enzyme digestion, fluorescently labelled reverse primer was added following 30 PCR cycles and a single extension reaction was performed. The restriction enzymes used for analysis of these mutations were *HpaII* (site gain for the T8993G/C mutation), *HaeIII* (site loss for G8994A polymorphism), *BglI* (site gain for A8344G) and *ApaI* (site gain for A3243G). Following digestion, fluorescent products were analysed using an ABI 3100 Genetic Analyser and the level of heteroplasmy determined by comparison of the cleaved and uncleaved peak areas. Non-fluorescent products were analysed using agarose gel electrophoresis.

DNA samples were randomised and re-analysed in a blinded fashion for all six mutations using Pyrosequencing™ and NanoChip® technology.

3.2 Pyrosequencing™ assays

Pyrosequencing™ technology is a real time sequencing method for the analysis of short to medium length DNA sequences (Ronaghi *et al.*, 1998). Four enzymes and specific substrates are used to produce light whenever a nucleotide forms a base pair with the complementary base in a DNA template strand. Biotinylated PCR products are converted to single stranded templates onto which a sequencing primer is annealed. Analysis begins as the enzymes and substrates are dispensed into the reaction, nucleotides are dispensed sequentially and a light signal is detected and the base registered. If the added nucleotide is not complementary to the next base in the template then no light is generated. The genotype of the mtDNA can be determined using the PSQ™ 96MA System and the percentage of mutated mtDNA present in the sample can be quantified using the Allele Frequency Quantification function of the SNP software (Biotage AB).

Inter- and intra-assay variation for each mutation was determined by analysing 15 normal control samples in 6 independent experiments and mutated samples were analysed in triplicate to determine reproducibility of results.

Mutation	Oligonucleotides	Sequence 5' to 3'	Sequence to analyse	Dispensation order
A3243G	RFLP Fwd PCR	AGGACAAGAGAAATAAGGCC		
MELAS	RFLP Rev PCR	TAGAAGAGCGATGGTGAGAG		
	Pyrosequencing Fwd PCR	CCTCCCTGTACGAAAGGACA		
	Pyrosequencing Rev PCR	Biotin-TGGCCATGGGTATGTTGTTA		
	Pyrosequencing Sequencing	GGTTTGTTAAGATGGCAG	**(A/G)**GCCCGGTAATC	*C***AG***T*CGTAT
A8344G	RFLP Fwd PCR	GTAGTATTTAGTTGGGGCATTTCACTGTAAAGCCGTGTTGG		
MERRF	RFLP Rev PCR	CTACCCCCTCTAGAGCCCAC		
	Pyrosequencing Fwd PCR	CATGCCCATCGTCCTAGAAT		
	Pyrosequencing Rev PCR	Biotin -TTTTATGGGCTTTGGTGAGG		
	Pyrosequencing Sequencing	TAAGTTAAAGATTAAGAGA	**(A/G)**CCAACACCT	*T***AG***T*CACAC
G3460A LHON	RFLP Fwd PCR	AGGACAAGAGAAATAAGGCC		
	RFLP Rev PCR	TAGAAGAGCGATGGTGAGAG		
	Pyrosequencing Fwd PCR	Biotin -ATGGCCAACCTCCTACTCCT		
	Pyrosequencing Rev PCR	TAGATGTGGCGGGTTTTAGG		
	Pyrosequencing Sequencing	TCTTTGGTGAAGAGTTTTAT	GG**(C/T)**GTCAG	*A*G**CT***C*GTCA
G11778A	RFLP Fwd PCR	CAGCCACAGAACTAATCATA		
LHON	RFLP Rev PCR	GTAAGCCTCTGTTCTCAGAT		
	Pyrosequencing Fwd PCR	Biotin -CAGCCATTCTCATCCAAACC		
	Pyrosequencing Rev PCR	CAGAGAGTTCTCCCAGTAGGTTAAT		
	Pyrosequencing Sequencing	AGTCCTTGAGAGAGGATTAT	GATG**(C/T)**GA	*C*GATG**CT***C*G
T14484C LHON	RFLP Fwd PCR	AGTATATCCAAAGACAGGCA		
	RFLP Rev PCR	GGTTTAGTATTGATTGTTAGC		
	Pyrosequencing Fwd PCR	CCCCACTAAAACACTCACCAA		
	Pyrosequencing Rev PCR	Biotin -TGGGTTTAGTAATGGGGTTTG		
	Pyrosequencing Sequencing	TGTAGTATATCCAAAGACA	ACCA**(T/C)**CATTC	*G*ACA**TC***G*AT
T8993G/C	RFLP Fwd PCR	CCGACTAATCACCACCCAAC		
NARP/Leighs	RFLP Rev PCR	TGTCGTGCAGGTAGAGGCTT		
	Pyrosequencing Fwd PCR	AGGCACACCTACACCCCTTA		
	Pyrosequencing Rev PCR	Biotin -TGTGAAAACGTAGGCTTGGAT		
	Pyrosequencing Sequencing	CATTCAACCAATAGCCC	**(T/G/C)**GGCCGTACG	*A***CTG***TA*CGTAC

Table 1: Sequences of oligonucleotides required for the Pyrosequencing and RFLP Assays. For the Pyrosequencing assays, the sequence to analyse is immediately 3' to the sequencing primer binding site on the biotinylated strand. The position of the mutation is shown in brackets (**bold font**). The dispensation order of the nucleotides includes several reference peaks, where no signal should be observed, these are shown in *italics* (T8993G/C assay has an additional reference peak included at dispensation 6 to detect the G8994A polymorphism). The dispensation orders used were those determined by the software in the Simplex SNP entry function.

3.2.1 PCR amplification and clean-up

The sequences of PCR and sequencing primers (Thermo Electron) used for each assay are listed in Table 1. Amplicons were generated in a 50µl reaction volume with 15pmol of forward and reverse PCR primers, 0.2mM dNTPs (Promega), 1.5mM $MgCl_2$, 1X Buffer II (Applied Biosystems), 1U AmpliTaq Gold (Applied Biosystems) using 10ng genomic DNA. PCR conditions for all reactions were 94°C for 7 min; 50 cycles with denaturation at 94°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 30s; 1 cycle at 72°C for 7 min; and a final hold at 4°C. Thermocycling was performed using a PTC-0225 DNA Engine Tetrad (MJ Research).

Single-stranded biotinylated PCR products were prepared for sequencing using the Pyrosequencing™ Vacuum Prep Tool. 3µl Streptavidin Sepharose™ HP (Amersham) was added to 37µl Binding buffer (10 mM Tris-HCl pH 7.6, 2M NaCl, 1 mM EDTA, 0.1% Tween 20) and mixed with 20µl PCR product and 20µl high purity water for 10 min at room temperature using a Variomag Monoshaker (Camlab). The beads containing the immobilised templates were captured onto the filter probes after applying the vacuum and then washed with 70% ethanol for 5 sec, denaturation solution (0.2M NaOH) for 5 sec and washing buffer (10 mM Tris-Acetate pH 7.6) for 5 sec. The vacuum was then released and the beads released into a PSQ 96 Plate Low containing 45µl annealing buffer (20 mM Tris-Acetate, 2 mM $MgAc_2$ pH 7.6), 0.3µM sequencing primer. For the T8993C/G assay, we found it necessary to add 1µl single stranded binding protein (Promega, 2.2µg/µl) to eliminate secondary structure in the template DNA. The samples were heated to 80°C for 2 min and then allowed to cool to room temperature.

3.2.2 Pyrosequencing reactions and data analysis

Pyrosequencing reactions were performed according to the manufacturer's instructions using the PSQ 96 SNP Reagent Kit which contained the enzyme and substrate mixture and nucleotides. Assays were performed using the nucleotide dispensation orders shown in table 1. The sample genotype and % heteroplasmy were determined using the Allele Frequency Quantification (AQ) function in the SNP Software (Biotage AB). Samples were considered to have the mutation if the value of % heteroplasmy was greater than three standard deviations from the mean value obtained from the normal replicates.

3.2.3 Threshold Detection of A3243G mutation

DNA samples with known levels of heteroplasmy were prepared to determine the lowest level of the A3243G mutation that could be detected reliably. The detection of low frequency heteroplasmy for this mutation is particularly important since it can be clinically significant. DNA from a MELAS patient was amplified using the primers 5' tgcagccgctattaaaggtt 3' and 5' ggttcggttggtctctgcta 3' (amplified region 3014 – 3894). The resulting 880bp amplicon was cloned into the vector pCR2.1 (Invitrogen) and several colonies were sequenced to identify 2 clones; one with a wild type genotype and the other with the MELAS mutation. DNA from each clone was quantified and diluted to a final concentration equivalent to 2×10^4 mitochondrial genomes / µl (diluted in 10ng salmon sperm DNA). The wild type and mutated DNA were mixed to generate samples with the A3243G mutation present at levels ranging from 1 – 100%. Each sample was analysed in triplicate using the Pyrosequencing assay, fluorescent and non-fluorescent PCR RFLP (using identical primer sets) and the detection threshold for each technique was determined.

3.3 NanoChip® Molecular Biology Workstation Assays

The NanoChip® Molecular Biology Workstation is an automated multi-purpose instrument that can be used for SNP detection using the NanoChip® Electronic Microarray. The Workstation consists of the NanoChip® Loader which electronically addresses negatively charged DNA molecules onto NanoChip® Cartridges, a NanoChip® Reader which contains a laser-based fluorescence scanner for detection of assay results and computer hardware and software. The NanoChip® Cartridge contains a microarray with a grid of 10x10 sites to which DNA samples can be addressed electronically. Biotinylated PCR amplicons are loaded onto the array by electronic activation of specific test sites and the amplicons are immobilised through interaction with streptavidin in the gel layer covering the array.

Hybridisation of wild type (Cy3) and mutant (Cy5) labelled reporter probes is made specific using thermal stringency. The fluorescence signals detected from the wild type and mutant reporter probes

Mutation	Oligonucleotides	Sequence 5' to 3'
3243G (MELAS)	Forward PCR primer	Biotin-5'cctccctgtacgaaaggaca 3'
	Reverse PCR primer	5'tggccatgggtatgttgtta 3'
	Wt reporter	Cy3-5'aatccgggc**t** 3'
	Mutant reporter	Cy5-5'atccgggc**c** 3'
	Wt ratio reference	Biotin-5'cccacccaagaacagggtttgttaagatggcag**a**gcccggtaatcgca 3'
	Mut ratio reference	Biotin-5'cccacccaagaacagggtttgttaagatggcag**g**gcccggtaatcgca 3'
	Stabiliser	5'ctgccatcttaacaaaccctgttcttggg 3'
A8344G (MERRF)	Forward PCR primer	5'catgcccatcgtcctagaat 3'
	Reverse PCR primer	Biotin-5'ttttatgggctttggtgagg 3'
	Wt reporter	Cy3-5'aaagattaagaga**a** 3'
	Mutant reporter	Cy5-5'aaagattaagaga**g** 3'
	Wt ratio reference	Biotin-5'tagttggggcatttcactgtaaagaggtgttgg**t**tctcttaatctttaactt 3'
	Mut ratio reference	Biotin-5'tagttggggcatttcactgtaaagaggtgttgg**c**tctcttaatctttaactt 3'
	Stabiliser	5'ccaacacctctttacagtgaaatgcccc 3'
G3460A (LHON)	Forward PCR primer	Biotin-5'atggccaacctcctactcct 3'
	Reverse PCR primer	5'tagatgtggcgggttttagg 3'
	Wt reporter	Cy3-5'agagttttatgg**c** 3'
	Mutant reporter	Cy5-5'aagagttttatgg**t** 3'
	Wt ratio reference	Biotin-5'aggcccctacgggctactacaacccttcgctgac**g**ccataaaactcttcaccaaa 3'
	Mut ratio reference	Biotin-5'aggcccctacgggctactacaacccttcgctgac**a**ccataaaactcttcaccaaa 3'
	Stabiliser	5'rtcagcgaagggttgtagtagcccgtagg 3'
G11778A (LHON)	Forward PCR primer	5'cagccattctcatccaaacc3'
	Reverse PCR primer	Biotin-5'cagagagttctcccagtaggttaat3'
	Wt reporter	Cy3-5'actcacagtc**g** 3'
	Mutant reporter	Cy5-5'cactcacagtc**a** 3'
	Wt ratio reference	Biotin-5'ggagtagagtttgaagtccttgagagaggattatgatg**c**gactgtgagtgcgttc 3'
	Mut ratio reference	Biotin-5'ggagtagagtttgaagtccttgagagaggattatgatg**t**gactgtgagtgcgttc 3'
	Stabiliser	5'yatcataatyctctctcaaggacttcaaactct 3'
T14484C (LHON)	Forward PCR primer	Biotin-5'ccccactaaaacactcaccaa 3'
	Reverse PCR primer	5'tgggtttagtaatggggtttg 3'
	Wt reporter	Cy3-5'taggggggaatg**a** 3'
	Mutant reporter	Cy5-5'aggggggaatg**g** 3'
	Wt ratio reference	Biotin-5'aatagccatcgctgtagtatatccaaagacaacca**t**cattccccctaaataa 3'
	Mut ratio reference	Biotin-5'aatagccatcgctgtagtatatccaaagacaacca**c**cattccccctaaataa 3'
	Stabiliser	5'tggttgtctttggrtatactacagcgatgg 3'
T8993G (NARP/Leighs)	Forward PCR primer	5'aggcacacctacacccctta 3'
	Reverse PCR primer	Biotin-5'tgtgaaaacgtaggcttgga 3'
	Wt reporter	Cy3-5'ccaatagccc**t**
	Mutant reporter	Cy5-5'ccaatagccc**g**
	Wt ratio reference	Biotin-5'ctgcagtaatgttagcggttaggcgtacggcc**a**gggctattggttgaa 3'
	Mut ratio reference	Biotin-5'ctgcagtaatgttagcggttaggcgtacggcc**c**gggctattggttgaa 3'
	Stabiliser	5'ggccgtacgcctaaccgctaacattac 3'

Table 2: Sequences of oligonucleotides required for the Nanogen Assays

are analysed to determine genotype and heteroplasmy can be estimated from a standard curve generated using ratio reference oligonucleotides. Background levels obtained for each mutation in the Nanogen® assays were determined by analysing the data from the non-mutated samples within the test population

3.3.1 PCR amplification and desalting

The sequences of PCR primers, reporter oligonucleotides and ratio reference oligonucleotides (Thermo Electron) used for each assay are listed in Table 2. Amplicons were generated in a 50µl reaction volume with 15pmol of forward and reverse PCR primers, 0.2mM dNTPs (Promega), 1.5mM $MgCl_2$, 1X Buffer II (Applied Biosystems), 1U AmpliTaq Gold (Applied Biosystems) using 100ng genomic DNA. PCR conditions for all reactions were 94°C for 7 min; 35 cycles with denaturation at 94°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 30s; 1 cycle at 72°C for 7 min; and a final hold at 4°C. Thermocycling was performed using a PTC-0225 DNA Engine Tetrad (MJ Research).

Amplicons were desalted using the Montage PCR_{96} Cleanup Kit (Millipore). PCR products were added to a $Multiscreen_{96}$ PCR plate and vacuum was applied at 8 inches Hg for 5-10 min. The filtration plate was washed with 100µl nuclease free water and vacuum was applied at 8 inches Hg for 5-10 min until the wells were empty. 100µl 50mM L-histidine (conductivity <100 µS/cm) was added to each well and the amplicon was recovered by vigorous pipetting of the solution across the membrane to ensure optimal recovery. The PCR products were then transferred to a clean 96-well plate for storage.

3.3.2 Ratio reference controls.

Biotinylated wild type and mutant oligonucleotides which contain the binding sites for the stabiliser and Cy3 and Cy5 labelled reporter oligonucleotides acted as reference controls and were used to generate a standard curve. The ratio references were mixed to give wt:mut ratios of 80:20, 60:40, 50:50, 40:60, 20:80 and were electronically addressed onto the same cartridge as the test samples. The standard curve generated from the reference ratios was used to determine the proportion of mutated mtDNA present in the test samples.

3.3.3 Cartridge loading.

All amplicons, ratio references and control samples were electronically addressed to pre-defined test sites on a NanoChip® Cartridge using the NanoChip Loader. Samples were addressed to the sites at 2 Volts for 120s. Double stranded amplicons were then denatured with a passive wash in 0.1M NaOH for 180s. The cartridge was then rinsed twice manually with 100µl high salt buffer (50mM sodium phosphate pH 7.4, 500mM NaCl).

3.3.4 Reporter hybridisation and detection.

0.5µM Cy3 labelled wild type reporter, 0.5µM Cy5 labelled mutant reporter and 1µM stabiliser oligonucleotide were prepared in high salt buffer. 100µl reporter/stabiliser mix was then introduced manually into the cartridge and incubated at room temperature for 3 min. The reporter/stabiliser mix was removed and the cartridge washed twice manually with 100µl high salt buffer. The NanoChip® Cartridge was placed in the NanoChip® Reader and the temperature of the microchip environment was increased to the appropriate discrimination temperature where the mismatched reporter probes were denatured and matched reporter probes remain hybridised (A3243G, 40°C; A8344G, 40°C; G3460A, 44°C; G11778A, 42°C; T14484C, 40°C; T8993G/C, 32°C). The cartridge was washed four times in low salt buffer (50mM Sodium phosphate pH 7) to remove unbound probes. The cartridge was cooled to 24°C and then each pad was excited by a red laser (635nm) and green laser (532nm). The photomultiplier tube gain and accumulation time settings used for detection were: A3243G, Medium 512µs; A8344G, Low 1024µs; G3460A, Low 256µs; G11778A, Low 256µs; T14484C Low 1024 µs; T8993G/C, Low 512. The fluorescence emitted from the hybridised Cy3 and Cy5 reporter oligonucleotides was detected at 550-600nm and 660-720nm respectively.

3.3.5 Data analysis.

The raw fluorescence signals were adjusted by subtracting background signals obtained from blank (histidine buffer only) addressed pads for both red and green fluorescence. The amount of red fluorescence obtained from the ratio reference samples was plotted as a proportion of total fluorescence and a standard curve was generated using a worksheet prepared in Microsoft Excel 2000. The relative red fluorescence reading for each sample was then converted to % heteroplasmy using the standard curve. Unless stated otherwise, samples were considered to have the mutation if the value of % heteroplasmy before correction was greater than three standard deviations from the mean value obtained from the normal samples.

4. RESULTS

4.1 Overview

Both the Nanogen and Pyrosequencing assays correctly identified each of the 31 mutations. No false positive results were seen, although analysis of the T8993G mutation using the Nanogen system required more stringent cutoff criteria. Broadly, there was very good correspondence between the level of heteroplasmy estimated by Pyrosequencing and the commonly used technique of fluorescent PCR followed restriction enzyme digestion. Different degrees of heteroplasmy were also quantified by the Nanogen system, although a greater degree of variation was observed for some mutations.

4.2 Pyrosequencing Assays

4.2.1 Sensitivity, specificity and reproducibility

To determine the background levels for each Pyrosequencing assay, 15 normal DNA samples were analysed in six independent experiments. The mean and standard deviation values for intra and inter assay analysis are shown in table 3. No differences were observed between the inter and intra assay analysis which suggests that the background variation seen in these assays is consistent and that the assays are robust. Figure 1 shows the genotype and % heteroplasmy obtained from the 50 patient DNA samples using the Pyrosequencing assays compared to the results obtained using the routine PCR RFLP method for all mutated samples. Samples were considered to harbour the mutation if the AQ value obtained was greater than three standard deviations from the mean value obtained from the normal replicated samples (table 3). The genotypes obtained using the Pyrosequencing assays were 100% concordant with those obtained using PCR RFLP and the levels of heteroplasmy detected using both techniques were essentially identical. Of the 50 patient DNA samples 13 were found to have one of the three LHON mutations, 10 had the A3243G MELAS mutation, four were positive for the MERRF A8344G mutation, four carried the NARP/Leighs T8993G/C mutation and no mutation was detected in 19 samples. None of the samples had the G8994A polymorphism. The Pyrosequencing assays were 100% sensitive and 100% specific taking into account the respective background values. The determination of the level of heteroplasmy using the Pyrosequencing assays was highly reproducible. For the mutated samples analysed in triplicate, no deviation from the mean was observed for the three LHON assays and the coefficient of variance for the 3243, 8344 and 8993 assays was 1.2 – 52.7, 1.65 – 5.47 and 0.6 – 3.5 respectively. Representative pyrograms obtained for a mutated sample from each assay are shown in figure 2.

	Inter assay analysis (n=15)		Intra assay analysis (n=6)		
Mutation	Mean	Standard Deviation	Mean	Standard Deviation	Mean + 3SD
A3460G LHON	2.86	1.62	2.88	1.62	7.72
G11778A LHON	0.05	0.14	0.06	0.22	0.72
T14484C LHON	2.13	0.63	2.13	0.98	5.07
A3243G MELAS	0.06	0.07	0.06	0.11	0.39
A8344G MERRF	0.13	0.32	0.13	0.50	1.63
T8993G/C NARP/Leighs	0.01	0.02	0.01	0.03	0.04

Table 3: Inter and Intra assay analysis of 15 normal samples tested in six independent experiments to determine level of background detection of mutant allele for each assay.

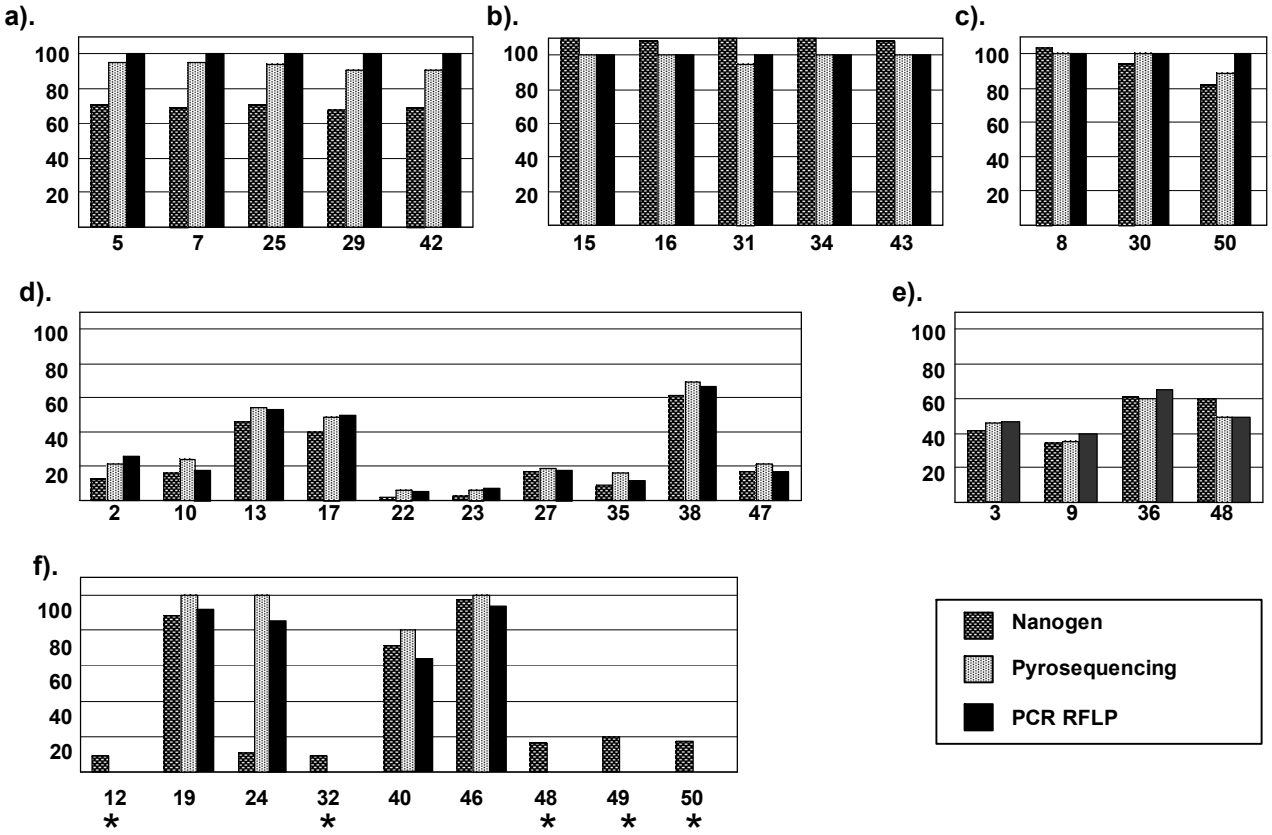


Figure 1: The level of heteroplasmy detected by the Nanogen and Pyrosequencing assays compared to the diagnostic result obtained using PCR RFLP for the assays: **a)** G3460A, **b)** T14484C, **c)** G11778A, **d)** A3243G, **e)** A8344G, **f)** T8993G/C. The asterisk indicates samples for which no mutation was detected in the PCR/RFLP assay. The x axis shows the patient sample number and the y axis shows the % heteroplasmy detected after correction for background.

4.2.2 Threshold Detection of A3243G mutation

The lowest level of detection of the A3243 mutation for the Pyrosequencing, non–fluorescent PCR RFLP and fluorescent PCR RFLP assay was determined by generating a standard curve from cloned wild type and mutated DNA samples which had been mixed to generate samples with the A3243G mutation present at levels of 0, 1, 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%. Figure 3 shows the standard curves for the Pyrosequencing and fluorescent PCR RFLP assays for identical samples analysed in triplicate. A level of 1% heteroplasmy was reliably detected with a mean AQ value of 1.73 (standard deviation 0.84). Results from the fluorescent PCR RFLP show that 5% heteroplasmy is the limit of detection for this technique. In contrast to the expected linear relationship between observed and expected heteroplasmy levels for the Pyrosequencing assay an apparent quadratic relationship was seen for PCR RFLP. This may reflect the under-representation of heteroplasmy detected due to the formation of heteroduplexes which cannot be cut by the restriction enzyme. Analysis of the same PCR products by agarose gel electrophoresis detected the mutation with a sensitivity of only 20% (data not shown) which is consistent with other studies (Hancock *et al.*, 2002).

4.3 Nanogen Assays

The genotypes obtained using the Nanogen assays for LHON, MELAS and MERFF were 100% concordant with those obtained using PCR RFLP, however the levels of heteroplasmy detected using this technique were variable. Of the 50 patient DNA samples 13 were found to have one of the three LHON mutations, 10 had the A3243G MELAS mutation and four were positive for the MERRF A8344G mutation. Five false positive results were obtained using the NARP/Leighs T8993G/C assay. Figure 1 shows the calculated % heteroplasmy obtained for both technologies compared to the results obtained using the routine PCR RFLP method. Table 4 shows the mean and standard deviation values obtained for the normal samples for each assay.

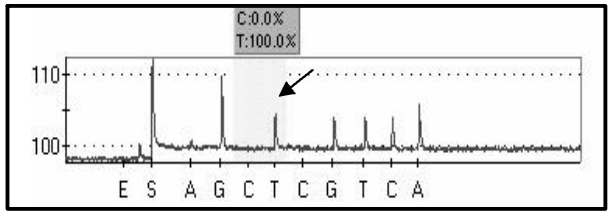
Assay	Mean (Standard Deviation)
3243 (n=40)	5.73 (0.11)
8344 (n=46)	19.36 (0.21)
8993G (n=46)	-10.91 (6.39)
3460 (n=45)	9.19 (0.23)
11778 (n=45)	16.2 (2.16)
14484 (n=47)	5.19 (1.32)

Table 4: Mean and Standard Deviation values (% heteroplasmy) obtained for normal samples analysed using the Nanogen assays.

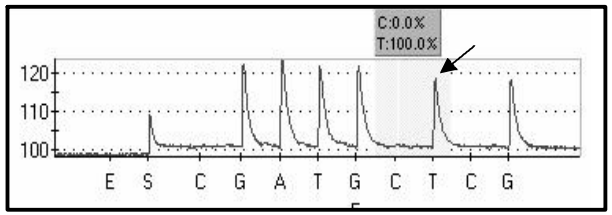
The G3460A Nanogen assays demonstrated an under-representation of mutational load by 30% when compared to PCR RFLP (after subtracting a mean % heteroplasmy value for normal samples of 9.19; Table 4). The G11778A Nanogen assay consistently produced an over-representation of approximately 10% heteroplasmy with a mean % heteroplasmy value of 16.2 seen in normal samples. For the T14484C assays the Nanogen assay gave results of 104% and 94% respectively in the Nanogen assay, compared to 100% by Pyrosequencing and the PCR RFLP assay. The A3243G assay detected levels of heteroplasmy ranging from 2 – 61% (after subtracting a mean % heteroplasmy value for normal samples of 5.73%, table 4). Levels of heteroplasmy were detected from 34% - 61% for the A8344G assay (after subtracting a mean % heteroplasmy value for normal samples of 19.4 %, table 4) which correlated well with the Pyrosequencing and PCR RFLP data.

Four out of the 50 patient DNA samples were found to be positive for the NARP/Leighs T8993G/C mutation using PCR RFLP and no samples were found to have the G8994A polymorphism. Three positive T8993G samples were successfully identified, however 5 false positive samples were also found by the Nanogen assay using a cutoff of >3 standard deviations from the mean value obtained from the normal samples. We tried several different reporter oligonucleotides and ratio reference controls but were unable to improve the background noise. When the cutoff was increased to 5 standard deviations the false positives were eliminated. The one positive T8993C patient (no. 24) was identified by Pyrosequencing but this mutation was not specifically tested for using the Nanogen system. The reporter oligos were designed so that the 8994 polymorphism was not included in the reporter oligonucleotide binding region and therefore the presence of the polymorphism should not affect the assay sensitivity.

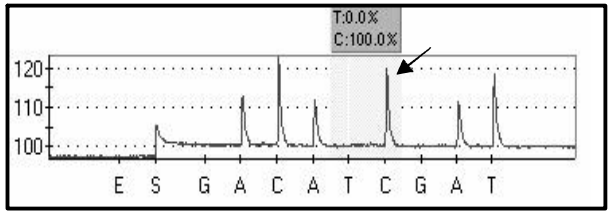
a)



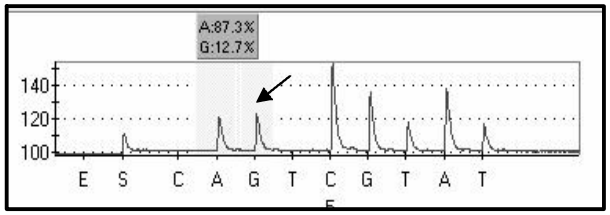
b)



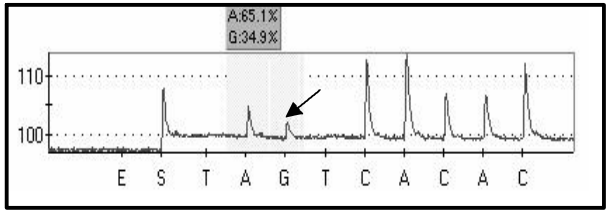
c)



d)



e)



f)

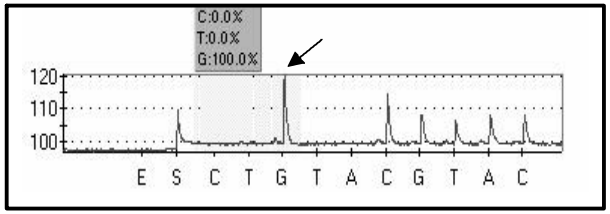


Figure 2: Representative pyrograms for representative patient samples which are heteroplasmic for a) G3460A b) G11778A c) T14484C d) A3243G e) A8344G and f) T8993G. The arrows mark the position corresponding to the mutated base. The boxes show the AQ values obtained for each allele, the value for the mutated allele represents the level of heteroplasmy present in the sample.

4.4 Costings and Speed of Analysis.

Table 5 shows costings for the instrumentation needed for the two technologies and also costings for the analysis of 96 samples including consumables and personnel. Costs correspond to UK list prices at December 2003 and are in pounds sterling and a workload unit (WLU) equals one minute of personnel time. The Pyrosequencing system is the least expensive technology to use with the system costs and analysis costs being 36% and 58% less expensive than the Nanogen Molecular Biology Workstation system respectively.

	Nanogen		**Pyrosequencing**	
System Costs	Loader, Reader, Software System, Colour monitor, Installation, Training, 1 year warranty	£83,777	PSQ 96MA System, Vacuum Prep Workstation, Thermoplate Low, computer with preinstalled software, training, 1 year warranty	£54,060
Cost for analysis of 96 samples				
Consumables	PCR, Sample Clean up and all additional reagents	£433.40	PCR, Sample Clean up and all additional reagents	£183.74
Personnel	160 WLU MTO2 30 WLU Clinical Scientist Grade B(16)	£25.60 £9.00	58 WLU MTO2 10 WLU Clinical Scientist Grade B(16)	£9.28 £3.00
	Total	£468.00	**Total**	£196.02

Table 5: Costings for the purchase of equipments and analysis of 96 samples including all reagents and personnel time but excluding maintenance contracts.

Costings for the Pyrosequencing assays and fluorescent PCR RFLP using 2004 list prices were comparable with the cost per sample being £1.20 (GBP) and £1.17, respectively, excluding system costs and machine maintenance contracts. After the PCR, the analysis time is faster using the Pyrosequencing system with a result being available in 30 minutes as compared to 2 hours for fluorescent PCR RFLP.

	Nanogen		**Pyrosequencing**	
PCR	PCR Set up	15	PCR Set up	15
	PCR Run	105	PCR Run	143
Preparation	50mMHistidine	10	Wash buffer, NaOH, 70% Ethanol	5
PCR Clean up and Sample preparation	Millipore Clean Up	20	Preparing samples and sequencing reactions	10
	Preparation of Ratio References	5	Pyrosequencer Clean up	5
	Pipetting samples onto 96 well plate	5	Preparation of cartridge	1
	Preparing NanoChip	1		
Machine Time	Priming Loader and Reader	5	Setting up assay sheet	4
	Setting up Loader Protocol	12	Setting up run	3
	Loading Samples onto NanoChip	360	Pyrosequencing run time	10
	Washing and Incubation with reporter oligos	15		
	Setting up Reader protocol	5		
	Loading Chip into Reader and aligning pads	2		
	Reading Chip	17		
	Priming Loader and Reader	5		
Analysis	Data Analysis	60	Data Analysis	15
	Total Time	636	**Total Time**	211

Table 6: Breakdown of the time taken to analyse 96 samples for one mitochondrial assay in minutes

Figure 3: Standard curves from cloned wild type and mutated DNA samples which have been mixed to generate samples with the A3243G mutation present at levels of 0, 1, 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% (x axis). Error bars indicate the standard deviation for triplicate samples

a) Standard curve for the Pyrosequencing assay showing a linear relationship between the % heteroplasmy present in the samples and the % heteroplasmy detected by the Pyrosequencing assay. The lowest reliable level of A3243G detection was 1% (0.5% not tested).

b) Standard curve for the PCR RFLP assay showing the linear (solid line) and quadratic (dotted line; $y = 0.005x^2 + 0.42x - 0.94$, $R^2 = 0.99$) relationships between the % heteroplasmy present in the samples and the % heteroplasmy determined by analysis of the cleaved and uncleaved peak areas after restriction digestion. The lowest reliable level of A3243G detection was 5%.

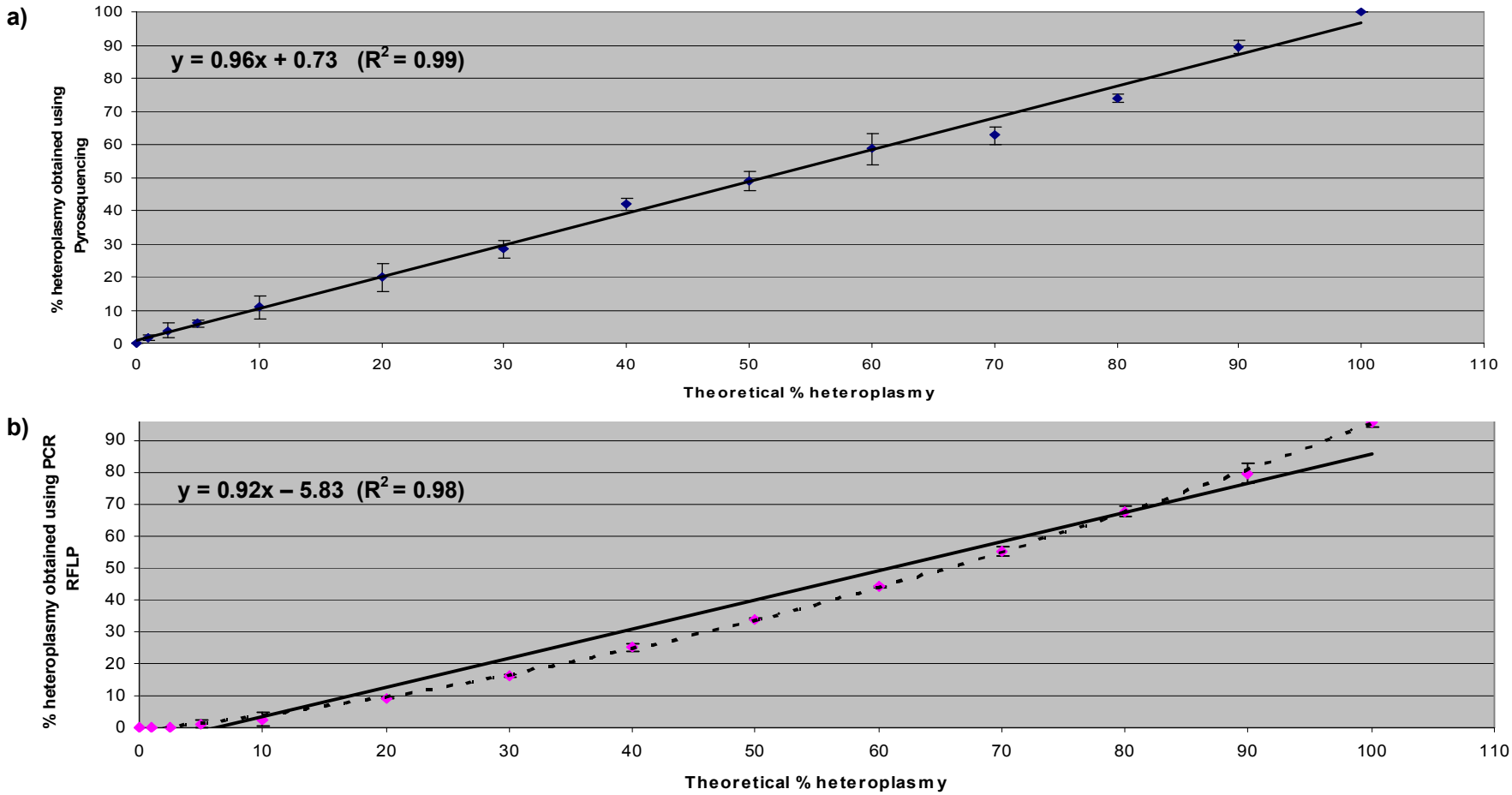


Table 6 shows the breakdown of the time taken for the two technologies to analyse 96 samples for one mitochondrial mutation. The Pyrosequencing system total run time is 67% faster than the Nanogen system. For detection of multiple mutations, eg. the three changes in LHON, it would be possible in principle to multiplex the analysis on either system.

5. DISCUSSION

Many commonly used molecular biological techniques have been adapted to characterise and quantitate mitochondrial mutations. With the continuing discovery of new mitochondrial mutations and increasing demand for molecular testing, it is important that clinically relevant levels of heteroplasmy can be detected in a reliable and time- and cost-effective manner. It is technically possible to undertake prenatal diagnosis and genetic counselling for mitochondrial DNA mutations but predicting the severity and type of symptoms has been hampered by the problems associated with interpreting the level of heteroplasmy. Here, we have examined how Pyrosequencing and Nanogen technologies can be used to genotype and estimate the level of heteroplasmy for six mitochondrial mutations compared to the widely used diagnostic technique PCR RFLP analysis.

95% of LHON patients harbor one of three mtDNA point mutations which affect genes encoding complex 1 subunits of the respiratory chain; G11778A (Wallace *et al*., 1988), T14484C (Johns *et al.*, 1992) and G3460A (Huoponen *et al.*, 1991). They differ from other pathogenic mitochondrial mutations since in the majority of pedigrees affected family members are homoplasmic for these mutations (Thornburn and Dahl, 2001). In our study thirteen samples were identified as having one of the LHON mutations by PCR RFLP. The Pyrosequencing assays correctly genotyped the 13 samples and also produced reliable estimates of heteroplasmy with a sensitivity and specificity of 100%. The assay for the G3460A mutation showed the highest level of background signal for normal samples with a mean AQ value for the A allele of 2.88% (standard deviation 1.62) which precludes the detection of samples with levels of heteroplasmy below 8%. However, since LHON mutations are usually homoplasmic this does not represent a significant problem when using this assay in a diagnostic setting. The assays for G11778A and T14484C were very robust and reproducible and had low background signals for normal samples.

Using the NanoChip® Molecular Biology Workstation all of the LHON samples were correctly genotyped but the level of heteroplasmy was not accurately determined by any of the assays. Therefore, although this technique is acceptable for genotyping it was less reliable for calculating the level of heteroplasmy. The accuracy of this analysis depends critically on the accuracy of the standard curve, which in our system depends on accurate quantitation of the ratio reference oligonucleotides. Construction of standard curves by mixing patient and control DNA is hampered by the need to know precisely beforehand the level of heteroplasmy (which is rarely or never truly 100%) in the patient DNA, plus the need for accurate amplifiable DNA quantitation. In practice this is likely to lead to variations in results between laboratories in the absence of widely available validated reference materials.

The tRNA gene mutation A3243G was first described by Ciafaloni *et al.* in a MELAS patient (Ciafaloni *et al.*, 1992). 80% patients with MELAS have the A3243G mutation which is the most common mtDNA point mutation detected in ~ 2000 patients with suspected mtDNA disorders (Wong and Senadheera, 1997; Liang *et al.*, 1998). The mutation is associated with stroke-like episode and mitochondrial myopathy (MELAS) when present at > 85% mutant load but is associated with maternally inherited diabetes mellitus and deafness when present at a lower percentage (5 – 30%). The mutation is usually present in low amounts in tissues that can be sampled in a non-invasive manner (e.g. blood) and it appears that levels of the mutation decrease in blood and increase in muscle over time. The detection of low frequency heteroplasmy for this mutation is particularly important since it can be clinically significant. In our study ten patients (9 peripheral blood samples and one muscle biopsy) were found to have the A3243G mutation. The data from the Pyrosequencing assays correlated well with results obtained using PCR RFLP and the assay had a sensitivity and specificity of 100% with a cut off AQ value of 0.39%. The Pyrosequencing assay had very low level background signals with a mean AQ value for the G allele of 0.06% (standard deviation 0.11) determined by the analysis of the normal samples. Using samples with known mutation loads it was established that the lowest level of heteroplasmy that could be detected reliably was 1% which produced a mean AQ value of 1.73% (standard deviation 0.84) in the Pyrosequencing assay. This threshold level of detection should be adequate for most clinical diagnostic laboratories.

Using the NanoChip® Molecular Biology Workstation the MELAS samples were correctly genotyped and the level of heteroplasmy was accurately determined. We are unable to report the threshold level of detection of the MELAS mutation using this system since the plasmid standards were not available whilst we were evaluating this platform.

The A8344G mutation in the tRNA Lys gene was first described by Silvestri *et al.* in association with myoclonic epilepsy with red ragged fibres (Silvestri *et al.*, 1993). The pathogenic MELAS and MERRF mutations both affect the mitochondrial tRNA genes and they have similar effects on the respiratory chain *in vitro*. As for the MELAS A3243G, there is a correlation between % heteroplasmy and clinical features in muscle biopsy samples where patients with > 90% mutated DNA in muscle are more likely to have deafness, ataxia and myoclonus than patients who have mutated DNA present at < 80% (Chinnery *et al.*, 1997). Unlike the A3243G mutation individuals with A8344G often have similar levels of mutant mtDNA in their blood and muscle. Four patients in this study were found to have the MERRF A8344G mutation present in peripheral blood samples using Pyrosequencing, Nanogen and PCR RFLP assays. Therefore both technologies were equally suited to the analysis of this mutation both for genotyping and estimation of mutational load. The Pyrosequencing assay had very low background with the mean AQ value for the G allele in normal samples being 0.13 (standard deviation 0.5) and the assay results were reproducible with a sensitivity and specificity of 100%.

The T8993G/C mutation affects the ATP synthase 6 subunit resulting in reduced ATP synthesis and is invariably heteroplasmic. It was first reported in 1990 in a family with NARP syndrome (Holt *et al.*, 1990). The T8993G and T8993C mutations are among the most common mtDNA mutations reported in children and the T8993C mutation is generally considered to be a milder variant (Rahman *et al.*, 1996; Santorelli *et al.*, 1997). Variable clinical expression within families has been reported and two main phenotypes have been identified; NARP syndrome and maternally inherited Leigh syndrome which can be distinguished by different degrees of heteroplasmy of the T8993G mutation. Symptoms usually appear when the mutant load is greater than 60% with retinal dystrophy related visual loss being the prevalent symptom in the 60-75% range; NARP syndrome usually occurs between 75% and 90% heteroplasmy. The more severe phenotype of Leigh syndrome occurs when levels increase above 95%. The probability of having severe symptoms is low until the mutant load reaches 60-70% for T8993G and 80-90% for T8993C when there is a rapid increase in severity of symptoms with increased mutant load (White *et al.*, 1999).

In this study three patients were found to have the T8993G mutation (two peripheral blood sample and one muscle biopsy), one carried the T8993C mutation and no samples had the G8994A polymorphism. The Pyrosequencing assay had 100% sensitivity and specificity when the cut off level for detection of mutated samples was 0.04% and the quantitation was reproducible with CVs ranging from 0.6-3.5. The T8993G/C mutation is usually detected by PCR followed by digestion of the product with *HpaII*, where a site gain is created by both sequence changes. The polymorphic G to A transition at 8994 will abolish this recognition site and therefore patients who have the 8994 polymorphism and the 8993 mutation will be given a false negative diagnosis using this methodology. The 8994 polymorphism is commonly tested for using the enzyme *HaeIII* since this site is destroyed by the polymorphism. In patients with the 8994 polymorphism the PCR product is usually sequenced to exclude the 8993 mutation. With Pyrosequencing assays the disease associated genotype and % heteroplasmy were accurately determined using a single dispensation order. For individuals with the G8994A polymorphism the analysis will fail due to an unrecognized reference pattern as indicated in (figure 4). These samples can be re-screened using the dispensation order ACGTCAGCGT (figure 4b).

The T8993G Nanogen assay showed a high assay variation and false positive data could only be excluded using stringent analysis parameters where the mutation was considered to be detected only if the fluorescence signal obtained exceeded 5 standard deviations from the mean of normal samples. Hence the sensitivity of the assay is compromised. We designed the binding region for the reporter oligos 5' to the mutation in order to avoid the 8994 polymorphism. It is possible that other configurations might have performed better, but a more complicated and time consuming analysis would be required.

Overall, the six mitochondrial assays on the NanoChip® Molecular Biology Workstation provided accurate genotyping but had limitations when determining the level of heteroplasmy present in samples. The Nanogen system has been designed for classical SNP detection and the analysis and interpretation of results is intended for the identification of classical heterozygous and homozygous mutations. The software could not be adapted to manipulate the data to determine the %

heteroplasmy directly and so it was necessary to export the raw data and generate standard curves using ratio reference oligonucleotides which had been mixed at various ratios. Although this was successful for the MELAS and MERRF assays results were more variable for the LHON mutations and also in particular the T8993G mutation. The processing of samples and use of the machine was fairly undemanding but relatively labor intensive, and the analysis costs were relatively high.

The Pyrosequencing assays provided both accurate genotyping and determination of % mutation load. The Allele Frequency Quantification function of the SNP software allows automated calling of % heteroplasmy at the mutated base and a confidence score is given for each sample analysed (either passed, checked or failed) which alerts the user to the quality of the assay data. The parameters taken into account are the agreement between the observed and expected sequence, the signal to noise ratios and also the peak width. The mutations are presented in sequence context and therefore polymorphic variants will be identified and several 'reference peaks' are also incorporated into the analysis that add confidence to the data collection. This provides additional benefit when compared to PCR RFLP or techniques that rely upon hybridisation where false positive and negative results can be obtained in patients with polymorphisms which disrupt the hybridisation or restriction enzyme sites or where incomplete restriction enzymes digestion can occur (e.g. Kirby *et al*., 1998, White *et al*., 1998).

The Pyrosequencing assays were also easy to design and optimise and sample processing and use of the machine were straightforward. The incorporation of new and existing mutations into mitochondrial point mutation screening would be relatively undemanding with the advantage that the assays would not have to be designed to include restriction enzyme sites. The platform is very flexible and is amenable to the analysis of either individual mutations in single wells or the analysis of multiple samples for the same mutation within the same run. This is an advantage over PCR RFLP where different, and sometimes multiple, restriction digests are required for the analysis of each mutation. Pyrosequencing is more sensitive and rapid when compared to fluorescent PCR RFLP with costings for each technique being similar. We conclude therefore that Pyrosequencing is a robust, effective and efficient means of detecting and quantifying mitochondrial mutations in a clinical laboratory setting.

6. REFERENCES

Bai RK, Wong LJ. Detection and quantification of heteroplasmic mutant mitochondrial DNA by real-time amplification refractory mutation system quantitative PCR analysis: a single-step approach. Clin Chem 50:996-1001, 2004.

Boles RG, Chaudhari D, Soderkvist J, *et al* Quantification of mitochondrial DNA heteroplasmy by temporal temperature gradient gel electrophoresis. Clin Chem 49:198-200, 2003.

Chinnery PF, Howell N, Lightowlers RN, *et al*. Molecular pathology of MELAS and MERRF. The relationship between mutation load and clinical phenotypes. Brain120:1713-1721, 1997.

Ciafaloni E, Ricci E, Shanske S, *et al.* MELAS: clinical features, biochemistry, and molecular genetics. Ann Neurol 31:391-398, 1992.

Conley YP, Brockway H, Beatty M, *et al.* Qualitative and quantitative detection of mitochondrial heteroplasmy in cerebrospinal fluid using denaturing high-performance liquid chromatography. Brain Res Brain Res Protoc 12:99-103, 2003.

DiMauro S, Schon EA. Nuclear power and mitochondrial disease. Nat Genet19:214-215, 1998.

Hancock DK, Schwarz FP, Song F, *et al.* Design and use of a peptide nucleic acid for detection of the heteroplasmic low-frequency mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) mutation in human mitochondrial DNA. Clin Chem 48:2155-2163, 2002.

He L, Chinnery PF, Durham SE, *et al.* Detection and quantification of mitochondrial DNA deletions in individual cells by real-time PCR. Nucleic Acids Res 30(14):e68, 2002.

Holt IJ, Harding AE, Petty RK, *et al.* A new mitochondrial disease associated with mitochondrial DNA heteroplasmy. Am J Hum Genet 46:428-433, 1990.

Huoponen K, Vilkki J, Aula P, *et al.* A new mtDNA mutation associated with Leber hereditary optic neuroretinopathy. Am J Hum Genet 48:1147-1153, 1991.

Johns DR, Neufeld MJ, Park RD. An ND-6 mitochondrial DNA mutation associated with Leber hereditary optic neuropathy. Biochem Biophys Res Commun 187:1551-1557, 1992.

Juvonen V, Huoponen K, Syvanen AC, *et al.* Quantification of point mutations associated with Leber hereditary optic neuroretinopathy by solid-phase minisequencing. Hum Genet 93:16-20, 1994.

Kirby DM, Milovac T, Thorburn DR. A False-Positive Diagnosis for the Common MELAS (A3243G) Mutation Caused by a Novel Variant (A3426G) in the ND1 Gene of Mitochondria DNA. Mol Diagn 3:211-215, 1998.

Lagerström-Fermér M, Olsson C, Forsgren L, *et al.* Heteroplasmy of the Human mtDNA control region remains constant during life. Am J Hum Genet 68:1299-1301, 2001.

Liang MH, Wong LJ. Yield of mtDNA mutation analysis in 2,000 patients. Am J Med Genet 77:395-400, 1998.

Macmillan C, Lach B, Shoubridge EA. Variable distribution of mutant mitochondrial DNAs (tRNA(Leu[3243]) in tissues of symptomatic relatives with MELAS: the role of mitotic segregation. Neurology 43:1586-90, 1993.

Mashima Y, Nagano M, Funayama T, *et al.* Rapid quantification of the heteroplasmy of mutant mitochondrial DNAs in Leber's hereditary optic neuropathy using the Invader technology. Clin Biochem 37:268-276, 2004.

Mashima Y, Saga M, Hiida Y, *et al.* Quantitative determination of heteroplasmy in Leber's hereditary optic neuropathy by single-strand conformation polymorphism. Invest Ophthalmol Vis Sci 36:1714-1720, 1995.

Rahman S, Blok RB, Dahl HH, *et al.* Leigh syndrome: clinical features and biochemical and DNA abnormalities. Ann Neurol 39:343-351, 1996.

Ronaghi M, Uhlén M, Nyrén P. A sequencing method based on real-time pyrophosphate. Science 281:363-365, 1998.

Santorelli FM, Tanji K, Shanske S, *et al.* Heterogeneous clinical presentation of the mtDNA NARP/T8993G mutation. Neurology 49:270-273, 1997.

Silvestri G, Ciafaloni E, Santorelli FM, *et al.* Clinical features associated with the A-->G transition at nucleotide 8344 of mtDNA ("MERRF mutation"). Neurology 43:1200-1206, 1993.

Suomalainen A, Syvanen AC. Quantitative analysis of human DNA sequences by PCR and solid-phase minisequencing. Mol Biotechnol 15:123-131, 2000.

Szuhai K, Ouweland J, Dirks R, *et al.* Simultaneous A8344G heteroplasmy and mitochondrial DNA copy number quantification in myoclonus epilepsy and ragged-red fibers (MERRF) syndrome by a multiplex molecular beacon based real-time fluorescence PCR. Nucleic Acids Res 29(3):E13, 2001.

Tanno Y, Yoneda M, Tanaka K, *et al.* Quantitation of heteroplasmy of mitochondrial tRNA(Leu(UUR)) gene using PCR-SSCP. Muscle Nerve 18:1390-1397, 1995.

Thornburn DR, Dahl H-HM. Mitochondrial Disorders: Genetics, Counseling, Prenatal Diagnosis and Reproductive Options. Am J Med Genet 106:102-114, 2001.

Tully LA, Parsons TJ, Steighner RJ, *et al.* A sensitive denaturing gradient-Gel electrophoresis assay reveals a high frequency of heteroplasmy in hypervariable region 1 of the human mtDNA control region. Am J Hum Genet 67:432-443, 2000.

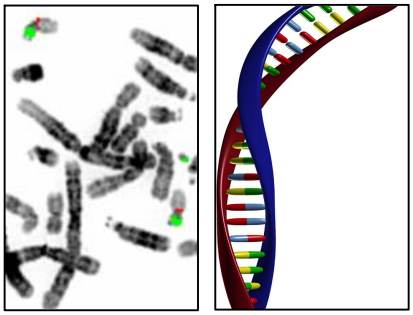
Wallace DC, Singh G, Lott MT, *et al.* Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 242:1427-1430, 1988.

Wallace DC. Mitochondrial Diseases in Man and Mouse. Science 283:1482-1488, 1999.

White SL, Collins VR, Wolfe R, *et al.* Genetic counseling and prenatal diagnosis for the mitochondrial DNA mutations at nucleotide 8993. Am J Hum Genet 65:474-482, 1999.

White SL, Thorburn DR, Christodoulou J, *et al.* Novel Mitochondrial DNA Variant That May Give a False Positive Diagnosis for the T8993C Mutation. Mol Diagn 3:113-117, 1998.

Wong LJ, Senadheera D. Direct detection of multiple point mutations in mitochondrial DNA. Clin Chem 43:1857



National Genetics Reference Laboratory (Wessex)
Salisbury District Hospital
Salisbury SP2 8BJ, UK

www.ngrl.org.uk