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Accurate detection and quantitation of heteroplasmic mitochondrial point mutations by Pyrosequencing

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ABSTRACT

Disease causing mutations in mitochondrial DNA are typically heteroplasmic and therefore interpretation of genetic tests for mitochondrial disorders can be problematic. Detection of low level heteroplasmy is technically demanding and it is often difficult to discriminate between the absence of a mutation or the failure of a technique to detect the mutation in a particular tissue. 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 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. Results obtained from the 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 Pyrosequencing assays provided accurate genotyping and quantitative determination of mutational load with a sensitivity and specificity of 100%. The MELAS A3243G mutation was detected reliably at a level of 1% heteroplasmy. We conclude that Pyrosequencing is a rapid and robust method for detecting heteroplasmic mitochondrial point mutations.

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™ 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.

MATERIALS AND METHODS

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; 50 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 either agarose gel electrophoresis.

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 (Pyrosequencing AB).

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

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.

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 (Pyrosequencing 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.

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.

Results

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 2. 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. Error bars for the Pyrosequencing data indicate the standard deviation for triplicate analysis. 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 2). 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 0.4 - 4.1, 0.8 – 3.5 and 0.6 – 3.5 respectively. Representative pyrograms obtained for a mutated sample from each assay are shown in figure 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).

Cost-effectiveness and Speed of Analysis

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.

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 can be used effectively 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 theses 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.

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. hair, blood and buccal swabs) and it appears that levels of the mutation decrease in blood and increases 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.

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 both Pyrosequencing and PCR RFLP. The level of heteroplasmy determined by Pyrosequencing assay correlated well to that detected using PCR RFLP. Again, 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 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 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 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 restricition 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.

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Figure 1: Percentage heteroplasmy detected by the Pyrosequencing assays compared to the diagnostic result obtained using non-fluorescent PCR RFLP a) G3460A, (b) G11778A, (c) T14484C and fluorescent PCR RFLP (d) A3243G, (e) T8993G/C (f) A8344G. The x axis shows the sample number and the y axis shows the % heteroplasmy detected. In graphs a) to c) the 100 % heteroplasmy obtained for PCR RFLP indicates that no mutated product was viewed on a 3% agarose gel after restriction enzyme digestion.

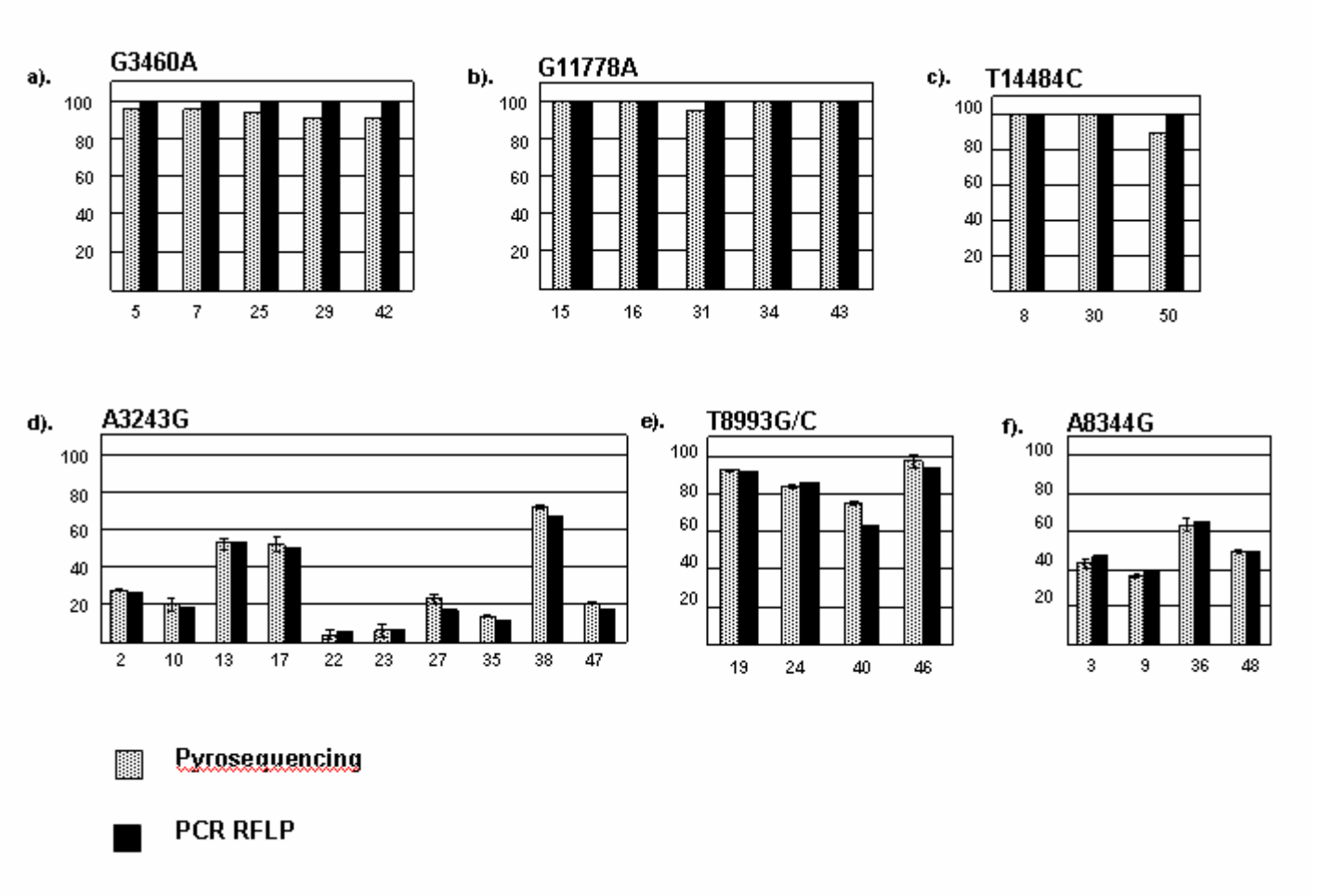
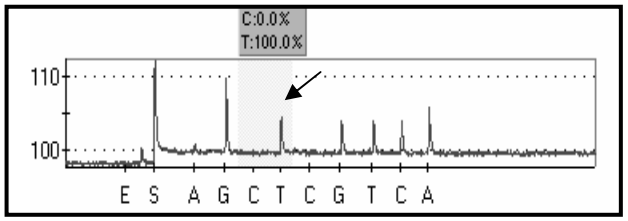
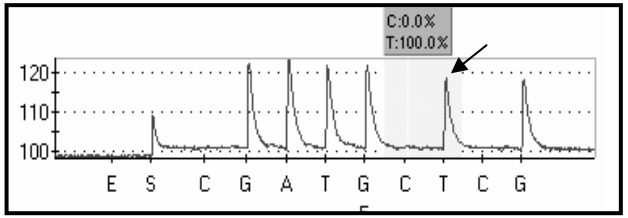


Figure 2: Representative pyrograms for representative patient samples which are heteroplasmic for a) G3460A b) G11778A c) T14484C d) A3243G e) A8344G and f) T8993G. The arrows mark the position corresponding to the mutated base. The boxes show the AQ values obtained for each allele, the value for the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

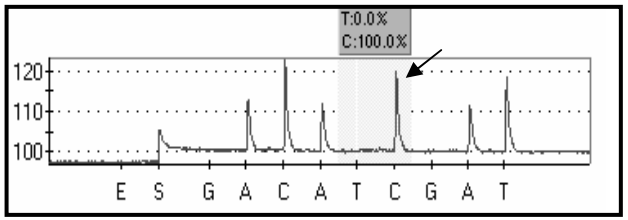
a)



b)



c)



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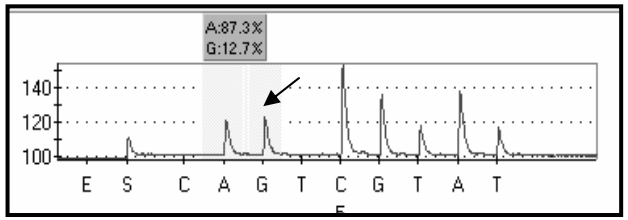
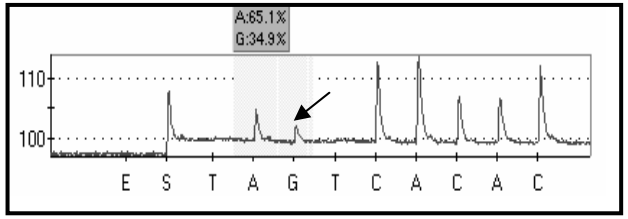


e)



f)

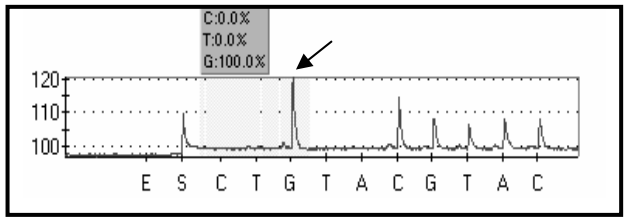


Figure 3: Standard curves from cloned wild type and mutated DNA samples which have been mixed to generate samples with the A3243G mutation present at levels of 0, 1, 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% (x axis). Error bars indicate the standard deviation for triplicate samples

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%.

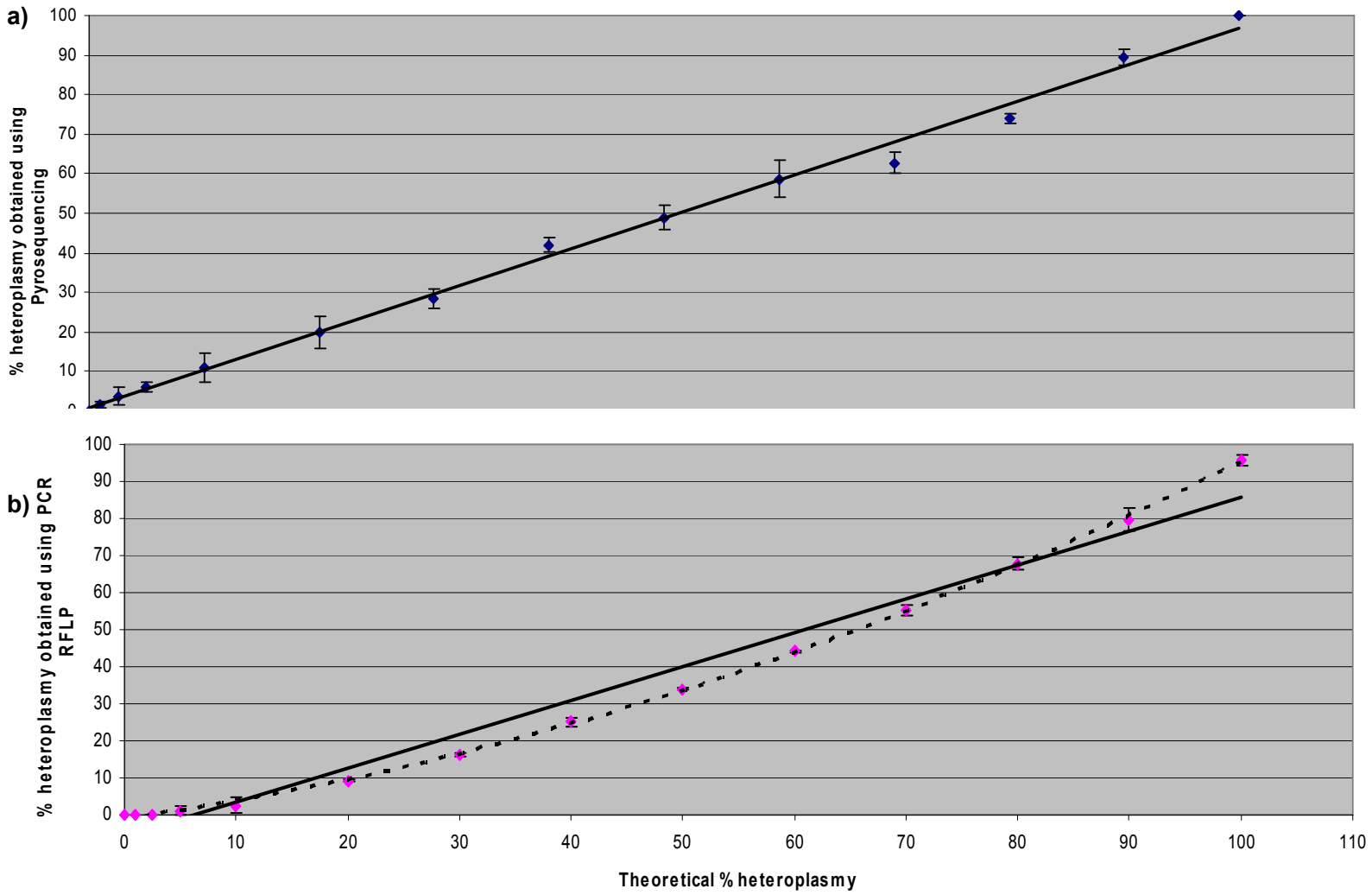

a)
% heteroplasmy obtained using Pyrosequencing
100
90
80
70
60
50
40
30
20
10
b)
% heteroplasmy obtained using PCR RFLP
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
Theoretical % heteroplasmy

Figure 3

Figure 4: Pyrosequencing histograms showing results expected for samples with i) and iv) 50% heteroplasmy for T8993C; ii) and v) 50% heteroplasmy for T8993G; iii) and (vi) no mutation. Reference peaks where no signal is expected are indicated by #.

a) Dispensation order used to detect the T8993G/C mutation. This dispensation order will determine the percentage heteroplasmy and mutation status for samples without G8994A polymorphism. Samples with a G8994A polymorphism will cause an A peak to occur at dispensation 5 (reference peak indicated by *). Since the resulting reference pattern will be unrecognised the sample will be reported as failed. Therefore, manual examination of all failed samples is necessary to determine whether samples are polymorphic at this position.

b) Dispensation order to be used on samples with a known G8994A polymorphism. The percentage heteroplasmy and mutation status can be determined using this assay only when the G8994A polymorphism is present.

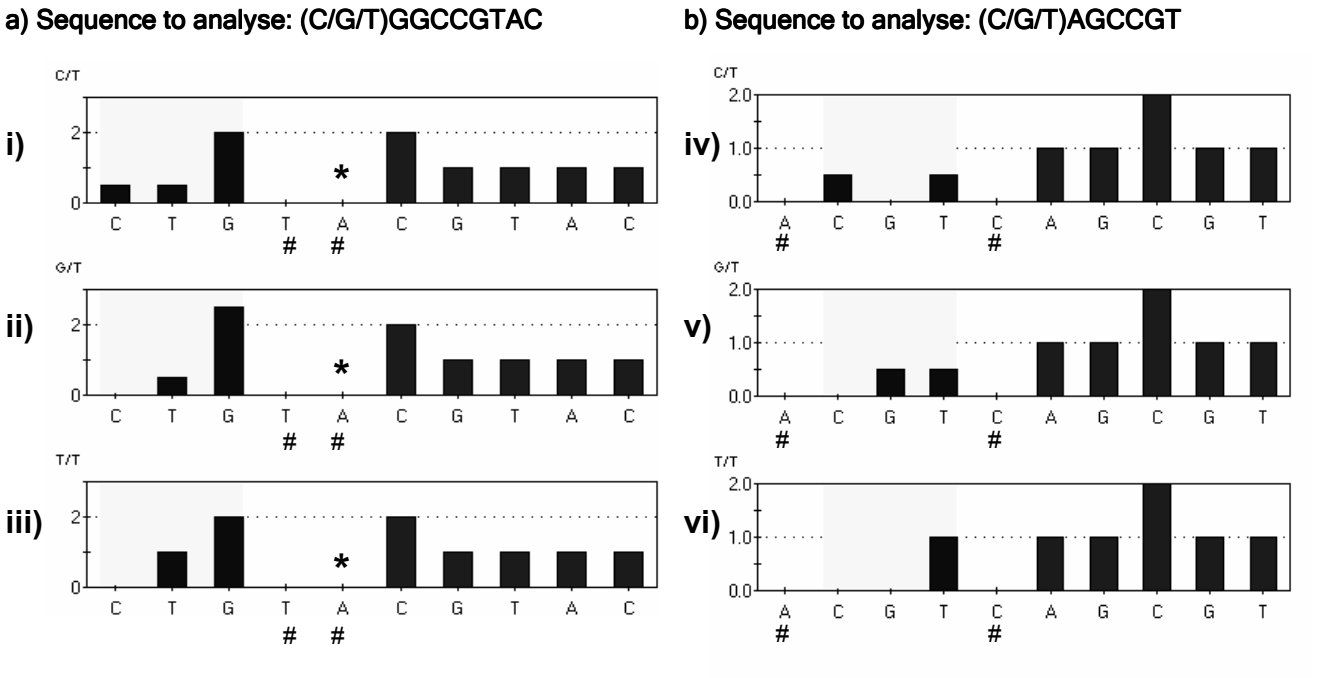


Table 1: Sequences of oligonucleotides required for the Pyrosequencing and RFLP Assays. For the Pyrosequencing assays, the sequence to analyse is immediately 3' to the sequencing primer binding site on the biotinylated strand. The position of the mutation is shown in brackets (**bold font**). The dispensation 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.

Mutation	Oligonucleotides	Sequence 5′ to 3′	Sequence to analyse	Dispensation order
A3243G MELAS	RFLP Fwd PCR	AGGACAAGAGAAATAAGGCC		
	RFLP Rev PCR	TAGAAGAGCGATGGTGAGAG		
	Pyrosequencing Fwd PCR	CCTCCCTGTACGAAAGGACA		
	Pyrosequencing Rev PCR	Biotin-TGGCCATGGGTATGTTGTTA		
	Pyrosequencing Sequencing	GGTTTGTTAAGATGGCAG	**(A/G)**GCCCGGTAATC	*C***AG***T*CGTAT
A8344G MERRF	RFLP Fwd PCR	GTAGTATTTAGTTGGGGCATTTCACTGTAAAGCCGTGTTGG		
	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 LHON	RFLP Fwd PCR	CAGCCACAGAACTAATCATA		
	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 NARP/Leighs	RFLP Fwd PCR	CCGACTAATCACCACCCAAC		
	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 2: 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.

Mutation	Inter assay analysis (n=15)		Intra assay analysis (n=6)		
	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