Rapid and Sensitive Real-Time Polymerase Chain Reaction Method for Detection and Quantification of 3243A>G Mitochondrial Point Mutation

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Maternally inherited diabetes and deafness and mitochondrial encephalomyopathy, lactic acidosis with stroke-like episodes result from the 3243A>G mitochondrial point mutation. Current methods to detect the presence of the mutation have limited sensitivity and may lead to potential misclassification of patients with low levels of heteroplasmy. Here, we describe development and validation of a rapid real-time polymerase chain reaction (PCR) method for detection and quantification of levels of heteroplasmy in a single assay. Standard curve analysis indicated that the sensitivity of detection was less than 0.1%. Time from sample loading to data analysis was 110 minutes. We tested 293 samples including 23 known positives, 40 known negatives, and 230 samples from patients clinically classified as having type 2 diabetes. All positive samples were correctly detected, and of those samples previously quantified, heteroplasmy levels determined using the real-time assay correlated well ($r^2 = 0.88$ and 0.93) with results from fluorescently labeled PCR-restriction fragment length polymorphism and pyrosequencing methods. Screening of 230 patients classified as having type 2 diabetes revealed one patient with 0.6% heteroplasmy who had previously tested negative by PCR-restriction fragment length polymorphism. Real-time PCR provides rapid simultaneous detection and quantification of the 3243A>G mutation to a detection limit of less than 0.1%, without post-PCR manipulation. (*J Mol Diagn* 2006, 8:225–230; DOI: 10.2353/jmoldx.2006.050067)

The mitochondrial m.3243A>G point mutation is associated with multiple clinical presentations including maternally inherited diabetes and deafness (MIDD)¹ and mitochondrial encephalomyopathy, lactic acidosis with stroke-like episodes (MELAS).² The phenotypes can be extremely variable, making mutation analysis important for diagnosis and management. Pathogenic mitochondrial mutations are present only in a proportion of, rather than all, mitochondrial DNA (mtDNA) because of heteroplasmy, having both wild-type and mutant mtDNA.³ Be-

cause of random segregation of mutant and normal mtDNA into daughter cells, the high rate of replication in rapidly dividing tissues such as blood cells tends to reduce the proportion of mutated mtDNA in comparison with wild type. Slower dividing (postmitotic) tissue such as muscle, neurons, and endocrine organs, usually show higher levels of mutated mtDNA and are often clinically affected.⁴ Unfortunately, tissues (eg, pancreas) containing the highest levels of heteroplasmy are not easily accessible for diagnostic testing.

There are three main reasons for determining the heteroplasmy level of m.3243A>G: relevance to clinical phenotype, genetic counseling for disease transmission, and sensitivity of genetic diagnosis. No correlation with diabetes onset or severity can be elicited from the level of heteroplasmy detected in a blood sample; however, there is some correlation of degree of heteroplasmy with the age of onset⁵ and severity of deafness.⁶ Heteroplasmy levels in muscle may better correlate with the degree of age-corrected hearing loss,⁷ but this is not a practical assessment for most patients. In very rare cases of Leigh's syndrome (a subacute encephalopathy with prominent brainstem signs) associated with m.3243A>G mutation, levels of heteroplasmy in excess of 80 to 90% in most tissues have been found, correlating with the most severe phenotype of this mutation.^{8–10}

In genetic counseling, the frequency of affected offspring at a population level may be roughly predicted from maternal blood heteroplasmy level^{9,11,12}; however, more studies are required using accurate methods of heteroplasmy quantification before this information may be helpful on an individual basis.

Current methods for detecting m.3243A>G are either simple but insensitive or laborious and overly sensitive. The relatively simple but insensitive methods include densitometry of ethidium bromide-stained polymerase chain reaction (PCR) products,¹³ dot blotting/Southern blotting of restriction digestion fragments,¹⁴ denaturing high-performance liquid chromatography, and pyrosequencing.

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Table 1. Sequence of Primers and Probes Used in Quantitative Real-Time PCR

Primer/probe	Sequence	mtDNA region
Forward primer	5'-CCA CAC CCA CCC AAG AAC AG-3'	3206 to 3225
Reverse primer	5'-AGG AAT TGA ACC TCT GAC TGT AAA GTT T-3'	3268 to 3295
Wild-type probe	VIC-CCG GGC TCT GCC AT-MGB	3237 to 3250
Mutant probe	6FAM-CCG GGC CCT GCC AT-MGB	3237 to 3250

quencing.^{15,16} A sensitive but radioactive method is radiolabeled PCR-restriction fragment length polymorphism (PCR-RFLP).¹³ Highly sensitive but time-consuming methods, such as ligation-mediated PCR,¹⁷ amplification refractory mutation system (ARMS),¹⁸ or peptide nucleic acid binding assays,¹⁹ have been used in the research setting, but they have the disadvantage of being too sensitive, detecting such low levels of m.3243A>G (<0.01%) that they are thought to represent levels of somatic mitochondrial mutation present even in unaffected individuals.¹⁸

Here, we describe a real-time PCR approach to the simultaneous detection and quantification of the m.3243A>G mutation that can be applied to any diagnostically relevant sample without the need for any post-PCR manipulation. This is a rapid, sensitive, and accurate technique that promises to improve detection levels (within the diagnostically important range of above 0.1%) and should allow rapid and high throughput diagnostic testing.

Materials and Methods

DNA Samples and DNA Isolation

Total DNA was isolated from peripheral blood leukocytes using the Promega Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI) and stored at -20°C. A total of 23 known positive samples isolated from patients with clinical manifestations of MIDD or MELAS were analyzed along with 40 known negatives. Levels of heteroplasmy had previously been determined by White et al¹⁶ using pyrosequencing and fluorescently labeled PCR-RFLP methods for eight positive samples. An additional 230 samples were also analyzed from patients with clinically classified type 2 diabetes, absent autoantibodies, and undetectable m.3243A>G using PCR-RFLP with Gelstar (BioWhittaker, Walkersville, MD) staining. The study was approved by the local ethics committee, and informed consent was obtained from all individuals.

Assay Development

We obtained primers and probes (Assays-by-Design) from Applied Biosystems (Foster City, CA) to identify wild-type (A) and mutant (G) sequences at nucleotide 3243 of the mitochondrial genome. Our assay uses a single set of primers, so mutation discrimination is achieved by the use of probes that differ only at the position of the mutation (see Table 1 for probe and primer sequences). The labeling of mutant and wild-type

probes with different fluorophores allows quantification of m.3243A>G mutation in single-tube analysis. Probes were labeled at the 5' end with either 6-FAM or VIC and at the 3' end with a minor groove binding protein. The use of the minor groove binding protein moiety increases melting temperature of the probe/template duplex and allows the use of shorter, more specific probes.²⁰

The approach was validated by mixing experiments and standard curve analysis. Wild-type and mutant plasmids were mixed to generate samples with the m.3243G (mutant) target at concentrations ranging from 0.01 to 100%, which were used to determine the lowest level of mutant detection using a standard curve.

PCR reaction mixture contained 10 µl of Taqman Universal PCR MasterMix (Applied Biosystems), 0.36 µmol/L of each primer, 0.08 µmol/L of each of wild-type- and mutant-specific probe, and 60 ng of DNA in a total volume of 20 µl. Real-time PCR conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of denaturation for 15 seconds at 95°C and annealing/extension for 60 seconds at 60°C. The fluorescent signal intensities were recorded and analyzed during PCR in an ABI Prism 7000 sequence detector system (Applied Biosystems) using the SDS (ver. 1.0) software. The total time required from loading of samples to data analysis was 1 hour and 50 minutes. Average time taken for data analysis for 96 samples was 30 minutes.

Allele-Specific Relative Quantification

We determined the crossing points for mutant and wild-type alleles in triplicate from each sample. These values were used to calculate the difference between wild-type and mutant crossing points (ΔC_t), which is a direct measure of the relative abundance of each target (Figure 1). These results were then normalized to a known mixture of 50% heteroplasmy to account for differences in probing efficiency ($\Delta\Delta C_t$) as described previously.²¹

The ratio of mutant to wild-type alleles was calculated by inserting the $\Delta\Delta C_t$ value into the equation $2^{-\Delta\Delta C_t}$, which relies on the fact that a 100% efficient PCR reaction produces a doubling of product with each cycle. The percentage of heteroplasmy could then be determined from the ratio of alleles.

Results

Assay Validation

Standard curve analysis indicated that the detection of mutant and wild-type alleles was equally efficient, with

Figure 1. Crossing points of wild-type and mutant alleles in strong positive (heteroplasmy level, 40%) (a), weak positive (heteroplasmy level, 9%) (b), and normal samples (c).

curve gradients of -3.02 and -2.79 respectively (Figure 2). By generating serial dilutions of the 100% mutant plasmid with 100% wild-type plasmid to generate samples with the 3243 mutant alleles present at levels of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.1, 0.05, 0.02, and 0.01%. A concentration of mutant mtDNA in samples as low as 0.01% was detected by this method (Figure 3); however, the accuracy of quantification is reliable down to 0.1%. The extent of cross-hybridization was determined by analysis of the mutant probe with a pure sample of normal plasmid. Low levels of cross-hybridization are a common observation where sequences differ by single bases. Such “creeping curves” are easily distinguished from genuine low positives (Figure 4) and are inherent to an exonuclease assay where discrimination is at the 1-bp level. Proper placement of analysis threshold is nevertheless very important.

Figure 2. Standard curves generated with plasmids containing 100% mutant and 100% wild-type sequences.

Detection: Testing Samples

All 23 known positives were identified by the quantitative real-time PCR method. None of the known negatives were

Figure 3. Mixing experiment showing limit of detection of the real-time PCR assay. 1, 50% mutant; 2, 40% mutant; 3, 30% mutant; 4, 20% mutant; 5, 10% mutant; 6, 1% mutant; 7, 0.1% mutant; 8, 0.01% mutant.

Figure 4. Results obtained with mutant probe tested on triplicate samples of 100% normal plasmids, a normal patient, and a case of known low positive m.3243A>G (9% heteroplasmy).

Table 2. Real-Time PCR A3243G Heteroplasmy Levels Compared with Those Obtained by White et al¹⁶ Using Fluorescent PCR-RFLP and Pyrosequencing

Patient samples	Real-time PCR % heteroplasmy (error range)*	Pyrosequencing % heteroplasmy ¹⁶ (error range)*	Fluorescent PCR-RFLP % heteroplasmy ¹⁶
2	20.2 (19.8 to 20.7)	27.6 (26.7 to 28.5)	26.0
10	30.8 (28.1 to 33.5)	21.6 (20.4 to 22.8)	18.0
13	52.8 (47.9 to 57.6)	52.6 (49.5 to 55.7)	53.0
17	65.7 (60.7 to 70.4)	52.2 (48.1 to 56.3)	50.0
22	6.6 (6.2 to 7.3)	3.8 (1.8 to 5.8)	5.0
23	3.7 (3.2 to 4.3)	5.9 (2.2 to 9.6)	7.0
27	20.8 (19.2 to 22.5)	23.1 (21.2 to 25)	17.5
35	11.3 (10.3 to 12.5)	13.9 (12.8 to 15)	11.3

*Mean and error range (± 1 SD) derived from triplicate determinations of each identical sample (pyrosequencing error ranges courtesy of Dr H. White).

scored as positive. Testing 230 patients clinically classified as having type 2 diabetes revealed one positive sample for the m.3243A>G mutation.

Quantification of Positive Results

We used the crossing point data to calculate the percentage of heteroplasmy for the positive samples. Of the positive samples, eight had been previously quantified by White et al¹⁶ using two independent methods (fluorescently labeled PCR-RFLP and pyrosequencing technology). Levels of heteroplasmy ranged from 3 to 60%, as determined by our method, and compared favorably with results obtained with fluorescently labeled PCR-RFLP and pyrosequencing ($r^2 = 0.88$ and 0.93 , respectively; Table 2).

Our results were reproducible within each test plate with triplicate measurements generating CT values within 0.5 SD and also from plate to plate with Δ CT values within 0.5 SD. Although the absolute CT values were not numerically identical from plate to plate (because these depend on various experimental parameters including amount of DNA in the sample), the $\Delta\Delta$ CT values altered much less, because this reflects the constant proportion of different mtDNA alleles present.

The single positive sample from 230 patient samples clinically classified as having type 2 diabetes showed 0.6% heteroplasmy. This sample came from a 77-year-old man with a body mass index of 29.7 kg/m^2 who was diagnosed with diabetes at 63 years of age and was insulin treated. He had no parental history of diabetes, but two siblings also had diabetes.

Discussion

We have produced a new real-time quantitative PCR assay for simultaneous detection and quantification of m.3243A>G mutation heteroplasmy that is rapid and simple to use. It provides accurate results down to a level of 0.1% mutant mitochondria, provided that a standard sample is used in every run to overcome any differences in master mix or probe efficiency. Using this method, we were able to identify all samples testing positive by Gelstar staining of PCR-RFLP products and to detect accurately and quantify samples comparable with other

more expensive and time-consuming methods. In addition, we were able to identify one case of low-level heteroplasmy for m.3243A>G who was previously classified as having type 2 diabetes after testing negative for the mitochondrial mutation by PCR-RFLP with Gelstar staining. Although we used blood samples, this technology can easily be applied to any tissue sample.

Currently the most common method for detection of m.3243A>G mutation in blood leukocytes uses PCR amplification of both mutant and wild-type alleles, followed by restriction enzyme analysis and ethidium bromide or Gelstar staining. In our laboratory, Gelstar staining shows only a very faint band with m.3243A>G mutation loads of 5 to 10%, below which they are undetectable. A more sensitive method uses a radiolabeled assay with the addition of isotope for the last cycle of PCR amplification and detects levels of heteroplasmy down to 1%.¹³ Neither of these methods are quantitative, and the latter involves radioactivity along with its associated risks and costs.

The sensitivity of detection by Southern blotting or dot-blot hybridization has been reported as 2%.¹⁴ New methods such as denaturing high-performance liquid chromatography have a limit of 3 to 10% heteroplasmy detection, comparable with 5% heteroplasmy discrimination of m.3243A>G by pyrosequencing.¹⁵

Other new, highly sensitive methods for detecting low-level m.3243A>G mutations used in the research setting are semiquantitative because they rely on allele-specific amplification of the mutant mtDNA with suppression of wild-type amplification using the ARMS method,¹⁸ peptide nucleic acid binding assays,¹⁹ and ligation-mediated PCR.¹⁷ Heteroplasmy levels of m.3243A>G below 0.01%, using the ARMS method, have been found in more than 90% of both healthy and diabetic subjects, indicating somatic mutation,^{17,18} and should hence be considered diagnostically negative.

Our single 77-year-old patient with 0.6% heteroplasmy for m.3243A>G, out of a cohort of 230 patients with type 2 diabetes, did not have other features of mitochondrial diabetes such as deafness or maternal inheritance of diabetes, but we were not able to analyze his mother's or siblings' blood. This case probably represents levels of somatic mitochondrial mutation at the m.3243A>G posi-

tion in the presence of longstanding oxidative stress associated with diabetes; however, it is possible that it could be the pathogenic cause of diabetes if levels are higher in the pancreas.¹⁹ The highest level of m.3243A>G heteroplasmy detected from previous studies of somatic mutations in subjects with type 1 and type 2 diabetes has been 0.04%.^{18,22,23} Clinical features of three diabetic patients with 0.01 to 0.1% heteroplasmy did not include additional mitochondrial features such as maternal inheritance, muscle weakness, and deafness, which have been present in many of those with levels above 0.1%.²⁴ More clinical studies with sensitive and accurate methods of detecting m.3243A>G are required to clarify the significance of finding patients with levels of heteroplasmy in the 0.1 to 1% range within blood.

It is well established that the heteroplasmy load of m.3243A>G mutation in blood declines with age at a mean rate of 1.4% per year,^{11,25–28} and levels of heteroplasmy in blood do not necessarily reflect levels in affected tissues such as the pancreas.²⁹ In confirming obligatory carrier status in first-degree maternal relatives of probands with MIDD or MELAS in a large-scale Finnish epidemiological study, highly sensitive PCR using allele-specific primers was used when standard PCR-RFLP methods did not show presence of the mutation, indicating minimum heteroplasmy levels in some oligosymptomatic individuals.³⁰ With our sensitive method for routinely determining heteroplasmy levels, the clinical significance of diabetic patients with levels of m.3243A>G in the previously undetected lower range of 0.1 to 1% will become clearer. In the interim, diagnostic results in this range will have to be interpreted cautiously in light of clinical circumstances.

By using our real-time PCR method, it is possible to rapidly and accurately screen a large number of samples for m.3243A>G mutation, improving our diagnostic sensitivity and offering a possible application in population screening. Because detection and quantification of heteroplasmy levels occurs simultaneously, this information can be gained without further processing. Using the sensitive real-time allele-specific assay, the proportion of false-negative results from clinically positive individuals should be reduced, allowing more accurate measurement of the prevalence of the m.3243A>G mitochondrial mutation.

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