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Multiplex fluorescence-based primer extension method for quantitative mutation analysis of mitochondrial DNA and its diagnostic application for Alzheimer's disease

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ABSTRACT

A sensitive and highly reproducible multiplexed primer extension assay is described for quantitative mutation analysis of heterogeneous DNA populations. Wild-type and mutant target DNA are simultaneously probed in competitive primer extension reactions using fluorophor-labeled primers and high fidelity, thermostable DNA polymerases in the presence of defined mixtures of deoxy- and dideoxynucleotides. Primers are differentially extended and the resulting products are distinguished by size and dye label. Wild-type:mutant DNA ratios are determined from the fluorescence intensities associated with electrophoretically resolved reaction products. Multiple nucleotide sites can be simultaneously interrogated with uniquely labeled primers of different lengths. The application of this quantitative technique is shown in the analysis of heteroplasmic point mutations in mitochondrial DNA that are associated with Alzheimer's disease.

INTRODUCTION

Quantitative mutation detection techniques are valuable genetic tools for analysis of heterogeneous DNA populations that are typically seen in mitochondrial diseases and in drug-resistant bacterial and viral strains. We recently reported the use of a fluorescence-based primer extension method for quantitating mutational burden in a Leber hereditary optic neuropathy (LHON) family that carried the pathogenic nt 3460 mutation in the mitochondrial genome (1). The approach exploited the high fidelity of certain DNA polymerases for simultaneously detecting mutant and wild-type DNA by competitive primer extension. Methods like the described procedure are essential for studying diseases that are governed by the principles of population genetics. Such diseases are exemplified by metabolic and neurodegenerative disorders of mitochondrial DNA (mtDNA) origin (2).

The 16.5 kb human mtDNA is maternally transmitted and codes for 13 protein subunits of the electron transport chain and for the structural RNAs required for protein synthesis within the mitochondria (3). Wild-type and mutant mtDNA can co-exist within the same cell in a condition called heteroplasmy and the ratio of mutant to wild-type mtDNA can drift throughout life across different tissues and organ systems. The phenotypic expression of a mitochondrial disease occurs once mutant mtDNA exceeds a critical threshold in an affected tissue, resulting in bioenergetic failure and, eventually, cell death (4–6). We have described specific nucleotide substitutions that could lead to amino acid replacement in mitochondrial-encoded subunit genes 1 and 2 of cytochrome c oxidase (CO) that are associated with a form of late onset Alzheimer's disease (AD) (7). Sequencing analysis of multiple clones of these genes revealed that these AD-associated mutations reside within the same mtDNA molecule, physically distinct from the more prevalent wild-type mtDNA molecule. Importantly, the allelic abundance of this mutant DNA was found to be significantly higher in AD cases as compared with cognitively normal, neurodegenerative or metabolic disease controls. These findings suggest an elevated mutational burden in late onset AD that is associated with impaired cellular energy metabolism and the disease phenotype. Therefore, accurate quantitation of heteroplasmy can provide valuable information for diagnosis and may be relevant for determining relative risk of developing the disease and assessing the progress and severity of the disease (8).

A variety of techniques have been employed for quantifying heteroplasmy in mtDNA. These methods include restriction fragment length polymorphism (RFLP), single-strand conformational polymorphism, multiple clonal analysis, allele-specific oligonucleotide hybridization and single nucleotide primer extension (9–15). Some of the drawbacks of the reported procedures that restrict their scope are the use of radioisotopic labels, absence of appropriate restriction sites or unique DNA conformations in the target sequences, lack of discrimination in detecting subtle sequence variations in DNA by hybridization and

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limited multiplexing potential. These factors preclude their use for high volume genetic testing.

In this report we discuss the design and features of our reported fluorescence-based primer extension method (1). The key advantages of the method are its ease of design, accurate quantitation of heteroplasmy, potential for multiplexing and automation and wide applicability. The utility of the method is shown in detecting AD-associated mutant mtDNA containing missense mutations at nt 6366, 7146, 7650, 7868 and 8021 and a silent mutation at nt 6483. Related radiosotopic procedures for simultaneously detecting mutant and wild-type DNA have been described elsewhere (16–17).

MATERIALS AND METHODS

Thermo Sequenase™, dNTPs and ddNTPs were purchased from Amersham. Calf intestine alkaline phosphatase and biochemical reagents were obtained from Boehringer Mannheim and QIAquick PCR purification kits from Qiagen. Accuspin™ tubes and HISTOPAQUE® 1077 were purchased from Sigma and EDTA vacutainers from Beckton Dickinson. UlTma™ DNA polymerase, AmpliTaq® DNA polymerase and reagents for DNA synthesis were purchased from Perkin Elmer. Oligonucleotides were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer (Perkin Elmer) using standard phosphoramidite chemistry. Fluorescein 5′-labeled oligonucleotide primers were obtained using the 6-FAM or HEX Amidite reagent in the last step of the automated synthesis. Tritylated and fluorescein-labeled oligonucleotides were purified by standard reverse phase chromatography procedures. The homogeniety of the fluorescein-labeled oligonucleotides was assessed by electrophoresis on an Applied Biosystems Model 373 Sequencing System.

DNA isolation

After IRB approval and informed consent, fresh venous blood samples were drawn from 65 patients with clinical diagnosis of probable Alzheimer's disease (AD mean age = 74.7 ± 1.1 years) and 73 controls (67 ± 1 years; cognitively normal age-matched n = 43; cortico-basal ganglionic degeneration $n = 2$; Pick's disease $n = 2$; Parkinson's disease $n = 16$; non-insulin-dependent diabetes $n = 10$). AD patients met the National Institute of Neurological, Communicative Disorders and Stroke and Alzheimer's Diseases and Related Disorders Association (NINCDS-ADRDA) criteria of probable Alzheimer's disease (18).

Blood samples were collected in EDTA vacutainers and kept at 4°C for no more than 24 h. The platelet/white blood cell fraction was isolated in Accuspin™ tubes (Sigma Diagnostics) using the following procedure. Three milliliters of HISTOPAQUE® 1077 was added to the upper chamber of an Accuspin™ tube and the device was centrifuged at 1000 *g* for 30 s. Three to four milliliters of blood were then introduced into the upper chamber and separated by centifugation at 1000 *g* for 10 min at room temperature. After centrifugation, the plasma and white blood cell layers were transferred to a new tube and the white blood cells were sedimented by centrifugation at 7000 *g* for 10 min. The white cell pellet was resuspended in 0.4 ml of a solution containing 0.9% sodium chloride, 1 mM EDTA and stored at –80°C until use.

Frozen white blood cells (0.2 ml) were thawed and sedimented by centrifugation at 12 000 *g* for 5 min. The white cell pellet was washed with 0.6 ml Dulbecco's phosphate-buffered saline (PBS; Gibco BRL) and resuspended in 0.2 ml water. The cells were lysed by incubation in a boiling water bath for 10 min. After cooling to room temperature, the cellular debris was sedimented by centrifugation at 14 000 *g* for 2 min. The supernatant was transferred to a new vial and the approximate concentration of the crude DNA preparation was estimated from its A_{260} absorbance. The DNA sample was stored at –80°C.

PCR of target DNA

PCR amplification of cellular DNA was carried out in a total volume of 50 µl using the primer pair sets listed in Table 1. Reactions contained ~1 µg cellular DNA, 2.5 U AmpliTaq® DNA polymerase, 20 pmol each of the light strand primer and the heavy strand primer and 10 nmol each dNTP in PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$). After an initial denaturation step at 95°C for 10 s in a Gene Amp PCR System 9600 thermal cycler (Perkin Elmer), amplification was carried out for 30 cycles under the following conditions: 95°C for 1 min, 60°C for 1 min, 72°C for 1 min. After the last cycle reaction tubes were kept at 72°C for 4 min to ensure extension of incomplete strands to full length products. Following amplification, the PCR products were analyzed by electrophoresis on a 0.8% agarose gel.

Table 1. PCR primers for preparing primer extension templates

Gene	Sequence (5′→3′)	Strand[a]	PCR length	Base analysis[b]
CO1	GAGCCTCCGTAGACCTAACCATCT	L	246	6366, 6483
CO1	GGTCGAAGAAGGTGGTGTTGAG	H		
CO1	CCATCATAGGAGGCTTCATTCACTG	L	200	7146
CO1	TGATAGGATGTTTCATGTGGTGTATGC	H		
CO2	CATGCAGCGCAAGTAGGTCTACAAGAC	L	255	7650
CO2	TGTTATGTAAAGGATGCGTAGGGATGG	H		
CO2	CCTGCCCGCCATCATCCTAGT	L	308	7868, 8021
CO2	AGCCTAATGTGGGGACAGCTCATG	H		
ND1	CAGTCAGAGGATCAATTCCTC	L	298	3460
ND1	GAGGGGGGATCATAGTAGAGG	H		

[a]L and H refer to light and heavy strands of mtDNA (3).
[b]PCR products containing AD-associated nucleotide positions in the *CO1* and *CO2* genes and 3460 LHON mutation in the *ND1* gene.

Table 2. Primer and nucleotide combinations for mtDNA mutations

Entry	Gene	Base	Substitution[a]	Associated Disease	Sequence (5'→3')	Strand	5'-Label[b]	Length	Product size[c]	dNTPs	ddNTPs
1	CO 1	6366	G→A (Val/Ile)	AD	TGATGAAATTGATGGCCCCTAAGATAGAGGAGA	H	FAM	33	w34, m35	T	A, C
2	CO 1	6483	C→T (silent)	AD	AGGACTGGGAGAGATAGGAGAAGTA	H	FAM	25	w28, m26	G	A
3	CO 1	7146	A→G (Thr/Ala)	AD	ACCTACGCCAAAATCCATTTC	L	FAM	21	w22, m23	G	A, C
4a	CO 2	7650	C→T (Thr/Ile)	AD	TATGAGGGCGTGATCATGAAAG	H	FAM	22	w23, m25	A, T	G
4b	CO 2	7650	C→T (Thr/Ile)	AD	TCCCCTATCATAGAAGAGCTTATCA	L	FAM	25	w28, m26	C	T
5	CO 2	7868	C→T (Leu/Phe)	AD	GGCCAATTGATTTGATGGTAA	H	FAM	21	w22, m23	A	G, T
6a	CO 2	8021	A→G (Ile/Val)	AD	TTATTATACGAATGGGGGCTTCAA	H	FAM	24	w25, m27	C	G, T
6b	CO 2	8021	A→G (Ile/Val)	AD	ATGTAATTATTATACGAATGGGGGCTTCAA	H	FAM	30	w31, m33	C	G,T
7	ND1	3460	G→A (Ala/Thr)	LHON	GCTCTTTGGTGAAGAGTTTTATGG	H	FAM	24	w27, m26	A, C	G, T
8	ND1	3460	G→A (Ala/Thr)	LHON	GCTACTACAACCCTTCGCTGAC	L	HEX	22	w23, m27	A, C	G, T
9	ND1	3460	G→A (Ala/Thr)	LHON	GCTCTTTGGTGAAGAGTTTTATGG	H	FAM	24	w28, m26	C, G	A, T
10	ND1	3460	G→A (Ala/Thr)	LHON	GCTACTACAACCCTTCGCTGAC	L	HEX	22	w26, m23	C, G	A, T

[a]Base and codon change in mtDNA.
[b]Light or heavy strand primers were synthesized with FAM or HEX dyes at their 5′-termini.
[c]Sizes of primer extension products. w, product from wild-type DNA template; m, product from mutant DNA template.

Residual nucleotides that persisted after the PCR reaction were dephosphorylated by adding 1 U calf intestine alkaline phosphatase (CAP) in 5 µl 10× CAP buffer (100 mM Tris–HCl, pH 8.3, 10 mM $MgCl_2$, 10 mM $ZnCl_2$) to the PCR reaction mixture and incubating for 30 min at 37°C in a thermal cycler. Then 1.1 µl 0.25 M EDTA, pH 8.0, was added and the alkaline phosphatase was denatured at 75°C for 10 min.

Double-stranded PCR products were separated from primers, nucleosides and enzymes using QIAquick™ columns (Qiagen, Chatsworth, CA) and the recommended procedure of the manufacturer. The PCR products were eluted with 50 µl 10 mM Tris–HCl, pH 8.5, dried in a Savant SpeedVac Concentrator and then reconstituted in 20 µl water.

Primer extension assay

The fluorescein-labeled primers and nucleotide combinations for analysis of AD-associated mutations are shown in Table 2. Stock solutions of each dNTP and ddNTP were prepared by mixing equimolar amounts of the nucleotides with $MgCl_2$ and diluting the mixture to the desired concentration with TE. The fluorescein-labeled primers were diluted in TE to provide final stock concentrations of 40 fmol/µl. One micrliter of the purified PCR-amplified DNA fragment was used as template for each assay.

Primer extension reactions were performed in a total volume of 8 µl. The UlTma™ DNA polymerase-catalyzed reactions contained template, 20 fmol fluorescein-labeled primer, 400 µM ddNTPs/25 µM dNTPs of the appropriate nucleotide combination and 0.6 U enzyme in buffer containing 10 mM Tris–HCl, pH 8.8, 10 mM KC1, 0.002% Tween 20, 2 mM $MgC1_2$. Thermo Sequenase DNA polymerase-catalyzed reactions were performed with 20 fmol fluorescein-labeled primer, 25 µM each of the appropriate ddNTP/dNTP combination and 0.64 U enzyme in buffer containing 10 mM Tris–HCl, pH 9.5, 5 mM KC1, 0.002% Tween 20, 2 mM $MgC1_2$. Each set of primer extension assays included control template preparations that had been amplified from homoplasmic wild-type and mutant DNA. After an initial denaturation step at 95°C for 2 min, the reaction conditions comprised 20 cycles of 95°C for 20 s and 55°C for 40 s. The samples were concentrated to ~1 µl by heating open reaction tubes at 94°C for 7 min. After the concentration step, 8 µl loading dye (0.5% blue dextran in 83% formamide, 8.3 mM EDTA, pH 8.0) were added.

The products of the primer extension reaction were analyzed on an ABI 373 Sequencer using a 12% denaturing polyacrylamide gel and Tris–borate, EDTA as running buffer. Prior to electrophoresis, the samples in loading dye were denatured for 3 min at 85°C. Three microliter aliquots of the standards (primer with no added template, reaction products from homoplasmic wild-type and mutant DNA templates) and each unknown reaction mixture were then loaded and electrophoresed according to the manufacturer's instructions. Fluorescent band intensities associated with wild-type- and mutant DNA-derived extension products were estimated by the GENESCAN™ 672 software program (Applied Biosystems Division, Perkin Elmer). Quantitative analysis of heteroplasmy was carried out by correlating the fluorescent band intensities of wild-type- and mutant DNA-derived extension products from unknown samples with those from homoplasmic wild-type and mutant DNA control templates.

RESULTS

Competitive primer extension strategy for multiplexed mutational analysis

Simultaneous detection of wild-type and mutant DNA is accomplished using a thermostable DNA polymerase with a fluorophor-labeled primer and a selected mixture of deoxynucleotides (dNTPs) and dideoxynucleotides (ddNTPs). Figure 1 illustrates the approach for interrogating the G→A point mutation at nt 6366 in the mitochondrial *CO1* gene, one of several allelic variations associated with late onset Alzheimer's disease. The mutant and wild-type DNA compete as templates in the primer

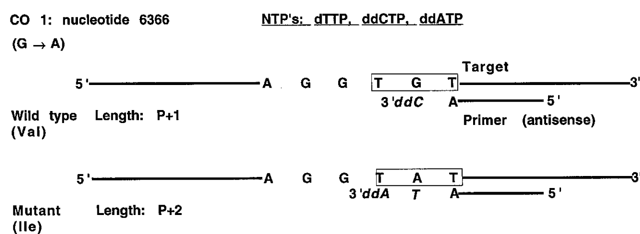

Figure 1. Discrimination and quantitation of wild-type and mutant alleles at AD-associated nt 6366 in the *CO1* gene by multiplexed primer extension assay. A thermostable DNA polymerase is utilized with a nucleotide combination of dTTP, ddATP and ddCTP to extend a fluorescein 5′-labeled primer by either 1 (wild-type sequence) or 2 nt (for mutant sequence). After gel electrophoretic separation, fluorescence intensities associated with wild-type and mutant products were used to quantitate levels of heteroplasmy.

extension reaction and are distinguished by differential extension of the primer. Thus, with a nucleotide combination of dTTP, ddATP and ddCTP, the extended primer is 1 nt longer when the template is the mutant rather than wild-type DNA. Typically, the reactions are performed with multiple thermal cycles to ensure quantitative conversion of primers to extension products. The degree of heteroplasmy at the monitored site is estimated by comparing the ratio of fluorescence intensities of the gel-separated extension products with a standard curve generated from known wild-type/mutant template mixtures.

Templates for the primer extension reaction are generated by PCR amplification of cellular DNA. A two-step protocol is utilized for template purification since residual PCR nucleotides transferred into the primer extension reaction compromise the accuracy of the assay. Purification entails alkaline phosphatase treatment of the PCR product mixture followed by passage through QIAquick® columns. The thermostable *AmpliTaq*, *UlTma*™ and *Tth* DNA polymerases were studied in PCR amplification of heteroplasmic target DNA and were found to provide product mixtures of very similar compositions (results not shown).

Preliminary experiments revealed some extension past the designated final base when thermostable DNA polymerases lacking 3′→5′ exonuclease activity were used in primer extension reactions. This problem was encountered with *Taq*, *Tth*, Exo⁻ *Pfu*, Vent® (exo⁻) and Thermozyme DNA polymerases and arises from base misincorporation. In addition, fluorescein-labeled primers were degraded by *Taq* polymerase when >0.2 U enzyme were used in the reactions. The misincorporation problem was rectified with the use of high fidelity polymerases and *UlTma*™ DNA polymerase was selected for subsequent studies since its 3′→5′ exonuclease activity enables removal of base mismatches. Thermo Sequenase was also noted to be very accurate because of its high specificity for dNTP and ddNTP substrates (19). As seen in Table 3, primer extension with the two enzymes provided very similar estimates of mutant allelic frequencies of heteroplasmic mtDNA samples isolated from blood.

Next, reaction cycles and input target concentrations for the PCR step were varied to evaluate the influence of these parameters on reproducing the heteroplasmy of input DNA mixtures. Plasmids containing wild-type and mutant *CO1* or *CO2* gene inserts were mixed in 4:1 molar ratios and 0.1, 1 and 10 fmol of the DNA mixtures were used in the study. It should be noted that 1 µg total cellular DNA was typically used as input target amount in our AD studies (7; see also Materials and Methods). This pool contains ~1 fmol mtDNA and falls within the copy number range of target mentioned above. Table 4 shows that the mutant:wild-type DNA ratios determined by the assay for various input target concentrations and PCR cycles were in excellent agreement with the level of heteroplasmy of starting DNA mixtures. Further, mutant allele frequencies were unaltered when the annealing temperatures for PCR and the primer extension reaction were varied between 50 and 60°C (data not shown).

Figure 2 shows a representative standard curve derived from *UlTma* DNA polymerase-catalyzed primer extension assays. These assays used templates containing known mixtures of mutant and wild-type DNA. There is a direct linear relationship between percent mutant DNA in the assay and the amount that is detected (coefficient of correlation $r = 0.99$). The assay reliably detects mutant alleles present at relative ratios between 1 and 3%. The variability in the assay is typically ≤5%. The reproducibility of the assay is illustrated by the high positive correlation between two independent assays of heteroplasmic mtDNA samples (Figure 2, inset; $r = 0.98$).

Table 3. Heteroplasmy analysis by primer extension using *UlTma* and Thermo Sequenase DNA polymerases[a]

Patient	Nucleotide 6483		Nucleotide 7146		Nucleotide 7650	
	UlTma	Thermo Sequenase	*UlTma*	Thermo Sequenase	*UlTma*	Thermo Sequenase
1	14.5	17.0	11.4	11.1	17.5	18.6
2	10.2	13.6	7.4	9.0	17.6	16.7
3	12.7	14.6	8.0	7.8	14.8	14.6
4	21.8	26.1	15.2	16.0	21.3	20.8
5	4.5	6.7	1.9	3.4	5.4	6.6
6	43.7	48.1	40.6	39.2	45.4	43.8

[a]Mutant allele frequencies at AD-associated positions determined by primer extension using *UlTma* and Thermo Sequenase DNA polymerases. Reported values are means of duplicates.

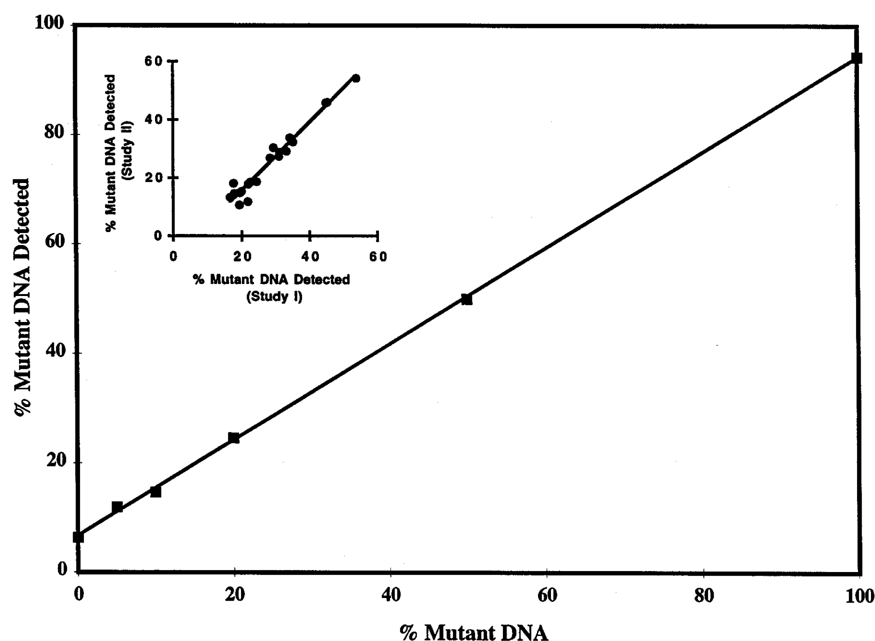

Figure 2. Standard curve for the multiplexed primer extension assay. Quantitated plasmids containing wild-type and mutant *CO1* gene inserts were mixed in known ratios and amplified by PCR. Nucleotide 6366 was interrogated in the PCR products and the mutant allele frequency detected by the assay was plotted against percent mutant DNA in the plasmid mixture. Linear regression analysis yielded a coefficient of correlation (r) of 0.99. (Inset) Reproducibility of the assay for two independent analyses of 6366 mtDNA mixtures. Duplicate PCR amplifications were performed on the test samples followed by primer extension analysis. Linear regression analysis provided an r value of 0.98.

Table 4. Influence of PCR conditions on heteroplasmy at AD-associated bases

	Nucleotide position		
	6843	7650	8021
Input DNA for PCR[a] (fmol)			
0.1	25.4	24.7	24.7
1	24	25.8	24.5
10	27.3	22.5	23.2
PCR cycles[b]			
15	17.6	21.4	
20	24.3	24.1	
25	27.1	19.5	
30	26.9	21.9	

Mutational analysis of PCR products obtained from amplification of 20% mutant/plasmid co-mixes with [a]different initial PCR target concentrations and [b]different PCR cycles.
Heteroplasmy at AD-associated nucleotide posistions reported as mutant allele frequencies and are means from duplicate experiments.

The differential extension approach offers considerable flexibilty in designing assays and several strategies have been employed for simultaneously interrogating nucleotide status at multiple sites. These include use of primers of different length, primers carrying distinct fluorophor labels or primers which are distinguished by size and dye label. Figure 3 shows electrophoretic analysis of two sets of multiplexed primer extension reactions. In the first set, nt 6366, 6483 and 7146 of the *CO1* gene were interrogated using the nucleotide combination dGTP, dTTP, ddCTP and ddATP and locus-specific FAM primers 33, 25 and 21 bases in length respectively (Table 2). The multiplexed reactions produce extension products from wild-type and mutant DNA templates that are unique in size and were easily resolved with single base resolution. A similar separation of reaction products was observed with the second set of reactions, which was designed to estimate heteroplasmy at nt 7650, 7868 and 8021 of the *CO2* gene using primers 25, 21 and 30 bases in length respectively. PCR products from two mtDNA samples isolated from blood were included as templates in both sets and show heteroplasmy at the monitored sites. Mutant allele frequencies reported by the multiplex procedure were identical to those obtained from the individual reaction format (results not shown).

Multiplexed mutational analysis using different dyes is illustrated by the following example. Light and heavy strand primers carrying separate dye labels were designed to detect the mtDNA 3460 mutation associated with LHON (20). The primer/nucleotide combinations in Table 2 (entries 7–10) provide four independent ways of determining the frequency of the 3460 mutant allele. Both strands of mtDNA isolated from LHON family members were simultaneously analyzed by primer extension assay using strand-specific primers in two multiplexed reactions. The two nucleotide combinations (Table 2) provide products which are distinguished by size and fluorescent label. Table 5 shows a close correlation of mutant allele frequencies reported by the two primers within the same reaction as well as in the separate multiplexed reactions and demonstrates the multiplexing potential of the assay.

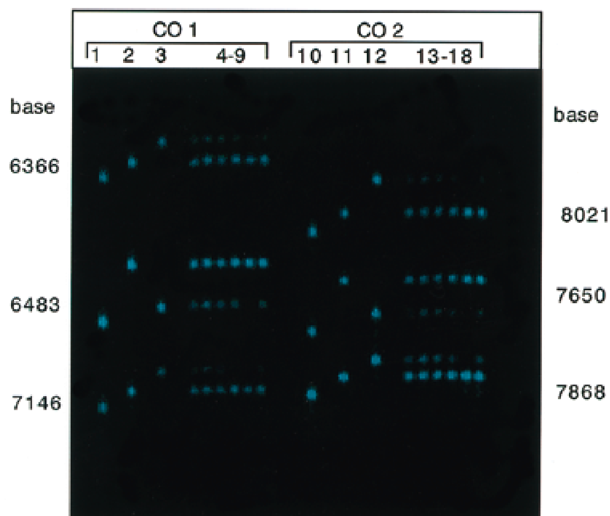

Figure 3. Analysis of multiple nucleotide sites using multiplexed primer extension. In the first set of reactions (lanes 1–9), nt 6366, 6483 and 7146 of the *CO1* gene were simultaneously monitored using primer entries 1–3 of Table 2 and a nucleotide mix of dTTP, dGTP, ddATP and ddCTP. Lane 1, primers; lane 2, products from homoplasmic wild-type DNA control; lane 3, products from homoplasmic mutant DNA control; lanes 4–9, products from cellular DNA from patient samples. The second set of multiplexed reactions (lanes 10–18) analyzes nt 7650, 7868 and 8021 of the *CO2* gene using primer entries 4b, 5 and 6b of Table 2 and a nucleotide mix of dATP, dCTP, ddGTP and ddTTP. Lane 10, primers; lane 11, products from homoplasmic wild-type DNA control; lane 12, products from homoplasmic mutant DNA control; lanes 13–18, products from cellular DNA from patient samples.

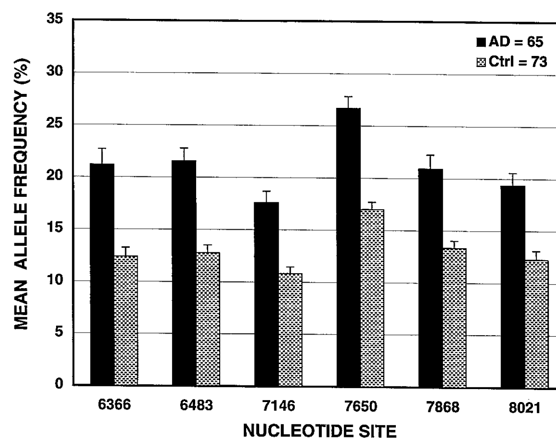

Figure 4. mtDNA heteroplasmy at AD-associated nucleotide sites in the *CO1* and *CO2* genes for AD patients and controls. Each bar represents the group mean percentage of the mutant base relative to the wild-type base. Error bars represent the standard errors of the mean (SEM) for each group. At each site AD cases had significantly higher levels of the mutant base than controls, as determined by independent *t*-tests ($P < 0.001$).

Table 5. Frequency of the 3460 mutant allele by primer extension using a dye-based multiplexing strategy

Patient ID	Multiplexed reaction A		Multiplexed reaction B	
	FAM primer	HEX primer	FAM primer	HEX primer
20	35.0	38.3	32.1	32.8
21	35.0	40.1	37.1	43.3
30	11.6	14.2	6.3	11.6

The 3460 LHON mutation in the mitochondrial *ND1* gene was interrogated with primers that target either the light or heavy DNA strand. Strand-specific primers were labeled with different dyes (Table 2). In multiplexing reaction A both strands of template were probed simultaneously with the primers and the nucleotide mix dATP, dCTP, ddGTP and ddTTP. In multiplexing reacton B the template strands were probed with a nucleotide mix of dCTP, dGTP, ddATP and ddTTP. Heteroplasmy in the DNA samples are reported as mutant allele frequencies. Reported values are means of duplicates.

Analysis of mtDNA from Alzheimer's disease patients and controls

Total cellular DNA was isolated from blood of AD patients, cognitively normal age-matched controls, neurological controls and patients with non-insulin-dependent diabetes. DNA samples were prepared by isolating the platelet-rich white blood cell fraction of whole blood followed by lysing cells by boiling. This protocol is a critical step in isolating mtDNA. Extraction of DNA by standard SDS/proteinase K, phenol/chloroform treatments proved inadequate, as this results in nearly quantitative loss of mutant mtDNA (unpublished observations). Since mtDNA is prone to oxidative damage and therefore susceptible to cleavage (21–22), small fragments of the *CO1* and *CO2* genes were amplified by PCR (Table 1). The multiplexed primer extension assay was used to quantify the relative proportion of mutant to wild-type bases at nt 6366, 6483 and 7146 of the *CO1* gene and nt 7650, 7868 and 8021 of the *CO2* gene. These mutations occur at low frequency in most controls, but are elevated in a subset of Alzheimer's disease patients. Cohort analysis (Fig. 4) revealed that AD patients exhibited statistically significant increases in mutational burden at each of the six nucleotide sites as compared with normal, aged-matched and other disease controls.

The mutational burden data for AD patients and controls were further analyzed by the Fisher multivariate discriminant analysis technique (23). This method transforms multivariate observations (percent heteroplasmy at the six nucleotide sites) to univariate Fisher scores. The calculated Fisher score is reflective of the overall mutational burden for each individual. The mean Fisher score of the AD group was significantly higher than the control group (Fig. 5, inset). A receiver operating characteristic (ROC) curve was then generated to evaluate the sensitivity and specificity of this assay. A ROC curve represents the distribution of scores within a study population (24). Sensitivity was defined as percent AD cases with a given or higher Fisher score, while specificity was defined as percent controls with a given or lower Fisher score. The resulting ROC curve (Fig. 5) shows that it is common for AD cases to have a high mutational burden, whereas it is rare for controls to share this characteristic. As seen in Figure 5, the assay captures 30% of the Alzheimer's disease patients with absolute specificity at the very high end of the mutational burden spectrum. High mutational burden therefore represents a strong positive risk factor for AD. In contrast, it is rare for AD cases to have low mutational burden and it is quite common for controls to show this genetic pattern. The profiles of the ROC curves generated from Fisher scores and from mutational burden values from a representative AD-associated nucleotide position (nt 7650) were superimposable (Fig. 5), illustrating linkage of the AD-associated mutations. High levels of these

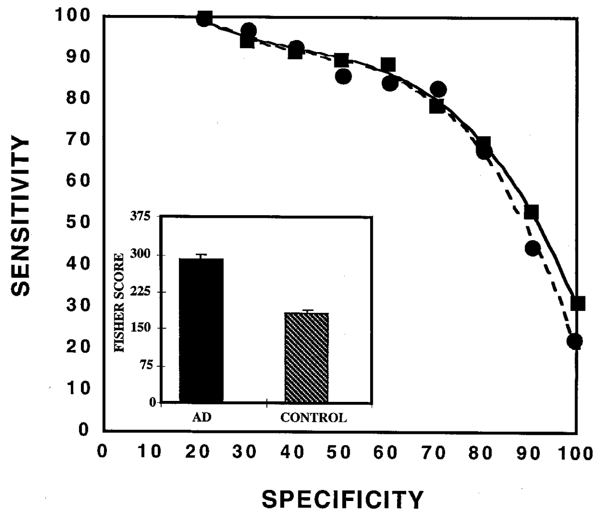

Figure 5. ROC curves presenting sensitivity (percentage of AD cases with mutational burden above a selected value) and specificity (percentage of non-AD cases with mutational burden below a selected value). The data were subjected to a curve fitting program (Microsoft Excel v.5.0a). ■, ROC curve generated from Fisher score values; ●, ROC curve generated from mutational burden values at AD-associated nt 7650 (see text for details). (Inset) Group mean Fisher scores for AD cases and controls. Error bars represent the SEM for each group.

mutations are relatively specific for AD. Mutational burden is not elevated above age-matched control values in patients with non-insulin-dependent diabetes mellitus or in neurological disease controls, including Parkinson's disease cases (7).

DISCUSSION

We describe a non-isotopic, competitive primer extension-based method for multiplex mutation analysis and its applicability for the quantitative detection of mtDNA mutations associated with Alzheimer's disease and other mitochondrial diseases. The mutational burden estimated by the method faithfully mirrors the composition of the DNA template population. Standard curves are linear and the method provides a 1–3% threshold of detection for rare mutant alleles. In an earlier study we reported an excellent agreement between the present method and nucleotide sequencing in the quantitation of heteroplasmy of DNA samples harbouring the LHON 3460 mutation (1).

Primer extension assays are easy to design and are readily configured to detect mutations at multiple nucleotide sites. This can be accomplished using primers of different sizes and multiple fluorophor labels. There are two variations for multiplexed analysis with primers of differing length and nucleotide combinations: (i) primer extension reactions using matched primer/nucleotide combinations can be carried out in the same reaction tube (Fig. 3); (ii) primer extension with incompatible nucleotide combinations can be performed separately and the products can be pooled prior to gel analysis. Thus, by judicious grouping of mutations and selection of primer lengths and dyes it is possible to quantitatively detect a number of mutations in a conventional automated DNA sequencer. We have also realized increased throughput by batch loading of samples in the same gel from different primer extension reactions. Typically, gel electrophoresis is performed with four to five sample loadings at timed intervals and a fluorescent electrophoretogram is obtained with clearly resolved product bands.

A key advantage of this diagnostic approach is that both the wild-type and mutant DNA are probed in the same reaction. This minimizes the possibility of tube-to-tube variability inherent in related solid phase minisequencing approaches and time-resolved fluorometric hybridization methods which detect mutant and wild-type DNA in separate reactions (9–10). Misincorporation of nucleotides during primer extension is a potential drawback that can compromise quantitative mutation analysis. This problem is sometimes encountered in single nucleotide primer extension assays (25–27) and is most evident with thermostable polymerases lacking proofreading function (see above). Another contributing factor is the poor selectivity exhibited by *Taq* polymerase for modified deoxy- or dideoxynucleotides in assays that use fluorophor-labeled nucleotides as substrates, which can result in false extension products (28). These issues can influence the quantitative potential of related multiplex single nucleotide primer extension formats that detect multiple mutations using labeled nucleotides and/or primers of different sizes (29–30). In the assay described above the choice of high fidelity polymerases and a competitive reaction format significantly reduces erroneous nucleotide incorporation.

The multiplexed method circumvents some of the shortcomings of the existing technologies for heteroplasmy analysis. Traditional hybridization-based methods, such as allele-specific oligonucleotide (ASO) hybridization and allele-specific PCR, are limited in their scope for detecting subtle differences in mutant allele frequencies. Typically, the utility of these methods is sequence dependent. When applicable, the methods often require tailored optimization conditions for interrogating each mutation site for efficient discrimination of wild-type and mutant alleles. Mutation analysis by RFLP is best applicable when mutations cause a gain or loss of a restriction site. However, the technique lacks sensitivity in detecting mutant alleles that appear at low frequencies. Mispairing PCR–RFLP has been used to engineer a restriction site when a convenient site is absent in wild-type and mutant DNA (31). The application of this variation of RFLP can be compromised by differences in the PCR amplification efficiencies of wild-type and mutant DNA (32). In contrast, the high fidelity DNA polymerases used in the primer extension assay ensure exquisite discrimination of wild-type and mutant DNA with accurate quantitation.

The utility of the multiplexed primer extension assay is demonstrated in the analysis of mtDNA mutations associated with Alzheimer's disease. A set of six mutations in the *CO1* and *CO2* genes, which appear linked in a unique mtDNA molecule, were screened in DNA extracted from blood of Alzheimer's disease patients and controls. Two important findings emerge from the mutation study. First, quantitative analysis revealed that the mutations were usually heteroplasmic in blood tissue. This implies that heteroplasmic alleles are common in the general population, the implications of which require further investigation. Second, the allelic frequency of the mutant mtDNA is significantly elevated in clinically defined cases of AD but not in cognitively normal, age-matched controls, neurological controls or patients with non-insulin-dependent diabetes mellitus. Mutational burden values for the AD and control groups were transformed into an ROC curve to illustrate the sensitivity and specificity of the assay. The data indicate that a high mutational burden is a strong positive risk factor for AD and a subset of AD cases can be identified with absolute specificity.

In conclusion, the multiplexed primer extension assay is a valuable diagnostic tool that can be used for a variety of applications in molecular medicine. These include the study of tissue distribution of heteroplasmic mtDNA mutations and association of heteroplasmy with clinical phenotype in mitochondrial diseases, analysis of heterogeneous viral populations in infected individuals who are on antiviral drug regimens and quantitation of tissue mosaicism in tumor biopsies (33–34). The assay is well suited for automation, a critical requirement for high volume genetic screening. Reactions can be set up by automated stations equipped with pipetting and thermocycling functions, while capillary array electrophoresis is an attractive approach for automating the separation step. The development of multiple capillary array systems with high throughput capacities provides a unique opportunity to introduce the technology for DNA diagnostic analysis.

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