



Rapid screening of the entire mitochondrial DNA for low-level heteroplasmic mutations

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Abstract

Alterations of the mitochondrial DNA (mtDNA) are implicated in various pathological conditions. In this study, we used denaturing high performance liquid chromatography (DHPLC) as a method to rapidly screen the entire mtDNA for mutations. Overlapping DNA fragments, amplified by one single cycling protocol from frozen pre-formulated PCR mixes, were subjected to DHPLC analysis. Single DHPLC injections of fragments yielded straightforward interpretation of results with a detection limit down to 1% mtDNA heteroplasmy. Furthermore, collection and re-amplification of low degree heteroduplex peak-fractions allowed sequence analysis of mtDNA mutations down to the detection limit of the DHPLC method. In order to demonstrate that the method has diagnostic value, we analyzed and confirmed known mtDNA mutations in patient samples. © 2005 Elsevier B.V. and Mitochondria Research Society. All rights reserved.

Keywords: DHPLC; MtDNA; Mutation; Heteroplasmy

1. Introduction

During the last decades, numerous mitochondrial point mutations have been identified in patients with diverse clinical phenotypes (Jaksch et al., 2001; Lenaz et al., 2004; Moslemi et al., 2004; Nishigaki et al., 2003). Although clinical manifestations of disorders that are related to mtDNA mutations are extremely variable, progress in genetic testing and identification

of mitochondrial diseases has been achieved. A cell contains hundreds of mitochondria and thousands of copies of mtDNA molecules. Both normal and mutated mtDNA may coexist within a tissue, a condition known as heteroplasmy, whereas the presence of identical mtDNA molecules is called homoplasmy. Heteroplasmic pathogenic mutations can change over the course of time and exceed the pathogenic threshold in a particular tissue. Recent studies demonstrated that mtDNA mutations accumulate with increasing age (Jaksch et al., 2001; Murdock et al., 2000), that the level of heteroplasmy is associated with the age of onset and progression of chronic conditions, such as diabetic complications (Iwase et al., 2001) and that the decline in mitochondrial function can be due to

Abbreviations: DHPLC; denaturing high-performance liquid chromatography; mtDNA; mitochondrial DNA.

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the accumulation of mutations in mtDNA (Singh, 2004). Somatic homoplasmic and heteroplasmic changes have also been reported in a wide variety of cancers suggesting an influence on carcinogenesis (Singh, 2004; Yang et al., 2004; Zhu et al., 2005).

Denaturing high performance liquid chromatography (DHPLC), a method which separates heteroduplex and homoduplex DNA molecules on a stationary phase under partially denaturing conditions has already been shown to successfully detect unknown alterations in nuclear encoded genes (O'Donovan et al., 1998; Torrente et al., 2004; Xiao and Oefner, 2001) and the mtDNA (Conley et al., 2003; Danielson et al., 2003; LaBerge et al., 2003; Liu et al., 2002; van Den Bosch et al., 2000). DHPLC based methods are probably the most efficient approach for the detection of mutations and were shown to achieve highly reliable results (Torrente et al., 2004). In addition, detection and quantification of low-level heteroplasmy has been shown to be feasible by DHPLC (Conley et al., 2003). However, the methods to analyze the entire mtDNA have used either large amplicons in combination with a restriction digest or DHPLC analysis of single PCR fragments with indefinite resolution (Biggin et al., 2005; van Den Bosch et al., 2000). In addition, no simple method for sequence analysis of low-level heteroplasmy was provided.

Here, we describe a new DHPLC approach that allows and simplifies rapid screening of the entire 16.5 kb mtDNA genome, by using a combination of frozen pre-tested PCR mixes, a single PCR protocol for DNA amplification and DHPLC analysis. In addition, we developed a protocol to subsequently sequence low-level heteroplasmy.

2. Materials and methods

2.1. Patients

Samples from patients and family members were collected after informed consent was given.

2.2. PCR amplification of the entire mitochondrial genome

DNA of tissues, cells or blood leukocytes was extracted by proteinase K digestion followed by

phenol/chloroform extraction. The whole human mtDNA was amplified by 48 overlapping DNA fragments ranging from 200 to 600 bp (Table 1). The fragments overlapped by at least 40 bp. PCR mixtures contained 10× PCR buffer, 0.4 μM of each primer, 120 μM of each deoxynucleotide triphosphate and 0.45 U of Optimase® DNA polymerase (Transgenomic San Jose, CA, USA). Pre-aliquoted reaction mixes (30 μl) were stored at –20 °C. After thawing, 60 ng of genomic DNA was added and all fragments were amplified with identical conditions: 95 °C for 2 min, 35 cycles of 95 °C for 15 s, 57 °C for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. PCR products were visualized by gel electrophoresis on 2% agarose gels stained with ethidium bromide. *Pfu* DNA polymerase (Promega, Madison, USA), Discoverase™ (Invitrogen, Santa Cruse, USA) and AmpliTaq Gold® DNA polymerase (Applied Biosystems, Warrington, UK) were used according to manufacturer's protocols.

2.3. Mutation detection by DHPLC

Denatured and re-annealed amplicons (5 μl; heated at 98 °C for 2 min and cooled to 70 °C at a rate of 0.05 °C/s) were directly injected into the dual plate WAVE 3500 apparatus equipped with a Hitachi D-7000 Interface, L-7100 Pump, L-7300 Oven, L-7400 UV Detector, DNASep® cartridge and the navigator™ software version 1.5.4 (Transgenomic San Jose, CA, USA). The melting temperatures of the fragments and time shifts are given in Table 1. The gradient for the elution of the fragments (buffer A: 0.1 M triethylammonium acetate, B: 0.1 M triethylammonium acetate, 25% acetonitrile) was created automatically with the navigator™ software.

Oven temperature for optimal heteroduplex separation under partial DNA denaturation was determined for each PCR fragment using the navigator software. Heteroplasmic peaks that were lower than the homoplasmic peak were manually collected and re-amplified.

2.4. Introduction and analysis of artificial mutations

PCR fragments of mtDNA regions containing an endogenous restriction site and an artificial mismatch 2–9 bp distant from the respective restriction site were

Table 1

Primer sequences used to amplify the entire mtDNA in 48 overlapping fragments and DHPLC analysis temperatures as calculated by navigator software

Fragment	Position	Sequence forward primer	Sequence reverse primer	bp	Temperatures (°C)	
1	108–638	AGCACCTATGTCGCAGTATC	GGTGATGTGAGCCCGTCTAAAC	531	58.3	59
2	548–964	CCAACCAAACCCCAAAGAC	GGGAGGGGGTGATCTAAAAC	417	58.4	60
3 ^a	871–1250	GCCCCGCCACAGGGTTGGT- CAATTTTCGTGCC	GAGCAAGAGGTGGTGAGGTTG	390	58.8	
4	1172–1612	CCTGGCGGTGCTTCATATCC	GCTACACTCTGGTTCGTCCAAG	441	59.1	
5	1485–1950	GCCCGTACCCCTCCTCAAG	ACGGGTGTGCTCTTTAGCTG	466	57.9	
6	1883–2433	GGAGAGCCAAAGCTAAGACCC	GTGTTGGGTTGACAGTGAGGG	551	56.6	57.8
7	2182–2722	GCAGCCACCAATTAAGAAAGCG	TCTCGTCTTGCTGTGTCATGC	541	58	60.3
8	2676–3225	AAATTGACCTGCCCGTGAAGAG	CCTGTTCTTGGGTGGGTGTG	550	58.2	
9	3079–3505	GGAGTAATCCAGGTCGGTT	TAGATGTGCGGGTTTTAGG	427	58	60.5
10	3438–3893	GCTACTACAACCCCTTCGCTGAC	GTTCGGTTGGTCTCTGCTAGTG	456	60	
11	3703–4203	CTGCGAGCAGTAGCCCAAAC	TGCTAGGGTGAGTGGTAGGAAG	501	58	61.3
12	4183–4552	TTCCTACCACTCACCTAGCA	AAAAATCAGTGCAGCTTAGC	370	56	59.2
13	4505–5003	CATCTTTGCAGGCACACTCATC	GATTTTGCCTAGCTGGGTTTGG	499	58	
14	4955–5483	CATAGCAGGCAGTTGAGGTGG	AGGTAGGAGTAGCGTGGTAAGG	529	56.3	58.7
15	5428–5926	CAAAACCCACCCATTCTCC	AATAGTCAACGGTCCGCGAAC	499	57.7	58.6
16	5861–6345	ACAGTCCAATGCTTCACTCAGC	AGATGGTTAGGTCTACGGAGGC	485	59.7	
17	6266–6669	AGCAGGAACAGGTTGAACAGTC	GGGAGATTATCCGAAGCCTGG	404	60	
18	6572–6831	CGGAGGAGGAGACCCCATC	TGGTAGCGGAGGTGAAATATGC	260	57.3	
19	6747–7088	TTCCTAGGGTTTATCGTGTGAGC	GTGAATGAAGCCTCCTATGATGG	342	60	
20	6954–7491	GGTGGCCTGACTGGCATTG	GTTGGCTTGAACCAGCTTTGG	538	58	
21	7403–7682	ACCCTACCACACATTTCG	GGAAA ATGATTATGAGGGCG	280	55.9	57.3
22	7612–8091	ACAAGACGCTACTTCCCCTATC	CCTAATGTGGGGACAGCTCATG	480	57.5	59.5
23	8011–8560	AGTACTCCCGATTGAAGCCCC	GGGCAATGAATGAAGCGAACAG	550	56.1	59
24	8466–8925	ACCTACCTCCCTACCAAAGC	TGTGCCTTGTGGTAAGAAGTGG	460	57.8	
25	8854–9076	GCGGGCACAGTGATTATAGG	TGGTTGATATTGCTAGGGTGGC	223	60	
26	9001–9335	CGCCTAACCGCTAACATTACTG	GAGGAGCGTTATGGAGTGGAAG	335	58.5	59.5
27	9271–9793	TCTCAGCCCTCCTAATGACCTC	GTTGAGCCGTAGATGCCGTC	523	58	60
28	9742–9988	CAGAGTACTTCGAGTCTCCCTTC	GACCCTCATCAATAGATGGAGAC	247	59.9	
29	10,083–10,407	ATCAACACCCTCCTAGCCTTAC	CCAATTCGGCTCAGTCTAATCC	325	57	60
30	10,279–10,634	CCCTACCATGAGCCCTACAAC	TAAGAGGGAGTGGGTGTTGAGG	356	55.5	
31	10,535–10,734	TCGCTCACACTCATATCCTCC	AGCTTAGGCCATATGTGTTGGAG	200	58.5	61
32	10,690–10,925	GCCTAGCCCTACTAGTCTCAATC	AGGTTGGGGAACAGCTAAATAGG	236	57	
33	10,832–11,315	ATCAACACAACCACCCACAGC	GTTCTTGGGCAGTGAGAGTGAG	484	59	
34	11,187–11,719	TGAACGCAGGCACATACTCC	GCCGTGGGCGATTATGAGAATG	533	58.6	
35	11,654–12,195	ACAGCCATTCTCATCCAAACCC	GGTCGTAAGCCTCTGTTGTCAG	542	58	59
36	12,009–12,462	GCTCACTACCCACCACAT	GGATGCGACAATGGATTTTA	454	57.3	
37	12,358–12,848	ACCACCCTAACCCCTGACTTCC	GCTTGAATGGCTGCTGTGTTG	491	56.8	58
38	12,806–13,311	GATGATACGCCCGAGCAGATG	TGCTAGGTGTGGTTGGTTGATG	506	60.2	
39	13,249–13,785	TCCACTTCAAGTCAACTAGGAC	GGGGATTGTTGTTTGAAGGG	537	58.6	
40	13,708–14,070	GCAGCCGGAAGCCTATTCCG	TGAGGTGATGATGGAGGTGGAG	363	58.4	
41	13,930–14,371	ATCACACACCCGACAATCCC	ATTGGTGTGTTGGGTGAAAAGAG	442	57.3	

60

42	14,261–14,706	TCCTCCGAATCAACCCTGAC	TCATTGGTCGTGGTTGTAGTCC	446	59.2	60.6
43	14,548–14,992	AATAACACACCCGACCACAC	AAGGTAGCGGATGATTCAGC	445	57.2	
44	14,936–15,341	TCATCAATCGCCACATCACTC	ATAGGAGTGGAGTGTGCTAG	406	59	
45	15,256–15,743	AGACAGTCCACCCTCACAC	GGAGGCTGCGGCTAGGAG	488	58.9	
46	15,587–16,185	CTCCGATCCGTCCCTAACAAAC	GGTTTGTATGTTGGATTGGGT	599	56.6	
47	16,098–16,456	ACATTACTGCCAGCCACCATG	CCGGAGCGAGGAGAGTAGC	359	59.4	
48	16344–276	CAGTCAAATCCCTTCTCGTCCC	TCTGTGTGGAAAGTGGCTGTG	502	59.8	61.6

^a A GC-clamp was added to this primer to enable clear peaks.

generated (Table 2). Since the mismatch position of the primers consisted of a 1:1 mixture of two bases (A/C, A/G, C/T; Table 2) mtDNA fragments with 50% heteroplasmy were amplified (Table 2). The affinity of the Optimase[®] DNA polymerase to the ends of PCR fragments prevents the restriction digest close to the ends of the amplicons. Therefore, a phenol-chloroform extraction of the PCR products was performed prior to restriction digest. Additives of the restriction digest reaction were separated from the DNA fragments via S400 columns (Amersham, Buckinghamshire, UK).

2.5. Sequencing

Not incorporated primers and nucleotides of the PCR reaction were removed by treatment with the EXOSAP-IT[®] kit (USB Corporation, Ohio, USA). The PCR product (2 µl) and EXOSAP-IT (0.5 µl) were incubated at 37 °C for 15 min and inactivated at 83 °C for 15 min (total volume 4.5 µl).

The sequencing reaction (10 µl) included: 0.5 µl of PCR product, 5 pmol primer, 3 µl Quick Start Mix (10× sequencing reaction buffer, 10 µl dNTPs, 20 µl of each ddUTP, ddGTP, ddCTP, ddATP dye terminator and 10 µl DNA polymerase; SNP-kit#390280, Beckman Coulter, Fullerton, CA). Cycle sequencing conditions were as follows: denaturation at 98 °C for 40 s, followed by 30 cycles of 96 °C for 20 s, 57 °C for 20 s, 63 °C for 3 min, all with a heating rate of 3 °C/s. Sequencing reactions were analysed on a Beckman Coulter CEQ[™] 8000 system (Fullerton, CA, USA). Exact positions of mutations and amino acid changes were defined with the [mitoAnalyser](#) tool, using the reference mtDNA sequence J01415.1.

2.6. Determination of heteroplasmy of pathogenic mutations

Restriction digests of the respective PCR fragments were carried out with the enzymes DdeI, HaeIII and MspI which cut specific the mutations 3302G, 3243G and 8993G. The degree of heteroplasmy was detected by densitometry of ethidium-bromide stained agarose gels (Uziel et al., 1997; van den Bosch et al., 2004).

Table 2
Primer sequences used to amplify mtDNA fragments with mismatches distant to the restriction site of DdeI, TaqI and AluI as indicated

Fragment	Position	Sequence forward primer	Sequence reverse primer	bp ^a	°C
AluI 3 bp	9271–9658	See Table 1 forward primer number 27	AGACTATGGTGAG↓CTRAGGTGATTG ^b	376	58.0
AluI 4 bp	9271–9658	See Table 1 forward primer number 27	AGACTATGGTGAG↓CTCYGGTGATTG ^c	376	58.0
AluI 5 bp	9271–9658	See Table 1 forward primer number 27	AGACTATGGTGAG↓CTCAMGTGATTG ^d	376	58.0
TaqI 3 bp	8947–9335	CTAGTTATTATCG↓AAAYCCATCAGCC ^c	See Table 1 reverse primer number 26	376	58.0
TaqI 4 bp	8947–9335	CTAGTTATTATCG↓AAAYCATCAGCC ^c	See Table 1 reverse primer number 26	376	58.0
TaqI 5 bp	8947–9335	CTAGTTATTATCG↓AAAGRATCAGCC ^b	See Table 1 reverse primer number 26	376	58.0
DdeI 3 bp	14598–14992	TAGGAGAAGGCTTA↓GAYGAAAACCC ^c	See Table 1 reverse primer number 43	381	57.2
DdeI 4 bp	14598–14992	TAGGAGAAGGCTTA↓GAAMAAAACCC ^d	See Table 1 reverse primer number 43	381	57.2
DdeI 6 bp	14598–14992	TAGGAGAAGGCTTA↓GAAGAYAACCC ^c	See Table 1 reverse primer number 43	381	57.2
DdeI 8 bp	14598–14992	TAGGAGAAGGCTTA↓GAAGAAAACCC ^c	See Table 1 reverse primer number 43	381	57.2
DdeI 9 bp	14598–14992	TAGGAGAAGGCTTA↓GAAGAAAARCC ^b	See Table 1 reverse primer number 43	381	57.2

^a Size after restriction digestion.

^b R = A + G.

^c Y = C + T.

^d M = A + C.

3. Results

3.1. Amplification of the entire mtDNA

PCR reactions were optimized to amplify the entire mtDNA in 48 overlapping fragments with one single cycling protocol (Table 1). The proof-reading Opti-mase[®] DNA polymerase, which is specifically designed for DHPLC analysis, was necessary to produce clear peaks with high amplitude (Fig. 1A,B). Other enzymes tested, like AmpliTaq Gold[®] DNA polymerase and the proofreading *Pfu* and Discoverase[™] polymerases showed a wide spectrum of pre-peaks and shoulders in the DHPLC chromatogram leading to difficulties in detection of low-level heteroplasmies (Fig. 1C–H). In addition, the two latter enzymes need additives, which if not eliminated before DHPLC analysis, lead to early column decay.

Freezing of pre-formulated and pre-aliquoted PCR mixes did not change performance of amplification and DHPLC analysis but saved time and enhanced reproducibility (Fig. 2).

3.2. Sensitivity of DHPLC analysis

PCR amplicons of homoplasmic mtDNA displayed clear cut single homoduplex peaks in the DHPLC chromatogram. Heteroplasmic mutations lead to at least two peaks, the first one representing the heteroduplexes and the second one the homoduplexes (Figs. 3–6). Mixture of different quantities of two homoplasmic mtDNA variants revealed a detection limit of heteroplasmy down to 1%, visible as a shoulder (Fig. 4). In cases of unclear low-level heteroplasmy, the heteroduplex peak was re-amplified. Presence of a mutation resulted in an equal mixture of heteroduplex and homoduplex peaks after DHPLC analysis (Fig. 5A,B).

Sensitivity of DHPLC analysis for mutations close to the end of a DNA fragment is an important issue if restriction digest of larger PCR products is used for mutation detection. By the use of degenerated primers we have introduced mismatches 2–9 bp distant from naturally occurring restriction sites leading to sticky or blunt ends of the DNA fragment (Table 2). All mutations in the fragments containing the AluI restriction site (blunt end) with mismatches in the primers at position three to five bases distant from

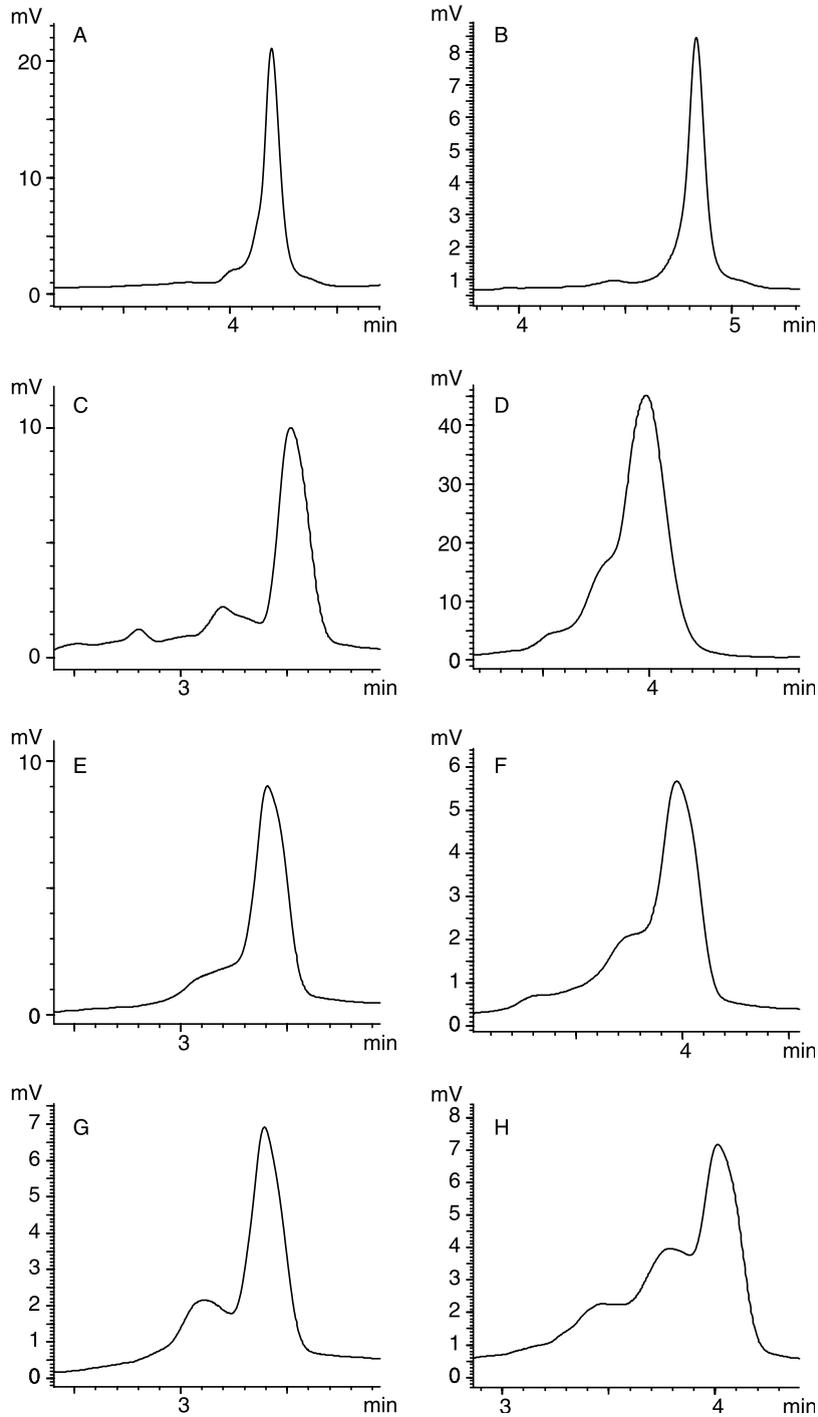


Fig. 1. DHPLC analysis of mtDNA fragment 7 (A,C,E,G) and fragment 8 (B,D,F,H) amplified with the following DNA polymerases: Optimas[®] DNA polymerase (A,B), Discoverase[™] (C,D), *Pfu* DNA polymerase (E,F) and Ampli Taq Gold[®] DNA polymerase (G,H).

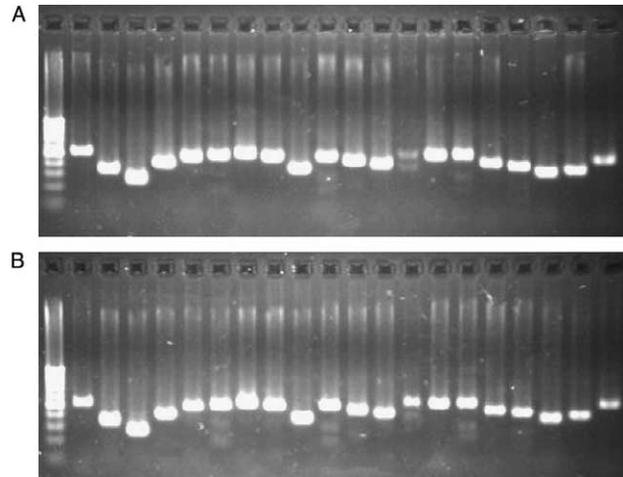


Fig. 2. Comparison of performance of fresh (A) and frozen (B) pre-aliquoted PCR reaction mixes for amplification of the first 20 mtDNA fragments using a single cycling protocol and primers shown in Table 1.

the restriction site were identified correctly (Fig. 3A). The mutations in the TaqI fragment (sticky end) were not detected clearly up until the fifth base pair from the restriction site (Fig. 3B). In the DdeI fragment (sticky end), the mutation was undetectable from base pair 2–8 distant from the restriction site (Fig. 3C).

3.3. Sequence analysis of heteroplasmic mtDNA

Sequence analysis of heteroplasmy around 50% gave clear results (Fig. 5D). As shown in other studies, low-level heteroplasmy was undetectable by direct sequencing (Fig. 5C). To allow sequence analysis of low-levels of polymorphic mtDNA, fractions of the small-sized heteroduplex peak were collected. After re-amplification the heteroplasmy increased to about 50%, now allowing sequence analysis (Fig. 5A,B,D).

3.4. Mutation analysis of patient samples

To test the applicability of the protocol for detection of mtDNA variations by DHPLC established in this study, we analysed several samples from patients with proven pathogenic mtDNA mutations like A3302G, A3243G and T8993G. All tested mutations were detected correctly by our protocol (Fig. 6). A crude estimation of the degree of heteroplasmy ($\pm 10\%$) is possible by visual

examination of the DHPLC chromatograms. In the samples investigated, the degree of heteroplasmy of DHPLC analysis corresponds well with the degree of heteroplasmy determined by restriction digest.

4. Discussion

In this study, a DHPLC-based protocol for the rapid detection of unknown variations of the entire human mtDNA was developed. To ensure clean homospecies peaks it was necessary to use the DHPLC optimized enzyme Optimase[®] DNA polymerase. This enzyme has the advantage that it does not need additives, which if not eliminated, would cause early column abrasion. A recent study also used DHPLC analysis of single PCR fragments to screen for mtDNA alterations (Biggin et al., 2005). *Pfu* DNA polymerase was used for mtDNA amplification, which created substantial pre-peaks in DHPLC analysis in about 20% of the fragments, a finding that is in agreement with our data. In general, pre-peaks in DHPLC analysis might be caused by the use of non-proofreading enzymes, suboptimal conditions for DNA polymerases as well as unwanted tailing of the PCR products. Secondary structures of certain PCR fragments might also lead to a proportion of incorrectly annealed fragments. Even in the course of the present study some fragments initially showed

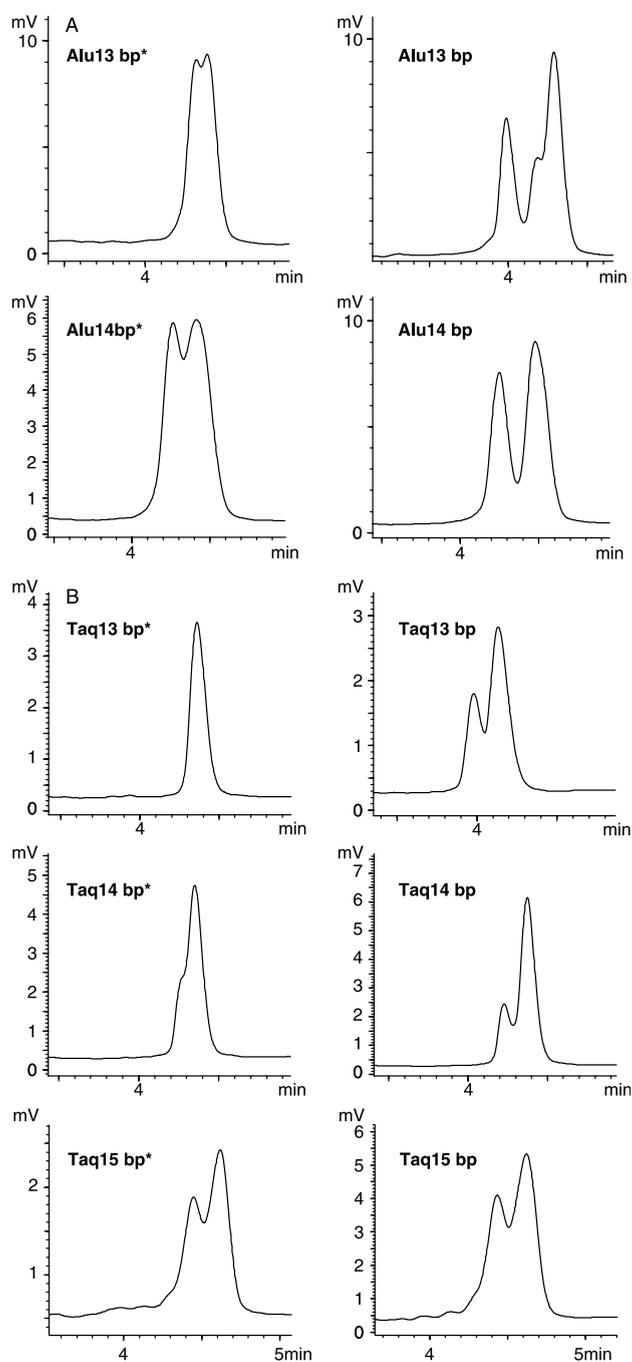


Fig. 3. Sensitivity of DHPLC analysis in detecting artificial heteroplasmy at certain distances from the end of a DNA fragment. Restriction sites and mutations were introduced into a PCR product with primers (Table 2) that contain the corresponding endogenous restriction site and mismatched bases at various distances to the restriction site (A, AluI; B, TaqI; C, DdeI; as indicated in each figure). The distance of the heteroplasmy position to the end of fragment after restriction digest is indicated. DHPLC analysis was carried out with undigested and digested (*) PCR fragments. Since the heteroplasmy of the fragment was more than 10 bp away from the end of the amplicon in the undigested DNA all mutations in the undigested PCR product were detected.

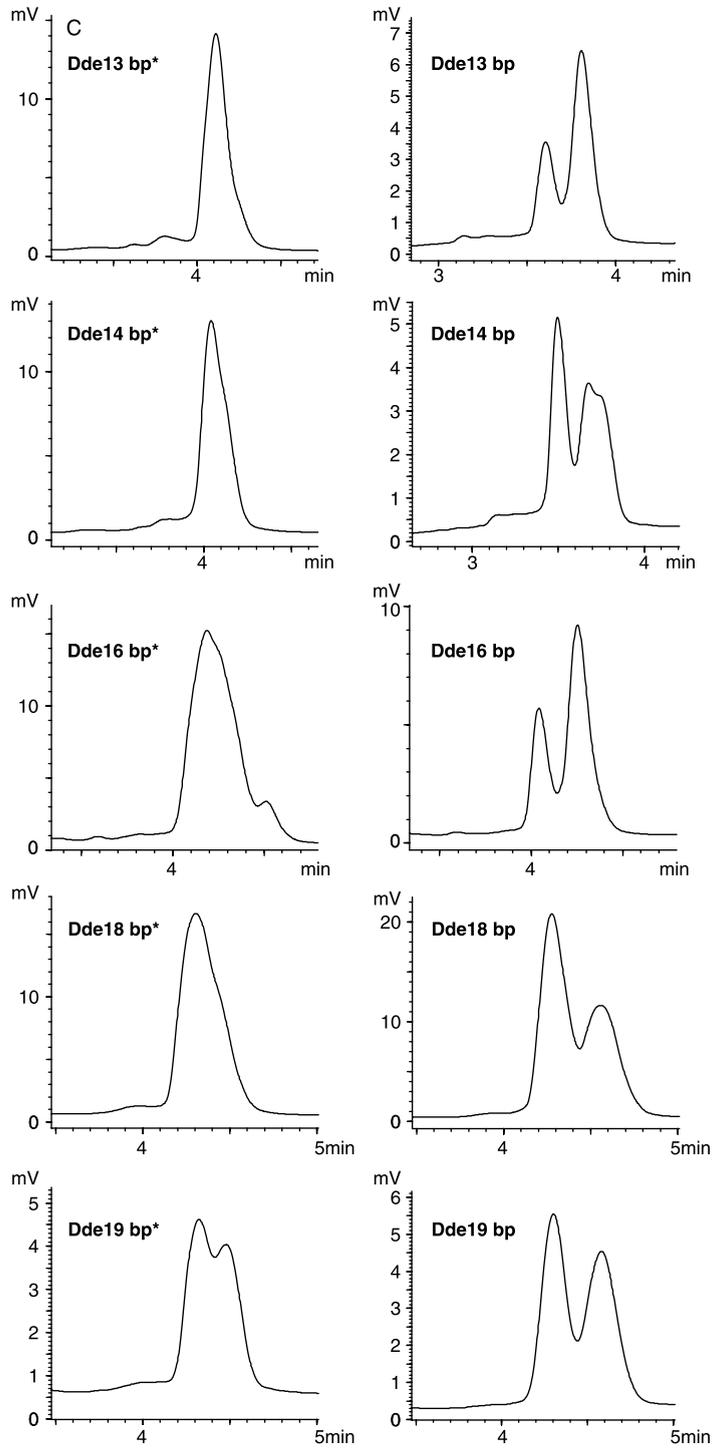


Fig. 3 (continued)

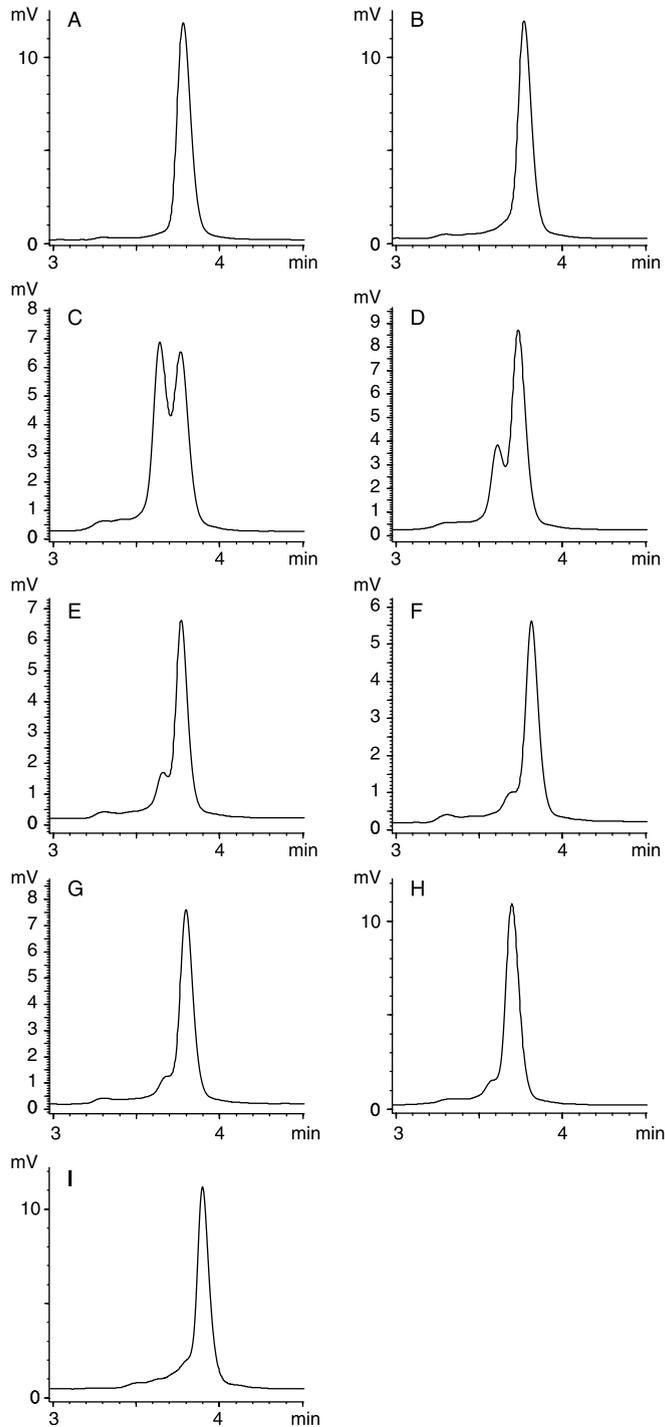


Fig. 4. Sensitivity of detection of heteroplasmy by DHPLC analysis of fragment 9. A, homoplasmic fragment with the A3305G mutation; B, same fragment of an unaffected control. Mixture of homoplasmic A3305G (I) and control (II) fragment: C, 50% I and 50% II; D, 90% I and 10% II; E, 95% I and 5% II; F, 97.5% I and 2.5% II; G, 99% I and 1% II; H, 99.5% I and 0.5% II; I, 99.9% I and 0.1% II.

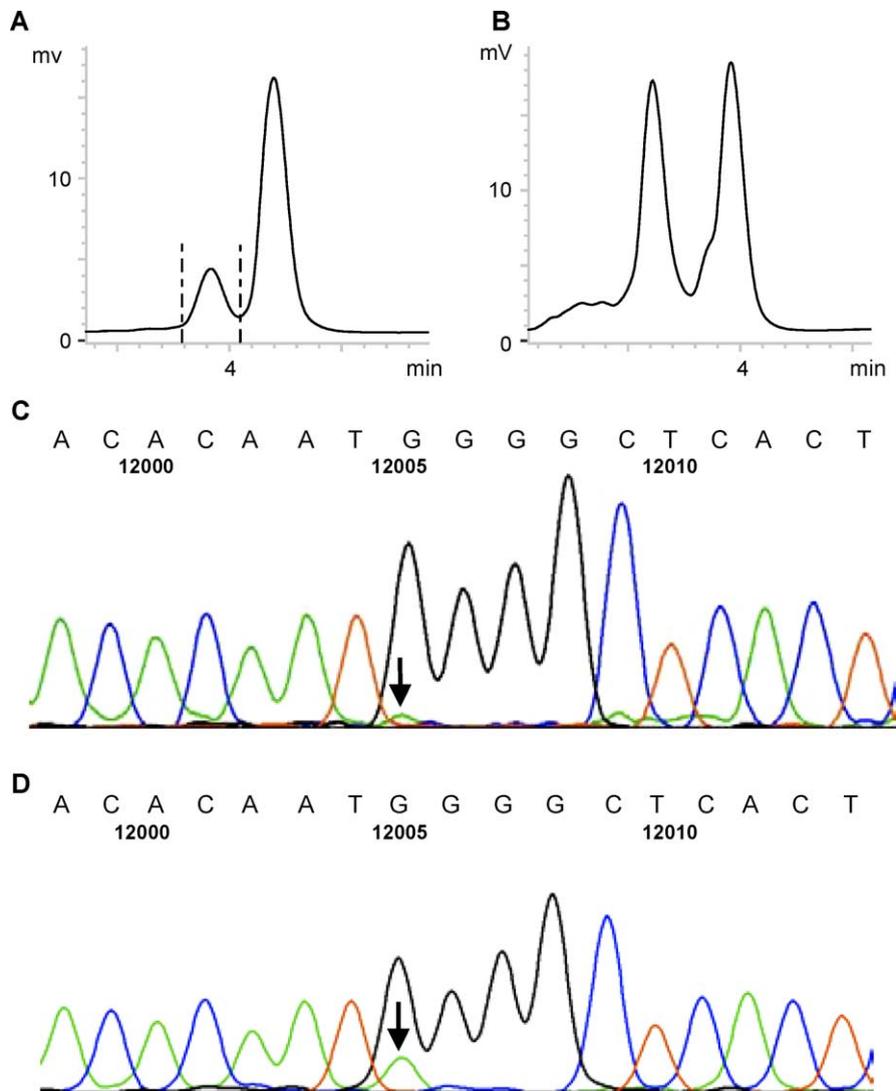


Fig. 5. A, DHPLC analysis of fragment 35 with a somatic heteroplasmy of less than 10%. B, Re-amplification of the collected heteroplasmatic fraction results in one hetero- and one homoduplex peak of the same height. C, Direct sequence analysis of the PCR product gave no indication of a mtDNA variation. D, Sequence analysis of the re-amplified PCR fragment clearly shows the heteroplasmy at position 12005.

heteroduplex formation. Re-positioning of primers solved the problem.

van Den Bosch et al. (2000) analysed the entire mtDNA from 13 PCR fragments followed by restriction digest and multiplex DHPLC analysis at 5 different temperatures for each fragment. Since fragments lose the size-dependent separation at temperatures exceeding 53 °C (Xiao and Oefner, 2001), an interpretation of the DHPLC chromatograph is difficult, because peaks can overlap and low-level

heteroplasmic peaks are lost in other peaks. In addition, polymorphisms in restriction sites further complicate the interpretation of the results.

We analysed artificially introduced mutations two to nine bp distant of a restriction site to determine the detection limit of the DHPLC analysis at the ends of digested fragments. We show for the first time, that detection of mutations close to the end of a PCR fragment is sequence and restriction enzyme specific. Mutations that are located up to 8 bp away from

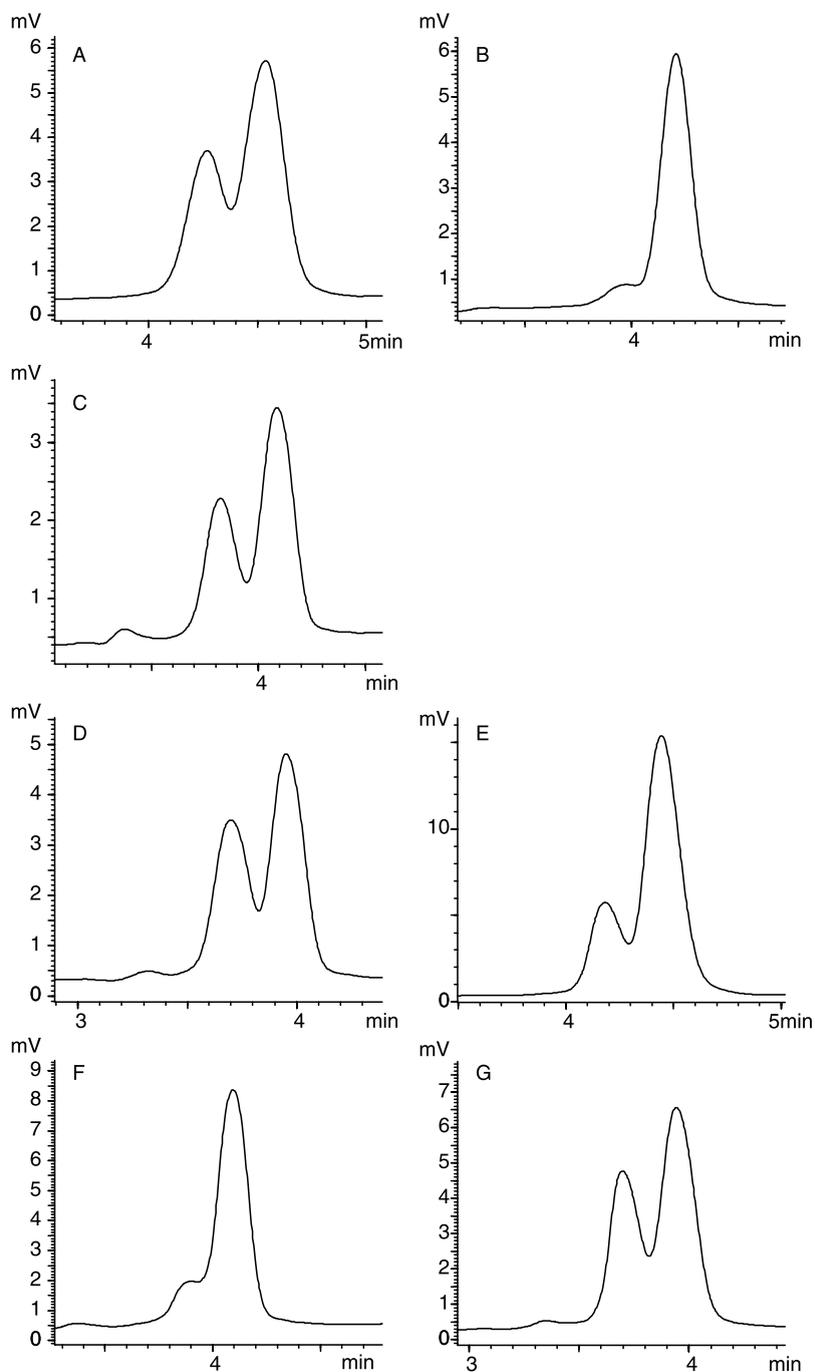


Fig. 6. DHPLC analysis of known mutations in the mtDNA of patients with different levels of heteroplasmy of mutations A3302G (A–C), A3243G (D–H) and T8993G (I–K). DHPLC analysis of A3302G mutation in fragment 9 of A, leukocytes of mother (35%); B, muscle of son (96%); C, leukocytes of son (60%). DHPLC analysis of A3243G mutation in fragment 9 of D, leukocytes of mother (31%); E, fibroblasts of son I (89%); F, muscle of son I (92%); G, leukocytes of son II (46%); H, leukocytes of daughter (31%). DHPLC analysis of T8993G mutation in fragment 25 of I: fibroblasts of patient (>99%), J: unaffected control, K: mixture of I and J. The degree of heteroplasmy, as determined by densitometry of restriction digest of the PCR fragment, is given in brackets.

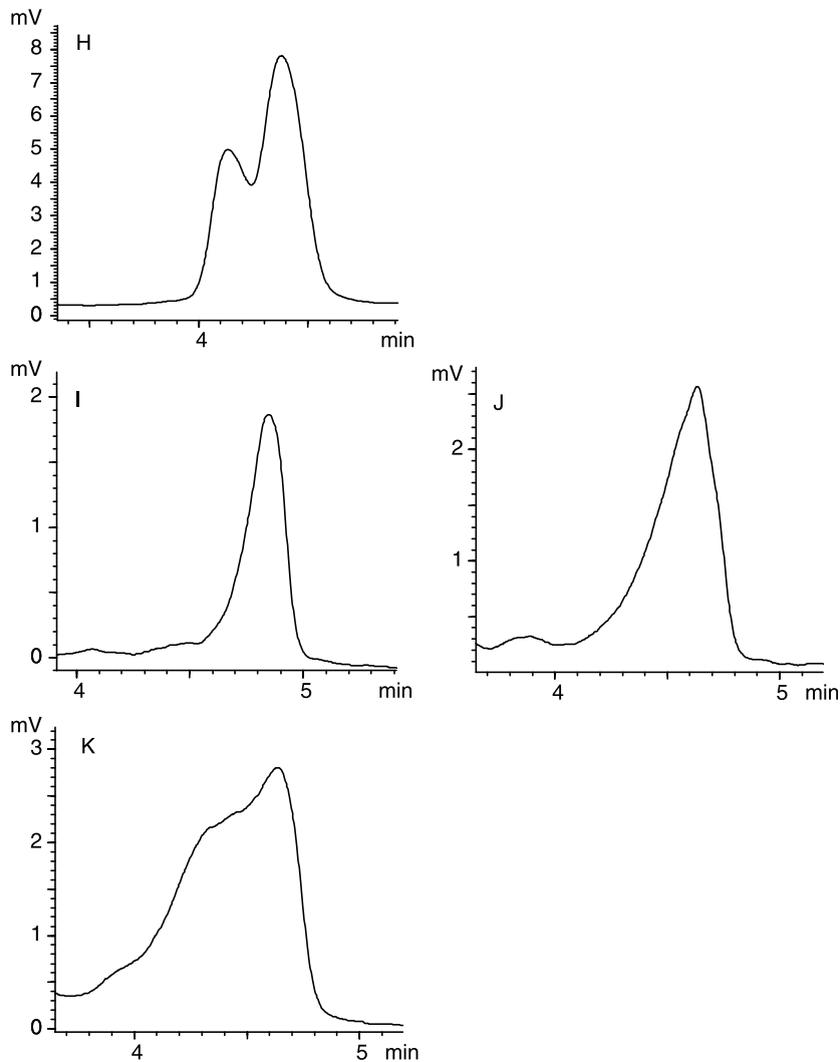


Fig. 6 (continued)

the end of the fragment can be missed. Depending on the fragment size this could account for 3–10% uncertainty of the analysed region.

Jones et al. (2001) sequenced DNA fragments of low-level mosaicism, which showed low heteroduplex peaks in DHPLC analysis. However, he was not able to detect the heteroplasmic mutation by direct sequence analysis. Finally, cloning of the fragments resulted in a few clones displaying the mutation. Here, we avoid time-consuming cloning of heteroduplex fragments

for sequence analysis simply by collection and re-amplification of the heteroduplex peak. The re-amplification of low heteroplasmic peaks for sequence analysis could also be an important tool in diagnosis of mtDNA-related disorders. Since mtDNA mutations can segregate and accumulate in certain tissues, the level of heteroplasmy is frequently low in blood leukocytes compared to affected tissues. In cases of suspected pathological mtDNA mutations investigation of leukocytes, buccal swabs or urine

sediment could be adequate for the detection of a pathological mutation. Another important application of the presented method will be the determination of a pathogenic mutation in the blood of maternal relatives. As a consequence appropriate genetic counselling of families with affected patients could be offered.

Pre-aliquoted PCR master mixes not only save time, but also lead to more reproducible results (Klauegger et al., 1999). Reactions for screening the entire mtDNA of one individual can be prepared within minutes simply by adding the template DNA. The mixes for screening one sample can be supplied in convenient 48 well plates. The possibility of semi-automated analysis of patient samples in 48 or 96 well plates within 24 h and comparable low cost make the proposed DHPLC method an ideal tool for the investigation of mtDNA variations.

In summary, we have developed a rapid protocol for the identification of variations of the entire mtDNA, which will allow high throughput screening for diseases with suspected involvement of mtDNA mutations.

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