

A rapid and accurate approach to identify single nucleotide polymorphisms of mitochondrial DNA using MALDI-TOF mass spectrometry

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Abstract

Background: Single nucleotide polymorphisms (SNPs) of mitochondrial DNA (mtDNA) are involved in physiological and pathological conditions. We developed a rapid, accurate, highly sensitive and high-throughput approach with low cost to identify mtDNA SNPs.

Methods: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to detect 18 SNPs of mtDNA by uniplex and multiplex assays. The sensitivity and specificity of the MALDI-TOF MS were evaluated. The accuracy of the approach was validated by the comparison of using the robust sequencing analysis.

Results: The detection limit achieved with the assays corresponded to the identification of five-genome equivalence of mtDNA per reaction after first round PCR amplification. The testing system enabled the discrimination of as little as 5% of mtDNA polymorphism in the predominating background of mtDNA not containing the SNP. No false positive and false negative results were obtained using the uniplex and multiplex MALDI-TOF MS assays for the analysis of the 18 SNPs compared with those obtained by sequencing analysis.

Conclusions: Possible fields which could benefit from this powerful and sensitive tool include forensic medicine, tracing of matrilineage, transplantation immu-

nology, transfusion medicine, the diagnosis of mtDNA mutation related disorders, and the research regarding aging, apoptosis and carcinogenesis based on physiologic and pathogenic alterations of mtDNA for the analysis of large-scale samples, multiple SNPs or rare mtDNA.

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Introduction

Mitochondrial DNA (mtDNA) is a circular cytoplasmic double stranded DNA of 16.6 kb, including the coding regions and the hypervariable non-coding D-loop region (1, 2). The features of mtDNA, such as matrilineal inheritance, high copy numbers per cell and lack of recombination make it a powerful tool in life science.

Physiologically, mtDNA typing has been applied routinely in forensic medicine (3). Maternal inheritance of mtDNA enables the tracing of matrilineage far back in time (4). Single nucleotide polymorphisms (SNPs) can also be used as markers in transfusion and transplantation medicine (5).

Pathologically, the properties of mtDNA, such as reactive oxygen species enhanced aggression, high sensitivity to damage, deficient repair of damage and high rate of mutations suggest their potential importance in mtDNA related disorders, aging, apoptosis and carcinogenesis (6–8). Somatic mutations in mtDNA have been identified in various human cancers and in a large spectrum of clinically important disorders (9, 10).

Based on the importance of mtDNA SNPs in life science, a rapid, accurate, highly sensitive and high-throughput approach with low cost to identify physiologic and pathogenic mtDNA variants is needed for the analysis of large-scale samples, multiple SNPs or rare mtDNA.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) proved to be a superior, high-throughput technology for the robust detection of single nucleotide variations with low cost on nuclear DNA (nDNA). It could permit the reliable detection of SNPs (11) from a very rare target, including fetal point mutations from cell-free fetal

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DNA in maternal plasma and donor derived cell-free DNA in organ transplant recipients urine, which cannot be reliably detected by more conventional PCR-based approaches (12, 13).

In this study, we developed a MALDI-TOF MS-based method to detect 18 SNPs in mtDNA on mtDNA hypervariable regions in platelet-apheresis products by uniplex and multiplex assays. This novel application enables the rapid, sensitive and accurate identification of single or multiple mtDNA SNP variants in a single reaction on mtDNA hypervariable regions.

Materials and methods

DNA extraction

Platelet-apheresis products containing mtDNA from three blood donors with informative mtDNA SNPs were provided by the Institute for Clinical Transfusion Medicine, Staedtisches Klinikum Braunschweig gGmbH, Germany. This study was approved by the Local Ethics Committee "Ethikkommission beider Basel". Written informed consent was obtained from all three individuals. A total of 18 mtDNA SNPs on three hypervariable regions (HVR1, HVR2 and HVR3) within the displacement loop (D-loop) region of the mtDNA were identified using a traditional sequencing approach by the Institute (5).

For our study, mtDNA was isolated from 800 μ L of platelet-apheresis products using the MagNA LC Isolation Kit-Large Volume protocol with the MagNA Pure LC Instrument (Roche Applied Science, Basel, Switzerland) according to the manufacturer's introduction. The DNA preparations were eluted in 200 μ L elution buffer according to this protocol.

Quantification of mtDNA and contamination of nDNA in the samples

The amounts of nDNA and mtDNA were quantified by multiplex TaqMan real-time PCR for both glyceraldehyde-3-phosphodehydrogenase (GAPDH) gene and mtDNA sequence.

The GAPDH and mtDNA primer and probe sequences are shown as follows: GAPDH (14): forward, 5'-CCCCACACACATGCACTTACG-3', reverse, 5'-CCTAGTCCCAGGGCTTTGATT-3', probe, 5'-(MGB) GTGAACGTGGATGAAGTTGG (VIC)-3'; mtDNA (15): forward, 5'-AATATTAAACACAACTACCACC-TACC-3', reverse, 5'-TGGTTCTCAGGGTTTGTATA-3', probe, 5'-(MGB) CCTCACCAAAGCCATA (FAM)-3'.

The PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, ABI, Rotkreuz, Switzerland). DNA (5 μ L) was used as template for the PCR analysis. The TaqMan assays were carried out in 25 μ L of total reaction volume using 2 min incubation at 50°C, followed by an initial denaturation step at 95°C for 10 min, and 40 cycles of 15 s at 95°C and 1 min at 60°C.

To determine the quantification of mtDNA and nDNA present in platelet-apheresis product samples, standard dilution curves using HPLC-purified single-stranded synthetic DNA oligonucleotides (Microsynth, Balgach, Switzerland) specifying a 79-bp mtDNA amplicon and a 97-GAPDH amplicon with concentration ranging from 5×10^7 copies to 5×10^2 copies were used. Absolute concentrations of mtDNA and GAPDH were expressed as genome equivalence (GE/mL) of platelet-apheresis products.

Detection of mtDNA polymorphisms on hypervariable regions using MALDI-TOF MS

The PCR and extension primers used to analyze the mtDNA SNPs were designed using MassArray Assay Design v.3.1 (Sequenom, San Diego, CA, USA) and are listed in Table 1.

The uniplex and multiplex PCR reactions were carried out in 50 μ L PCR cocktail mixes containing 5 μ L DNA, 1.626 mM MgCl₂, 500 μ M dNTP mix, 0.5 U Hot-star Taq Gold polymerase and primer pairs. The amplification was performed under the following conditions: incubation at 94°C for 15 min, followed by 50 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 1 min and final extension at 72°C for 5 min.

To remove the non-incorporated dNTPs, a shrimp alkaline phosphatase treatment was performed after the PCR reaction under the following conditions: 37°C for 20 min, 85°C for 5 min and cooling to 4°C.

The iPLEX reaction was performed using the iPLEX cocktail mix (Sequenom, San Diego, CA, USA), which contains buffer, termination mix, enzyme and extension primers, under the following conditions: 94°C for 30 s, followed by 40 cycles of 94°C for 5 s, 52°C for 5 s, 80°C for 5 s, final extension at 72°C for 5 min, then cooling to 4°C.

The iPLEX reaction products were desalted using clean resin to optimize mass spectrometric analysis and dispensed onto a 384-format SpectroCHIP bioarray using a nanodispenser for MALDI-TOF MS analysis. MassARRAY Workstation version 3.4 software (Sequenom) was used to process and analyze iPLEX SpectroCHIP bioarrays. The call rate, extension rate and peak area for all allele-specific analyses in any given assay were calculated using the software provided by the manufacturer. Positive and negative control samples were run at each step and on each chip.

The specificity of the assay for mtDNA was assessed through the use of a mitochondria negative cell line, which was obtained by subculturing that cell line in the presence of low concentration of ethidium bromide until the cells were devoid of any mitochondria. The sensitivity of the assay for detecting mtDNA polymorphisms was assessed through the five-fold dilution of mtDNA samples containing informative polymorphisms with concentrations as low as 1 GE/ μ L, as well as through mixing known mtDNA SNPs into a predominant background of mtDNA molecules not containing those polymorphisms.

Results

Co-extraction of mtDNA and nDNA from platelet-apheresis

mtDNA and nDNA were co-extracted from the platelet-apheresis products (Table 2). The GAPDH level, representing cell-free total nDNA, ranged from 1073 to 4039 GE/mL of platelet-apheresis concentrate in the platelet-apheresis samples, and the mtDNA level ranged from 869,667 to 3,492,084 GE/mL of platelet-apheresis products. The levels of mtDNA were found to be at least 774- up to 2713-fold higher than nDNA levels.

Specificity of MALDI-TOF MS for detecting mtDNA polymorphisms

The specificity of the MALDI-TOF MS for detecting the 18 mtDNA polymorphisms was assessed through the use of a mitochondria negative cell line (143b rho0).

Table 1 SNPs in the study cases and sequences of primers.

	Sequencing	PCR primers
1	263 A-G	Forward primer: ACGTTGGATGTTAAGTGCTGTGGCCAGAAG Reverse primer: ACGTTGGATGATAACAATTGAATGTCTGC Extension primer: TGAATGTCTGCACAGCC
	309 C-CC	Reverse primer: ACGTTGGATGCCACTTCCACACAGACATC Forward primer: ACGTTGGATGTTAAGTGCTGTGGCCAGAAG Extension primer: TTTCCACCAAACCCCC
	315 C-CC	Forward primer: ACGTTGGATGGCTGGTGTAGGGTTCTTTG Reverse primer: ACGTTGGATGAAATTTCCACCAAACCCCC Extension primer: CAAACCCCCCTCC
2	16189 T-C	Forward primer: ACGTTGGATGGGGTTGATTGCTGTACTTGC Reverse primer: ACGTTGGATGCTTGACCACCTGTAGTACAT Extension primer: CAATCCACATCAAACCC
	152 T-C	Forward primer: ACGTTGGATGGTTCGCCTGTAATATTGAACG Reverse primer: ACGTTGGATGCCTATGTCGCAGTATCTGTC Extension primer: TGATTCCTGCCTCATCC
	263 A-G	Forward primer: ACGTTGGATGTTAAGTGCTGTGGCCAGAAG Reverse primer: ACGTTGGATGATAACAATTGAATGTCTGC Extension primer: TGAATGTCTGCACAGCC
3	16069 C-T	Forward primer: ACGTTGGATGGTGGCTGGCAGTAATGTACG Reverse primer: ACGTTGGATGGAAGCAGATTTGGGTACCAC Extension primer: CCACCAAGTATTGACT
	16324 T-C	Forward primer: ACGTTGGATGTCATCCATGGGGACGAGAAG Reverse primer: ACGTTGGATGAACCTACCCACCCTTAACAG Extension primer: ATTTACCGTACATAGCACAT
	16366 C-T	Forward primer: ACGTTGGATGGCGGGATATTGATTTACGG Reverse primer: ACGTTGGATGGCACATTACAGTCAAATCCC Extension primer: AAATCCCTTCTCGTCCC
	16390 G-A	Forward primer: ACGTTGGATGAGTCAAATCCCTTCTCGTCC Reverse primer: ACGTTGGATGGCGGGATATTGATTTACGG Extension primer: GATGGTGGTCAAGGGAC
	73 A-G	Forward primer: ACGTTGGATGTATTAACCACTCACGGGAGC Reverse primer: ACGTTGGATGAGCGTCTCGCAATGCTATCG Extension primer: TCGCAATGCTATCGCGTGCA
	185 G-A	Forward primer: ACGTTGGATGGTGCAGACATTCAATTGTTA Reverse primer: ACGTTGGATGTATCGCACCTACGTTCAATA Extension primer: CACCTACGTTCAATATTACAG
	188 A-G	Forward primer: ACGTTGGATGCGCACCTACGTTCAATATTA Reverse primer: ACGTTGGATGGCTGTGCAGACATTCAATTGT Extension primer: TTAACACACTTTAGTAAGTATGT
	228 G-A	Forward primer: ACGTTGGATGAAGTGGCTGTGCAGACATTC Reverse primer: ACGTTGGATGGGCGAACATACTTACTAAAG Extension primer: TGTGTTAATTAATTAATGCTGTAG
	263 A-G	Forward primer: ACGTTGGATGTTAAGTGCTGTGGCCAGAAG Reverse primer: ACGTTGGATGATAACAATTGAATGTCTGC Extension primer: TGAATGTCTGCACAGCC
	295 C-T	Forward primer: ACGTTGGATGTTAAGTGCTGTGGCCAGAAG Reverse primer: ACGTTGGATGCCACTTCCACACAGACATC Extension primer: ACAGACATCATAACAAAAATTT
	315 C-CC	Forward primer: ACGTTGGATGGCTGGTGTAGGGTTCTTTG Reverse primer: ACGTTGGATGAAATTTCCACCAAACCCCC Extension primer: CAAACCCCCCTCC
	462 C-T	Forward primer: ACGTTGGATGTGTGTGTGCTGGGTAGGATG Reverse primer: ACGTTGGATGTTAACAGTACCCCCCAAC Extension primer: AACACATTATTTCCCTC
	489 T-C	Forward primer: ACGTTGGATGTTAGCAGCGGTGTGTGTGTG Reverse primer: ACGTTGGATGTTATTTCCCTCCCCTCC Extension primer: CCCATACTACTAATCTCATCAA
	522 C-del	Forward primer: ACGTTGGATGTCTCATCAATACAACCCCG Reverse primer: ACGTTGGATGTTGGTTGGTTGGTTGGTTGATG Extension primer: TATGGGGTTAGCAGCGGT
	523 A-del	Forward primer: ACGTTGGATGTTGGTTGGTTGGGGTATG Reverse primer: ACGTTGGATGTCTCATCAATACAACCCCG Extension primer: CTACCCAGCACACAC

Table 2 Quantities of mtDNA and nDNA in the samples of platelet-apheresis products.

Case	mtDNA quantities, GE/mL	GAPDH, GE/mL	Ratio mtDNA/nDNA
1	2,911,332	1073	2713
2	869,667	1123	774
3	3,492,084	4039	865

No positive amplifications were obtained from the cell line using the 18 primer pairs listed in Table 1.

Sensitivity of MALDI-TOF MS for detecting mtDNA polymorphisms

To determine the sensitivity of MALDI-TOF MS for analyzing mtDNA SNPs, we designed experiments by diluting known amounts of mtDNA five-fold with informative polymorphisms in a series from 5×10^4 GE to 5 GE (1 GE/ μ L) per PCR reaction. Analysis for informative polymorphisms was performed using MALDI-TOF MS. The assay enabled the reliable identification of the polymorphisms using as little as 5 GE per PCR reactions. In our other study, where we used MALDI-TOF MS to identify point mutations on single cell level, close to 70% of the successful analysis was achieved (data not shown).

A dilution series (2.5×10^4 , 5×10^4 , 10×10^4 , 25×10^4 , 50×10^4 GE) of mtDNA with an informative polymorphism was mixed with mtDNA molecules (1×10^6 GE) not carrying the same polymorphism, to give a ratio ranging from 2.5%, 5%, 10%, 25% and to 50% of mtDNA molecules with the polymorphism to those without the polymorphism. MALDI-TOF MS could discriminate down to the level of 5% of polymorphism-containing mtDNA in a 95% background of mtDNA lacking the polymorphism (Figure 1). We compared the sensitivity using MALDI-TOF with those using sequencing analysis (Figure 2).

Detection of mtDNA polymorphisms in three individuals using uniplex and multiplex assays

From three individuals, 18 mtDNA polymorphisms were identified by sequencing analysis. The positions and nature of the polymorphisms in the three individuals are listed in Table 1.

A total of 18 uniplex assays for each polymorphism and 3–12 multiplexed assays using MALDI-TOF MS were developed. The results were compared with those obtained by sequencing analysis. No false positive and false negative results were obtained using the MALDI-TOF MS assays. The uniplex assays and the multiplex assays showed good concordance regarding the determination of the polymorphisms.

Discussion

Rapid and accurate identification of physiologic and pathogenic mtDNA variants is an important issue in life science. In this study, we evaluated the sensitivity

and specificity of MALDI-TOF MS for the detection of SNPs mtDNA on hypervariable regions.

To evaluate the specificity of MALDI-TOF MS for detection of mtDNA SNPs, the assays for 18 mtDNA SNPs in this study were tested by using a mitochondria negative cell line. No mtDNA was detectable from the cell line by both MALDI-TOF MS and sequencing. In our study, we co-extracted mtDNA with nDNA using commercial kits. The contaminated co-extraction of nDNA did not alter our results regarding the detection of mtDNA SNPs.

The detection limit achieved with the assay corresponded to the identification of 5 GE of mtDNA per reaction after first round PCR amplification. As a human eukaryotic cell contains hundreds or thousands of mtDNA (16), the sensitive MALDI-TOF MS established in our study could enable the analysis of mtDNA SNPs from less than one cell.

In our study, MALDI-TOF MS enabled the discrimination of as little as 5% of the mtDNA polymorphism in the predominating background of mtDNA not containing the SNP. Several studies using MALDI-TOF MS for genetic analysis support our results regarding the sensitivity of this assay for mtDNA detection. In our group, Li et al. could detect very rare fetal genetic materials in maternal blood (12). The concentration of fetal DNA in maternal circulation is considerably low, comprising 3.7% of total DNA as quantified by real-time quantitative analysis (17). A previous study from our laboratory also showed that donor-derived genetic materials could be sensitively detected in the urine of kidney transplant recipients using MALDI-TOF MS (13).

In our group, Li et al. compared the MALDI-TOF MS assay with a well-established TaqMan real-time PCR assay for the detection of rare fetal genetic material in the maternal circulation. The MALDI-TOF MS assay and the TaqMan real-time PCR assay had similar detection rates in terms of non-invasive prenatal identification of fetal gender (18). However, identification of multiple SNPs by individual allele-specific TaqMan real-time PCR is a time-consuming and laborious process. Furthermore, SNPs, which only differ minimally from the wild-type DNA of interest, make the design of allele-specific PCR especially challenging (19).

The iPLEX Assay developed by Sequenom, Inc. enables up to 36–40 multiplex PCR and primer extension reactions per assay, so that 36–40 mutations can be detected simultaneously in a single reaction. The multiplexed genotyping assays rely on the natural molecular weight differences of DNA bases. In our study, we analyzed the 18 SNPs using uniplex and multiplex assays in parallel. The precision and accuracy of the mass determination by multiplex iPLEX assay at 3–12 levels were comparable with those by uniplex assay. There were no false positive or false negative signals from the MALDI-TOF MS method, as compared to the results obtained by sequencing. The method demonstrated a high concordance of results and 100% accuracy when compared with capillary sequencing, suggesting that

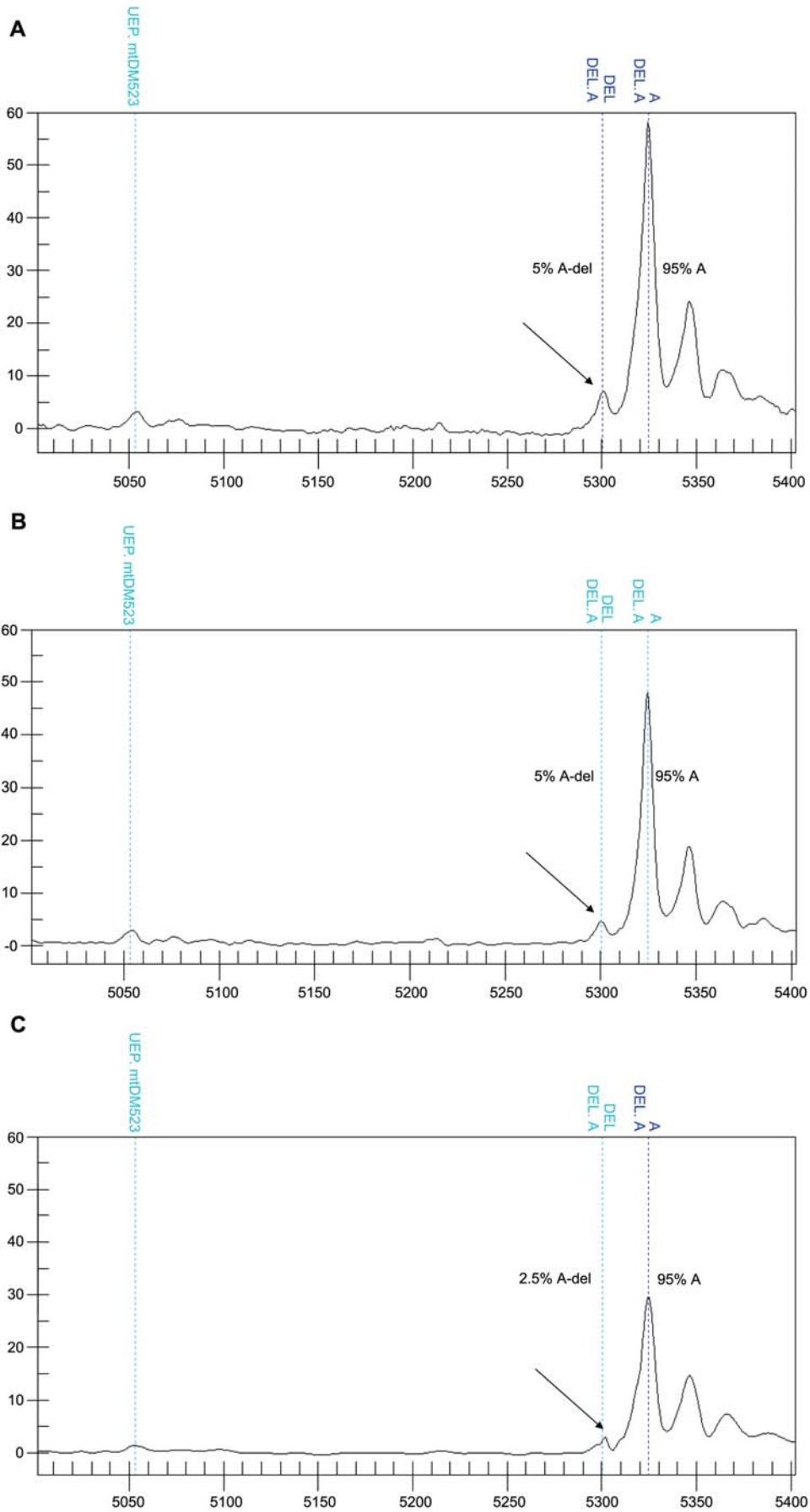


Figure 1 MALDI-TOF MS discriminates rare SNP-containing mtDNA in the background of mtDNA lacking the polymorphism. mtDNA sample containing the SNP of an “A-deletion” at the position of 523 was mixed into the mtDNA sample without an “A-deletion” at the same position. Panels (A) and (B) show the reproducible detections of as little as 5% of the “A-deletion”-containing mtDNA in the 95% background molecules by MALDI-TOF MS. Panel (C) shows a mini-peak on MALDI-TOF MS for the detection of as little as 2.5% of the “A-deletion”-containing mtDNA in the 97.5% background molecules.

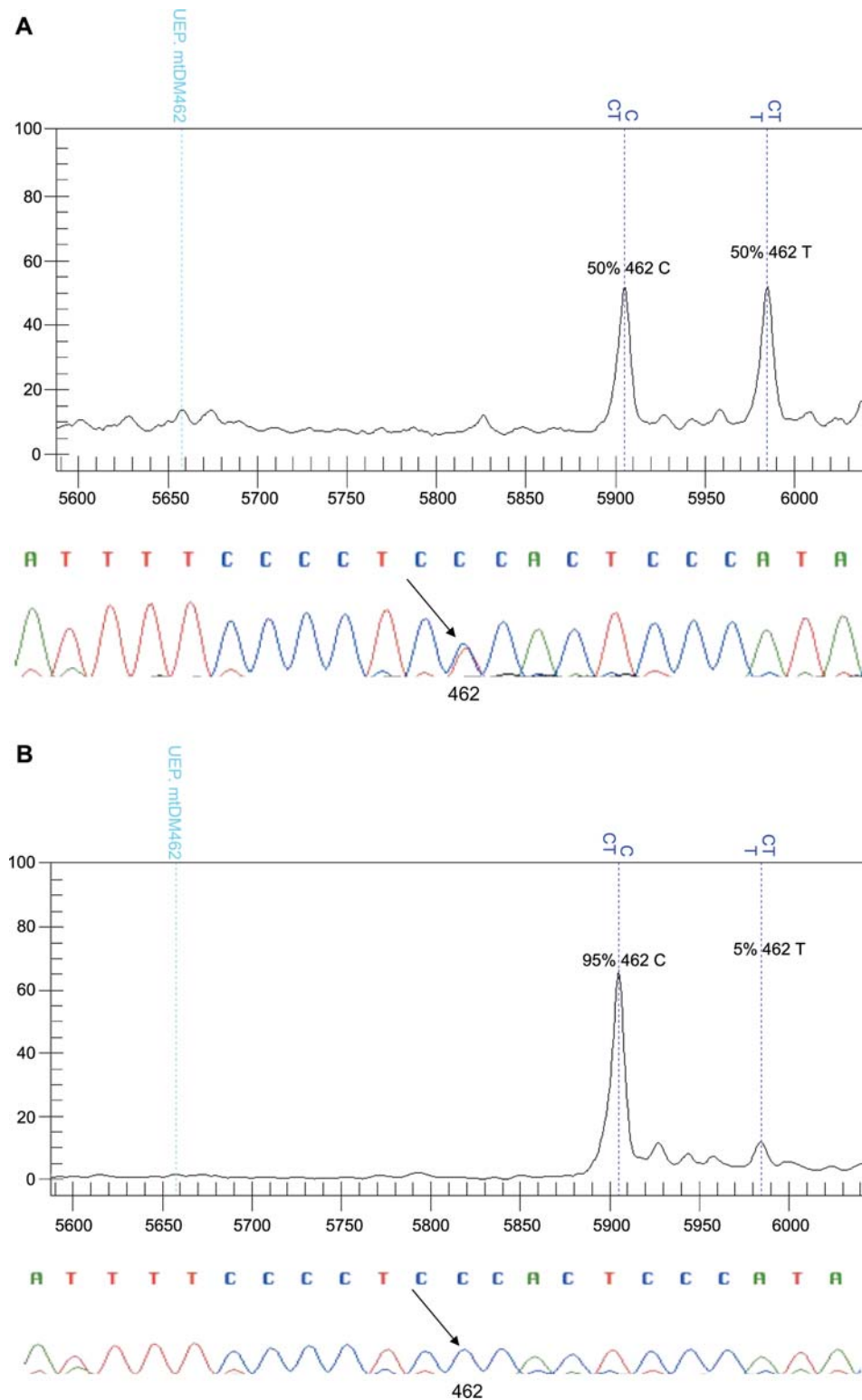


Figure 2 Comparison of MALDI-TOF and sequencing analysis for the sensitivity test. mtDNA sample containing the SNP of "T" at the position of 462 was mixed into the mtDNA sample containing a "C" at the same position. Panel (A) shows the results by MALDI-TOF MS and sequencing analysis for the mixture of 50%–50%. Panel (B) shows the results by MALDI-TOF MS and sequencing analysis for the mixture of 5%–95%.

mass spectrometric analysis of SNPs is a robust and reproducible technique for the detection of mtDNA mutations. Multiplex assays enable the rapid detection of polymorphisms and reduce the amount of DNA input required and the costs for multiple genotyping. The possibility of analyzing up to 384 samples

on a single chip enables high-throughput detections in large-scale studies.

Possible fields which could benefit from this powerful and sensitive tool include forensic medicine and transplantation immunology, particularly when the target mtDNA input is limited (20). This approach can

also be used for the tracing of matrilineage with large-scale multiple genotyping. Further potential applications may be possible in the field of transfusion medicine, in the diagnosis of mtDNA mutation related disorders, and in research regarding aging, apoptosis and carcinogenesis based on physiologic and pathogenic alterations of mtDNA.

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References

- Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. *Nature* 1981;290:457–65.
- Fernandez-Silva P, Enriquez JA, Montoya J. [Replication and transcription of mammalian mitochondrial DNA](#). *Exp Physiol* 2003;88:41–56.
- Andreasson H, Nilsson M, Budowle B, Lundberg H, Allen M. Nuclear and mitochondrial DNA quantification of various forensic materials. *Forensic Sci Int* 2006;164:56–64.
- Alonso A, Albarran C, Martin P, Garcia P, Capilla J, Garcia O, et al. Usefulness of microchip electrophoresis for the analysis of mitochondrial DNA in forensic and ancient DNA studies. *Electrophoresis* 2006;27:5101–9.
- Garritsen HS, Hellenkamp F, Hoerning A, Mittmann K, Sibrowski W. Identifying allogeneic platelets by resolution of point mutations in mitochondrial DNA using single-stranded conformational polymorphism PCR. *Transfusion* 2001;41:1531–8.
- Augenlicht LH, Heerdt BG. [Mitochondria: integrators in tumorigenesis?](#) *Nat Genet* 2001;28:104–5.
- Bartnik E, Lorenc A, Mroczek K. Human mitochondria in health, disease, ageing and cancer. *J Appl Genet* 2001;42:65–71.
- Bianchi NO, Bianchi MS, Richard SM. Mitochondrial genome instability in human cancers. *Mutat Res* 2001;488:9–23.
- Zhu W, Qin W, Bradley P, Wessel A, Puckett CL, Sauter ER. Mitochondrial DNA mutations in breast cancer tissue and in matched nipple aspirate fluid. *Carcinogenesis* 2005;26:145–52.
- Filosto M, Tomelleri G, Tonin P, Scarpelli M, Vattemi G, Rizzuto N, et al. Neuropathology of mitochondrial diseases. *Biosci Rep* 2007;27:23–30.
- Huang DJ, Nelson MR, Zimmermann B, Dudarewicz L, Wenzel F, Spiegel R, et al. Reliable detection of trisomy 21 using MALDI-TOF mass spectrometry. *Genet Med* 2006;8:728–34.
- Li Y, Wenzel F, Holzgreve W, Hahn S. [Genotyping fetal paternally inherited SNPs by MALDI-TOF MS using cell-free fetal DNA in maternal plasma: influence of size fractionation](#). *Electrophoresis* 2006;27:3889–96.
- Li Y, Hahn D, Wenzel F, Holzgreve W, Hahn S. Detection of SNPs in the plasma of pregnant women and in the urine of kidney transplant recipients by mass spectrometry. *Ann NY Acad Sci* 2006;1075:144–7.
- Zhong XY, Ladewig A, Schmid S, Wight E, Hahn S, Holzgreve W. Elevated level of cell-free plasma DNA is associated with breast cancer. *Arch Gynecol Obstet* 2007;276:327–31.
- Walker JA, Hedges DJ, Perodeau BP, Landry KE, Stoilova N, Laborde ME, et al. Multiplex polymerase chain reaction for simultaneous quantitation of human nuclear, mitochondrial, and male Y-chromosome DNA: application in human identification. *Anal Biochem* 2005;337:89–97.
- Robin ED, Wong R. Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. *J Cell Physiol* 1988;136:507–13.
- Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998;62:768–75.
- Li Y, Holzgreve W, Kiefer V, Hahn S. [MALDI-TOF mass spectrometry compared with real-time PCR for detection of fetal cell-free DNA in maternal plasma](#). *Clin Chem* 2006;52:2311–2.
- Ding C, Lo YM. MALDI-TOF mass spectrometry for quantitative, specific, and sensitive analysis of DNA and RNA. *Ann NY Acad Sci* 2006;1075:282–7.
- Alonso A, Martin P, Albarran C, Garcia P, Garcia O, de Simon LF, et al. Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. *Forensic Sci Int* 2004;28:139:141–9.