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Mitochondrial single nucleotide polymorphism genotyping by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using cleavable biotinylated dideoxynucleotides

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ABSTRACT

Characterization of mitochondrial DNA (mtDNA) single nucleotide polymorphisms (SNPs) and mutations is crucial for disease diagnosis, which requires accurate and sensitive detection methods and quantification due to mitochondrial heteroplasmy. We report here the characterization of mutations for myoclonic epilepsy with ragged red fibers syndrome using chemically cleavable biotinylated dideoxynucleotides and a mass spectrometry (MS)-based solid phase capture (SPC) single base extension (SBE) assay. The method effectively eliminates unextended primers and primer dimers, and the presence of cleavable linkers between the base and biotin allows efficient desalting and release of the DNA products from solid phase for MS analysis. This approach is capable of high multiplexing, and the use of different length linkers for each of the purines and each of the pyrimidines permits better discrimination of the four bases by MS. Both homoplasmic and heteroplasmic genotypes were accurately determined on different mtDNA samples. The specificity of the method for mtDNA detection was validated by using mitochondrial DNA-negative cells. The sensitivity of the approach permitted detection of less than 5% mtDNA heteroplasmy levels. This indicates that the SPC–SBE approach based on chemically cleavable biotinylated dideoxynucleotides and MS enables rapid, accurate, and sensitive genotyping of mtDNA and has broad applications for genetic analysis.

The human mitochondrial genome is a double-stranded, circular DNA molecule of 16,569 bp that encodes 13 structural proteins, 2 ribosomal RNAs (rRNAs),[1] and 22 transfer RNAs (tRNAs) [1]. The mitochondrial DNA (mtDNA) is maternally inherited, has a high copy number per cell, and appears as a mosaic of mutant and wild-type genomes (heteroplasmy) [2]. More than 200 pathogenic point mutations in mitochondrial tRNA and rRNA genes as well as protein coding regions have been reported, and the hypervariable region of mtDNA is also highly polymorphic [3,4]. These features demand precise interpretation of mtDNA mutation data for disease treatment, population association studies, and forensic investigations [5–9].

Several technologies have been developed for mtDNA genotyping, including the MitoChip [10], PCR–RFLP (polymerase chain reaction followed by restriction fragment length polymorphism) analysis [11], TaqMan real-time genotyping assay [12], and direct Sanger sequencing [10,13], yet each has some drawbacks [14,15]. Most are time-consuming and fail to detect low heteroplasmy levels (<10%), PCR–RFLP and TaqMan assays are labor-intensive when detecting multiplex single nucleotide polymorphisms (SNPs), and false positives often exist due to the limitations of the detection mechanisms. Next-generation sequencing [14–16] is still too costly, inefficient, and error prone for routine targeted SNP genotyping and low-level heteroplasmy detection. Thus, a more rapid, accurate, sensitive, and cost-effective method for SNP genotyping of mtDNA is needed.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) allows rapid and accurate sample

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[1] *Abbreviations used:* rRNA, ribosomal RNA; tRNA, transfer RNA; mtDNA, mitochondrial DNA; PCR–RFLP, polymerase chain reaction followed by restriction fragment length polymorphism; SNP, single nucleotide polymorphism; MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SBE, single base extension; SPC, solid phase capture; TCEP, tris(2-carboxyethyl)phosphine; MERRF, myoclonic epilepsy with ragged red fibers; WT, wild type; MT, mutant type; PT, point mutation; MSBE, multiplex single base extension; B/W, binding and washing; EB, ethidium bromide.

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measurement and provides high resolution and sensitivity. It has been used for SNP genotyping coupled with hybridization [17], strand invasion-dependent cleavage of oligonucleotides [18], and single base extension (SBE) [19–23]; the latter has emerged as a very powerful method for multiplex SNP detection. In single base extension, SBE primers designed to anneal adjacent to targeted SNP sites are extended by dideoxynucleotides that are complementary to the nucleotides at the polymorphic positions. The nucleotides can be identified by measuring the molecular weight of primer extension products. It is critical to stringently purify DNA primer extension products from excess primers in the reaction mixture for high-fold multiplex SNP analysis and unambiguous MS-based detection. Therefore, in previous work, we incorporated a solid phase capture step after single base extension (SPC–SBE) reactions using biotinylated dideoxynucleotides, with isolation of SBE products on streptavidin-coated beads [23–26]. However, the release of biotinylated DNA products from streptavidin-coated surfaces requires high temperature formamide treatment or other harsh conditions to break the biotin–streptavidin bond and the subsequent requirement for extra ethanol precipitation steps, which are tedious and can result in sample loss. Furthermore, the relatively small mass differences between each dideoxynucleotide reduce the effective peak resolution, posing challenges for low-level heteroplasmy detection due to potential peak overlaps. To address these problems, we have developed a novel set of chemically cleavable biotinylated dideoxynucleotides, ddNTPs-N_3-biotin (ddATP-N_3-biotin, ddGTP-N_3-biotin, ddCTP-N_3-biotin, and ddUTP-N_3-biotin; depicted in Fig. 1), whose synthesis is described in detail in our accompanying article [27]. In these modified ddNTPs, the biotin moiety is attached to the 5 position of C and U and to the 7 position of A and G via a cleavable azido linker. The azido linker between the biotin and the nucleotide is cleavable with high efficiency by treatment with tris(2-carboxyethyl)phosphine (TCEP) under DNA-compatible aqueous conditions. In addition, the length of the linker is adjusted to increase the mass differences among these nucleotide analogs for high-resolution MS measurement. These modifications of the ddNTPs have led to improved sensitivity, accuracy, and efficiency of SPC sequencing [27] and multiplex genotyping reported here.

We validated this SPC–SBE approach using cleavable biotinylated ddNTPs in testing mtDNA samples for MERRF (myoclonic epilepsy with ragged red fibers) syndrome. MERRF syndrome, like many other mitochondrial diseases [28,29], is a maternally inherited multisystemic disorder. Among the most commonly used point mutations for clinical diagnosis of MERRF syndrome are m.8344A>G (A8344G) [30], m.8356T>C (T8356C) [31], and m.8363G>A (G8363A) [32] in the mitochondrial MT-TK gene encoding $tRNA^{Lys}$ and m.3243A>G (A3243G) [33] and m.3255G>A (G3255A) [34] in the mitochondrial MT-TL1 gene encoding $tRNA^{Leu}$. A8344G is present in more than 80% of MERRF-affected individuals [28], whereas A3243G and G3255A are mutations shared with MELAS (myopathy, encephalopathy, lactic acidosis, and stroke) syndrome [13,34,35]. We have developed a 5-plex genotyping assay to simultaneously identify variants at all five of these sites in mtDNA samples. We demonstrate that the SPC–SBE approach using cleavable biotinylated ddNTPs could quantify heteroplasmy levels and detect as low as 5% heteroplasmy. This enables rapid and accurate identification of mtDNA SNPs at high sensitivity and specificity, offering great potential for mitochondrial disease diagnosis.

Materials and methods

DNA samples were extracted from homoplasmic 8344 wild-type (8344WT), homoplasmic 8344 mutant-type (8344MT), and homoplasmic 3255 mutant-type (3255MT) cybrids, a mitochondrial DNA-negative cell line (rho^0), a MERRF patient with the 8344 point mutation (8344PT), and an anonymous healthy donor (8344WT-2). The cleavable dideoxynucleotides were synthesized according to the procedures described in the accompanying article [27].

PCR amplification

The PCR primers were initially designed with Primer3 (http://www.frodo.wi.mit.edu/primer3) but further adjusted after a BLAST search in order to avoid nonspecific amplification of nuclear DNA. A uniplex PCR was performed to amplify region 1 containing three of the SNP sites (A8344G, T8356C, and G8363A), whereas a 2-plex PCR was used to amplify regions 1 and 2 simultaneously, the latter of which covers the other two SNP sites (A3243G and G3255A). The primers were 5′-GACCGGGGGTATACTACGGT-3′ (region 1, forward), 5′-GGAGGTAGGTGGTAGTTTGTG-3′ (region 1, reverse), and 5′-TTAGTATTATACCCACACCCACCCAAG-3′ (region 2, forward), 5′-ATTAGAATGGGTACAATGAGGAGT-3′ (region 2, reverse) (purchased from Integrated DNA Technologies, Coralville, IA, USA, or Eurofins MWG Operon, Huntsville, AL, USA). Therefore, the DNA fragments that are amplified from the 2-plex PCR include all five of these frequently mutated sites used for the clinical diagnosis of MERRF syndrome. The uniplex PCR was carried out in a 30-μl PCR cocktail mixture containing 100 ng of mtDNA, 10 nmol of dNTPs, 8 pmol each of forward and reverse primers (region 1, forward and reverse), 0.5 U of JumpStart REDTaq DNA Polymerase (Sigma–Aldrich, St. Louis, MO, USA), and the corresponding 1× polymerase reaction buffer. The 2-plex PCR consisted of 100 ng of mtDNA, 12 nmol of dNTPs, 7 pmol each of forward and reverse primers, 0.5 U of JumpStart REDTaq DNA Polymerase, and 1× polymerase reaction buffer in a total volume of 30 μl. Amplification was performed using the following PCR profile: incubation at 94 °C for 2 min, followed by 25 cycles of 94 °C for 20 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 3 min. After PCR, 1 μl of PCR product from each sample was run on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to visualize the product quality. To remove excess primers and dNTPs, PCR products were subsequently treated with ExoSAP-IT (USB/Affymetrix, Cleveland, OH, USA), with incubation at 37 °C for 15 min and enzyme deactivation at 80 °C for 15 min.

Relative quantification of mtDNA in the samples

The relative amounts of mtDNA in the homoplasmic samples, 8344MT and 8344WT, were quantified by TaqMan real-time PCR. Region 1, which is specific to the mitochondrial genome, was chosen to compare the relative quantity of mtDNA between these two samples. Here 10-fold serial dilutions from 10 to 10^{-5} ng/μl were carried out on both 8344MT and 8344WT samples before real-time PCR. The real-time PCR cocktail consisted of 1 μl of diluted DNA sample, 4 pmol each of forward and reverse primers, and 10 μl of diluted SYBR Green mixture (Life Technologies, San Diego, CA, USA) in a total volume of 20 μl. The reaction was performed under the following conditions: incubation at 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Threshold cycle (C_t) values for each dilution of both samples were collected, and the graph of C_t values against concentrations was plotted to determine the relative amount of mtDNA in samples 8344MT and 8344WT. Based on the quantification results, the 8344MT and 8344WT samples were mixed in a series of ratios (2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, and 97.5% mutant type) for heteroplasmy quantitation and sensitivity analysis, targeting the SNP at position A8344G.

Taking into account the potential for preferential PCR amplification, purified homoplasmic PCR products instead of the original