

Highly efficient semi-quantitative genotyping of single nucleotide polymorphisms in mitochondrial DNA mixtures by liquid chromatography electrospray ionization time-of-flight mass spectrometry

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Abstract. The advantages of ion-pair reversed-phase high-performance liquid chromatography hyphenated to electrospray ionization quadrupole time-of-flight mass spectrometry for the semi-quantitative genotyping of single nucleotide polymorphisms were evaluated. Artificially prepared and naturally occurring mitochondrial DNA mixtures showing different levels of heteroplasmy at nucleotide position 16519 served as reference samples. The observed assay performance regarding accuracy, reproducibility, and sensitivity suggests that the described technique represents one of the most powerful assays available today for the determination of allelic contents. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

Sanger sequencing represents the “golden standard” for the typing of mitochondrial DNA (mtDNA). Accordingly, sequencing is commonly used for the detection and the relative quantification of heteroplasmic mixtures. Typically, a minimum contribution of 20% of the minority allele is required to unequivocally reveal the presence of

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heteroplasmy, which clearly restricts the use of this method for the quantification of allelic contents. A number of different technologies have been introduced for the determination of allelic frequencies in DNA mixtures including allele-specific oligonucleotide hybridization, minisequencing, denaturing gradient gel electrophoresis, real-time PCR, and denaturing high-performance liquid chromatography. Here, the hyphenation of ion-pair reversed-phase high-performance liquid chromatography and electrospray ionization quadrupole time-of-flight mass spectrometry (ICEMS) is presented as an efficient method for the assessment of allelic proportions of single nucleotide polymorphisms (SNPs) in mixtures.

2. Materials and methods

The primers 16493F (5'-TCC GAC ATC TGG TTC CTA CTT CA-3') and 16543R (5'-CGT GTG GGC TAT TTA GGC TTT ATG-3') were used to generate 51-bp-long amplicons as described in Oberacher et al. [1]. The chromatographic system consisted of an ultimate fully integrated capillary HPLC system (LC-Packings, Amsterdam, The Netherlands) and a Famos micro autosampler (LC-Packings) equipped with a 1 µl injection loop. PCR amplicons were injected onto the 60 × 0.2 mm I.D. monolithic capillary column without any prior sample preparation step. ESI-MS was performed on a QSTAR XL mass spectrometer (Applied Biosystems, Foster City, CA). The exit of the capillary column was connected directly to the transfer line by means of a microtight union (Upchurch Scientific, Oak Harbor, WA, USA).

3. Results and discussion

The mitochondrial locus 16519T/C [2] was used as target for the evaluation of the benefits of ICEMS for the determination of allelic frequencies in DNA mixtures. 16519 represents a highly polymorphic marker within the mitochondrial genome. Both allelic states are common within populations and the probability of observing heteroplasmic mixtures is rather high [2].

The PCR represents the initial analysis step. In comparison to many common technologies, unmodified primers can be used for amplification, which keeps assay costs low. The next step is the analysis of the PCR-amplified sequences by ICEMS. The nucleic acids are chromatographed as single strands in a capillary column under completely denaturing conditions (70 °C) and characterized on-line by ESI-MS in a quadrupole-quadrupole-time-of-flight (QqTOF) mass spectrometer.

The obtained raw mass spectrum shows characteristic series of multiply charged ions each representing the molecular mass of the intact molecule. The mass-to-charge ratios corresponding to a certain molecular species are deconvoluted yielding its molecular mass. The identification of the present allelic state is based on the comparison of the measured molecular mass to all theoretically possible masses calculated from the nucleotide compositions of anticipated alleles. Taking advantage of the high mass spectrometric performance of the applied QqTOF mass spectrometer, a single strand is assigned to an allele, if the relative difference between the measured and theoretical molecular mass is within ± 50 ppm [1]. Finally, for each observed single-strand allele-specific peak intensities are extracted out of the deconvoluted mass spectrum. Strand-specific allelic frequencies were calculated by applying the following formulas: content of allele 1 in % = $100 \times \text{intensity allele 1} / (\text{intensity allele 1} + r \times \text{intensity allele 2})$, content of allele 2 in % = $100 - \text{content of allele 1 in \%}$. The ratio of the signal intensities ($r = \text{intensity allele 1 (50\%)} / \text{intensity allele 2 (50\%)}$) serves for the correction of experimental biases caused by differential

PCR amplification and/or preferential ionization of single alleles. Strand-specific allelic frequencies are averaged to yield the final result.

Evaluation of the accuracy of ICEMS for the determination of allelic frequencies was accomplished by measuring the allelic frequencies of artificially constructed T/C-mixtures. Thirteen different samples with allelic frequencies in the range of 1.0% C up to 99.0% C were prepared by mixing different amounts of homoplasmic mtDNA and analyzed. Measured and theoretical allelic frequencies correlated well ($R^2=0.9983$). A calibration curve with an intercept of 0.20 and a slope of 1.01 was obtained, which clearly supports the hypothesis that in general no calibration curve is needed for accurate determination of allelic frequencies. An average standard deviation of 2.45% and a maximum deviation of 5.35% proved the reproducibility of the assay. Due to the sensitivity of the applied mass spectrometric detection system, alleles occurring at a frequency of 1.0% were unequivocally detected. The limit of quantification was found in the range of 5% minority component. An average deviation of 1.2% and a maximum deviation of 2.2% were examined, which suggests that ICEMS represents one of the most accurate assays for the determination of allelic frequencies available today.

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