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ORIGINAL ARTICLE

Factors affecting the detection and quantification of mitochondrial point heteroplasmy using Sanger sequencing and SNaPshot minisequencing

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Abstract Mitochondrial DNA analysis plays an important role in forensic science as well as in the diagnosis of mitochondrial diseases. The occurrence of two different nucleotides at the same sequence position can be caused either by heteroplasmy or by a mix of samples. The detection of superimposed positions in forensic samples and their quantification can provide additional information and might also be useful to identify a mixed sample. Therefore, the detection and visualization of heteroplasmy has to be robust and sensitive at the same time to allow for reliable interpretation of results and to avoid a loss of information. In this study, different factors influencing the analysis of mitochondrial heteroplasmy (DNA polymerases, PCR and sequencing primers, nucleotide incorporation, and sequence context) were examined. BigDye Sanger sequencing and the SNaPshot minisequencing were compared as to the accuracy of detection using artificially created mitochondrial DNA mixtures. Both sequencing strategies showed to be robust, and the parameters tested showed to have a variable impact on the display of nucleotide ratios. However, experiments revealed a high correlation between the expected and the measured nucleotide ratios in cell mixtures. Compared to the SNaPshot minisequencing, Sanger sequencing proved to be the more robust and reliable method for quantification of nucleotide ratios but showed a lower detection sensitivity of minor cytosine components.

Keywords Mitochondrial DNA · Sequence heteroplasmy detection · Sanger sequencing · Minisequencing · SNaPshot · Artificial mixtures

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Introduction

The analysis of mitochondrial DNA (mtDNA) plays an important role in forensic genetics especially if the detection of nuclear DNA fails [1–4]. Moreover, it is a valuable tool in the diagnosis of mitochondrial diseases [5].

Mitochondrial heteroplasmy is present if two or more mtDNA populations are found within an individual, tissue, cell, or even within the same mitochondrion or mitochondrial particle, respectively [6–10]. It can be differentiated into point and length heteroplasmy. The detection of heteroplasmic positions extends sequence information and thus increases the significance of a match [7], as in the case of the identification of the remains of Tsar Nicholas II [11, 12]. However, their discrimination from sequence background, from phantom mutations [13], and from sample mixtures often is difficult, and the interpretation of mtDNA results might be complicated. The frequency of heteroplasmy in humans is still under debate [14–17]. Compounding the problem, the heteroplasmy level, i.e., the ratio of nucleotides, seems to vary within ill or healthy individuals [7, 8, 16–18], as well as with age [19–21]. Moreover, to allow for profound interpretation of sequencing results, stable and sensitive heteroplasmy detection just as well as accurate determination of the nucleotide ratios is necessary. Common methods of heteroplasmy analysis in forensic laboratories are the Sanger

sequencing (SEQ) [22] and the SNaPshot minisequencing (SNAP) [23, 24]. The choice of method depends on the problem to be solved. The Sanger sequencing leads to a continuous nucleotide analysis of a DNA stretch of up to 500 bp. In case of highly degraded DNA, the SNaPshot minisequencing is advantageous, and a single reaction results in the analysis of a single nucleotide position. However, multiplex assays have been developed [25]. Apart from the analysis method used, different factors may influence the displayed level of heteroplasmy. For example, Brandstätter and Parson demonstrated that the amplification strategy itself has a substantial impact on the appearance of heteroplasmy [26]. We also observed an interrelationship between the measured nucleotide ratios and the sequencing primers used [15], but other factors also have to be considered.

To gain insight into the robustness of the system, we investigated the effects of the DNA polymerase used, of PCR and sequencing primers, of preferential nucleotide incorporation and sequence context, as well as of the capillary electrophoretic process with regard to their influence on the appearance of a given mitochondrial heteroplasmy. These factors were studied using BigDye Terminator v1.1 chemistry for Sanger sequencing and SNaPshot multiplex kit for minisequencing by analyzing nucleotide positions 16093 and 146 of the mitochondrial genome as they represent hotspot positions with a high mutation rate [16].

Also, both methods were compared as to correctly depicting nucleotide ratios at positions 16093, 146, and 152, analyzing different artificial mtDNA mixtures.

Materials and methods

Samples and DNA extraction

Blood from five and oral swabs from another two individuals, who had given their informed consent, served as DNA source. Individuals were chosen according to their mtDNA sequences to obtain special nucleotide combinations for creation of artificial mixtures (16093 T or C, 146 T or C, 152 T or C). DNA for cloning experiments was extracted from buccal swabs using the automated QuickGene-810 system (FUJIFILM Corp., Tokyo, Japan) and from 200 μL of EDTA-treated blood samples using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols.

Standard PCR conditions

Unless stated otherwise, PCRs were performed in 10-μL volumes containing 10× PCR buffer (Clontech, BD Biosciences, Palo Alto and Mountain View, CA, USA), 200 μM of each deoxyribonucleotide triphosphate, 0.2 μM of each primer (Biomers.net, Ulm, Germany), 50× Advantage 2 Polymerase Mix (Clontech), and plasmid or cell DNA. Reactions were cycled with an initial denaturation step at 95°C for 2 min followed by 32 cycles with 95°C for 15 s, 56°C for 30 s, 72°C for 90 s, and a final extension step at 72°C for 10 min according to the manufacturer's protocol. PCRs serving as template for SEQ or SNAP were purified enzymatically adding 2 μL ExoSap-IT (USB, Cleveland, OH, USA) followed by incubation at 37°C for 30 min and 80°C for 15 min.

Sanger sequencing

The BigDye™ v1.1 Terminator kit (Applied Biosystems, Foster City, CA, USA) was used for direct sequencing in final volumes of 5 μL consisting of 5× BD v1.1 Terminator Ready Reaction Mix, 5× BD Dye Terminator Sequencing Buffer, 0.2 μM primer (Table in Online Resource 1), and 1 μL of PCR product or 100 ng of plasmid DNA. Cycling was performed according to the manufacturer's protocol. Sequencing reaction products were purified using the DyeEx96 column plates or the DyeEx2 columns (both: Qiagen) according to the manufacturer's recommendations. Capillary electrophoresis (POP6 polymer, 36 cm capillary, 60 s injection time, 1.2 kV injection voltage) was either run on a 3130*xl* or on a 3100*Avant* Genetic Analyzer (Applied Biosystems) with Foundation Data Collection software v3.0 or v2.0. Experiments of the same type were analyzed using the same Genetic Analyzer. The obtained sequences were compared to the rCRS [27] using the Sequencher 4.9 software (GeneCodes, Ann Arbor, MI, USA). All peak heights (*peak*) at the respective heteroplasmic positions were measured starting from the baseline with ImageJ (National Institutes of Health, Bethesda, MD, USA). A "background threshold" was not applied. For comparison, the ratios of the cytosine nucleotides were calculated as

$$C\% = \text{peak C}/(\text{peak C} + \text{peak T}) \times 100\%$$

using Excel 2003 (Microsoft Corporation, Redmond, VA, USA).

Minisequencing

Minisequencing was performed using the ABI Prism SNaPshot™ Multiplex Kit (Applied Biosystems) in total reaction volumes of 5 μL containing 1 μL SNaPshot reagent and 0.05 μM primer for positions 146 and/or 152 and 0.02 μM primer for position 16093 (Online Resource 1), respectively. Depending on the experiment, either 1 μL of PCR product or 100 ng of plasmid DNA was used, respectively. Cycling was performed according to manufacturer's protocol for all primers and templates. Reactions were purified adding 1 μL alkaline phosphatase (Roche, Mannheim,

Germany) followed by incubation at 37°C for 60 min and 75°C for 15 min. Reaction products (2 μL of minisequencing products or 5 μL of minisequencing products from non-preamplified plasmid DNA) were added to 10 μL formamide and 0.5 μL Liz-120 internal standard (Applied Biosystems). Fragments were separated on a 3100*Avant* Genetic Analyzer (POP6 polymer, 36 cm capillary, 3 s injection time, 3 kV injection voltage) and analyzed using GeneMapper® ID v3.2 (Applied Biosystems). All peak heights of the respective heteroplasmic positions were used to calculate the cytosine ratios as given above. A "background threshold" within the expected range was not applied. With minisequencing primer H153 (Online Resource 1), the complementary nucleotide at position 152 was detected, and accordingly, the guanine ratio (corresponding to the cytosine ratio) was calculated as

$$G\% = \text{peak G}/(\text{peak G} + \text{peak A}) \times 100\%.$$

Plasmid DNA experiments

Generation of artificial heteroplasmy using different plasmids

The whole mitochondrial control region of the mtDNA was amplified with primers L15900/H00599. After purification (QIAquick® Kit, Qiagen), the PCR fragments were ligated into a pCR4-TOPO TA cloning vector using the FastPlasmid Cloning kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was prepared with the Fast Plasmid MiniPrep or Perfectprep Plasmid 96VAC Kit (both: 5 PRIME GmbH, Hamburg, Germany) according to the manufacturer's protocols. Sanger sequencing was performed to revalidate the cloned sequences. Also, the homoplasmic state of the sampled individuals was confirmed by sequencing of 50 clones each. DNA concentrations were measured using the NanoDrop 1000 (Thermo Scientific, Hamburg, Germany). Mixtures of two plasmids differing at the position(s) of interest were produced to obtain the artificial heteroplasmies 16093 T/C or 146 T/C. DNA concentration was adjusted to 500 pg/μL when serving as template for PCR and to 100 ng/μL for sequencing reactions without any preceding PCR.

Robustness and reproducibility

Plasmid mixtures of 146 T and 146 C were used to study the variability caused by the reaction processes for SEQ and SNAP themselves. The intra-assay variability of these reactions was assessed ten times taking triplicates of the same PCR and sequencing approach. The inter-assay variability of the PCR was assessed calculating the standard deviation from ten independent triplicate PCRs of each 50% 146 T/C samples, which were then sequenced using the same SEQ or SNAP master mixes. The inter-assay variability of the sequencing reaction was calculated analyzing six replicate sequencing reactions of ten PCR products generated from samples with different nucleotide ratios. Additionally, we analyzed six repeated injections of the same sequencing product from plasmid mixtures (ten samples) with different nucleotide ratios in different plate wells.

Investigation of effects caused by the DNA polymerase used for PCR

The Advantage 2 Polymerase Mix (Clontech), AmpliTaq Gold (Applied Biosystems), and *Pfu*Turbo hotstart polymerases (Stratagene, La Jolla, CA, USA) were compared as to their influence on the detected nucleotide ratios. Plasmid mixtures of each 50% 146 T/C and of each 50% 16093 T/C, respectively, were analyzed in ten independent experiments using triplicates. PCR primers L15900/H16186 were used for position 16093 and primers L16517/H00293 for position 146. Standard PCR conditions were adjusted due to a higher activation time needed for the AmpliTaq Gold, extending initial denaturation to 10 min. An adverse effect on the Advantage 2 was not observed. For sequencing, primers L15989, L16019, or L16092-1 and L16517, L00015, or L145-2 were used, respectively (Online Resource 1).

The lowest needed DNA template amount for a successful DNA amplification by the three enzymes was tested for plasmid DNA amounts between 0.005 and 500 pg (corresponding to 800 and 8×10^7 copies of the mtDNA fragment, respectively) amplifying 15 mixtures per DNA amount with each of the polymerases. The enzyme sensitivity was determined by analyzing the readouts after capillary electrophoresis: A successful result was assigned if base calls were given by the Sequencing analysis software 5.1 (Applied Biosystems) requiring a quality value over 20 (SEQ), or if only the expected peaks of the SNP were clearly identified (visually without background threshold) within the expected range (SNAP), respectively.

Investigation of effects caused by the PCR and sequencing primers

To investigate any primer-related effects, three different forward primers for each position (L15900, L15989, and L16019 for position 16093; L16450, L16517, and L00015 for position 146; Online Resource 1) were used for PCR and sequencing. Also, different SNaPshot primers were tested (L16092-1 and L16092-2 for position 16093; L145-1 and L145-2 for position 146; Online Resource 1). Reverse primers (H16186 and H00293, respectively) were not varied due to the nearby lying C-stretches. Additionally, plasmid DNA was sequenced without any preceding PCR. For each primer set, experiments were done five times

using triplicates, and standard conditions for PCR and sequencing were applied.

Investigation of effects caused by preferential nucleotide incorporation

Typically, only T-to-C transitions exist at the examined positions. To also obtain A and G nucleotides at position 16093, mismatch primers overlapping this region were used for amplification (Online Resource 1) with a reduced annealing temperature of 55°C. The resulting 200-bp fragments (L15900/H16100) were cloned as described above. Plasmids were mixed to obtain all the nucleotide combinations possible. In five experiments, triplicates of mixed and duplicates of non-mixed samples were sequenced using primers L15989 (SEQ) and L16092-1 (SNAP).

Cell mixture experiments

Isolation of T lymphocytes

For cell sorting, leukocytes from EDTA-treated blood samples were separated using Leucosep centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) according to the manufacturer's recommendations. T lymphocytes were fluorescently labeled with fluorescein isothiocyanate-labeled Anti $CD3^+$ antibodies (BD, Heidelberg, Germany).

Generation of artificial heteroplasmy using cell mixtures

Defined cell mixtures of two different individuals at a time were created by flow cytometry for each position of interest. A MoFlo cell sorter (DakoCytomation, Fort Collins, CO, USA) was used to deposit different amounts of T lymphocytes onto 96-well reaction plates (100 cells per well) yielding cell ratios of 0%, 5%, 10%, 20%, 30%, and 50% for each of the tested individuals for the positions 16093 or 146/152. Moreover, low ratios of 1%, 2%, 3%, 4%, 7%, and 9% were created from 16093 C and 16093 T T lymphocytes to test the sensitivity of heteroplasmy detection. Instrument settings and the cell selection process were described previously [15], but selection of T lymphocytes was facilitated by fluorescent labeling. One row per plate was omitted to allow for positive and negative PCR and sequencing controls, respectively.

Analysis of cell mixtures

Sorted cells were lysed by adding water, 10× PCR reaction buffer (Clontech), and 20 μg Proteinase K (Qiagen) into the plate wells yielding a total volume of 7.2 μL. Plates were incubated at 56°C for 10 min followed by enzyme inactivation at 95°C for 15 min.

PCR was directly performed in the plate wells using the standard conditions given above. Primers L15989/H16186 were used to amplify the sequence containing position 16093 and primers L16450/H00293 for positions 146/152, respectively. Sequencing and calculations were done as described using primers L15989 or L00015 (SEQ) and L16092-1 or L145-2/H153 (SNAP).

Visual recognition experiment

For qualitative identification of heteroplasmic positions, the 16093 T/16093 C T cell mixtures (eight samples per ratio ranging from 0% to 50%) were analyzed by SEQ as well as by SNAP. A random sequence stretch (25 bp) of the obtained sequence chromatogram (including position 16093) and the SNP electropherograms were shown to five individuals with a background in molecular biology. They were given the task to qualitatively identify any mixtures without knowing the actual nucleotide ratios.

Statistics

Statistical analysis and creation of diagrams were done using the software packages SPSS 15 and PASW 18 (SPSS, Chicago, IL, USA). Intra- and inter-assay variabilities of the "system robustness experiments" were determined calculating standard deviations for the triplicates and the repeated experiments, respectively. For non-parametrical testing of experimental results, the exact Kruskal–Wallis test (two-tailed) and the exact Mann–Whitney *U* test (two-tailed) were applied. Performance of different sequencing primers on the same PCR product was tested with the exact Friedman test and the Wilcoxon rank test (two-tailed). Statistical significance was accepted for $p<0.05$. Cell experiments were analyzed using linear regression models.

Results

Robustness and reproducibility

The ratios of the heteroplasmic positions were calculated for SEQ and SNAP results. Intra-assay variabilities ranged from 0.03% to 1.38% for SEQ and from 0.19% to 0.86% for SNAP, respectively. The inter-assay standard deviation for the PCR reactions was 0.65% (SEQ) vs. 0.86% (SNAP), and the average standard deviations for independent sequencing reactions were 0.81% (SEQ) vs. 4.61% (SNAP). Additionally, repeated injections revealed very low variations through the injection process (0.88% for SEQ and 0.1% for SNAP). Additional out-of-range peaks ("background") occurred more often in the SNAP electropherograms, complicating the interpretation of results.

Influences of the reagents used

Variability caused by different DNA polymerases for PCR

Three different DNA polymerases representing different characteristics (mixed, proofreading, and non-proofreading) were tested for their impact on the measured cytosine peaks of each 50% C/T mixtures at positions 16093 and 146, respectively (Fig. 1). Calculated ratios ranged around the expected 50%, and DNA polymerases showed less impact than the sequencing primers used especially in the case of SNaPshot primer L16092-1, which led to an overestimation of the cytosine amount (Fig. 1a).

Regarding the amplification sensitivity test with different amounts of plasmid DNA (800 to 8×10^7 copies of the mtDNA control region fragment), 100% of the samples with 8×10^7 copies were successfully analyzed with all three DNA polymerases and both sequencing techniques. Advantage 2 Mix showed the highest sensitivity (>70% positive results for both methods using 800 copies of template DNA) followed by AmpliTaq Gold and *Pfu*Turbo hotstart polymerase. SNAP analysis of 800 template copies using *Pfu*Turbo hotstart polymerase failed completely.

Variability caused by different PCR and sequencing primers

Again, each 50% C/T mixtures at positions 16093 and 146 were analyzed (Fig. 2). In SEQ of 16093 C/T non-preamplified plasmid DNA, primers L15989 and L16019 led to an overestimation of C nucleotides compared to primer L15900, whereas any preceding PCR resulted in a (slightly) underestimated C ratio (Fig. 2a). Regarding SEQ analysis of position 146, the opposite applied to primer L16517 (Fig. 2b). For both positions, SEQ of non-preamplified plasmid DNA showed the highest variability in calculated C ratios (Fig. 2a, b).

Regarding SNAP analysis of position 16093, calculated C ratios were generally overestimated compared to the actual 50% C/T ratios. No significant differences were observed for either PCR primer as well as for analysis of non-preamplified plasmid DNA (Fig. 2c). Analyzing position 146, SNaPshot primer L145-1 resulted in overestimation and L145-2 in slight underestimation of C ratios when combined with either PCR forward primer (Fig. 2d).

Effects of the incorporated nucleotides

The peak heights representing position 16093 in the chromatogram (SEQ) as well as in the electropherogram (SNAP) differed depending on the incorporated nucleotide (A ≈ G > T > C; Online Resource 2a, b). The 50% C/T mixtures were closely matched by the displayed peaks with both techniques (approximately 45% to 55%), and the 50% C/A mixtures were displayed the worst (approximately 35% to 65% in SEQ and approximately 32% to 68% in SNAP, respectively; Online Resource 2c, d). A relevant interference with the incorporation of the other nucleotide present in the respective heteroplasmic mixture was not observed.

Cell mixture experiments

Visual recognition experiment

Mixtures of 16093 T and 16093 C T lymphocytes with mixture ratios between 0% and 100% 16093 C were

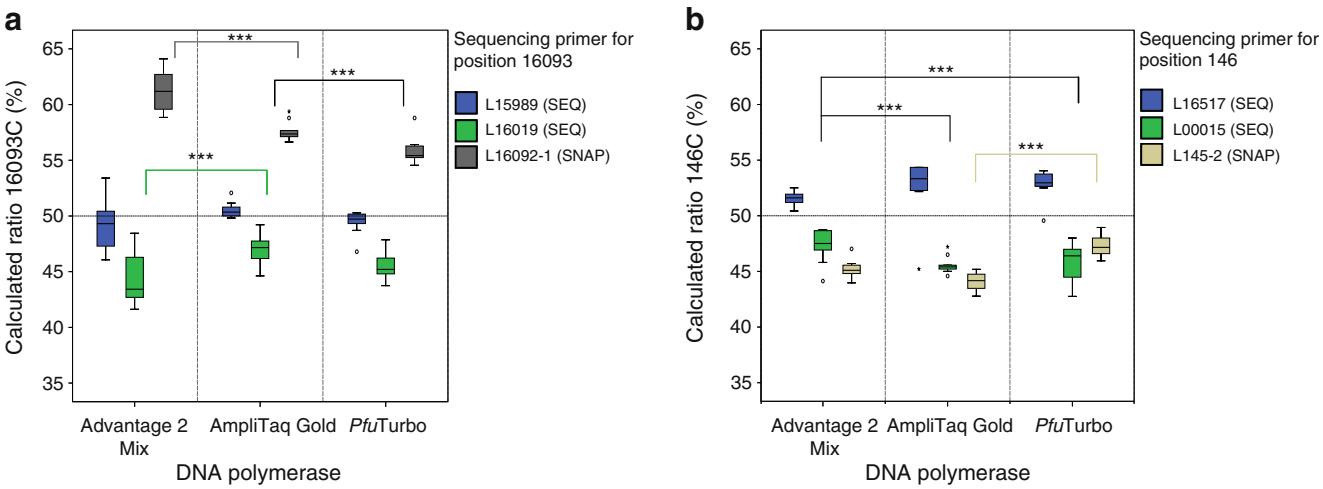


Fig. 1 Influence of DNA polymerase on calculated mixture ratios. *Box-and-whisker plots* of calculated cytosine ratios (percent) at positions 16093 (**a**) and 146 (**b**), respectively, for different enzymes and primers. The *dashed line* depicts the expected nucleotide ratio of 50%. The *boxes* comprise 50% of all data points—defined by the first and third quartiles—with a *black bar* indicating the median. *Circle* outliers. *Asterisk* extreme values. *SEQ* Sanger sequencing, *SNAP* SNaPshot minisequencing. ***$p<0.05$

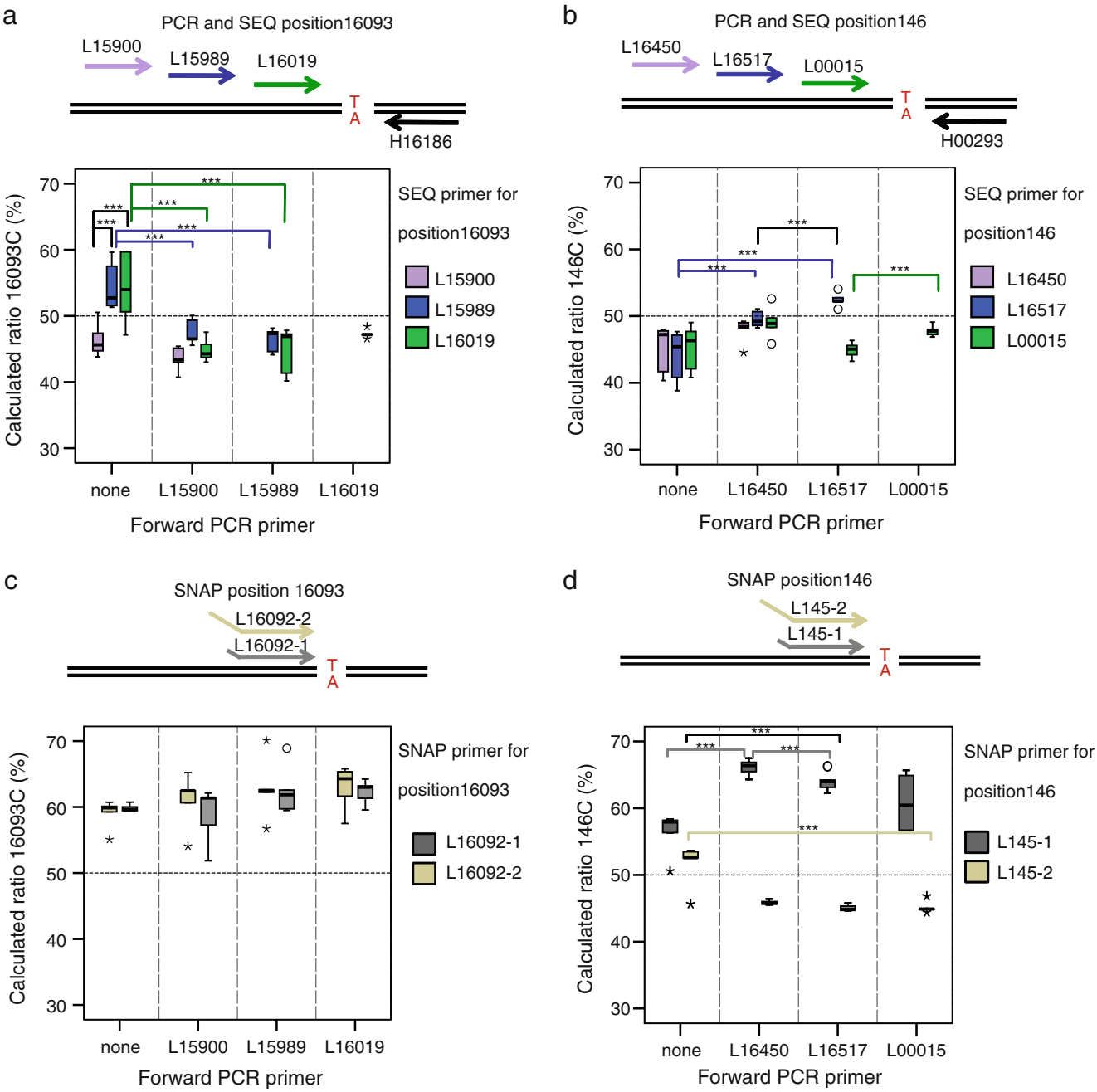


Fig. 2 Influence of PCR and sequencing primers on calculated mixture ratios. *Box-and-whisker plots* of calculated cytosine ratios (percent) at positions 16093 (**a**, **b**) and 146 (**c**, **d**), respectively, for different PCR and sequencing primers. The *dashed line* depicts the expected nucleotide ratio of 50%. *Circle* outliers. *Asterisk* extreme values. *SEQ* Sanger sequencing, *SNAP* SNaPshot minisequencing. ***$p<0.05$

analyzed as to the possibility of visual recognition and compared to the calculated nucleotide ratios (Fig. 3). Compared to SEQ, SNAP allowed for reliable identification even of low-level heteroplasmies (<7% 16093 C lymphocytes), what was mainly due to an overrepresentation of minor cytosine components. With SEQ, low cytosine ratios (<10%) were mostly not identified. However, low thymine components were easily identified with both methods (cf. Fig. 3).

Quantification of the heteroplasmy level

To test the possible predictability of the actual heteroplasmy level in unknown samples, calculated nucleotide ratios at positions 16093, 146, and 152 were compared to the respective expected ratios, and a strong linear correlation was found (Fig. 4a, b). As Fig. 4a shows, a constant might be deduced to adjust for an over- or underestimation.

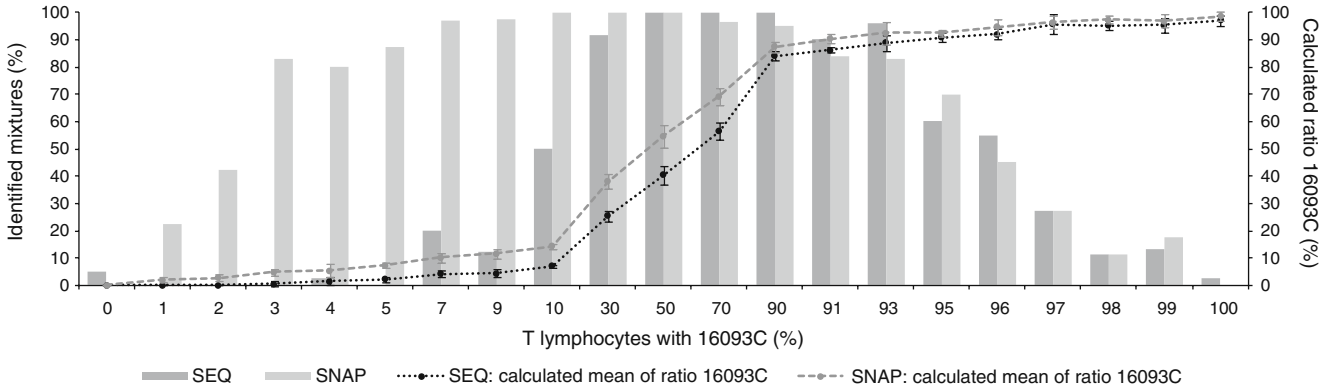


Fig. 3 Visual recognition of heteroplasmic samples (*bars*) and calculated mixture ratios (*dotted and dashed lines*) as obtained from analysis of different 16093 T/16093 C T lymphocyte mixtures. *Error bars* indicate the standard deviations. *SEQ* Sanger sequencing, *SNAP* SNaPshot minisequencing

However, low-level mixtures of 5% and below displayed less accurately (data not shown).

Discussion

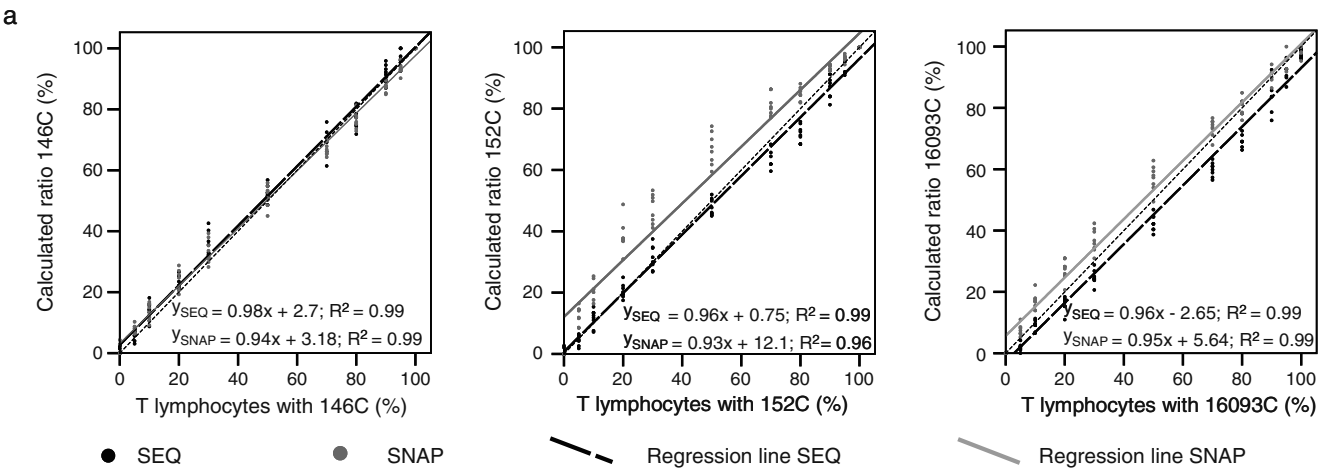
Most crucial for the evaluation of mitochondrial heteroplasmy in a forensic and diagnostic context as well as in research is the congruence of a detected heteroplasmic state and the given situation in vivo. In this study, we assessed the influence of different reagents and of two different analysis methods on the detection and accurate display of mitochondrial heteroplasmy. Results showed that diverse effects of a respective analytical setting have to be considered.

Surprisingly, the use of a proofreading polymerase did not increase the detection accuracy of the heteroplasmy level. The highest sensitivity was observed for Advantage 2 Polymerase Mix. Since no information on enzyme activity could be obtained, it cannot be excluded that this higher sensitivity was caused by a higher concentration of the polymerase. Altogether, the use of different polymerases showed only minor effects with SEQ and stronger effects with SNAP.

Comparing different PCR and sequencing primers, the most relevant finding was a systematic overestimation of C nucleotides with SNAP, excluding primer L145-2 (Fig. 2), which was the longest of the minisequencing primers (60 mer), but a satisfying explanation for this phenomenon was not found. This finding might be of biological relevance, whenever diagnostic thresholds have to be inferred from molecular testing procedures [28]. Regarding PCR and SEQ, analysis results varied depending on the primers used, which meets the experiences made previously [15], but no consistent effects were observed. Nevertheless, for each experimental design, different primers and enzymes should be tested and validated for each heteroplasmic position to be analyzed.

The PCR and sequencing DNA polymerase as well as the labeling of the nucleotides can influence the process of nucleotide incorporation. This was also observed in this study and had a noticeable impact on the display of heteroplasmic nucleotide levels (Online Resource 2). It is known that DNA polymerases show different affinities for the different nucleotides depending on their hydrophobicity, e.g., an increasing affinity in the order G = A > T > C was found for DNA polymerase I (Klenow fragment) [29]. Also, Parker et al. showed an influence of the nucleotides lying upstream the examined base [30]. All experiments in this study were performed using the BigDye® terminator mix v1.1 and the SNaPshot™ Multiplex kit including the labeled nucleotides as well as the sequencing polymerase. The BigDye chemistry uses energy transfer dyes, which are coupled to the accordant nucleotide by a propargylamino (ddATP) or propargyl ethoxyamino linker (ddCTP, ddGTP, ddTTP). This type of labeling is supposed to yield homogenous peak patterns [31]. However, equality is not yet fully achieved since different sterical conformations still occur. Also, dye-related effects cannot fully explain the observed differences in nucleotide incorporation at position 16093 (G ≈ A > T > C) as the labeling of cytosine and guanine is contrarian in BigDye and SNaPshot assays.

The study demonstrated a good correlation of the calculated nucleotide ratios and the actual mixture ratios for both techniques. To assess the predictability of a given heteroplasmic state from the situation displayed in a chromatogram or electropherogram, a linear regression analysis was performed (Fig. 4). The quantification results obtained with SEQ as well as SNAP showed a linear correlation, with a higher variability for SNAP. For minor components of 5% and less, only a qualitative analysis can



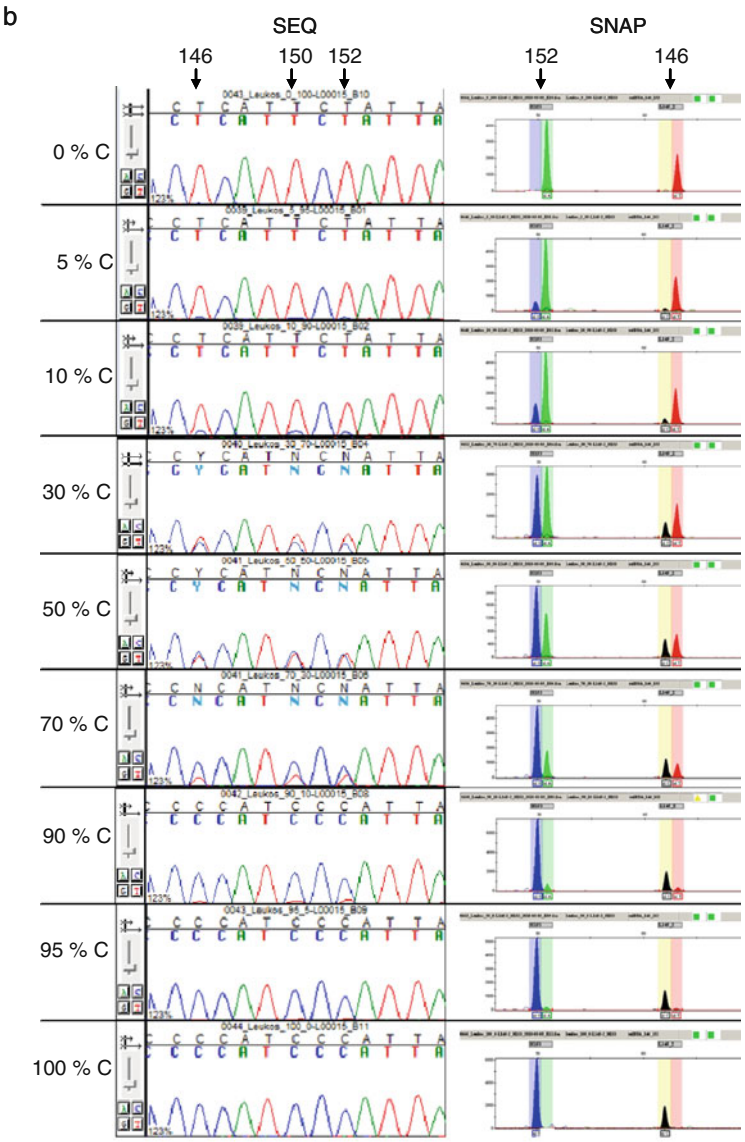


Fig. 4 **a** Linear regression analysis of calculated C ratios and T lymphocyte mixtures mimicking different levels of heteroplasmy at positions 146, 152, and 16093. Positions 146 and 152 were determined from the same sample. *SEQ* Sanger sequencing, *SNAP* SNaPshot minisequencing. **b** Comparison of exemplary SEQ and SNAP analysis results. An additional heteroplasmy at position 150 was detected with SEQ

be recommended since the variability of calculated C ratios was too high for a reliable quantification (data not shown).

In summary, the SEQ reaction seemed to be more robust, showing, e.g., in a lower process-related variability, in higher detection sensitivity for low DNA amounts, and in less occurring background. On the other hand, SNAP detected minor cytosine components with a higher sensitivity (due to the observed and systematic overestimation of C nucleotides) thus facilitating visual recognition, whereas the heteroplasmic C in SEQ was often interpreted as background. Naturally, visual recognition also strongly depends on the software settings used to identify any mixtures.

Other methods for analysis of mitochondrial heteroplasmy as denaturing gradient gel electrophoresis [14], pyrosequencing [32, 33], real-time PCR-based assays [34], and mass spectrometry [35] offer not only advantages but also drawbacks. The method of choice depends on the question to be solved. Essential prerequisites are a highly sensitive detection of heteroplasmy as well as a sufficiently exact quantification. The latter one is especially difficult in cases of low-level mixtures with a minor component of 5% or less. In these cases, the detection will often only be possible when the minor component is overrepresented, thus impairing an accurate determination of nucleotide ratios, as shown for the SNAP analysis of C/T mixtures in this study.

Some methodological limitations have to be considered. Artificially heteroplasmic samples were generated by cell sorting or plasmid mixtures. However, mixture ratios may have varied since within and between individuals, cells can contain different amounts of mitochondria as well as of mtDNA copies per mitochondrion [36]. Also, the created plasmid mixtures may not have been absolutely precise due to variability in the determination of DNA concentrations. Cell mixtures were investigated exemplarily, and plate-to-plate differences were higher for SNAP than for SEQ (data not shown).

As mentioned before, the best performing primer set has to be validated for each position of interest. On the other hand, exemplary SEQ analysis of positions lying in close proximity to positions 16093 and 146 showed good accordance (e.g., position 150, Fig. 4b). However, neighboring mutations may hamper the SNaPshot reaction due to primer mismatches leading to dropouts. This problem may occur when using a reverse primer for analysis of position 146 if there is a heteroplasmy at position 152.

In future experiments, plasmid DNA amounts should be reduced to template amounts expectable in forensic samples. However, preliminary tests in the course of this study did not show any differing results (data not shown). Also, reverse primers for sequencing and other dye chemistries might be evaluated. Beside a detailed assessment of the methods used to exactly quantify mitochondrial heteroplasmy, a critical debate of its relevance in forensics as well as in clinical diagnostics is necessary. The intra-individual variability of the heteroplasmy level over time as well as in different tissues has to be considered more thoroughly. As yet, successful and sensitive detection also of low-level heteroplasmy would by far be more important than the exact determination of its level.

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