Mass spectrometric base composition profiling: Implications for forensic mtDNA databasing

Mayra Eduardoff [a], Gabriela Huber [a], Birgit Bayer [b], Dagmar Schmid [b], Katja Anslinger [b], Tanja Göbel [c], Bettina Zimmermann [a], Peter M. Schneider [c], Alexander W. Röck [a], Walther Parson [a,d,*]

[a] Institute of Legal Medicine, Innsbruck Medical University, Innsbruck, Austria
[b] Institute of Legal Medicine, University of Munich, Munich, Germany
[c] Institute of Legal Medicine, Faculty of Medicine, University of Cologne, Cologne, Germany
[d] Eberly College of Science, Penn State University, University Park, PA, USA

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ABSTRACT

In forensic genetics mitochondrial DNA (mtDNA) is usually analyzed by direct Sanger-type sequencing (STS). This method is known to be laborious and sometimes prone to human error. Alternative methods have been proposed that lead to faster results. Among these are methods that involve mass-spectrometry resulting in base composition profiles that are, by definition, less informative than the full nucleotide sequence. Here, we applied a highly automated electrospray ionization mass spectrometry (ESI-MS) system (PLEX-ID) to an mtDNA population study to compare its performance with respect to throughput and concordance to STS. We found that the loss of information power was relatively low compared to the gain in speed and analytical standardization. The detection of point and length heteroplasmy turned out to be roughly comparable between the technologies with some individual differences related to the processes. We confirm that ESI-MS provides a valuable platform for analyzing mtDNA variation that can also be applied in the forensic context.

1. Introduction

Sanger-type sequencing (STS) has been the state of the art method for analyzing the mitochondrial DNA (mtDNA) control region (CR) in forensic genetics. Some alternative approaches have been introduced, such as single base extension assays [1], hybridization methods [2] and pyrosequencing [3]. It has also been shown that electrospray-ionization mass spectrometry (ESI-MS) can be successfully applied to analyze mtDNA variation in the forensic context [4]. Hall et al. [5] performed these experiments with an instrumental set-up that is highly automated and provided customized analysis software that is missing in other approaches especially those that use STS-based technology. This is the reason for numerous earlier error reports criticizing the quality of mtDNA work, not only in the forensic but general scientific field [6].

Advancements have been made since then to improve the production of high quality mtDNA data [7]. The main sources of error were found in human transcription, mix-up of samples and low-quality sequencing raw data [8,9] that could be avoided by an automated and customized software-controlled process. The PLEX-ID system (Abbott Laboratories, Abbott Park, IL, USA) was designed with such features in mind and was employed here to perform a side-by-side comparison with STS data from a population study of South Germany (Munich). Population data were derived from both technologies and discussed in the forensic context with special attention to concordance and the detection of point and length heteroplasmy.

2. Materials and methods

2.1. Samples and DNA extraction

For this study 200 blood samples were taken post-mortem in the course of routine autopsies at the Institute of Legal Medicine, University of Munich, from individuals originating from Munich, Germany in agreement with the ethics commission of the University of Munich. DNA was extracted using the Qiagen BioRobot M48 Robotic Workstation (Qiagen, Hilden, Germany)

* Corresponding author at: Institute of Legal Medicine, Innsbruck Medical University, Innsbruck, Austria. Tel.: +43 512 9003 70640; fax: +43 512 9003 73640.
E-mail address: walther.parson@i-med.ac.at (W. Parson).

following the manufacturer's protocol. Samples were anonymized prior to the analysis so that no link to the donor can be established.

2.2. Amplification and Sanger-type sequencing

Amplification and Sanger-type sequencing were performed according to the EMPOP protocol updated in [10]. Sequencing raw data were interpreted independently by three different scientists using Sequencher v4.10 and v5.0 (Gene Codes Corporation, Ann Arbor, MI, USA). Haplotypes are provided for forensic searches under the accession number EMP00482 in the EMPOP database (www.empop.org; [7]).

2.3. Amplification and mass spectrometric analysis

ESI-MS analysis was performed on the same DNA extracts using the PLEX-ID instrument and the PLEX-ID mtDNA assay v2.0 (Abbott Laboratories, Abbott Park, IL, USA) following the manufacturer's recommendations. This process was highly automated and required only minimal hands-on interaction by the operator. In brief, the DNA extracts (1.5 ml tubes) and sealed 96-well plates including the ready-to-use PCR master-mix were placed onto an automated liquid handling device (Tecan Trading AG, Switzerland) and pipetting was performed with a customized software script. PCR for each sample was set up in eight independent wells (one column of a 96-well plate) with a total of 24 primer pairs. The amplicons were designed to cover the entire mtDNA CR from nucleotide position (ntp) 15,924–576 in an overlapping manner with the exception of nucleotide positions (ntps) 16,251–16,253 and 16,428–31 that were not targeted by the approach. Plates were resealed after pipetting using a variable temperature sealer (Integrated Technologies Ltd., Kent, UK) and PCR was performed on an Eppendorf Mastercyclers Pro S (Eppendorf, Hamburg, Germany) with an initial denaturation temperature of 95 °C for 10 min, 36 cycles of 95 °C for 20 s, 50 °C for 90 s (5% ramp speed), and 72 °C for 5 s, followed by 72 °C for 4 min, 99 °C for 10 min and a final hold at 4 °C. The 96-well plates including the amplification products were then directly loaded onto the PLEX-ID instrument and the run was started. On the PLEX-ID the plate was first directed to an automated sample purification using magnetic beads and then amplicons were automatically subjected to electrospray ionization time-of-flight mass spectrometry following the principle described earlier [5]. Negative and positive controls were carried through the entire analytical process. After the run, raw data were automatically analyzed using the software package IbisTrack using default settings (Ibis Biosciences, Inc., Carlsbad, CA) to calculate and interpret the resulting base composition profiles (BCPs).

A full BCP of a sample consisted of the array of all base compositions (BCs) resulting from each of the analyzed amplicons. A single point heteroplasmy (PHP) in an amplified region was called, when two amplicons were generated by the same primer pair and gave identical total nucleotide counts (i.e. lengths), but differed by a single base state in their deduced base compositions (e.g. PHP in the region amplified by PP2897: 15A 8G 15C 14T; 15A 8G 14C 15T). Length heteroplasmy (LHP) was identified as two or more BCs resulting from amplicons of a single primer pair with different nucleotide counts (e.g. PP2908: A42 G16 C39 T32; A42 G16 C40 T32).

2.4. Comparison of STS and ESI-MS derived haplotypes

ESI-MS-derived BCPs were exported into Extensible Markup Language (XML) format for data handling and interpretation with the software package R (version 2.14.1 (2011-12-22) on Platform: i386-pcmingw32/i386 (32-bit) [19]). Each XML BCP was converted into a list object in R. Its abundance measurement was parsed from the data files generated by the IbisTrack software and stored thereto.

Sanger-type sequences were converted into FASTA format. BCPs were calculated in R by counting the number of A, T, G and Cs contained in mtDNA regions identical in range to the PLEX-ID amplicons (disregarding primer sequences). PHP was called using IUPAC code and for each variant an individual BCP was determined. Samples with length heteroplasmic C-stretches were called by the dominant (most abundant) variant using non-repetitive bases as reference as described in [11] and therefore yielded a single BCP for the corresponding primer pair. Peak height ratios for PHP detected by STS were determined manually and classified into three categories, low level (<20%), 20–80% and >80%, taking into consideration that STS with dye terminators is not always displaying mixture ratios correctly. For all computational analysis steps involving BCPs only forward strands were used.

3. Results and discussion

3.1. Overall performance, success rate and analyzed regions

A total of 200 DNA extracts were subjected to STS and ESI-MS analysis. The PLEX-ID workflow for the analysis of the mtDNA CR was highly automated, standardized and fully supported by software including the analysis of the resulting BCPs of the analyzed PCR amplicons. The analysis of the 200 samples took a total of six working days, including a quick review of the individual chromatograms and the repetition of 18 samples that failed to give a full BCP at first attempt. The PLEX-ID setup outperformed manual STS by an experienced mtDNA sequencing laboratory, in which the analysis of the same sample set took 10 working days for wet-lab analysis and a total of 120 h for data interpretation.

Among the repeated samples in the PLEX-ID approach were 15 where three or less amplicons dropped out. Most often primer pairs 2893 and 2908 covering the homopolymeric C-stretches were affected. One sample (MUC089) yielded no results with both technologies due to insufficient amount of mtDNA and was therefore excluded from the study. Another sample (MUC093) gave a successful STS haplotype (16126C 16129A 16294T 16296T 16304C 16519C 73G 263G 315.1C 315.2C, Table S1), but resulted in only a partial BCP using the PLEX-ID (data not shown), even after repetition. For this sample, three amplicons (produced by primer pairs 2907, 2908 and 2923, Fig. S1) covering the C-stretch in hypervariable segment II (HVS-II) did not yield useful results. Two primers showed mismatches with respect to the STS haplotypes. The reverse primer 2908R mismatched at positions 315.1C 315.2C (insertions) and 330 (A/G, 5′-end), whereas forward primer 2923F mismatched at position 262 (C/T, 5′-end). PLEX-ID analysis of two similar samples outside this study that also harbored 315.1C 315.2C led to similar dropouts of amplicons 2908 and 2907 in both samples confirming our earlier observation, while 2923 gave a signal in one sample (data not shown). These results suggest that the amplification of this particular sequence motif – that is observed in 0.2% of all samples in EMPOP Release 9 ([7]; N = 28,518) – was suppressed. Sample MUC093 was therefore excluded from further comparative analysis between the two technologies.

The STS protocol covered the entire CR from ntps 16,024 to 576 (1122 base pairs for the rCRS haplotype). The PLEX-ID assay targeted 1049 bases of the CR covering all three hypervariable segments. The first hypervariable segment (HVS-I) was covered by ntps 15,924–16,428, excluding ntps 16,251–16,253. Those three positions seem conserved in the CR, only two out of 29,444 haplotypes (EMPOP R9) showed variation here (at ntp 16,252). The

second and third hypervariable segments (HVS-II and HVS-III) are covered by ntps 31–576 (Fig. S1).

3.2. Concordance study - direct comparison of STS and ESI-MS derived BCPs

The STS haplotypes were computationally transformed into BCPs and compared pair-wise with the corresponding BCPs from the PLEX-ID approach. For this purpose a common reading frame was set to 16,024–16,482 (excluding ntps 16,251–16,253) and 31–576 representing the maximum overlapping coverage of both assays. Two profiles deriving from a sample were considered concordant when BCPs calculated from the comparable range products were identical. For this purpose point and length heteroplasmy were not considered. They are discussed in detail later. This comparison led to concordant results confirming the expectation that mass-spectrometric BCPs match the corresponding sequence derived haplotypes (turned into BCPs). Such experiments and results serve as independent validation criterion for ESI-MS based mtDNA analyses.

3.3. Population study

A total of 188 (94.9%) distinct STS haplotypes were observed in the set of 198 samples of which 178 (89.8%) were unique (Table 1). When adjusted to the PLEX-ID range (excluding ntps 15,924–16,023) 185 (93.4%) STS haplotypes were distinct in the dataset with 173 (87.4%) singletons. With PLEX-ID 182 (91.9%) distinct haplotypes were detected, 171 (86.4%) of which were observed only once. We note that LHP was not considered as exclusion. Thus, only 3 (1.5%) samples could not be further discriminated with the MS-based method compared to STS. The corresponding statistical values can be found in Table 1. The relatively small loss in discrimination power put the PLEX-ID system at an advantage over STS in the light of gaining automation and reducing analysis time of BCPs.

In our sample set seven of the 24 PLEX-ID amplicons did not show variation, whereas the remaining amplicons included sequence or length variants (Fig. S2A). The most conserved regions were covered by primer pairs 2925, 2899, 2910 and 2916, which showed less than three variants in this sample set (Fig. S2B). The distribution of variants across amplicons seemed to indicate that most samples differed by one or two variants whereas the remaining variants were detected in few samples only (Fig. S2C). These data confirm earlier findings of high discrimination power of ESI-MS based systems in the CR [12].

3.4. Point heteroplasmy (PHP)

PHP is known as the detectable coexistence of two (rarely more) sequences that differ usually at only one position in the mtGenome [13]. It has been shown that the sensitivity of detecting PHP, or mixtures in general, is correlated with the technology and it seems that the abundance of PHP is positively correlated with sensitivity of detection [14]. STS using Dye Terminator sequencing chemistry is known to display a particular

PHP at varying peak height ratios depending on the position affected and primer used. Hence, in some cases peak height ratios do not correspond with the relative amount of the contributing sequence variants. For the purpose of this study, PHP was called in STS data when redundant sequences provided clear evidence of the presence of a mixture with respect to signal-to-noise ratios. In contrast, ESI-MS produces discernable signals for PHP variants (due to mass differences) and their mass peak heights are quantitatively correlated with the amount of contributing variants [15].

With STS 14 (7.1%) PHPs were called in 14 of 198 samples (Table 2, S1 and S2). The PLEX-ID assay detected 19 (9.6%) PHPs in 17 (8.6%) of these 198 samples, 11 of which were matching those found in STS.

Due to the overlapping design of amplicons PHP was detected multiple times in the PLEX-ID assay, if located in a region covered by more than one primer pair. Here we discern PHPs that were present in all corresponding BCPs (full detection) from those that were displayed only in some (partial detection). In this sample set four (~2%) PHPs were detected only partially in the ESI-MS data (Table S3: MUC045, MUC118, MUC169, MUC172) and two were not detected at first analysis at all (Table S3: MUC049, MUC059, MUC094 and Fig. S3) compared to STS data. The reasons for this were threefold. In samples MUC049, MUC045, MUC118, MUC172, and MUC059 one of the mass peaks was masked by salt adducts, in sample MUC169 one of the mass peaks was below the detection level and in sample MUC094 PHP was positioned in a region that also showed length heteroplasmy with three length variants, which is why the PHP variants were most probably "diluted" below detection level (Fig. S4).

With the PLEX-ID assay, seven PHPs were called that were primarily not identified with STS. One of these was found at position 16022 that resided outside the reported region for STS, but could be confirmed in the raw data (primer F15851, MUC013). Three PHPs were detected at low levels (<20%) (Fig. S5), and three remained undetected with STS even after review. MUC169 was the only sample showing mixed bases at three positions, two of which were detected only with PLEX-ID (Table S4).

After review of both datasets, 17 PHPs were detected in common between the two approaches. The major variants (dominant types) were concordant except for PHP at ntp 204 (Y) in sample MUC194, which showed opposite but almost equally high peak ratios in the two assays. In the other instances peak height ratios were comparable between the technologies (Table S5).

PHPs were most frequently observed in amplicons produced by PP2897 and PP2898, both including ntp 16,093 (Fig. 1), which was also found to be the CR hotspot for PHP occurrence in a much larger systematic STS study [13]. In further accordance with this reference our sample set showed PHPs in fragments produced by PP2904, PP2905 and PP2906, which included positions 146, 152, 195, 204 and 215. These particular sites are known for showing a higher PHP frequency.

The samples analyzed in this study showed PHP at expected positions. Also their frequency in STS data (7.1%) confirmed other studies ([13] and references therein). ESI-MS confirmed PHPs detected in STS with the exception of one instance and, in addition,

Table 1
Comparing population statistics for STS and PLEX-ID derived haplotypes.

	Full STS 16,024–576	Adjusted STS 16,024–16,428, 31–576	PLEX-ID 15,924–16,428, 31–576
No. of samples	198	198	198
No. of haplotypes	188	185	182
No. of unique haplotypes	178	173	171
Random match probability	0.006	0.006	0.006
Haplotype diversity	0.994	0.994	0.994
Power of discrimination	99.424	99.391	99.378

Table 2

Summary of PHPs found with STS and PLEX-ID typing. Number in parenthesis refer to PHP in the same coverage range.

	STS PHPs	PLEX-ID PHPs	Common PHPs	PLEX-ID PHPs reanalysis	STS PHPs reanalysis	Common PHPs reanalysis
Total # of samples	198	198	198	198	198	198
# of samples with PHPs	14	17 (16)	11	18 (17)	17	16
PHPs	14	19 (18)	11	20 (19)	17	16
% PHPs	7.1	9.6 (9.1)	5.5	10.1 (9.5)[a]	8.6[a]	8.1

[a] Not statistically significant at $\alpha = 0.05$.
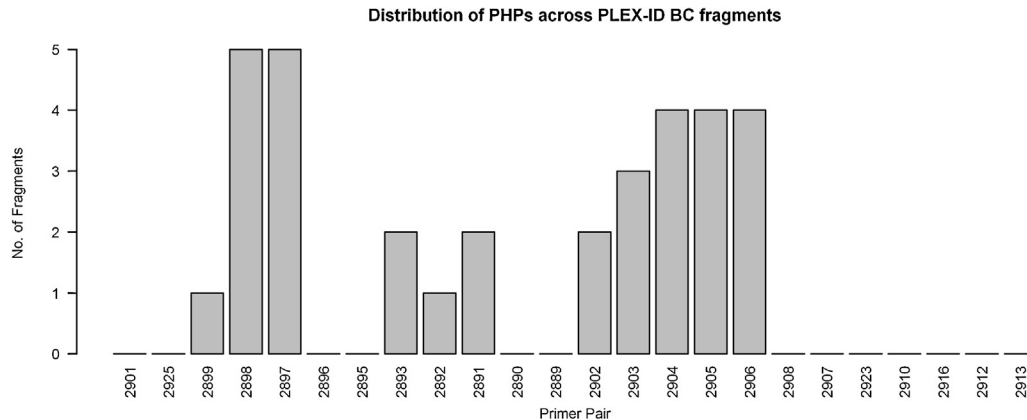
Fig. 1. Distribution of PHPs across the 24 amplicons targeted by the PLEX–ID assay, ordered 5′–3′ according to control region coordinates.

provided evidence for additional PHPs not detected or confirmed by STS. Excluding contamination the PLEX-ID based detection of PHP seems at least as sensitive as STS, in some instances even more sensitive. This has also been found in other studies [12] and seems to relate to the decreased sensitivity level of mixture detection in STS with Dye Terminator sequencing chemistry.

3.5. Length heteroplasmy (LHP)

LHP is more frequently encountered in the mtDNA CR than PHP and refers to the presence of multiple length variants in a single donor sample. Homopolymeric C-tracts are prone to LHP when more than 8 residues of a nucleotide are present in an uninterrupted stretch, e.g. around ntps 16,189, 310, and 573 in the CR [16]. The mechanisms are poorly understood and likely involve strand slippage phenomena introduced by the polymerase. Also other regions show LHP, such as the dimeric repeat region between positions 513 and 524, the T-tract between positions 451 and 456 and random positions when mixtures with indels are present. Most of the LHPs are instable between maternal members of a pedigree and even between tissues of an individual [16] and therefore not regarded as exclusive evidence in forensic mtDNA testing. It has been reported that the detection of LHP varies with respect to amplification and sequencing parameters [17], which is why variation in such regions either is completely disregarded or the dominant variant is called [11].

For the purpose of comparing LHP between the two technologies applied here, LHP was also determined in STS data by counting

the number of indicator bases at positions 16,196, 310 and 577 for the three hypervariable segments, respectively. In our sample set 25 (12.6%) samples displayed LHP in the HVS-I C-tract between positions 16,183 and 16,194 with the PLEX-ID assay (covered by amplicons 2893, 2895, and 2896). This was fully confirmed by STS data (Table S1). The HVS-II C-stretch between positions 302 and 310, which was also covered by three amplicons (2908, 2907, and 2906), was found to display LHP in 85 samples (42.9%) with PLEX-ID, which concurs with STS in 79 samples leaving six samples displaying LHP in the PLEX-ID assay only. Additional 35 samples showed LHP with the STS assay but not with PLEX-ID (Table 3), which may be due to differences in primers, polymerases and buffers between the technologies.

The AC dinucleotide repeat and the C-tract in HVS-III were both covered by a single amplicon (2913) in the PLEX-ID assay. Four samples showing LHP in the C-stretch were concordant between STS and PLEX-ID assays, two additional samples were detected by STS and one additional sample by the PLEX-ID assay. Differences in AC-repeat variants were more pronounced with only one overlapping sample between the two assays (Tables 3 and S6).

To examine differences in dominant C-variant calling between the two assays, all PLEX-ID BCs covering the length variation stretches HVS-I, HVS-II and HVS-III/AC were filtered for their most abundant BC measured for each amplified fragment. Overall, concordance was high between dominant length variant calls in these regions. 15 BCs out of 1386 BCs (1.08%) compared were discordant between the different assays In 11 of these BCs the dominant (most abundant) variant called by the PLEX-ID assay was

Table 3

Summary of LHP found with PLEX-ID and STS. Numbers in brackets indicate numbers of samples with low level (<20%) LHP.

	STS	PLEX-ID	PLEX-ID all fragments	PLEX-ID partial	Common
HVS-I (C-tract)	25	25	22	3	25
HVS-II (C-tract)	114 (13)[a]	85[a]	57	28	79
HVS-III (C-tract)	6	5	0	0	5
AC-Stretch	7	4	0	0	1
Total LHP	119	79	31		

[a] Not statistically significant at $\alpha = 0.05$.

HVS-I Stretch
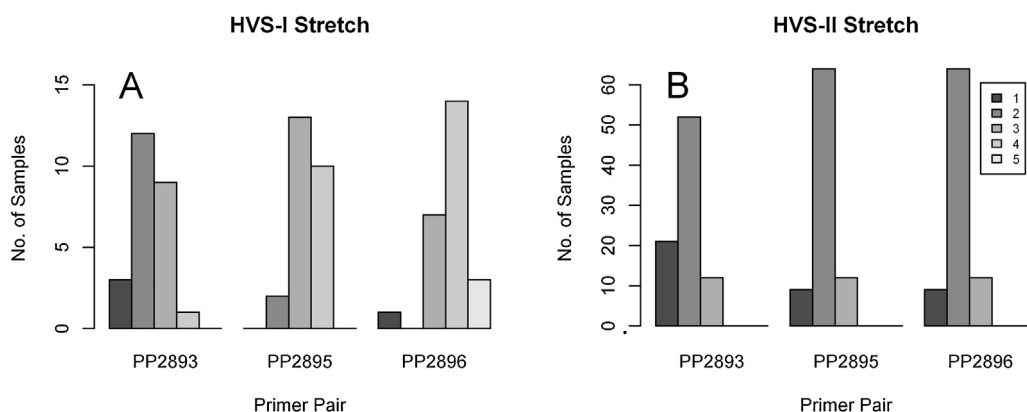
HVS-II Stretch

Fig. 2. Number of samples exhibiting different numbers of length heteroplasmic variation per amplicon covering C-stretches in HVS-I (A) and HVS-II (B).

closest to the peak representing the STS called dominant variant. In ten of these fifteen BCs, the abundance of the dominant variant called by the PLEX-ID assay lay within ±20% of the abundance of the peak representing the STS called dominant variant. These samples were repeated to see if the differences were consistent. One sample could not be analyzed further as the region of interest was not amplified in the repeat (Table S7, MUC164). For two repeated samples (Table S7, MUC12 PP29067 and MUC045 PP2895) the concordant STS variant was called as most abundant variant, whereas all other most abundant BCs called by the PLEX-ID remained the same as in the first analysis of the samples. Interestingly, nine of these reanalyzed primer pairs did not produce the same number of length variants as in the first analysis. Further studies would have to be conducted in order to show if the reason for this is decreased system sensitivity or any other cause for randomized LHP detection.

Finally, with the PLEX-ID assay the number of LHP variants was more heterogeneous in the HVS-I C-stretch (1–5) compared to the HVS-II C-stretch (2–4), which is concordant with STS data and previous findings using fragment size analysis [11] (Fig. 2).

4. Conclusions

The PLEX-ID system has been described and evaluated previously as a reliable and useful tool for mtDNA analysis [18]. In this study, these results were confirmed in terms of concordance of the data generated by the mtDNA Assay v2.0 on the PLEX-ID platform with STS data. Furthermore, this study shows that using PLEX-ID BCPs for population statistics is not only viable, but only results in a 1.5% loss of resolution compared to conventional STS analysis. This loss is counterbalanced by the high degree of automation of the system, encompassing shorter analysis time and sample traceability, as well as the potential for high throughput analysis. In terms of point heteroplasmy detection, the PLEX-ID system seems slightly more sensitive than conventional STS analysis, bearing in mind that automated detection levels are highly dependent on software settings in the PLEX-ID assay. LHP detection can be described as highly concordant in HVS-I, with more pronounced differences in HVS-II length variant detection, with the PLEX-ID system detecting less LHP variation in this region. Concordance of dominant variants called in both assays was high. Overall, the PLEX-ID mtDNA CR typing system has the potential to be an equivalent alternative to STS, offering a cost and time reducing, ease-of-use, standardized, automated sample preparation and analysis. The PLEX-ID instrument and chemistry are no longer commercially available and the service for forensic genetics has been discontinued. However, this does not diminish the scientific concept and methodology presented in this manuscript. On the contrary, our findings indicate the usefulness

of MS-based applications for mitochondrial forensics and continue to support its utility.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2013.05.007.

References

[1] A. Brandstätter, T.J. Parsons, W. Parson, Rapid screening of mtDNA coding region SNPs for the identification of west European Caucasian haplogroups, Int. J. Legal Med. 117 (2003) 291–298.

[2] M.N. Gabriel, C.D. Calloway, R.L. Reynolds, S. Andelinovic, D. Primorac, Population variation of human mitochondrial DNA hypervariable regions I and II in 105 Croatian individuals demonstrated by immobilized sequence-specific oligonucleotide probe analysis, Croat. Med. J. 42 (2001) 328–335.

[3] H. Andreasson, A. Asp, A. Alderborn, U. Gyllensten, M. Allen, Mitochondrial sequence analysis for forensic identification using pyrosequencing technology, Biotechniques 32 (2002) 124–133.

[4] T.A. Hall, B. Budowle, Y. Jiang, L. Blyn, M. Eshoo, K.A. Sannes-Lowery, R. Sampath, J.J. Drader, J.C. Hannis, P. Harrell, V. Samant, N. White, D.J. Ecker, S.A. Hofstadler, Base composition analysis of human mitochondrial DNA using electrospray ionization mass spectrometry: a novel tool for the identification and differentiation of humans, Anal. Biochem. 344 (2005) 53–69.

[5] T.A. Hall, K.A. Sannes-Lowery, L.D. McCurdy, C. Fisher, T. Anderson, A. Henthorne, L. Gioeni, B. Budowle, S.A. Hofstadler, Base composition profiling of human mitochondrial DNA using polymerase chain reaction and direct automated electrospray ionization mass spectrometry, Anal. Chem. 81 (2009) 7515–7526.

[6] P. Forster, To err is human, Ann. Hum. Genet. 67 (2003) 2–4.

[7] W. Parson, A. Dür, EMPOP-a forensic mtDNA database, Forensic Sci. Int. Genet. 1 (2007) 88–92.

[8] W. Parson, A. Brandstätter, A. Alonso, N. Brandt, B. Brinkmann, A. Carracedo, D. Corach, O. Froment, I. Furac, T. Grzybowski, K. Hedberg, C. Keyser-Tracqui, T. Kupiec, S. Lutz-Bonengel, B. Mevag, R. Ploski, H. Schmitter, P. Schneider, K. Szafranski, D. Syndercombe-Court, H. Thew, G. Tully, R. Scheithauer, The EDNAP mitochondrial DNA population database (EMPOP) collaborative exercises: organisation, results and perspectives, Forensic Sci. Int. 139 (2004) 215–226.

[9] A. Salas, A. Carracedo, V. Macaulay, M. Richards, H.J. Bandelt, A practical guide to mitochondrial DNA error prevention in clinical, forensic, and population genetics, Biochem. Biophys. Res. Commun. 335 (2005) 891–899.

[10] W. Parson, H.J. Bandelt, Extended guidelines for mtDNA typing of population data in forensic science, Forensic Sci. Int. Genet. 1 (2007) 13–19.

[11] C. Berger, P. Hatzer-Grubwieser, C. Hohoff, W. Parson, Evaluating sequence-derived mtDNA length heteroplasmy by amplicon size analysis, Forensic Sci. Int. Genet. 5 (2011) 142–145.

[12] H. Oberacher, H. Niederstätter, W. Parson, Liquid chromatography-electrospray ionization mass spectrometry for simultaneous detection of mtDNA length and nucleotide polymorphisms, Int. J. Legal Med. 121 (2007) 57–67.

[13] J.A. Irwin, J.L. Saunier, H. NiederstatterNiederstätter, K.M. Strouss, K.A. Sturk, T.M. Diegoli, A. Brandstätter, W. Parson, T.J. Parsons, Investigation of hetero-plasmy in the human mitochondrial DNA control region: a synthesis of obser-vations from more than 5000 global population samples, J. Mol. Evol. 68 (2009) 516–527.

[14] R.J. Steighner, L.A. Tully, J.D. Karjala, M.D. Coble, M.M. Holland, Comparative identity and homogeneity testing of the mtDNA HV1 region using denaturing gradient gel electrophoresis, J. Forensic Sci. 44 (1999) 1186–1198.

[15] H. Oberacher, H. Niederstätter, C.G. Huber, W. Parson, Accurate determination of allelic frequencies in mitochondrial DNA mixtures by electrospray ionization time-of-flight mass spectrometry, Anal. Bioanal. Chem. 384 (2006) 1155–1163.

[16] S. Lutz-Bonengel, T. Sänger, S. Pollak, R. Szibor, Different methods to determine length heteroplasmy within the mitochondrial control region, Int. J. Legal Med. 118 (2004) 274–281.

[17] J. Naue, T. Sänger, U. Schmidt, R. Klein, S. Lutz-Bonengel, Factors affecting the detection and quantification of mitochondrial point heteroplasmy using Sanger sequencing and SNaPshot minisequencing, Int. J. Legal Med. 125 (2011) 427–436.

[18] D.H. Warshauer, J. King, A.J. Eisenberg, B. Budowle, Validation of the PLEX-ID(TM) mass spectrometry mitochondrial DNA assay, Int. J. Legal Med. 127 (2013) 277–286.

[19] R Development Core Team, R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, 2011, ISBN 3-900051-07.