International Congress Series 1288 (2006) 1–3

www.ics-elsevier.com

Characterization of mtDNA SNP typing using quantitative real-time PCR with special emphasis on heteroplasmy detection and mixture ratio assessment

H. Niederstätter [a], M.D. Coble [b,c], T.J. Parsons [b], W. Parson [a,*]

[a] *Institute of Legal Medicine, Innsbruck Medical University, Müllerstrasse 44, 6020 Innsbruck, Austria*
[b] *Armed Forces DNA Identification Laboratory, Armed Forces Institute of Pathology, Rockville, MD, USA*
[c] *Biotechnology Division, National Institute of Standards and Technology, Gaithersburg, MD, USA*

Abstract. Using a plasmid DNA model and total genomic DNA (gDNA) reference samples, we examined the performance and forensic utility of a quantitative allele-discriminatory real-time PCR assay relying on TaqMan probes targeting the mitochondrial single nucleotide polymorphism (mtSNP) 16519T/C. For allele-calling and allele proportion assessment we used absolute quantification of both alleles as well as relative quantification based on endpoint fluorescence.

Keywords: Allele-discriminatory rtPCR; Allele proportion assessment; Forensic science

1. Introduction

The major limitation of forensic mitochondrial DNA (mtDNA) typing is the low power of discrimination that is obtained when common haplotypes are present. Current mtDNA testing typically targets one or two hypervariable regions in the non-coding control region of the human mitochondrial genome by sequencing. It is now increasingly recognized that assays targeting mtSNPs are well suited for gaining additional information in mtDNA testing. Our interest in real-time PCR (rtPCR) for mtSNP typing with duplexed allele-specific TaqMan probes targeting the highly informative mtSNP 16519T/C related to a number of potential advantages, such as quantitative nature, high sensitivity, broad linear

* Corresponding author. Tel.: +43 512 507 3303; fax: +43 512 507 2764.
E-mail address: walther.parson@uibk.ac.at (W. Parson).

0531-5131/
doi:10.1016/j.ics.2005.09.021

dynamic range, and homogeneous assay format. The multicolor capability of rtPCR instruments enables the simultaneous interrogation of both SNP alleles, which is particularly useful, as mtDNA has the potential to manifest single contributor mixtures in continuously varying proportions.

2. Materials and methods

We performed a concordance study on 405 total genomic DNA (gDNA) samples from 135 paternity trios from Tyrol (Austria) with known control region sequences using differently labeled (16519C: FAM; 16519T: VIC) allele-specific TaqMan hybridization probes with an attached minor groove binding moiety (MGB) and an Applied Biosystems 7700 sequence detection system. Protocols regarding PCR, rtPCR, cloning, and sequencing can be found in [1] and [2].

The cloned and sequence-verified alleles p16519T and p16519C were quantified and used as a pDNA model. Simultaneous absolute quantification of both alleles was based on threshold cycle (C_T) values with reference to the parameters of the plasmid DNA (pDNA) calibration curves. For relative quantification we used the percentage contribution of the MGB16519C TaqMan probe to the total PCR-related endpoint signal [$\%\Delta Rn_{FAM} = \Delta Rn_{FAM}/(\Delta Rn_{FAM}+\Delta Rn_{VIC})$]. For allele calling and to deduce the 16519T/C allele proportions from measured %ΔRn values, a calibration curve comprising defined mixtures of p16519T and p16519C (50,000 plasmids/reaction) was prepared.

For three heteroplasmic and two homoplasmic gDNA reference samples–as determined by sequencing analysis and rtPCR–the base state at nucleotide position 16519 was confirmed by cloning PCR products containing the SNP site and subsequent rtPCR typing of approximately 300 colonies per sample.

Automated data analysis was performed by means of an MS Excel spreadsheet template.

3. Results and discussion

The results of a concordance study on reference gDNA samples from 405 persons with known mitochondrial control region sequences [2] showed that both alleles of the mtSNP 16519 can be reliably and simultaneously typed with the TaqMan approach using either absolute [1] or relative quantification for data analysis.

For both base states the linear dynamic range covered at least 5 orders of magnitude with a sensitivity of approximately 10 double stranded DNA template molecules. Along with the short amplicons that are obtained (65 base pairs), this enables the analysis of a broad spectrum of samples differing in DNA quantity and quality under identical technical conditions.

The apparent single cycle PCR efficiencies for both alleles were close to 100% during the exponential phase of the rtPCR. As differences between the PCR efficiencies obtained for circular pDNA standards and genomic DNA were statistically insignificant ($p_\alpha > 0.05$), pDNA standard curves were used for absolute quantification.

Using absolute quantification for the assessment of allelic proportions, minor alleles could be detected down to the 10% level [1]. Correction of the systematic inter-well variation of C_T values we observed in initial experiments did not improve the performance of the assay (data not shown). However, when corrected %ΔRn values were used for data analysis, the minor component in defined mixtures of p16519T and p16519C (50,000 plasmids/reaction) could be detected and quantified

reliably down to the 5% level for both alleles without a need for replicate reactions. Heteroplasmy detection at the 2.5% level was possible when samples were run in replicates. This finding was also consistent with the calculated limits of detection (quantification) of ~2% (4%) minor contribution for both alleles (values based on corrected %ΔRn values). Using uncorrected %ΔRn values, the limits of detection (quantification) were ~3% (9%) and ~4% (13%) for minor T and C alleles, respectively. The mixture ratios found for three heteroplasmic (~2%, 6%, and 21% minor C allele) and two homoplasmic gDNA samples with the endpoint signal rtPCR approach were concordant with the results obtained by typing approximately 300 cloned PCR fragments per sample and those derived from the ratios of the peak heights in forward and reverse strand sequencing traces. The 2% 16519C minor allele was not detectable with Sanger type sequencing and absolute quantitative allele-discriminatory rtPCR [1]. However, neither cloning nor Phred quality value assisted analysis of sequencing traces facilitates automated detection and quantification of low level mixtures/point-heteroplasmy in real-world applications.

As in forensic casework specimens frequently contain only minute amounts of amplifiable DNA, we attempted to determine the minimum number of template molecules needed for accurate mtSNP typing and determination of mixture ratios by analyzing serial dilutions (50,000–100 plasmids/reaction) of defined mixtures (0%, 5%, 10% and 50% minor component) of both cloned alleles, and of homo/heteroplasmic gDNA reference samples. For quadruplicate measurements of the pDNA model as well as the complex gDNA reference samples, the low-end limit for accurate heteroplasmy detection and allele proportion quantification was for all tested ratios a total of 100–200 dsDNA molecules/reaction when corrected %ΔRn values were used for data analysis.

These results demonstrate the utility of the presented TaqMan approach for mtSNP typing. While absolute quantification might permit the formulation of objective criteria to distinguish genuine typing results from potential contamination, relative quantification based on endpoint fluorescence allows automated, sensitive and reliable allele calling, and detection/quantification of (heteroplasmic) mixtures. Furthermore, when absolute quantitative information is not needed, utilizing %ΔRn values for SNP typing facilitates the use of the high throughput post PCR plate-read assay format.

Acknowledgements

Part of this work was supported by the National Institute of Justice grant 2000-1J-CX-K010 to T.J.P. The opinions and assertions contained herein are solely those of the authors and are not to be construed as official or as views of the U.S. Department of Defense, the U.S. Department of the Army, or the U.S. Department of Justice.

References

[1] H. Niederstätter, et al., Characterization of mtDNA SNP typing and mixture ratio assessment with simultaneous real-time PCR quantification of both allelic states, Int. J. Leg. Med. (2005), doi:10.1007/s00414-005-0024-3, Electronic publication ahead of print.

[2] A. Brandstätter, H. Niederstätter, W. Parson, Monitoring the inheritance of heteroplasmy by computer-assisted detection of mixed basecalls in the entire human mitochondrial DNA control region, Int. J. Leg. Med. 118 (2004) 47–54.