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TECHNICAL NOTE

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Quantification of mtDNA mixtures in forensic evidence material using pyrosequencing

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Abstract Analysis of mtDNA variation using Sanger sequencing does not allow accurate quantification of the components of mtDNA mixtures. An alternative method to determine the specific mixture ratios in samples displaying heteroplasmy, consisting of DNA contributions from several individuals, or containing contamination would therefore be valuable. A novel quantification system for mtDNA mixture analysis has been developed based on pyrosequencing technology, in which the linear relationship between incorporated nucleotides and released light allows quantification of the components of a sample. Within five polymerase chain reaction fragments, seven variable positions in the mtDNA control and coding region were evaluated using this quantification analysis. For all single nucleotide polymorphisms quantified in this study, a linear relationship was observed between the measured and expected mixture ratios. This mtDNA quantification assay is an easy to use, fast and accurate quantification system, with the ability to resolve and interpret major and minor mtDNA components in forensic mixture samples.

Keywords Mixtures · Mitochondrial DNA · Pyrosequencing · Quantification · Forensic material

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Introduction

Forensic samples can be composed of a mixture of DNA from more than one individual, making the interpretation of typing results more complex. In analysis of multiple autosomal STR markers, more than two peaks or unequal peak areas being apparent for at least some markers indicates a mixture. In many cases, it is also possible to determine the profiles of a minor and a major contributor based on differences in allele peak area [1]. Forensic materials that contain very minute amounts or degraded DNA may not yield a STR result. In such situations, a mitochondrial DNA (mtDNA) analysis can be attempted [2–4]. The routinely used Sanger sequence analysis of mtDNA is robust and often successful due to the high copy number of mtDNA per cell [5]. However, the results of an mtDNA analysis may occasionally show a DNA mixture as a consequence of multiple contributors, heteroplasmy or contamination [6]. In contrast to STR analysis, quantification of mixed samples based on mtDNA sequence analysis is not feasible using the current sequencing methodology. The ABI PRISM BigDye Primer Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) is commonly used for mtDNA analysis. Although this chemistry can be used to easily and effectively determine the sequence in single-source samples, the uneven peak heights and sequence-dependent variation in dideoxynucleotide incorporation efficiency prevent reliable quantification of mixtures [7, 8]. Thus, mixtures can be detected and visualised using Sanger sequencing, but the information cannot be used to determine the relative quantities of the different mitochondrial types, and in most cases excluding heteroplasmy, the results are interpreted as inconclusive. Resolution of mtDNA mixtures has been demonstrated previously using alternative technologies such as cloning, denaturing high-performance liquid chromatography, fluorescent-labelled restriction fragments and real-time polymerase chain reaction (PCR) [9–13]. An alternative method to resolve and accurately quantify multiple mtDNA species contributing to a sample is pyrosequencing.

Table 1 PCR and pyrosequencing primers for quantification of seven single nucleotide polymorphism (SNP) positions

SNP positions	Primer	Sequence (5′–3′)	Fragment size
73	II 45 F	ATGCATTTGGTATTTTCGTCTG	
146	II 111 F[a]	ACCCTATGTCGCAGTATCT	
195	II 162 F[a]	CGCACCTACGTTCAATATTACA	
	II 287 R-B	TTGTTATGATGTCTGTGTGGAAAG	243 bp
3010	C 2988 F	CGATGTTGGATCAGGACA	
	C 3216 R-B	GGGTGGGTGTGGGTATAA	229 bp
7028	C 6990 F	CTAGACATCGTACTACACGACA	
	C 7008 F[a]	GACACGTACTACGTTGTAGC	
	C 7299 R-B	TTACTGCTGTTAGAGAAATGAA	310 bp
12372	C 12346 F	CACACTACTATAACCACCCTAA	
	C 12541 R-B	CTCAGTGTCAGTTCGAGATAA	196 bp
14766	C 14747 F	ATGACCCCAATACGCAAA	
	C 14949 R-B	TGGGCGATTGATGAAAAG	203 bp

II Hypervariable region II of the mtDNA control region, *F* forward, *R* reverse, *B* biotinylated 5′ end, *C* coding region
[a]Internal pyrosequencing primer

Pyrosequencing is a technology based on the release of pyrophosphate during strand elongation, which through an enzymatic cascade process produces light when a nucleotide is incorporated into the elongating DNA polymer. The light signal is proportional to incorporated nucleotides and can be quantified for each site utilising the PSQ 96MA SNP Software (version 2.02, Biotage, Uppsala, Sweden). The allele quantification capability has been previously demonstrated in a number of studies, including allele frequency measurements in pooled DNA samples [14, 15] and quantitative analysis of methylation status at CpG islands [16]. Other studies involved gene copy number determination [17, 18] and determination of allele-specific transcript expression [19].

In this study, PCR fragments previously utilised for a fast and simple pyrosequencing analysis of variation in the mtDNA control and coding regions were used to assess pyrosequencing-based quantification [20]. Seven polymorphic sites, three in the control region and four in the coding region, within five PCR fragments were selected and used to successfully quantify the mtDNA component species in mixture samples.

Materials and methods

PCR design

Three highly variable mitochondrial control region positions (73, 146 and 195) within a single PCR fragment were selected for the mixture quantification studies. In the Swedish population, the frequency of the 73G variant is 56%, 146C is 14% and 195C is 19% (based on analysis of 350 D-loop sequences, unpublished results). In addition, four coding region fragments containing highly variable sites at positions 3010, 7028, 12372 and 14766 were evaluated. Among 967 Caucasian coding region sequences, the frequency of the 3010A variant is 23%, 7028T is 45%, 12372A is 17% and 14766T is 51% (http://www.genpat.uu.se/mtDB/). Primers for PCR and pyrose-

Table 2 SNP variants and dispensation orders

SNP	Sequence (5′-3′)[a]	Dispensation order in SNP region[b]
73	GGGGGT**G**/**A**TGC	**C**GGTGA**C**TGC
146	GTCTTTGATTCCTGCC**T**/**C**CA	**C**GTCTGATCTGCTC**G**A
195	GGCGAACATAC**T**/**C**TA	**T**GCGACATACT**G**A
3010	TCCC**G**/**A**ATGGT	**G**TCGA**C**TGT
7028	**C**/**T**CACTTCCA	**G**TC**G**ACTCA
12372	CCCT**G**/**A**ACTTCCC	**G**CTGA**G**CTC
14766	A**C**/**T**TAACCCCCT	**G**ACT**G**ACT

[a]The SNP position is shown in bold
[b]Two additional control dispensations per reaction are shown in bold

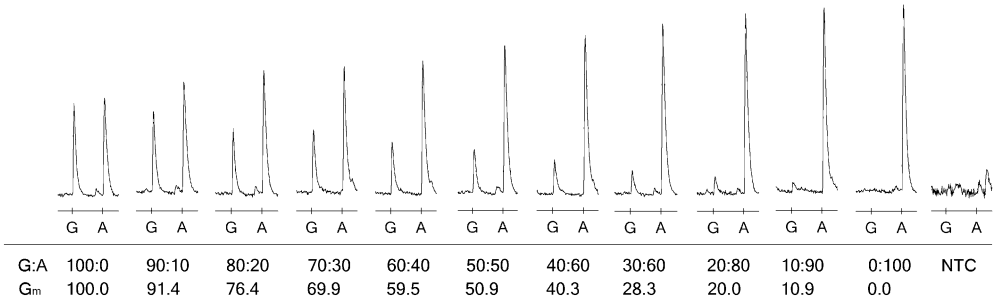

Fig. 1 Pyrosequencing-based quantification of SNP 3010 in the coding region mixture standard. Only the SNP position is shown from pyrograms of the different mixtures and a no-template control (NTC). The mixture standard ratio (G:A) and the experimentally measured outcomes of the G variant (G_m) are shown under the individual pyrograms

quencing were designed using the Primer Express software version 1.0 (Applied Biosystems). Previously designed PCR and sequencing primers were used for quantification of all SNPs except for site 7028 [20], where an internal sequencing primer was designed. All reverse PCR primers were 5′-labelled with a biotin molecule (Table 1).

DNA samples

DNA from the control individuals was extracted using a salting out procedure from peripheral blood lymphocytes [21]. Control samples for the mixture studies were selected from a database of 350 Swedish individuals, all sequenced for the mtDNA D-loop and a subset sequenced for mtDNA coding region. Two individuals, with the mtDNA variants 73G, 146C, 195C and 73A, 146T, 195T, respectively, were selected for the construction of a control region mixture standard. For the coding region SNPs, a mixture standard was generated using one individual carrying the 3010A, 7028C, 12372G and 14766C variants and another individual with the 3010G, 7028T, 12372A and 14766T variants. The DNA concentrations of the individual samples were determined by measuring six replicates using the NanoDrop ND 1000 spectrophotometer and NanoDrop 2.4.7c software (NanoDrop Technologies Inc., Wilmington, DE). Samples were diluted to a final concentration of 10 ng/μl. DNA from the two control individuals for each of the two mixture standards (control and coding region) were mixed in ratios of 0:100, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10 and 100:0. The mixtures were made in a total volume of 200 μl, and the final mixture standard DNA concentration of 10 ng/μl was verified by measurements of four replicates using the NanoDrop ND 1000 spectrophotometer. Although mtDNA quantification is not routinely performed in most forensic laboratories, quantification of mtDNA and nDNA using a previously described real-time PCR assay of the mixture standard series was performed [22]. The results were demonstrating consistency between mtDNA quantity and nDNA quantity in all mixtures of different ratios and unmixed samples. Furthermore, the total amount of nuclear DNA in unmixed samples and all mixtures in all ratios corresponded well with the NanoDrop quantification.

Amplification

Amplification of the mixture standard series was performed in a total volume of 35 μl containing 1× Gene-Amp PCR Buffer II (Applied Biosystems), 1.5 mM $MgCl_2$, 0.2 mM of the different deoxynucleotide triphosphates (dNTPs), 0.2 μM of each PCR primer, 1 U AmpliTaq Gold DNA polymerase, and 1 μl DNA (10 ng/μl). The amplification was performed in a GeneAmp PCR System 9600 (Applied Biosystems) with 10 min incubation at 95°C followed by

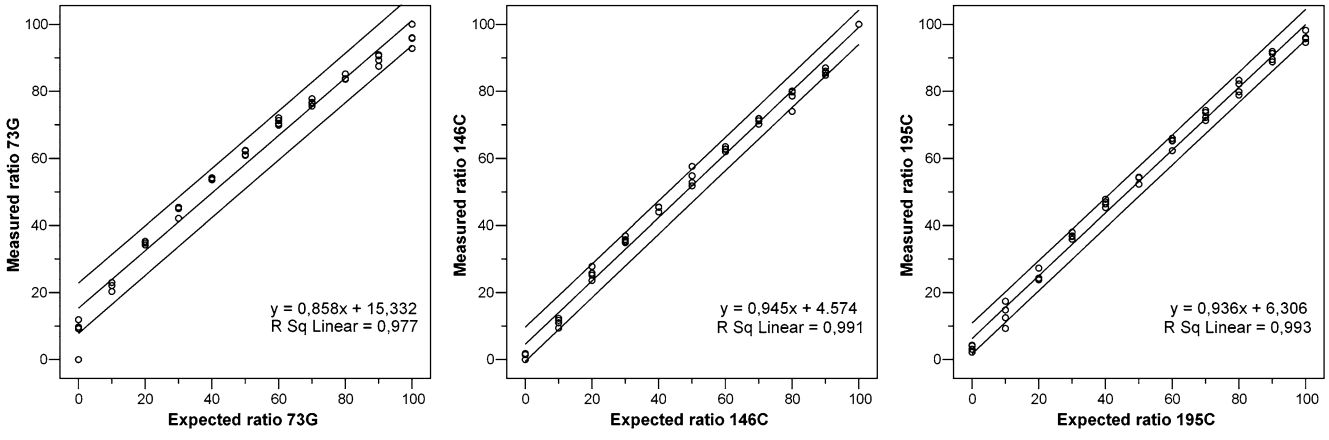

Fig. 2 Linear regression of the control region SNPs analysed in four replicates. The 95% confidence interval, based on individual estimates, is indicated in the figure

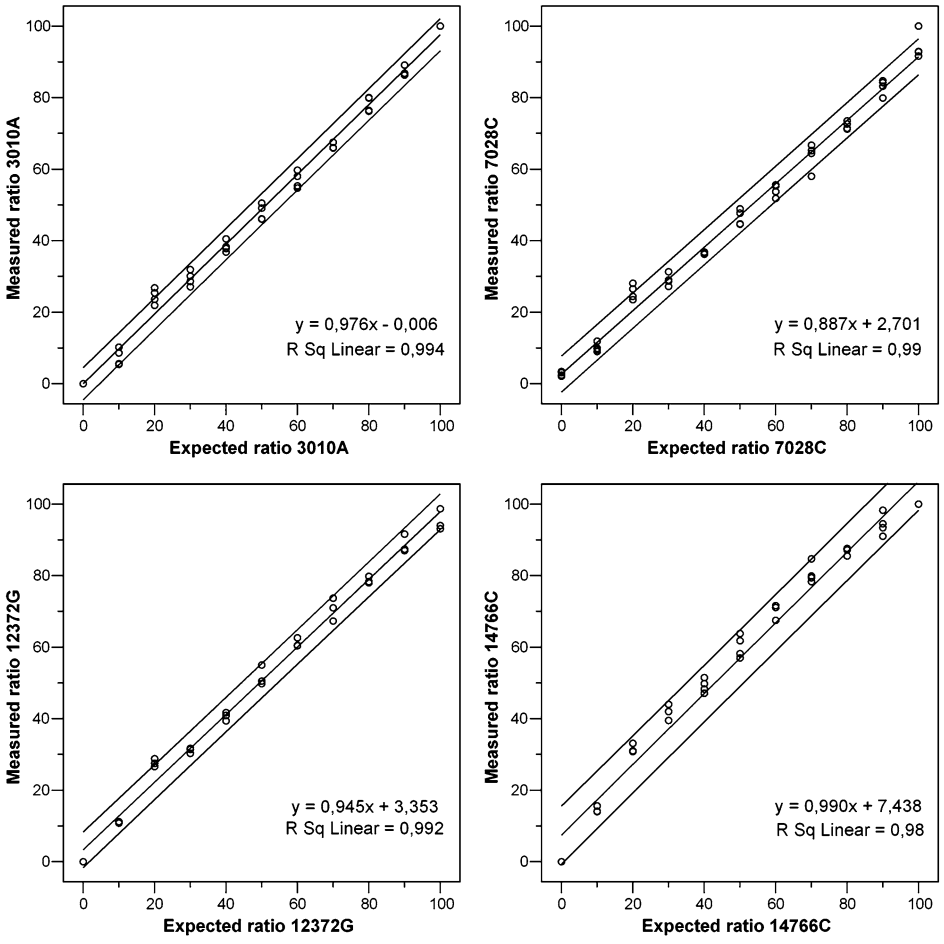

Fig. 3 Linear regression of the coding region SNPs analysed in four replicates. The 95% confidence interval, based on individual estimates, is indicated in the figure

cycling of 30 s at 95°C, 45 s at 53°C and 60 s at 72°C for 45 cycles. Finally, a 7-min elongation step at 72°C was carried out.

Pyrosequencing and mixture quantification

Using the PSQ 96 Sample Preparation Kit (Biotage), single-stranded DNA was purified from 25 μl of biotinylated PCR product through binding of the biotinylated strand to streptavidin-coated Sepharose beads. Purification was followed by annealing of 0.33 μM primer to 45 μl of the single-stranded DNA. Pyrosequencing quantification was performed using the PSQ96 Reagent Kit (containing enzyme, substrate, and dNTPs) in a PSQ 96MA system (Biotage). To the 73, 146, 12372 and 14766 SNP reactions, 2 μl (440 ng) SSB (single-stranded DNA binding protein, Amersham Biosciences, Uppsala, Sweden) was added. For

Table 3 Average measured mixture ratios and standard deviation values for all SNPs analysed in four replicate experiments

Expected	73G		146C		195C		3010A		7028C		12372G		14766C	
Mix ratio	Av.	SD	Av.	SD	Av.	SD	Av.	SD	Av.	SD	Av.	SD	Av.	SD
0:100	10.3	1.4	0.8	1.0	3.4	1.0	0.0	0.0	2.8	0.6	0.0	0.0	0.0	0.0
10:90	22.1	1.3	11.1	1.3	13.5	3.4	7.5	2.3	10.1	1.3	11.0	0.2	14.5	0.8
20:80	34.6	0.5	25.6	1.7	24.9	1.6	24.4	2.1	25.6	2.1	27.6	1.1	31.6	1.3
30:70	44.2	1.8	35.7	0.9	36.9	0.9	29.4	2.1	29.1	1.7	31.1	0.7	41.8	2.3
40:60	53.9	0.3	45.1	0.7	46.6	1.1	38.4	1.6	36.5	0.3	40.7	1.2	49.2	1.9
50:50	61.7	0.8	54.3	2.6	53.8	1.0	47.9	2.2	46.5	2.2	51.8	2.8	60.2	3.1
60:40	70.9	1.0	62.8	0.6	64.7	1.7	56.9	2.3	54.1	1.7	61.2	1.2	70.1	2.2
70:30	76.6	0.9	71.3	0.8	72.9	1.4	66.5	0.9	65.4	1.2	72.4	1.9	80.6	2.8
80:20	84.1	0.8	78.1	2.8	81.1	2.0	78.1	2.1	72.2	1.1	78.7	1.0	86.9	1.0
90:10	89.6	1.5	85.8	0.9	90.4	1.5	87.2	1.3	83.0	2.2	88.7	2.5	94.3	3.0
100:0	96.2	3.0	100.0	0.0	96.1	1.5	100.0	0.0	92.4	0.7	95.3	3.0	100.0	0.0

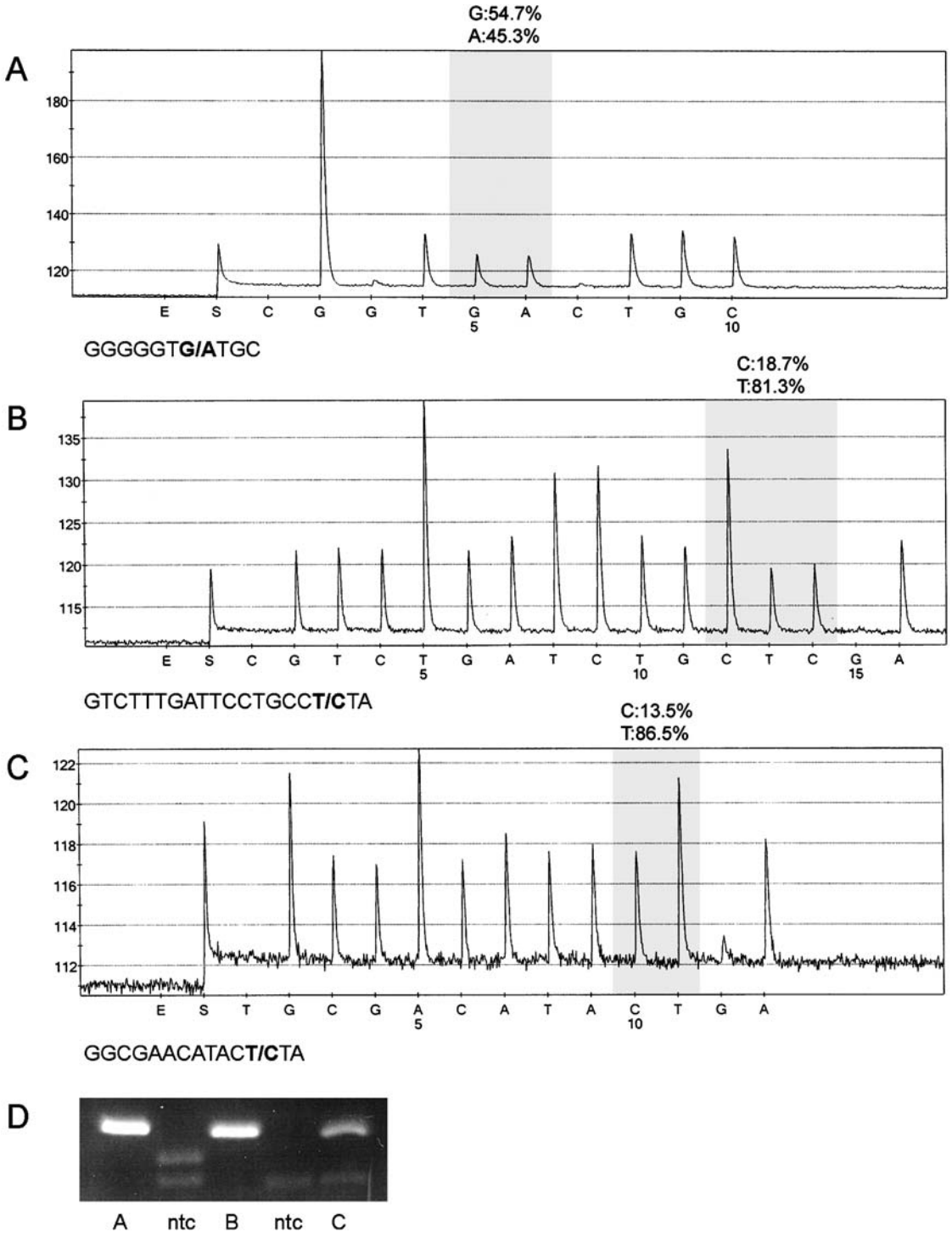

Fig. 4 Pyrosequencing mixture quantification at three control region SNP positions in three different forensic evidence materials. **a** DNA extracted from the lining of a wig. SNP 73 reveals, after correction to the standard curve, 46% G (95% CI, 36–56). **b** DNA extracted from a bandage. SNP 146 reveals 19% C (95% CI, 12–26). **c** DNA extracted from a piece of duct tape. SNP 195 reveals 14% C (95% CI, 8–20). **d** Agarose gel image of PCR products from the three different forensic samples and no-template controls

optimal quantification results, the most favourable dispensation order of nucleotides was determined (Table 2). The quantification was performed using the PSQ 96MA SNP Software version 2.02 (Biotage), and the results were calculated using the AQ mode. The software calculates the proportion of each nucleotide added during the synthesis reaction [minimum, maximum, mean frequency and standard deviation (SD)] and provides an overall quality label for each sample ranging from "passed" or "check" to "failed." The quality label check is shown, for example, if

the substrate peak is too high or if the initial baseline is abnormal. Samples showing low signal-to-noise ratios or wide peaks will show a quality label of check or failed. The data were exported to Microsoft Excel and SPSS (Version 12.0.1 SPSS Inc., Chicago, IL) for further statistical analysis.

Results

The two prepared mixture standard series, the control region standard and coding region standard, were quantified for the different ratios of the two variants of the seven selected SNPs. In Fig. 1, an example of quantification of the 3010 SNP is shown, illustrating that one of the SNP variants is slowly replaced (peak height relations are changing) by the other variant (here G to A) in these 11 mixtures consistent with their ratios. The standard mixture ratios and the experimentally measured quantity of the G variant in the mixture are shown. The A variant of the SNP is a part of a homopolymeric stretch with an A following the SNP (TCCCG/AATGGT). This is, as expected, resulting in unequal peak heights of the two variants in the 50:50 mixture.

For evaluation of the quantification precision, the measured mixture ratios in four replicate experiments were plotted against the expected mixture ratios (Figs. 2 and 3). Quantification of SNP 146 within the control region fragment showed a linear regression with a regression coefficient of 0.95×, an intercept of 4.6 and an R^2 of 0.99, although one of the variants is part of a four-nucleotide homopolymeric tract (GTCTTTGATTCCTGCCT/CCA). SNP 195 has an identical flanking nucleotide for both of its variants that, in theory, could complicate quantification (GGCGAACATACT/CTA). Despite this, the regression coefficient, the intercept and the R^2 value were 0.94×, 6.3 and 0.99, respectively. The quantification of SNP 73 (GGGGGTG/ATGC) resulted in a regression coefficient of 0.86×, an intercept of 15.3 and an R^2 value of 0.98 (Fig. 2). Although the R^2 value was close to 1 for this SNP, the slope of the linear regression and the intercept was not optimal. This might be corrected by an alternative assay design or by using the linear regression equation to adjust the unknown sample quantity according to the standard curve. Quantification results of SNP 146 and 73 were slightly improved by addition of SSB to the pyrosequencing reactions, while the quantification results of SNP 195 were optimal and did not require addition of SSB.

Quantification of the coding region SNP 3010 (TCCCG/AATGGT) showed a linear regression coefficient of 0.98×, an intercept of 0.0 and an R^2 value of 0.99. For SNP 7028, a new internal pyrosequencing primer had to be designed. Quantification results using the previously designed PCR primer were suboptimal, giving a regression coefficient of 0.65×. With the use of the new primer, the regression coefficient, the intercept and the R^2 value were 0.89×, 2.7 and 0.99, respectively, for this SNP (C/TCACTTCCA). SNP 12372 (CCCTG/AACTTCCC) resulted in a regression coefficient of 0.95×, an intercept of 3.4 and an R^2 value of 0.99 after addition of SSB. Also, SNP 14766 (AC/TTAACCCCCT) required addition of SSB to minimize a tendency for overestimated measurements of the C variant. With addition of SSB, a regression coefficient of 0.99×, an intercept of 7.4 and an R^2 value of 0.98 were observed (Fig. 3).

The linear regression coefficients for six of the seven different SNPs (excluding SNP 73) ranged between 0.89 and 0.99×, the intercepts between 0.0 and 7.4, and all the R^2 values were 0.98 or 0.99. This demonstrates that quantification of mixtures using this system and these SNPs can be done with high accuracy. In Table 3, the average measurements of the different mixture ratios are shown together with the corresponding SD values. The average SD per SNP varies between 1.2 and 1.7, ranging between 0.0 and 3.4.

The mixture quantification assay was further tested on a few nonprobative forensic materials displaying mixtures in mtDNA sequence analysis. The samples showed mixtures at several positions, but most sites involved rare variants. Therefore, only one SNP in each sample was analysed from the set of SNPs evaluated in this study. DNA material collected from the lining of a wig, a bandage and a piece of a duct tape were quantified by the real-time PCR to contain 1,810, 90 and 15 copies of input mtDNA, respectively (5/100 μl extract used in PCR). In Fig. 4, mixture ratios at the three control region SNPs (73, 146 and 195) are illustrated in analysis of the different samples. As SNP 146 and 195 are parts of homopolymeric stretches, the total peak heights at the positions involving the SNP do not correspond to the actual mixture ratio. The sample from the duct tape was from evidence material in a murder investigation from 1996. Although the amplification product from this old and limited material was weaker than for the other two materials, the mixture quantification analysis of all materials was successful and consistent with the detection of positions with mixtures in the sequence analysis.

Discussion

The pyrosequencing technology can be used for a wide range of different applications. Previously, pyrosequencing for sequence analysis of short stretches of mtDNA in forensic analysis was demonstrated [20, 23]. One of the advantages pyrosequencing technology offers over many other SNP detection systems is that it can yield a quantitative measure of multiple nucleotide variants at any site being assayed. In this study, we have utilised this quantification mode for determination of mtDNA contribution in simulated and real forensic samples, with one or several sites displaying mixtures in sequence analysis.

An assay for mixture quantification should be considered as an adjunct analysis to Sanger sequencing or other mtDNA typing assays. Forensic samples are often found in minute amounts and cannot be analysed many times. Therefore, amplicons previously generated for sequence analysis were also used in this study for quantification of certain SNP positions to consume less of the precious

evidence material [20]. For best quantification results, amplicons longer than 200 nucleotides and SNPs that are part of a homopolymer exceeding two nucleotides should, if possible, be avoided (manufacturer's instructions, Biotage). Furthermore, the SNP of interest should be located as close to the sequencing primer as possible. To achieve the dual use of the pyrosequencing system and due to the fact that highly polymorphic mtDNA variants are limited, some of the chosen SNPs reside within a homopolymeric tract, and most PCR fragments are longer than 200 base pairs. Despite this, five of the seven SNPs used in the system could be successfully quantified, while a correction factor could be applied for one SNP, and one SNP required design of an internal sequencing primer closer to the variable position. In the analysis of the two mixture series, linear relationships were seen for the SNPs quantified. The average SDs of each of the seven SNPs fell within 1–2% (for ten replicate reactions) which is expected for assays designed according to the manufacturer's instructions (Biotage). Consequently, this study illustrates that a mixture quantification of SNPs can, in most cases, be performed using previously developed PCR targets and primers often without redesign, thus saving time and valuable evidence material.

To limit reagent costs and handling, the non-biotinylated PCR primer was used as the pyrosequencing primer in the mixture quantification analysis. However, for SNP 7028, an internal pyrosequencing primer had to be designed for optimal quantification results. This SNP is located 17 nucleotides from the PCR primer, resulting in a 20-nucleotide-long dispensation order. Furthermore, one of the SNP variants is a part of a homopolymeric tract with a C nucleotide both prior to and following the variable position. By placing the internal sequencing primer next to the SNP, the homopolymer was limited to one identical flanking nucleotide, facilitating quantification. Initial analyses of SNP 14766 resulted in a high intercept due to higher-than-expected measurements of the C variant, likely related to the downstream homopolymeric C stretch (data not shown). However, addition of SSB overcame this problem, resulting in accurate and reliable quantification. Also, SNP 73 resulted in overestimated measurements for the G variants and a high intercept. SNP 73 has no flanking nucleotides identical to either of the two SNP variants and is located only a few nucleotides from the primer and should thus be an optimal SNP for quantification. However, a five-nucleotide-long G stretch immediately prior to the SNP site might have complicated the quantification in this case. For this SNP, the addition of SSB only slightly improved the results, and a corrective calculation had to be applied for quantification of unknown samples.

In forensic case-work investigations, it would be highly desirable to differentiate a DNA sample with multiple contributors from a single contributor sample displaying heteroplasmy. Pairwise comparisons of mtDNA D-loop sequences reveal on average eight nucleotide differences for Caucasians [24]. While some mixtures may result in no or a few sites displaying more than one nucleotide in the pyrogram, most multiple contributor samples will demonstrate mixtures at several sites. Heteroplasmy occurs most frequently in certain mutational hotspots, and the probability of showing heteroplasmy in more than one position is very small [25]. Multiple sites demonstrating a mixture can also be caused by contamination by exogenous DNA. Exogenous contamination adhering to the surface of evidence materials such as hair, bones or teeth can be removed by cleansing prior to DNA analysis [2, 26]. Other materials such as blood-stained and saliva-stained materials cannot be cleansed of exogenous DNA, and the results from an mtDNA analysis of these materials should be interpreted carefully. Moreover, it has been shown for Sanger sequencing that when the ratio of sample to contaminant is greater than 8:1, base-calling for the major component will not be attributed incorrectly. Therefore, a limit of a 10:1 ratio quantified by capillary electrophoresis has been adopted for acceptable amounts of background contamination [2]. With the accurate quantification provided by pyrosequencing technology, such thresholds should be more than adequate for monitoring and evaluating the effects of background assay contamination.

The results from this assay may be used to deconvolve mixtures and attribute the multiple SNPs to specific mtDNA types. In all cases when one SNP component was low, the corresponding SNPs from the same contributing DNA were concomitantly low (and similarly when high). Thus, as with STR analysis, when mixtures are composed of a minor and a major contributor, unequivocal determination of the two contributing mtDNA types should be possible. Also, consistent with STR analysis, when the contributions are more equal, deconvolution will not be possible based on quantification by pyrosequencing technology. In contrast, cloning of mtDNA mixtures can display the actual sequence of the different mtDNA species present in the sample. Therefore, although more laborious, cloning can resolve and quantify multiple mtDNA species when present in relatively equal ratios. When interpreting mixture results, one will assume the number of contributors, as is done in STR analysis and likelihood ratio (LR) calculations [27]. Because of the known average nucleotide differences among individuals, it may be possible to employ LRs to lend support or refute the assumption of the number of contributors.

This assay, as well as other assays developed for quantification of mtDNA mixtures, will contribute to additional costs and workload. However, mtDNA sequencing results from many samples that are mixtures currently are deemed inconclusive. A new design of primers followed by testing for other SNPs than previously used in an assay might be required in some instances. However, there are a few cases in which the cost and labour that has to be invested is worthwhile to obtain results, as opposed to calling them inconclusive. The capability of excluding individuals wrongfully associated with limited evidence as well as assisting in identifying true perpetrators can be enhanced by mixture deconvolution with pyrosequencing. In conclusion, this mtDNA mixture quantification system is an alternative application of the pyrosequencing technology that is useful in forensic DNA analysis. The microtiter plate

format in the pyrosequencing assay allows quantification of 96 or 384 SNPs within 10 min. Although this study involved a limited number of SNPs, other positions can easily be quantified following a few control experiments and likely will not require primer redesign in most cases. Pyrosequencing has been shown to provide a very rapid, accurate, automatable and easy to use quantification system that can be used in forensic case-work investigations to resolve and interpret major and minor mtDNA components from multiple individuals, determine heteroplasmy ratios and monitor contamination.

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References

1. Clayton TM, Whitaker JP, Sparkes R, Gill P (1998) Analysis and interpretation of mixed forensic stains using DNA STR profiling. Forensic Sci Int 91:55–70
2. Wilson MR, Polanskey D, Butler J, DiZinno JA, Replogle J, Budowle B (1995) Extraction, PCR amplification and sequencing of mitochondrial DNA from human hair shafts. Biotechniques 18:662–669
3. Allen M, Engstrom AS, Meyers S et al (1998) Mitochondrial DNA sequencing of shed hairs and saliva on robbery caps: sensitivity and matching probabilities. J Forensic Sci 43:453–464
4. Steighner RJ, Holland M (1998) Amplification and sequencing of mitochondrial DNA in forensic casework. Methods Mol Biol 98:213–223
5. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467
6. Calloway CD, Reynolds RL, Herrin GL Jr, Anderson WW (2000) The frequency of heteroplasmy in the HVII region of mtDNA differs across tissue types and increases with age. Am J Hum Genet 66:1384–1397
7. Parker LT, Zakeri H, Deng Q, Spurgeon S, Kwok PY, Nickerson DA (1996) AmpliTaq DNA polymerase, FS dye-terminator sequencing: analysis of peak height patterns. Biotechniques 21:694–699
8. Parker LT, Deng Q, Zakeri H, Carlson C, Nickerson DA, Kwok PY (1995) Peak height variations in automated sequencing of PCR products using *Taq* dye-terminator chemistry. Biotechniques 19:116–121
9. Walker JA, Garber RK, Hedges DJ, Kilroy GE, Xing J, Batzer MA (2004) Resolution of mixed human DNA samples using mitochondrial DNA sequence variants. Anal Biochem 325:171–173
10. LaBerge GS, Shelton RJ, Danielson PB (2003) Forensic utility of mitochondrial DNA analysis based on denaturing high-performance liquid chromatography. Croat Med J 44:281–288
11. Lutz S, Weisser HJ, Heizmann J, Pollak S (2000) Mitochondrial heteroplasmy among maternally related individuals. Int J Legal Med 113:155–161
12. Lutz-Bonengel S, Sanger T, Pollak S, Szibor R (2004) Different methods to determine length heteroplasmy within the mitochondrial control region. Int J Legal Med 118:274–281
13. Niederstatter H, Coble MD, Grubwieser P, Parsons TJ, Parson W (2005) Characterization of mtDNA SNP typing and mixture ratio assessment with simultaneous real-time PCR quantification of both allelic states. Int J Legal Med 120:18–23
14. Gruber JD, Colligan PB, Wolford JK (2002) Estimation of single nucleotide polymorphism allele frequency in DNA pools by using pyrosequencing. Hum Genet 110:395–401
15. Wasson J, Skolnick G, Love-Gregory L, Permutt MA (2002) Assessing allele frequencies of single nucleotide polymorphisms in DNA pools by pyrosequencing technology. Biotechniques 32:1144–1146, 1148, 1150 passim
16. Uhlmann K, Brinckmann A, Toliat MR, Ritter H, Nurnberg P (2002) Evaluation of a potential epigenetic biomarker by quantitative methyl–single nucleotide polymorphism analysis. Electrophoresis 23:4072–4079
17. Soderback E, Zackrisson AL, Lindblom B, Alderborn A (2005) Determination of CYP2D6 gene copy number by pyrosequencing. Clin Chem 51:522–531
18. Pielberg G, Day AE, Plastow GS, Andersson L (2003) A sensitive method for detecting variation in copy numbers of duplicated genes. Genome Res 13:2171–2177
19. Sun A, Ge J, Siffert W, Frey UH (2005) Quantification of allele-specific G-protein beta3 subunit mRNA transcripts in different human cells and tissues by pyrosequencing. Eur J Hum Genet 13:361–369
20. Andreasson H, Asp A, Alderborn A, Gyllensten U, Allen M (2002) Mitochondrial sequence analysis for forensic identification using pyrosequencing technology. Biotechniques 32:124–126, 128, 130–133
21. Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215
22. Andreasson H, Gyllensten U, Allen M (2002) Real-time DNA quantification of nuclear and mitochondrial DNA in forensic analysis. Biotechniques 33:402–404, 407–411
23. Balitzki-Korte B, Anslinger K, Bartsch C, Rolf B (2005) Species identification by means of pyrosequencing the mitochondrial 12S rRNA gene. Int J Legal Med 119:291–294
24. Budowle B, Wilson MR, DiZinno JA et al (1999) Mitochondrial DNA regions HVI and HVII population data. Forensic Sci Int 103:23–35
25. Budowle B, Allard MW, Wilson MR (2002) Critique of interpretation of high levels of heteroplasmy in the human mitochondrial DNA hypervariable region I from hair. Forensic Sci Int 126:30–33
26. Jehaes E, Gilissen A, Cassiman JJ, Decorte R (1998) Evaluation of a decontamination protocol for hair shafts before mtDNA sequencing. Forensic Sci Int 94:65–71
27. Weir BS, Triggs CM, Starling L, Stowell LI, Walsh KA, Buckleton J (1997) Interpreting DNA mixtures. J Forensic Sci 42:213–222