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# A novel method to quantify deleted mitochondrial DNA in a real time PCR

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**Abstract.** The quantification of 4977 bp deleted mitochondrial DNA (dmtDNA) may be of interest for the forensic as well as the clinical pathologist. However, the determination of dmtDNA in two separate PCR reactions may lead to imprecise if not false results due to pipetting inaccuracies, deviant PCR conditions, etc. A conventional duplex PCR with subsequent fragment analysis yields only relative quantities of dmtDNA based on the analysis of PCR end products. To eliminate these factors, we established a real time duplex PCR using FAM- and VIC-labeled MGB probes. The PCR was carried out on an ABI Prism 7000 Sequence Detection System using standard chemistries. Amplicon sizes were 123 bp for deleted and 113 bp for total mitochondrial DNA. Serial dilutions showed a detection limit of 100 fg for total mtDNA. Application of this method to clinically and forensically relevant samples proved the suitability of this duplex PCR for quantification of minute amounts of mtDNA as well as highly degraded material. © 2005 Published by Elsevier B.V.

*Keywords:* Real time PCR; mtDNA; 4977 bp deletion; MGB probe; Quantitative PCR

## 1. Introduction

The quantification of 4977 bp deleted mitochondrial DNA (dmtDNA) may be of importance for the forensic as well as the clinical scientist. Different PCR based methods are commonly used to determine dmtDNA quantities. However, the application of two independent singleplex PCRs to measure total and deleted mtDNA may lead to imprecise if not false results caused e.g. by inaccurate pipetting or deviant PCR conditions. Quantification by means of a conventional duplex PCR with subsequent fragment analysis, on the other hand, eliminates the problems mentioned above. Yet it only allows a relative quantification and is based on the end point measurement of PCR products,

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making reliable quantitative determinations somewhat difficult. To eliminate influences hampering the accurate quantification of dmtDNA, we established a real time duplex PCR using MGB probes and validated the new method by applying it to forensically and clinically relevant samples.

## 2. Material and methods

### 2.1. Primers and probes

Primers used yielded a 113 bp fragment for total mtDNA and a 123 bp fragment for deleted mtDNA. For primer sequences, see [1]. Probes applied were designed using the Primer Express software (Applied Biosystems, Foster City, USA); probe sequences were FAM-TTC ACC AAA GAG CCC CTA A-MGB for total mtDNA and VIC-ACC ACC TAC CTC CCT CAC-MGB for dmtDNA.

### 2.2. Reaction conditions

Total reaction volume was 25 μl. Final concentrations were 200 nM for each primer, 250 nM for each probe, 1.5 and 3.0 mM for manganese, and 200 μM for each dNTP. 1 unit of hot start polymerase was applied to each sample, polymerases used were Immolase (Bioline, Luckenwalde, Germany), Platinum Taq (Invitrogen, Karlsruhe, Germany), and AmpliTaq Gold (Applied Biosystems).

### 2.3. PCR conditions

PCR was carried out on an ABI Prism 7000 instrument (Applied Biosystems). Initial hot start enzyme activation was at 95 °C for 10 min, followed by 40 repeats of a two-step profile (denaturation at 95 °C for 30 min; annealing and elongation at 60 °C for 1 min). Experiments were done in triplicates.

### 2.4. Samples

Dilution series were performed with DNA extracted from a commercially available cell line (Promega, Mannheim, Germany), blood donated by laboratory personnel, and selected skeletal muscle tissues taken at autopsy. Standards for total and deleted mtDNA were synthesized as described elsewhere [2].

## 3. Results and discussion

Sensitivity of the real time duplex PCR was the same as that of a singleplex reaction. In dilution experiments with DNA extracted from cell lines, the detection limit was 100 fg, equivalent to 30–40 mitochondrial genomes. Threshold values ($C_T$) of the duplex PCR were the same as those of the respective singleplex PCRs. Comparison of $C_T$ values of sample triplets showed an excellent reproducibility (Fig. 1). Comparison of three different polymerases showed identical $C_T$ values for all three enzymes; AmpliTaq Gold was less sensitive than Platinum Taq and Immolase, with the latter one producing the best overall results.

Dilution experiments with standards for total and deleted mtDNA yielded exact results even with low copy numbers of dmtDNA (Fig. 1), the sensitivity of this method was also confirmed by detection of dmtDNA in blood samples, proving the suitability of the duplex PCR for quantification of minute amounts of mtDNA. In cases of degraded DNA, all samples were

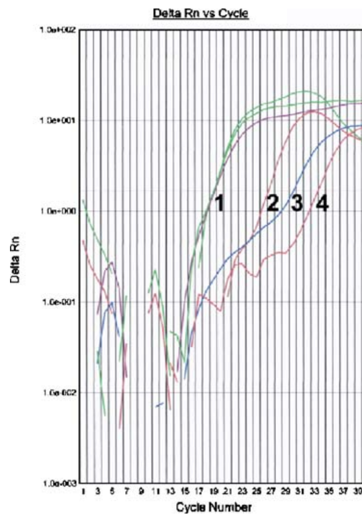


Fig. 1. Real time duplex PCR with standard fragments for total and deleted mitochondrial DNA. Total mtDNA specific fragments equivalent to 500 000 (1) copies of mtDNA were co-amplified with deletion specific fragments resembling 5000 (2), 500 (3), and 50 (4) copies of dmtDNA. $C_T$ values were reproducible in all three experiments. Serial dilution of 4977 bp deleted mtDNA over 3 orders of magnitude showed the expected interval of 3.3 cycles between samples, suggesting a PCR efficiency of >90%. Human mitochondrial fragments were mixed with mouse genomic DNA to simulate realistic conditions.

successfully amplified by real time duplex PCR, whereas a conventional duplex PCR [3] failed in every instance.

In conclusion, we believe that the real time duplex PCR presented here is an efficient means to reliably and easily quantify the mitochondrial 4977 bp deletion without the limitations of singleplex or conventional duplex PCRs. Our method is suitable for quantitative analysis of only minute amounts of dmtDNA (e.g. in blood) as well as highly degraded material, traits that make it an excellent application for forensic and clinical purposes.

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