



Multiplex–PCR of short amplicons for mtDNA sequencing from ancient DNA

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Abstract

We here describe a multiplex–PCR method to generate six overlapping short amplicons (100–130 bp) in two separate PCR reactions of non-overlapping fragments for full sequencing of the whole hypervariable region I (HV1). The performance of this multiplex system has been evaluated not only on ancient bone remains (5000–4000 BP) but also on different forensic samples with highly degraded DNA (bone remains, hair shafts, ...) that yielded negative PCR results with the mtDNA amplification strategies usually employed in forensic genetics. The multiplex–PCR methodology described in this study also illustrates a potential high throughput strategy to reconstruct the sequence of both coding and non-coding regions of the mtDNA genome from ancient DNA.

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1. Introduction

Amplification of each hypervariable region of the mtDNA Control Region in two separate PCR reactions (>400 bp) or using primers to amplify two overlapping segments

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of each region (>200 bp) are the strategies most employed in forensic mtDNA typing [1]. However, successful mtDNA analysis from old skeletal remains or other highly degraded DNA samples sometimes requires amplifications of shorter fragments [2]. The main trouble with the use of a singleplex short amplicons strategy is that multiple PCR amplifications are necessary to cover each hypervariable region (at least 4 or 6) and this is an expensive and time-consuming methodology. On the other hand, the amount of DNA extract is limited and in many cases can be exhausted after four or five PCR reactions. Trying to partially overcome these drawbacks, we have developed a multiplex-PCR system of short fragments (100–130 bp) using primers selected from previous ancient DNA studies [3–5] to cover the whole hypervariable region I (HV1). The performance of this multiplex-PCR methodology has been evaluated not only on ancient DNA samples (VI–VII and XI Centuries as well as Cooper age teeth samples) but also on different forensic samples (skeletal remains and hair shaft samples) that failed to amplify the fragment sizes commonly used by the forensic laboratories.

2. The multiplex-PCR approach for short amplicons: a simple way to evaluate mtDNA degradation

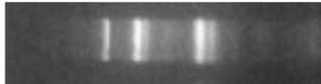
We have developed two multiplex-PCR reactions of non-overlapping short fragments to cover the whole hypervariable region using the primer sequences and PCR conditions described in Fig. 1. Multiplex-I allowed to amplify three non-overlapping fragments of 113, 126 and 131 bp while Multiplex-II allowed to amplify three non-overlapping fragments of 124, 133 and 93 bp that overlap the fragments obtained by Multiplex-I. After amplification, the PCR-products were purified by Centricon-100 and sequenced by Cycle sequencing with dichlororodamine or Bigdye terminator chemistry (Applied Biosystems). One interesting point of this multiplex-PCR approach of contiguous amplicons is that the degradation degree of the DNA template can be easily evaluated. As you can see in Fig. 1 when high molecular weight DNA template is PCR-amplified, in addition to the short amplicons, longer amplicons (around 400 and 250 bp) are formed because there are enough intact DNA template molecules for an efficient amplification between contiguous forward and reverse primers of different pairs (around 250 bp) and between the outside forward and reverse primers (around 400 bp). But if a highly degraded DNA sample is analysed only the short amplicons are generated.

3. Usefulness of the short amplicons strategy

This multiplex-PCR approach of short amplicons has been attempted for ancient DNA samples (five DNA samples obtained from 5000- to 4000-year-old teeth samples, three DNA samples obtained from 1500-year-old teeth samples, and two DNA samples obtained from 1000-year-old teeth samples), skeletal forensic samples (a 3-year-old skull recovered from the sea) and five telogenic hair and hair shafts that failed to amplify the fragment sizes commonly used by the forensic laboratories. In all cases successful PCR-amplification results were obtained with this shorter amplicons strategy. Our data indicate that the

15961 gaaaaagtct ttaactccac cattagcacc caaagctaag attctaattt aaactattct
16021 ctgttctttc atggggaagc agatttgggt accacccaag tattgactca cccatcaaca
16081 accgctatgt atttcgtaca ttactgccag ccaccatgaa tattgtacgg taccataaat
16141 acttgaccac ctgtagtaca taaaaaccca atccacatca aaacctcccc cccatgctta
16201 caagcaagta cagcaatcaa ccctcaacta tcacacatca actgcaactc caaagccacc
16261 cctcaccac taggatacca acaaacctac ccacccttaa cagtacatag tacataaagc
16321 catttaccgt acatagcaca ttacagtcaa atccccttct gtcccatagg atgaccccc
16381 tcagatagg gtcccttgac caccatcctc cgtgaaatca atatccgcga caagagtgct

MULTIPLEX I

Primer Set (0.5 μ M)	A1/A1R, 2F/2R, and 4F/4R
PCR Conditions (TaqGold)	10 min at 95°C followed by 36 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, and a final extension of 10 min. at 72°C
PCR PRODUCTS FROM HMW DNA	
PCR PRODUCTS FROM DEGRADED DNA (Cooper Age teeth sample)	

MULTIPLEX II

Primer Set (0.5 μ M)	1F/1R, 3F/3R, and 5F/5R
PCR Conditions (TaqGold)	10 min at 95°C followed by 36 cycles of 95°C for 10 s. 58°C for 30 s, and 72°C for 30 s. and a final extension of 10 min. at 72°C
PCR PRODUCTS FROM HMW DNA	
PCR PRODUCTS FROM DEGRADED DNA (Cooper Age teeth sample)	

Fig. 1. HIV1 primer sequences and PCR conditions for the Multiplex I and II reactions. Amplification results were different depending on the integrity of the DNA template as revealed by agarose gel electrophoresis. A1 primer sequence according to Wilson et al. [1]. 1F, 1R, 2F, 2R, 3F, 3R, 4F, 4R, 5F, and 5R primer sequences according to Handt et al. [3], Stone and Stoneking [4], and Di Benedetto et al. [5]. A1R primer sequence described in this study.

short amplicons strategy would be used for nearly 100% of Ancient DNA samples, 1–2% of forensic skeletal remains, and 12–15% of telogenic and hair shaft samples.

4. Preliminary validation data

The following preliminary validation studies were carried out:

- Monoplex–PCR amplifications at different annealing temperatures (from 55 to 60 °C) to determine the optimal annealing temperatures of each multiplex reaction.
- Duplicate (Mono- and Multiplex–PCR reactions) on the same set of samples (previously typed by standard mtDNA testing methods) to evaluate the performance of the multiplex system.
- Testing with non-probative DNA samples including ancient skeletal remains and hair shafts where previous testing gave negative results.

5. Advantages and future perspectives

The main advantages of this multiplex–PCR method are the following:

- It is an economical and effective strategy (compared with the singleplex approach)
- It is a simple PCR technique for the evaluation of mtDNA degradation
- It is a compatible methodology with different sequencing methods, different SNPs assays and with robotic implementation.

Regarding future perspectives on mtDNA typing from highly degraded samples, we are very interested in increasing the forensic discrimination of this multiplex approach by multiplexing HV1 and HV2 and also by multiplexing informative and silent SNPs on the coding region. At this point, we propose the necessity of an international collaborative effort for the analysis of the entire mtDNA genome around the world.

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