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High-throughput mitochondrial DNA cloning in forensic and anthropological studies

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Abstract. Mitochondrial DNA is widely used in forensic and anthropological investigations. Therefore, we developed an in-house high-throughput mitochondrial DNA cloning method targeting high speed at reduced costs. A home-made T/A cloning vector was obtained after ddTTP tailing of a *HincII* digested pUC19 vector. Due to this type of tailing, an extremely low background is found. Amplified mtDNA fragments were directly cloned into this vector after gel electrophoresis verification. PCR-grade plasmid purification was performed in 96-well blocks according to an adapted alkaline-lysis protocol. The obtained plasmids were further sequenced on both strands and resulting sequencing products were purified through ethanol precipitation. This method was applied in crime DNA mixture analysis and in a contamination check in an ancient sample from a Yakutia grave. Such a high-throughput method is performed in the same time required by commercial kits but with 20 times less costs. Thus, it opens possibilities for its routine use in forensic and anthropological laboratories. © 2005 Published by Elsevier B.V.

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1. Introduction

Mitochondrial DNA (mtDNA) is maternally inherited and used for forensic and anthropological studies despite some limitations. Common mtDNA analysis techniques are based on direct sequencing of hypervariable region I and II. But such an approach showed limits when analyzing mixture. Mitochondrial DNA mixture can be encountered

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in forensic cases. Moreover, most of ancient DNA samples are to be treated like a mixture in order to prove absence of contamination. Molecular cloning is a good way to separate the different haplotypes of these mtDNA mixtures. However, the generation of high numbers of clones is a limiting factor in the routine laboratory work. Thus, classically several steps of the cloning procedure are performed by means of expensive kits. Therefore, we developed an in-house high-throughput cloning procedure to introduce such method routinely in our laboratory at reduced costs.

2. Materials and methods

2.1. Samples

Two samples were investigated: a vaginal swab from a forensic rape case and an ancient bone from an anthropological study.

DNA from a vaginal swab was extracted using a differential lysis protocol used to separate the male sperm cells from the female epithelial cells. Only the male fraction was available for analysis. Blood sample gathered on the victim and hairs from a suspect were used as references of the mixture.

An ancient DNA sample was prepared from a femur of a male skeleton found in a grave in Yakutia (Oriental Siberia).

2.2. Cloning of mtDNA hypervariable regions

mtDNA hypervariable regions have been amplified as previously described [1] and further ligated into a ddTTP tailed *Hinc*II digested pUC19 vector. Plasmids were purified according to an adapted alkaline-lysis protocol. Insert presence in the plasmids was verified by a 15-cycle PCR, allowing semi-quantification against standards in a known range of concentrations. Plasmids were further sequenced using BigDye terminator kit v1.1 (Applied Biosystems, CA). The purified sequencing products were separated by capillary electrophoresis and read on an ABI prism 3100 genetic analyzer (Applied Biosystems, CA).

3. Results and discussion

3.1. Forensic rape case

An STR analysis of the male DNA fraction revealed the presence of two individuals in a proportion of 1:2. Due to the lack of reference samples from the suspect compatible with STR analysis, the mixture had to be investigated by means of mtDNA analysis. Amplification of both hypervariable regions I and II were carried out and further cloned. Plasmid DNA was prepared according to our 96-well adapted method and checked for size, concentration and PCR inhibition before sequencing.

48 HVI clones were sequenced and found to present the same haplotype: the one of the victim. We therefore investigated the proportion of offender and victim in the mixture. This was performed by means of restriction analysis on the mtDNA HVI amplification using an enzyme cutting once and twice, respectively, in the suspect and in the victim HVI region. The proportion of the offender was very low. Therefore, we specifically cloned the digested part of HVI corresponding to the suspect, but we only earned clones containing mutated HVI fragments derived from the victim haplotype and lacking the restriction site. Accordingly to the obtention of very rare mutated molecules, we can conclude that the content in offender's DNA in the sample was extremely poor. Having an almost

equal proportion for nuclear DNA and a very disequilibrium proportion for mtDNA lead to the questioning about the DNA sample on which the analysis have been performed. In fact Anslinger et al. [2] showed that during differential lysis, the spermatozooids lose their flagellum at the first lysis step, leading also to the loss of the mitochondria. In our sample, the loss was not complete; however, contrary of the male nuclear DNA which was enriched, the male mtDNA was impoverished.

Cloning of the HVII region was more successful since, after sequencing of 32 clones, a second haplotype was found, corresponding to the suspect. This result shows that even in extremely unbalanced mtDNA mixture, cloning can lead to the obtention of each constituent of the mixture. However, the number of clones to analyze in order to obtain each constituent is undetermined and can be, in some situations, very high.

3.2. Ancient DNA contamination check

To check the absence of contamination in DNA prepared from a femur of a male skeleton, HVI region of the mtDNA was amplified and cloned.

Sequencing of HVI clones resulted in one haplotype proving the absence of contamination. Several point mutations were found in different clones highlighting the error rate of the Taq polymerase used for amplification. Thanks to the number of sequences available, we were able to establish a consensus sequence within a confidence interval of 95% [3].

4. Conclusions

The developed high-throughput cloning technique turned out to be as fast and reliable as commercial kits. However, a larger number of clones is usually obtained and the costs of expensive kits are avoided (40% of the whole procedure). Thus, this method can now easily be used in our laboratory allowing us to perform the whole analysis within 5 days.

The result of the forensic case is consistent with the conclusions of Anslinger et al. [2], who underlined the loss of flagellum during the differential lysis extraction process and therefore obtention of samples with low concentration of spermatozoid mtDNA. Therefore, when analyzing mixtures recovered from sample prepared by differential lysis, the female fraction has to be considered for cloning.

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