

# Isopropanol precipitation removes PCR inhibitors from ancient bone extracts

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The emerging field of DNA extraction and PCR amplification from ancient samples is hampered by various methodological problems linked to post-mortem modifications of these remains. A major problem is the presence of some inhibitory activity preventing the PCR amplification process (1-6). Thus, it is very important to remove inhibitors present in ancient DNA samples to carry out efficient PCR amplifications. In this paper, we introduce an improved extraction method which allowed us to greatly diminish the amount of inhibitors present in ancient extracts.

The presence of an inhibitory activity in DNA extracted from an ancient ankle bone of 6000 years BP (from the Plots deposit at Berriac, Aude, France; 8) was demonstrated since 1 µl of this extract is able to inhibit PCR reactions using modern DNA as a template. If the ancient extract is progressively diluted (1/2, 1/4, 1/8) and added to the mixture containing modern DNA, normal *Taq* polymerase activity is progressively restored (not shown). The presence of such inhibitors strongly reduces the number of ancient bones from which we can obtain PCR amplification products.

We have tried to overcome this inhibitory activity by modifying the extraction procedure in order to separate inhibitors from DNA. The first step of our extraction procedure which involves powdering of bones and their incubation in a buffer containing EDTA 0.5 M (pH 8.0), sarcosyl 0.5%, Proteinase K 100 µg/ml followed by phenol-chloroform extractions remains unchanged (6). Classically, in a second step, the aqueous phase was dialyzed 4 h against a solution containing 100 mM Tris-HCl (pH 8.0) and 10 mM EDTA and then concentrated by centrifugation-driven dialysis using Centricon 30 micro concentrators from Amicon. As described in this paper, this concentration step is replaced by alcoholic precipitation (see below). The resulting pellet was dissolved in 30 µl of sterile water and the DNA visualized on 1% agarose gel. We often noticed the presence of a blurring of blue fluorescence which migrates at the level of a 500 bp DNA molecule on agarose gel illuminated by UV light. This colour can be observed without addition of ethidium bromide.

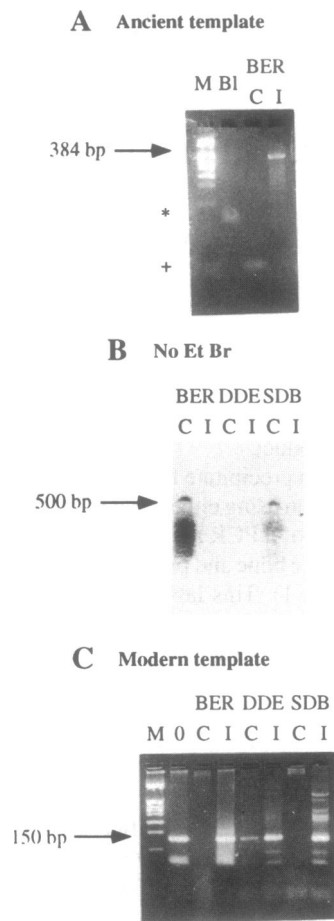
PCR amplifications were carried out in 100 µl final volume with 1 µl of ancient extract using a Perkin-Elmer Cetus DNA Thermal Cycler. *Taq* polymerase (10 U) and buffer (Amersham) were added as well as 25 mM of each dNTP (Pharmacia) and 200 µg/ml of bovine serum albumin (Boehringer Mannheim). The reaction was carried out with the following 40 cycles: 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. Two runs of PCR

amplifications were done consecutively to obtain sufficient amounts of PCR product.

We have chosen to precipitate DNA by the standard method of alcoholic precipitation using either ethanol or isopropanol. Figure 1A shows the result of a PCR amplification performed from two extracts of the Berriac bone and precipitated with ethanol (lane C) or isopropanol (lane 1). This last extract allows us to obtain an amplification product at the expected size. In the blank control, a 40 bp band which corresponds to oligonucleotide dimers can be detected. Therefore, PCR amplification succeeded in this control but, since there is no template DNA, only oligonucleotide dimers were generated. This result is in striking contrast with the result of the PCR amplification where 1 µl of the ethanol precipitated extract was used as a template. In this case, PCR reaction was completely inhibited since no amplification product is obtained either at the expected size or at the size of oligonucleotide dimers. This suggests that the reaction could not start due to the presence of *Taq* inhibitory activity as in the case of the dialysed extract (not shown). Isopropanol precipitation was successful according to the well-known property of isopropanol to be more 'selective' for DNA during precipitation (9). In addition to the blank control we also performed the amplification of an extract retrieved from an ancient cow bone extracted the same day and with the same reagents as our human bones (data not shown). Since our PCR primers are specific for human sequences, they cannot amplify any sequence of cow origin. With this control, we tested both the contamination of the PCR and extraction reagents as well as the contamination of the bone surface itself and we showed that there is no contamination of our samples.

We tested the efficiency of the isopropanol precipitation on two other neolithic samples, DDE and SDB (6) issued from different deposits. In the extracts realized with centrifugation-driven dialysis or by ethanol precipitation we always noticed a brown colour classically obtained after extraction of ancient remains (1,6,10). In contrast, the extracts precipitated with isopropanol do not display the same brown colour. Furthermore, these isopropanol extracts show a strong reduction of the blurring of blue fluorescence present on agarose gels illuminated with UV light. To separate this blue fluorescence from the orange fluorescence of the DNA, we performed our electrophoresis without addition of ethidium bromide (Fig. 1B). In these conditions, high amounts of blue fluorescence are observed in the control extracts (ethanol precipitated or centrifugation-dialysed) whereas this amount was greatly reduced in the isopropanol precipitated extracts. By PCR

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**Figure 1.** (A) PCR amplification of the first variable segment of mtDNA control region (7). PCR products were loaded on a 4% agarose gel. The blank (B1) corresponds to a negative control where no exogenous DNA was added. Lane C: 1  $\mu$ l of Berriac (BER) extract performed with our standard protocol (6) was added as a template; lane I: 1  $\mu$ l of the isopropanol precipitated Berriac extract was added. The 20 bp band (+) corresponds to the oligonucleotides, the 40 bp product (\*) to a dimer of oligonucleotides and the 384 bp product to the expected PCR product. (B) Visualization on a 3% NuSieve agarose gel not stained with ethidium bromide (which reveals only the blue fluorescence and not the DNA) of extracts from three neolithic bones (BER, DDE and SDB). The classical extraction procedure using centrifugation-driven dialysis (C) or with isopropanol precipitation (I) were compared for each sample. (C) Test of the inhibitory activity contained in the same extracts. The PCR was conducted using a modern DNA template with primers derived from the promoter of the thyroid hormone receptor  $\alpha$  gene (11). (O) modern DNA only; (C) addition of 1  $\mu$ l of centrifugated extracts; (I) addition of 1  $\mu$ l of isopropanol precipitated extracts. (M) molecular size marker.

amplification of a modern DNA template, we observed a strong correlation between the amount of blue fluorescence and the inhibitory activity (Fig. 1C). The case of the DDE sample is interesting since it contains only a small amount of blue fluorescence (Fig. 1B) and exhibits a small level of PCR inhibitory activity (Fig. 1C). On six other bone samples from human or animal origin we obtained the same effect of isopropanol precipitation on the inhibitory activity (data not shown).

In this paper we demonstrate a strong correlation between the brown colour, the blurring of blue fluorescence under UV light illumination and the *Taq* polymerase inhibitory activity. The fact that the inhibitor can be isolated from DNA further confirms that the inhibitory activity is not linked to the ancient DNA molecule itself but is an unknown product distinct from it. It is probable that, like the conservation of the DNA molecule itself, the presence of the inhibitor depends on the environment (oxidation, hydrolysis...). Some authors have hypothesized that in mummy extracts the inhibitors may be Maillard products of reducing sugars (10). The features of the inhibitors from ancient extracts (especially the brown colour) seem consistent with this hypothesis but remain to be addressed by chemical analysis.

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