A MULTIPLEX AMPLIFICATION APPROACH FOR SIMULTANEOUS TYPING OF FIVE LOCI IN DNA OF ANCIENT BASQUE POPULATIONS

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INTRODUCTION

We tried to test the commercial forensic kit worldwide known as "Polymarker" in cases of samples of very old DNA. We will try to type ancient DNA samples for five genetic systems (LDLR, GYPA, HBGG, D7S8 and GC) all of them comprised in this multiplex PCR commercial kit. Our hypothesis is that given that the amount of target ancient DNA is very small, only standardized systems already tested for succesful amplification of a few nanograms can be suitable for very scarce and damaged DNA. This kit, in the routine forensic casework, produces good results with a template DNA amount of 2-40 ngr in a final reaction volume of 100 μl.

Ancient DNA samples (teeth) from the Basque Country (Spain) were chosen for this study due to the special demographical and anthropological characteristics of this human population (Calderón, 1994).

MATERIAL

Samples were selected to cover a wide range of time periods, but only specially well preserved tooth samples were studied. The Basque sites studied included Atxuri (Vizcaya, Neolithic and Calcolithic period, 5000-3600 B.P.), Urbiola (Navarra, Early Bronze Age, 3700 B.P.) and Garai (Vizcaya, XI-XIII centuries A. D.). Samples from Atxuri are quoted AT-; Urbiola, UR-; and Garai, GA-.

DNA EXTRACTION

Teeth have been immersed in 15% chlorhydric acid (10') to remove dirt and carbonate deposits, 70% ethanol (30') to remove acid residues, distilled water (30') and posteriorly irradiated 10-15 minutes under UV light (254 nm). The methodology proposed for the treatment of the teeth is a modification of that of Ginther et al. (1992). The cleaning of the external surface of the ancient material is essential in order to eliminate possible exogenous DNA from the archaeologist and the curators and also to reduce the presence of soil inhibitors. The DNA extraction procedure mainly follows that described by Hagelberg (1994) with slight modifications. The samples were ground in a freezer mill (Spex Industries Inc.) refrigerated with liquid nitrogen, and the teeth powder was stored in sterile plastic tubes (Corning 50 mL) at -20ºC. The tubes and impactors of the Freezer mill were cleaned with ethanol and distilled water, and placed 10 minutes under UV for sterilization, between each grinding. Samples were washed by adding 0.5 M EDTA pH 8.0, and centrifugating them. The aquaeus fase was discarded and the washing repeated 2 or 3 times, for removing some of the brown coloration from the

sample. Ten mL lysis buffer, containing 8.5 mL 0.5 M EDTA pH 8.0 - 8.5, 1 mL 5% SDS, 0.5 mL 1M Tris and 100 µl. Proteinase K (1 mg/mL), was added to the tubes,and incubated overnight at 37ºC with agitation. After the incubation, the tubes were centrifugated 5' at 2000 rpm, and the supernatant stored in sterile tubes for the extraction with phenol/chloroform. Phenol was previously saturated with 0.02 M Tris (pH 8). After the addition of phenol to the sample, the tube is agitated and centrifugated for 5 minutes at 2000 rpm, until the phenol and the aqueous fases become separated. The aqueous fase is recuperated and extracted once more with phenol/chloroform (1:1), and finally only with chloroform. The 10 mL of the resultant sample were concentrated and purified with Centricon-30 microconcentrators (Amicon Inc.), by adding 6 mL of sterile water in 3 consecutive centrifugations during 30' at 4000 rpm. The final volume obtained was 100-300 µl. depending of the coloration of the sample. Dark brown samples were more diluted, to try to dilute the soil inhibitors, otherwise discarded.

AMPLIFICATION AND TYPING PROTOCOLS

The whole protocol was carried out in a sterile environment. Nine µl. of ancient DNA solution were added to a volume of 20 µl. of reaction mix, 20µl.of primers dilution and 1µl. of Bovine Serum Albumine (BSA) at a final concentration of 160 mgr/mL. The 50 µl. final volume was amplified in a first round of 94 1'/ 60 1'/ 72 1' 32 cycles. After checking the results in a 4% agarose gel electrophoresis (NuSieve 3: Seakem 1), a second round of amplification was started with 4 µl. of the last reaction product plus 5 µl. of distilled water, 20 µl. of mix, 20 µl. of primers dilution and 1 µl. of the former BSA dilution. Amplification conditions were 94 1'/ 60 1'/ 72 1' but this time for just 30 cycles. Definitive reaction products were checked in a 4% agarose gel electrophoresis before reverse dot blot typing, according to the manufacturer's protocol. DNA-1 Polymarker kit control (Perkin Elmer. USA) was used as a positive control for every amplification turn and subsequently sterile double distilled water was used as negative control. As recommended by the manufacturer, the control spot of the strips was considered as a reference of the quality of the amplification.

RESULTS AND DISCUSSION

Highly amplified bands were observed after the second PCR round in the case of the fresh DNA positive control . No signal was observed in the case of the negative control and DQA1 showed almost always a clear band, though this marker was not included in the typing strips. Damaged or not amplified systems produced always a lack of a band in the gel and subsequently no signal (hybridization) was observed in the corresponding site at typing strip. Reverse dot blot produced several kinds of results *(See table 1)*.

According to manufacturer's protocol, only the strips with hybridization in its control spot were considered for the study. A sample (AT-95) was correctly amplified and typed in the first PCR round and showed an invariant dot-blot pattern in three further repetitions. Other samples showed variation in some markers when repetition was performed, probably due to jumping PCR effect or inespecific hybridization.

All the samples were repeated (twice minimum) and at least one marker out of the five tested was persistently typed the same, the average being 1.8 markers per sample. This value scores below our previous results (Prieto et al., 1995). However, given that the intensity of the signal of the hybridization spots varies - and sometimes the typing remains unclear -, perhaps it would be better to try a sequencing approach given the high amount of DNA contained in the amplification band.

Table 1.

Sample	Amplified bands	Typed loci
UR-71	All but DQA1, GYPA	GC
UR-85	All but LDLR	GC
UR-95	All	All
UR-98	All but DQA1, LDLR	HBGG
AT-117	All but LDLR	GYPA
AT-118	All	GYPA, HBGG
AT-119	All	D7S8
AT-120	All but DQA1	GYPA
AT-121	All	LDLR, HBGG, D7S8
AT-122	All but HBGG	D7S8, GYPA
AT-123	All	LDLR, GYPA, GC
AT-124	All but LDLR	HBGG, D7S8
AT-125	All but DQA1, LDLR, GYPA	HBGG
AT-126	All	LDLR
GA-132	All	GYPA, D7S8
GA-133	All	HBGG
GA-136	All	All but GYPA
GA-138	All but GYPA	D7S8

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