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Reduction of shadow band synthesis during PCR amplification of repetitive sequences from modern and ancient DNA

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1. Introduction

Repetitive sequences like short tandem repeat (STR) loci are generally referred to as slippery DNA (1). They owe this nickname to a characteristic leading to slippage within the primer-template complex during PCR elongation of the new strand (2,3), resulting in the synthesis of byproducts shortened by one repeat unit compared with the original sequence. The generation of these so-called shadow bands (4) is a well-known problem connected with the amplification of repetitive DNA, complicating the genotype analysis of modern (e.g., ref. 5), forensic (6), and ancient (7,8) specimens. In some applications, the occurrence of this artifact makes it necessary to develop guidelines for allele designation (6,9).

The intensity of these byproducts increases with the degradation of the target DNA (8,10), and therefore represents a particular problem concerning the analysis of highly degraded DNA as in genotyping of forensic (e.g., refs. 11,12) and ancient DNA (7,13,14). In amplification products of highly degraded or ancient DNA, the intensity of a shadow band can exceed the peak height or band intensity of the original allele. As artifact alleles (7,15) they can lead to mistyping of amplification products (cf. also refs. 8,16). Because an amplification product is not necessarily affected in each case, this phenomenon can even result in seemingly different genotypes for independent amplification products of the same sample (7,15,16).

To improve the reproducibility of amplification results and consequently decrease the probability of mistyping by reducing the generation of this artifact, the optimization of the PCR amplification process itself represents the most important strategy besides optimization of the extraction of the DNA used as template (17–19).

The findings presented in the following resulted from a study within the scope of which different strategies to optimize PCR amplification of repetitive DNA were investigated with reference to their effect on the generation of shadow bands (cf. ref. 19 Schmerer, manuscript in preparation). The model locus investigated was HUMVWA31/A (20), the amplifications of which show a high tendency to accumulate shadow bands compared with other STR loci (11,12,21). Amplifications were performed on DNA Thermal Cycler (TC1, Perkin–Elmer Cetus). For a detailed presentation of the investigation concerned, please refer to Schmerer (19) and Schmerer (manuscript in preparation).

2. Materials

1. InViTAQ® DNA polymerase (Invitex).
2. NH₄ reaction buffer (Invitex). 10× buffer: 160 mM (NH₄)₂SO₄, 500 mM Tris-HCl (pH 8.3 at 25°C), Tween® 20.
3. Betaine (3 M solution with sterile water also used for set up of the reaction mix).
4. Betaine (2 M) + 10% dimethyl sulfoxide (DMSO; solution with sterile water).
5. Bovine serum albumin (BSA; 125 µg/mL solution with sterile water).
6. dNTP-mix composed according to the sequence amplified (e.g., for HUMVWA31/A with a A/T: G/C ratio of 1.9: 1, a stock of 121 µM each of dGTP, dCTP, and 220 µM each of dATP and dTTP were used).

3. Methods

Each of the following variations of a standard PCR amplification protocol resulted in reduced accumulation of shadow bands. They might be applied either singly or in any combination.

3.1. Denaturation Time

A reduction of denaturation time to 15 to 30 s results in a 28% decrease in shadow bands compared with the standard denaturation time of 1 min by decreasing the occurrence of additional degradation of the template DNA (cf. ref. 22) because of the shorter incubation period at the high temperature of 94° C.

3.2. Elongation Step

Applying an elongation time of 1 min, which is the standard used for synthesis of a PCR product up to 1 kb, the lowest intensity of shadow bands was found with an elongation temperature of 68°C, resulting in a 20.4% reduction compared with the generally applied temperature of 72°C. This reduction could be even increased to 24.7% by doubling the time for synthesis. The highest reduction of the generation of this artefact (–30.9%) could be achieved with an elongation at 70°C for 2 min.

3.3. Polymerase and Composition of Reaction Buffer

Comparing different polymerases and polymerase mixes respective, the lowest intensity of shadow bands was observed with InViTAQ® (Invitex) and the CombiPoi® polymerase mix (Invitex) in combination with OptiPerform™ Buffer (Invitex) with a reduction of the artefact by up to 24 and 21.4% compared with AmpliTaq™ Gold (Perkin–Elmer) in combination with GeneAmp® buffer (Perkin–Elmer). Applying InViTaq, a replacement of the generally used KCl-reaction buffer, by an optimized NH₄ buffer resulted in a 22% reduction in artefact accumulation applying the same polymerase. Further differences of the two buffer systems consist in a slightly elevated pH value of 8.8 (+0.5) and an addition of 0.01% Tween-20 in case of the NH₄ buffer.

The use of a polymerase displaying a 3'→5' exonuclease proof-reading ability showed no positive effects concerning the reduction of shadow band accumulation, neither applied alone, nor in combination with a Taq polymerase.

3.4. A/T : G/C Ratio of the dNTP-Mix

Changing the composition of the dNTP-mix from the usual equimolar concentration of nucleotides to a A/T:G/C ratio of 1.9:1 corresponding to the composition of the general sequence of HUMVWA31/A, the locus amplified, resulted in a decrease in shadow band generation by 7.3%. Changes in amplification efficiency were not observed, neither in processing modern nor ancient DNA. Equability of the amplification of both alleles belonging to a heterozygous genotype was slightly improved.

3.5. Betaine (N,N,N-trimethyl glycine)

The presence of betaine in a concentration of 0.5 to 2 M reduced the accumulation of shadow bands, with a maximum reduction of 15.5% at 0.5 M. Concentrations lower than 0.5 showed an increase in artifact production. Concerning the amplification of ancient DNA, low concentrations of betaine (0.25–1 M) resulted in an increase in product yield because of the neutralizing effect of this reagent against inhibitory substances (cf. ref. 23) frequently present within ancient DNA extracts. Beyond 1.5 M, an addition of the inhibitory effect of betaine at elevated concentrations (24) and the inhibition caused by co-extracted impurities occurred, resulting in partial inhibition of amplification.

3.6. Betaine Combined with DMSO

Like betaine, low concentrations of both betaine and DMSO resulted in a slight increase in intensity of shadow bands. A reduction of the artifact was observed at concentrations of 0.4 to 1 M betaine combined with 2 to 5% DMSO, with a maximum decrease of shadow band intensity by 12.6% at 0.8 M betaine and 4% DMSO. In ancient DNA amplifications, the presence of these reagents increased product yield and reproducibility between multiple amplifications of the same sample (see Note 1).

3.7. BSA

The addition of BSA in a concentration of 10 to 25 µg/mL resulted in reduced intensity of artifact bands with a maximum at 25 µg/mL by 21.2% accompanied by an increased equability of amplification of a heterozygote genotype. In addition to this, the presence of BSA showed to increase the efficiency of ancient DNA amplifications by neutralizing inhibitory substances (25) that consequently resulted in higher yield of specific product. A concentration of 25 to 50µg/mL was determined as optimal for the amplification of ancient DNA.

4. Notes

- 1. Also investigated concerning their impact in the generation of shadow bands were further reagents commonly used as PCR-enhancing additives, such as DMSO, glycerol, and formamide. In amplifications of the locus concerned the addition of DMSO, as well as formamide in different concentrations resulted in an increase in shadow band accumulation. Glycerol, however, did not show any effect, neither on modern, nor ancient DNA amplification.*

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