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Technical note

Optimized amplification of the polymorphic system COL2A1

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Summary. The influence of various amplification parameters on the demonstration of COL2A1 patterns was examined in serial experiments. The combination of 6 optimized parameters (concentration of primers, nucleotides, Taq polymerase, K^+, Mg^{2+}, number of cycles) led to an approximately tenfold increase in sensitivity and a decrease in allelic drop-out. In unequal mixtures of DNA from 2 individuals the weakest component was detectable in dilutions down to 1:20. In a small population sample ($n = 120$) 10 alleles could be demonstrated.

Key words: VNTR system COL2A1 – PCR amplification – Optimization

Zusammenfassung. In Reihenversuchen wurde der Einfluß verschiedener Amplifikations-Parameter auf den Nachweis von COL2A1-Mustern untersucht. Die Kombination von 6 optimierten Parametern (Konzentrationen der Primer, der Nukleotide, der Taq-Polymerase, von K^+, Mg^{2+}, Zahl der Zyklen) führte zu einem ungefähr zehnfachen Anstieg der Empfindlichkeit. Der Allelverlust schien besser vermeidbar. In ungleichen Mischungen von DNA von zwei Personen konnte die schwache Komponente noch in Verdünnungen bis 1:20 nachgewiesen werden. In einer kleinen populationsgenetischen Studie ($n = 120$) konnten 10 Allele nachgewiesen werden.

Schlüsselwörter: VNTR-Polymorphismus COL2A1 – PCR-Amplifikation – Optimierung

Introduction

The analysis of stains by means of PCR-based VNTR's such as YNZ22 (Horn et al. 1989), pMCT118 (Kasai et al. 1990), ApoB (Boerwinkle et al. 1989) and COL2A1 (Priestley et al. 1990) offers many more advantages than 'classical' RFLP analysis. But practical application can also suffer from limiting factors such as the amount of template DNA, extent of degradation, susceptibility to preferential amplification (Comey et al. 1991; Ruano et al. 1991). This investigation has been performed to further reduce the influence of these disadvantages.

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Materials and methods

DNA was extracted from blood by standard procedures (Brinkmann et al. 1991) or from cotton stains using 'single lysis' (Rand et al. 1991). The standard method of amplification for COL2A1 was as follows: primers after Wu et al. (1990); 50 ng template DNA; 2.5 U Taq polymerase (Promega Corporation, USA), 0.5 μ*M* of each primer; 200 μ*M* of each nucleotide; 5 μl buffer (Promega Corporation, USA – 500 m*M* KCl, 100 m*M* Tris-HCl, 15 m*M* $MgCl_2$, 0.1% (w/v) gelatine, 1% Triton X-100, pH 9.0) diluted to a total volume of 50 μl with distilled water. The reaction mixture was overlayed with 2–3 drops of oil.

Amplification (Triothermoblock, Biometra, FRG): (1) denaturation 94°C/60 s, (2) annealing 62°/60 s, (3) extension 70°/90 s; 25 cycles.

In the optimization series we selectively (only 1 parameter was varied in each step) varied these parameters as follows:

- Taq polymerase from 0.5–4.5 U in 0.5 U steps
- template DNA from 100 ng down to 1 ng
- number of cycles from 18–32
- Mg^{2+} with final concentrations from 0.5–3 m*M* in 0.5 m*M* steps
- K^+ final concentration from 35–70 m*M*
- primer concentration from 0.1–0.9 μ*M*

Electrophoresis. PAGE (6% T, 3% C, 400 μm, piperazine diacrylamide as cross linker), 10 cm separation distance; 140 m*M* Tris-borate, pH 9.0 as leading buffer and 35 m*M* Tris-sulfate, pH 9.0 as tracking buffer (Puers et al. 1992). Bands were visualized by silver staining (Budowle et al. 1991).

The optimized method was applied to 3 investigations:

- a population sample consisting of 120 unrelated Caucasians (Westphalia)
- unequal mixtures of DNA from 2 different persons
- a problematic rape case

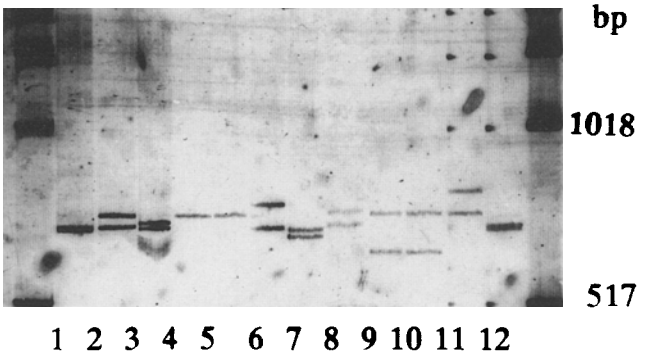
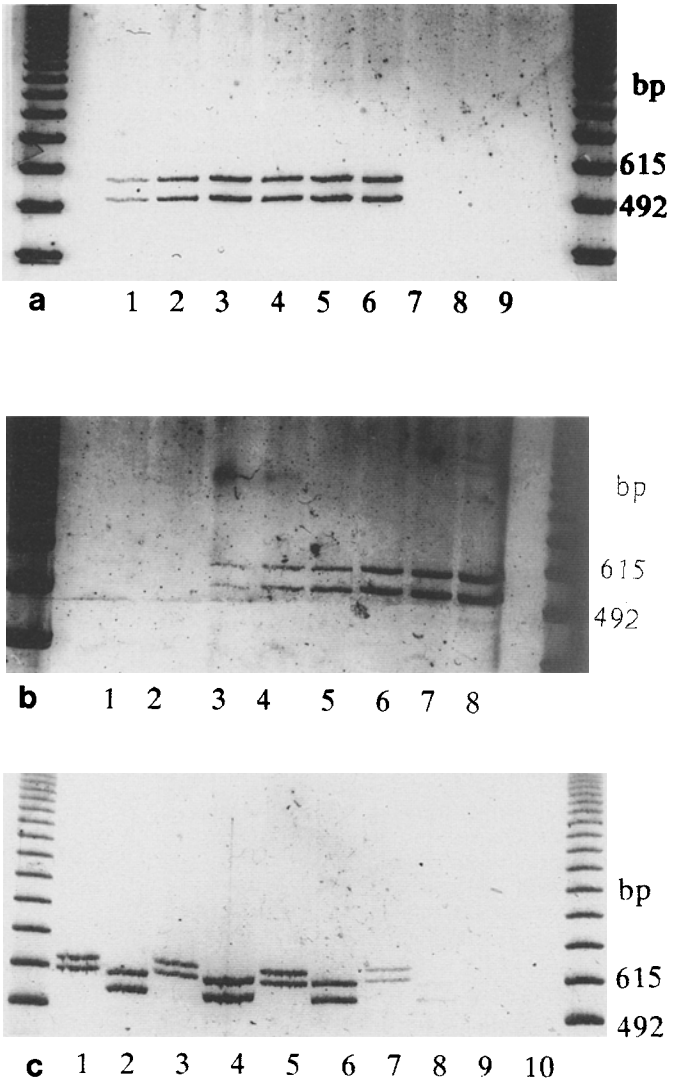


Fig. 1. COL2A1: PCR typing patterns of 12 unrelated individuals showing 10 alleles (first and last lane = 1 kb ladder; GIBCO-BRL, UK)

Results

Optimization studies. The following parameters were found to be optimal: 1.5 U Taq polymerase; 1.5 m*M* Mg^{2+}; 40–50 m*M* K^+; 0.5–0.9 µ*M* of each primer. – Within a certain range (Fig. 2a–f), changing the concentrations of these ingredients had little or no effect, but beyond this range, fading or disappearance of bands occurred either gradually or abruptly and was associated with either increasing or decreasing concentration of the parameter tested. – Interrelation between template DNA and the number of cycles showed that 20 ng/25 cycles was the optimal combination but 5 ng/29 cycles still gave results. Even smaller amounts of template DNA (range 1–2 ng) gave bands when 30/31 cycles were used.



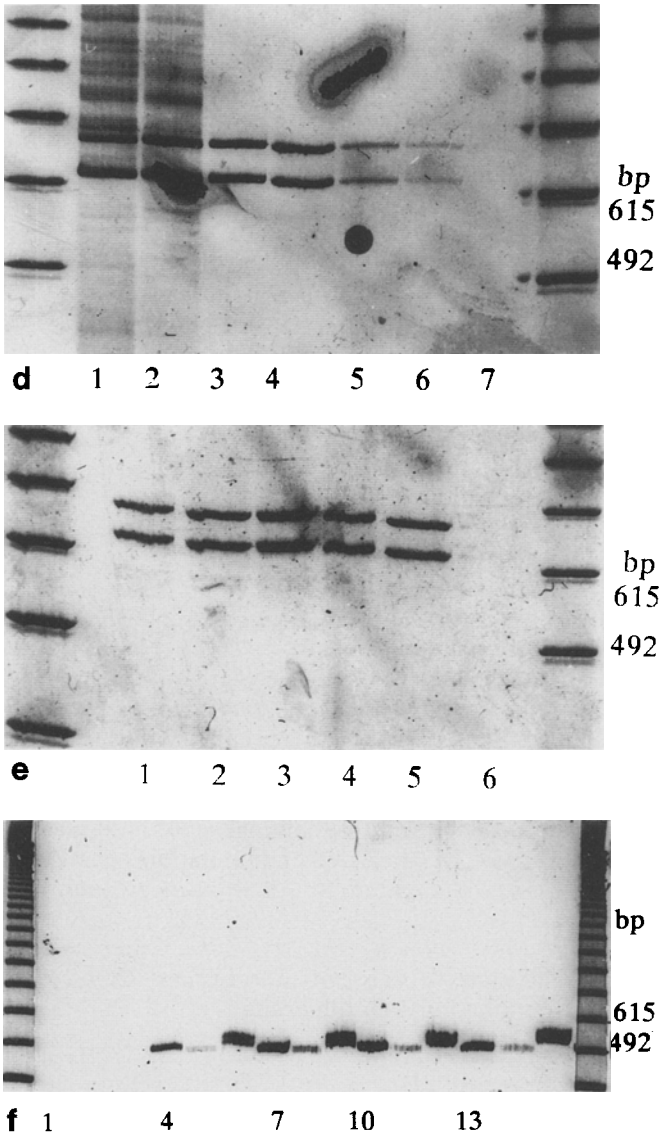


Fig. 2a–f. Effectiveness of defined parameters on the expression and intensity of COL2A1 patterns. First and last lane = 123 bp ladder (GIBCO-BRL; UK). **a** Variation of *Taq pol.;* 1–9: = 0.5–4.5 U in increasing order and 0.5 U steps. **b** Variation of *template*, 27 cycles; 1 = 1 ng, 2 = 2 ng, 3 = 5 ng, 4 = 10 ng, 5 = 20 ng, 6 = 40 ng, 7 = 80 ng, 8 = 100 ng. **c** Variation of *primers;* 1/2 = 0.9 µ*M*, 3/4 = 0.7 µ*M*, 5/6 = 0.5 µ*M*, 7/8 = 0.3 µ*M*, 9/10 = 0.1 µ*M*. **d** Variation of *cycle* numbers; 1 = 32, 2 = 30, 3 = 27, 4 = 25, 5 = 23, 6 = 20, 7 = 18. **e** Variation of Mg^{2+}; 1 = 0.5 m*M*, 2 = 1 m*M*, 3 = 1.5 m*M*, 4 = 2 m*M*, 5 = 2.5 m*M*, 6 = 3 m*M*. **f** Variation of K^+; 1–3 = 70 m*M*, 3–6 = 50 m*M*, 7–9 = 45 m*M*, 10–12 = 40 m*M*, 13–15 = 35 m*M*

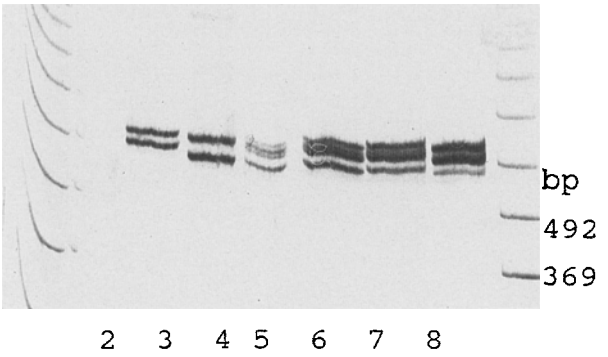


Fig. 3. COL2A1 pattern of unequal mixtures of 2 different blood samples 2 = negative control, 3 = person '1', 4 = person '2', 5 = ratio 7:3 (person '1':'2'), 6 = ratio 8:2, 7 = ratio 9:1, 8 = ratio 19:1

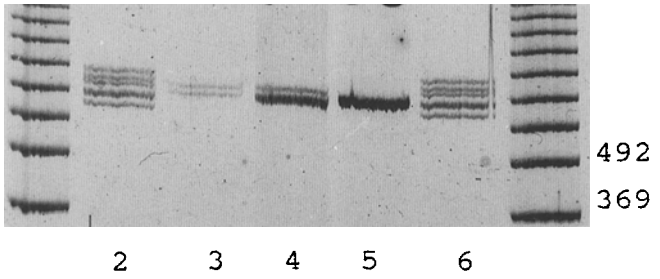


Fig. 4. COL2A1 patterns in a rape case; 2/6 = allelic cocktail, 3 = suspect, 4 = 'single lysis' amplification (optimized), 5 = victim

In the *population study* we observed 10 alleles (Fig. 1) in genetic equilibrium (further details see Rand et al. 1992).

Unequal mixtures of two different blood samples still showed amplification of the weak component when the ratio was 1:20 (Fig. 3).

In a rape case the extraction from a vaginal swab yielded only a few sperm heads in each microscope field. However, the number of vaginal cells was roughly ten times higher and the sperm heads showed signs of advanced autolysis. Single lysis yielded approx. 100 ng DNA (determined fluorimetrically). An aliquot of 10 ng was used for the first amplification, the product showing one missing band when compared to the suspect. A second investigation performed with optimized parameters showed a complete match with the suspect (Fig. 4) using again 10 ng. Even YNZ22 (template 50 ng) and pMCT118 (10 ng DNA) showed complete matches using optimized conditions[1]. The combined frequency of the male pattern was estimated to be 0.001%.

Discussion

This optimization experiment proved to be effective and the sensitivity of the COL2A1 system could be increased by about tenfold. This was obviously due to a combination of factors which influence the reaction kinetics even when modified in isolation. – Furthermore, the use of either small amounts of template or the optimized kinetics seemed to be accompanied by a reduction of preferential amplification. – Even the occurrence of 'ladder bands' as an artefact or side effect of over-amplification seemed to be reducible to some extent. – The study should only serve as an example to demonstrate that such serial studies are necessary for each PCR-based VNTR. From our experience, it is necessary to repeat such studies when another thermocycler is used which does not only show slight temperature differences but also (probably more effective) other velocities of temperature change in the reaction mixture. – We would also warn against the strong influence of small changes in concentrations if these are already at borderline levels and can lead to negative results. Borderline concentrations of any ingredient should therefore be avoided.

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[1] *pMCT118:* 1 ng template, 1.5 U Taq polymerase, 0.5 μ*M* each primer, 200 μ*M* each nucleotide (Budowle et al. 1991); other conditions as described above; 25 cycles. – *YNZ22:* 5 ng template, 2 U Taq polymerase, 0.5 μ*M* each primer, 200 μ*M* each nucleotide (Wiegand et al. 1992); other conditions as described above; 29 cycles.

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