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SHORT COMMUNICATION

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Modified primers for D12S391 and a modified silver staining technique

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Abstract In this paper we describe a new primer pair for the short tandem repeat (STR) D12S391 which makes it possible to obtain considerably shorter amplification fragments (125–173 bp), compared to the previously published primers (205–253 bp). The primers were tested on 70 samples with known genotypes, and no differing results were found. In sensitivity studies, forensic casework samples and DNA quality studies, we proved that the new primers can improve the efficiency of the amplification. Moreover, the resolution of this locus on denaturing PAGE followed by silver staining was dramatically improved. This improvement was found to be most valuable for typing the rare .3 variants known for this locus. We also present and propose a new method for silver staining denaturing acrylamide gels.

Key words D12S391 · Silver staining · Short tandem repeats

Introduction

The polymorphism D12S391 is one of the more interesting short tandem repeat systems (STR) in the spectrum of markers used for human identification. It is a compound STR with a consensus structure of (AGAT)7–17 (AGAC)6–10 (AGAT)0–1 (Lareu et al. 1996; Klintschar et al. 1998 b; Waiyawuth et al. 1998).

A rare occurrence of ".3 alleles" with incomplete (GAT) repeat units at position 2 of the tandem regions has also been described (Glock et al. 1997).

One disadvantage of this locus is the relatively large size of the amplified alleles (205–253 bp), whereas shorter amplification fragments are more prone to successful amplification in degraded DNA samples (Alvarez Garcia et al. 1998).

During routine casework, it was noted that for some of those samples where the PCR reaction for the amplification of D12S391 gave negative results, it was still possible to type these stains using "shorter" STR loci. We therefore constructed a new primer pair that produces shorter fragments (125–173 bp) than the one described earlier, and evaluated its forensic usefulness. We also evaluated whether the shorter fragments obtained by using the new primers were suitable for separation on native gels, which is not possible using the longer primers (Glock et al. 1997; Klintschar et al. 1998 a).

Additionally, we developed a new method for visualizing the alleles with silver staining, and tested its usefulness both on vertical denaturing and horizontal non-denaturing electrophoretic systems.

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Materials and methods

Blood samples were taken from resident unrelated donors and the DNA was extracted with a saline procedure (Miller et al. 1988) or an alkaline lysis protocol, as described by Klintschar et al. (1998 a). DNA was extracted from degraded forensic casework samples, with the Chelex method (Singer et al. 1989).

Primer design

In the NCBI site (National Center for Biotechnology Information www.ncbi.nlm.nih.gov/) the sequence for the D12S391 locus, deposited at the GenBank (Accession G08921), was examined. The forward primer anneals only 4 bases from the beginning of the repeat sequence, while the reverse primer anneals 106 bp from the repeat. For this reason, only the reverse primer was modified.

The following pair was used:

forward primer: 5′ AACAGGATCAATGGATGCAT 3′
reverse primer: 5′ AGCCTCCATATCACTTGAGC 3′

Amplification conditions

Amplification was conducted in two thermal cyclers as described (Ricci et al. 1998), but the annealing temperature was 62 °C.

Table 1 Proposed method for staining the denaturing and native polyacrylamide gels

	Gel 0.8 mm thin	Gel 0.4 mm thin
Fix solution A	3 min (repeat × 2)	3 min (repeat × 2)
Staining solution B	15 min	25 min
Wash	1 min (repeat × 2)	–
Development solution C	Add 0.15% formaldehyde 5–20 min	Add 0.1% formaldehyde 5–20 min
Stop solution D	10 min	10 min

Table 2 The reagents for proposed silver staining

Solution A – Fixing Solution 10% ethanol and 0.5% acetic acid	Prepare on the day of use
Solution B – Staining Solution 0.1% silver nitrate in bidistilled water	This solution can be reused for staining about 10 gels (45 cm long)
Solution C – Development Solution 1.5% sodium hydroxide and 0.01% $NaBH_4$	Prepare on the day of use, and immediately before the use add the appropriate quantity of formaldehyde indicated above
Solution D – Stopping Solution 0.75 sodium carbonate in bidistilled water or running water	

The amplified alleles were resolved by high resolution polyacrylamide gel electrophoresis using previously described method (Wiegand et al. 1993; Ricci et al. 1998).

Staining

After electrophoresis, the bands were visualized with a silver staining technique (Allen et al. 1989). Alternatively, we also used another system, proposed by Elles (1996), with some small changes, described in Tables 1 and 2.

Results and discussion

The ladder for D12S391, kindly donated by Lareu (Santiago de Compostela) and constructed for the original primer pair, could be efficiently amplified with the new primers, because the forward primer matched exactly with the original one and the new hybrid reverse primer matched internally with the target sequence.

The accuracy of the new primer pair was verified by amplifying 70 DNA samples where the genotype had been previously analysed for a population study (Klintschar et al.1998b). The results obtained using the new primer pair for D12S391 and the original primers were in complete agreement.

To check the assumption that the new primers increased the gel resolution, sequenced samples containing regular alleles and alleles 17.3, 18.3 and 19.3 were amplified using both primer pairs, and were run on the same type of gels as described above. As expected, the resolution of these alleles was greatly improved using our new primers.

Figure 1 shows a denaturing polyacrylamide gel where samples with the two primer pairs (the previous and the new one, separately amplified) of the same DNA were put in the same lanes. After 3 h of electrophoresis, the separation of the fragments obtained with the new primers was clearly better, and allowed the alleles to be interpreted by comparing them to the reference ladder, also when there were consecutive alleles containing a .3 variant.

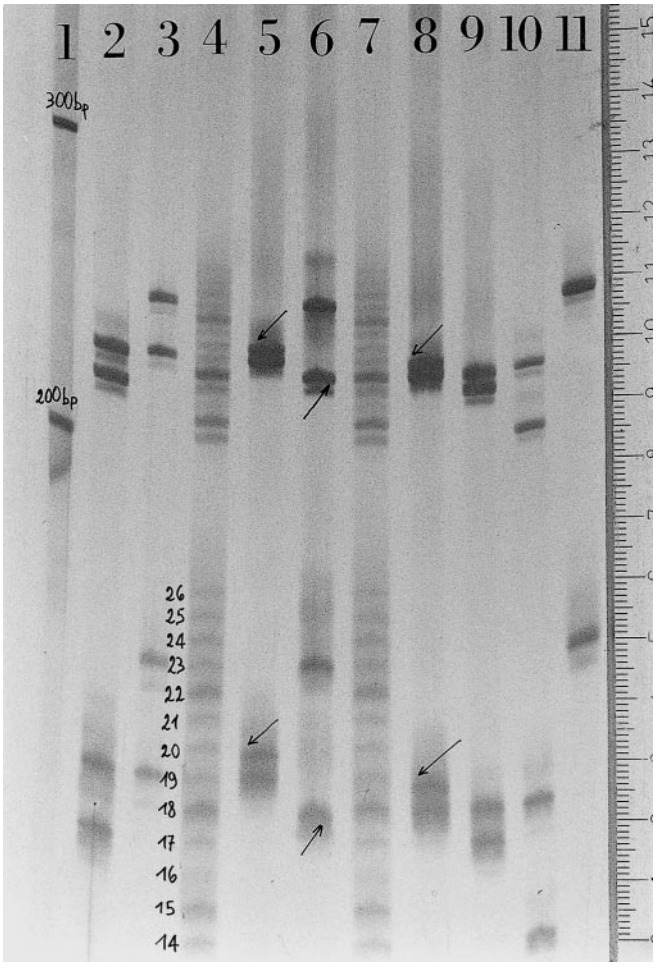

Fig. 1 Comparison of STR patterns with new and old primers in non-denaturing vertical system detected by the proposed silver staining technique. The amplified samples from same DNA were added together in the gel and run for 3 h. The amplified products obtained with the old primers are at the top; those with new primers are at the bottom. Line 1: 100 bp ladder; line 2: 17,19; line 3: 19, 23; line 4: ladder D12S391; line 5: 19,19.3; line 6: 17.3,23; line 7: ladder D12S391; line 8: 18,18.3; line 9: 17,18; line 10: 14,18; line 11: K562 (23,23). The arrows indicate the .3 variant alleles

One further attribute of the D12S391 locus is a sequence variation in the last repeat (Glock et al. 1997) which causes differences in electrophoretic mobility in native PAGE. Applying this method for typing is therefore not recommended (Lareu et al. 1996; Klintschar et al. 1998b). According to our results with native PAGE, it might be possible to achieve separation of these sequence variants on native gels under optimal conditions using our primers. Nevertheless we strongly recommend not to use this for additional information from casework, since the exchange of data between different laboratories would be handicapped.

Forensic casework

To check whether our short primers could actually improve the forensic suitability of the D12S391 locus, we analysed 32 samples of DNA, extracted from "problematic" stains (i.e. either stains with minimal DNA content or with partially degraded DNA) which were stored at –20 °C between 2 and 12 months. The samples consisted of 21 bloodstains, 3 semen stains, 5 hair roots, 3 saliva stains. Only 40% of the extracts (i.e. 13 out of 32), were successfully typed with the original primer pair. Using the new primers, positive results were obtained not only for those samples, but for 6 additional ones as well (1 semen stain, 1 hair root, 1 minimal blood stain on a knife and 3 blood stains mixed with plaster). The number of positive results could therefore be increased to approximately 60% (19 out of 32).

Although according to our experience in forensic casework with STRs, the sensitivity of a PCR reaction not only depends on the sequence of the primers or on the length of the amplified fragments, but also on individual differences in the quality of the primer synthesis, these results seem to support the assumption that our new primers are at least as sensitive as the old ones.

Silver staining

The new method described here proved to be advantageous, at least for staining vertical gels of denatured and non-denatured polyacrylamide. The development with the solution of 1.5% sodium hydroxide allowed regulating the intensity of the bands without an excessive background being formed. Adding 0.01% of $NaBH_4$ increased the contrast of the staining. The possibility of reusing the silver nitrate solution for more than ten times and the final arrest that can be performed also in running water, let to a allowed saving of reagents. The only inconvenience of this method is that it made the gels become brown-yellow. This discoloration is quite visible for native gels on gel-bond sheets, whereas it is not that strong in denaturing gels. A possible explanation might be a reaction of the gel-bond sheet and the highly alkaline solution.

Conclusions

We have shown that the D12S391 locus can be typed using a primer pair that produces shorter fragments (125–173 bp), for which an increased resolution using denaturing PAGE is possible. Nevertheless, reliable typing on native gels could not be achieved. In casework samples, the new primers show a higher sensitivity when compared with the original primers. Furthermore, we present a new method for visualising the DNA bands to by means of silver staining, which can be used for electrophoretic systems in which the gels are in direct contact with the glass plates.

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