Int J Legal Med (2002) 116:165–169

TECHNICAL NOTE

T. Lederer · G. Braunschweiger · P. Betz · S. Seidl

Purification of STR-multiplex-amplified microsamples can enhance signal intensity in capillary electrophoresis

Received: 30 May 2001 / Accepted: 26 October 2001

Abstract In this study, the effect of sample purification on total signal intensities of samples amplified with genRES MPX-2 (nine-locus multiplex) prior to capillary electrophoretic analysis has been investigated. Sample purification with the Qiaquick PCR purification kit led to an increase of the relative fluorescent signal intensity by a factor of 3.8 ± 0.8. In contrast, the application of larger sample volumes led to a decrease of signal intensities from 20% to 80%, depending on whether the samples were purified or not. In addition, increase of injection time showed a linear increase of signal intensity between 3 s and 10 s. Increasing the number of PCR cycles from 30 to 33 also led to a significant increase of signal intensities. Nevertheless, this increase greatly depended on the fragment lengths and was sometimes accompanied by the appearance of non-specific signals. In combination, optimisation of sample preparation and increase of injection time may intensify signals up to 12-fold, thereby increasing the overall sensitivity of the assay. This may be of special interest for forensic analysis of microspecimens containing limited amounts of DNA.

Keywords Purification · Multiplex · STR · Allelic drop-out

Introduction

Due to reproducibility of fragment sizing, high sensitivity and the possibility of considerable automatisation, capillary electrophoresis (CE) is a widely used technique for the sizing of PCR-amplified short tandem repeat (STR) polymorphisms [1, 2, 4, 10]. Compared to slab-gel electrophoresis, data obtained by CE show a somewhat higher reproducibility in sizing precision [4, 8]. Unlike traditional gel electrophoresis, samples are introduced into the sieving matrix by electrokinetic injection. Therefore, the CE requires a smaller sample volume and the sample can be automatically reinjected for re-running or method development purposes [1, 11]. Nevertheless, analysis of microspecimens containing limited amounts of DNA may lead to an overall low signal intensity or, even worse, nondetection of alleles, complicating the correct interpretation of the results. Because it is well known that the conductivity of applied samples greatly influences the electrokinetic injection of fragments [3], it was obvious to postulate that purification of samples might enhance the efficiency of fragment injection.

While several methods, such as ultrafiltration and high performance liquid chromatography (HPLC) have been described to separate PCR-amplified products from salts, unused dNTPs and unincorporated primers for direct sequencing of PCR samples [11], none of these purification methods has been outlined to be an effective tool for the optimisation of CE performance for multiplexed STR loci in forensic casework. Therefore, in this study the influence of post-PCR sample purification has been systematically investigated with regard to signal intensities of samples containing confined DNA amounts.

Material and methods

DNA extraction

For extractions from bloodstains and buccal cell swabbings, the Chelex 100 extraction method (Bio-Rad) was used [15]. Extraction from fresh blood was done using a standard phenol-chloroform method with ethanol precipitation. DNA solutions were diluted with distilled water to a concentration of < 20 pg/µl. The DNA amount contained in 5 µl was verified with the Quantiblot Human DNA Quantitation Kit (Applied Biosystems).

T. Lederer (✉) · P. Betz · S. Seidl
Institut für Rechtsmedizin, Universität Erlangen-Nürnberg,
Universitätsstrasse 22, 91054 Erlangen, Germany
e-mail: thomas.lederer@recht.imed.uni-erlangen.de,
Tel.: +49-9131-8522272, Fax: +49-9131-8522274

G. Braunschweiger
Serac GmbH, Bad Homburg, Germany

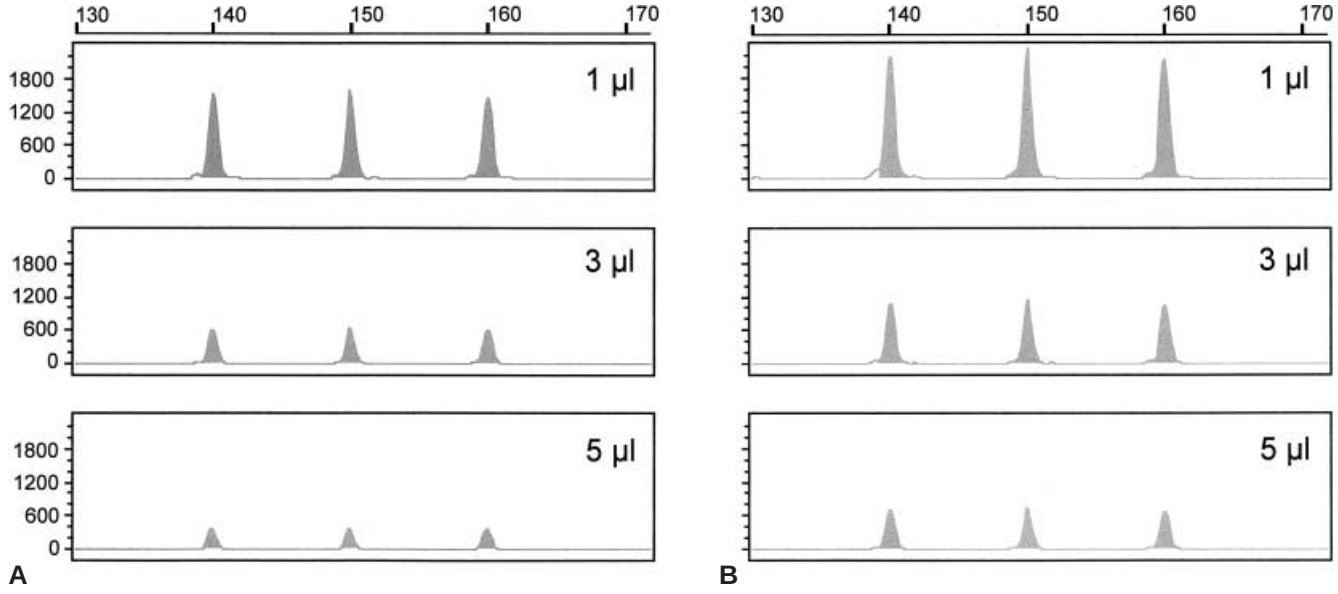

Fig. 1 Internal standard signal intensities (140 bp, 150 bp and 160 bp) using different applied volumes of **A** non-purified and **B** purified samples (all electrophoretic runs were performed with 0.5 µl of LS500 ROX and an injection time of 3 s)

PCR amplification and sample purification

The multiplex PCR was performed using the genRES MPX-2 amplification kit (Serac, Bad Homburg, Germany). With this kit, the loci vWA, SE33, TH01, D21S11, D8S1179, D3S1358, FGA, D18S51 and the gender-specific amelogenin locus are simultaneously amplified. PCR was done with a 5 µl aliquot of sample DNA (less than 100 pg) in a total volume of 25 µl using the following parameters: hot start (12 min at 95 °C), 30/33 cycles (1 min at 93 °C, 1 min at 59 °C, 1 min 30 s at 72 °C), final step (45 min at 60 °C). Two amplifications were performed in parallel for each sample which were then pooled to give a total volume of 50 µl and 30 µl was purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions (eluted volume 30 µl). Prior to electrophoresis samples were prepared as follows: 1–5 µl amplified DNA, 12 µl formamide and 0.5 µl of internal lane standard LS 500 ROX (Serac). Sample denaturation was done at 95 °C for 2–3 min. The detection of the signals was performed with the ABI Prism 310 Genetic Analyzer according to the manufacturer's instructions (run module GS STR POP-4 F).

Results

Sample purification and applied volume

The effects of post-PCR sample purification of six multiplex-amplified DNA samples have been studied with regard to the total signal intensity of the internal lane standard LS500 ROX as well as of allelic signals. From all of the samples less than 100 pg was used for the amplification reaction to simulate microspecimens containing limited DNA amounts. In all cases, partial non-detection of allelic signals was observed using electrophoretic conditions as recommended by the manufacturer.

After purification of the samples, signal intensities of the internal standard fragments were increased by a factor of 2.5 ± 0.8. A strong tendency to higher factors was observed when higher volumes of the amplified sample were applied to the capillary.

To check the influence of larger sample volumes, 1 µl, 3 µl and 5 µl of the amplified DNA fragments were used for electrophoretic separation. Generally, increasing the sample volume had a negative effect on standard signal intensities. Without purification, an increase of the sample volume from 1 µl to 3 µl and 1 µl to 5 µl led to a decrease of the signal intensity of about 40% (factor 0.6 ± 0.2) and 80% (factor 0.2 ± 0.0), respectively (Fig. 1 A). This decrease was less pronounced after sample purification (Fig. 1 B). In this case, the factors were 0.8 ± 0.1 (3-fold applied volume) and 0.4 ± 0.1 (5-fold applied volume).

For specific allelic signals, post-PCR sample purification resulted in an increase of signal intensities by a factor of 3.8 ± 0.5. As already seen for internal lane standard signals, this factor depended slightly on the applied sample volume. The higher the volume, the higher was the positive effect of sample purification. In contrast to observations with standard signals, larger applied volumes can result in higher signals of sample-specific fragments. Nevertheless, this effect was only slightly pronounced. Increasing the applied volume from 1 µl to 3 µl led to signal intensities which were enhanced by a factor of 1.6 ± 0.3 for purified samples and 1.3 ± 0.2 for non-purified ones. No further effect was observed when even higher volumes (5 µl) were applied to the system, indicating that there is no reason to apply more than 3 µl of amplified DNA.

In Fig. 2, two identical samples are shown, one of which was purified prior to electrophoretic analysis. As marked by asterisks, 12 out of a total of 17 allele signals were not detected by the analysis software (Fig. 2 A). Of course, some of these signals can still be distinguished from the background noise and, therefore, may be considered as real allele signals. Nevertheless, at least five of them were not detected at all, whereas a strong dependence of the total fragment length was observed. After sample purification, the complete profile was detectable (Fig. 2 B).

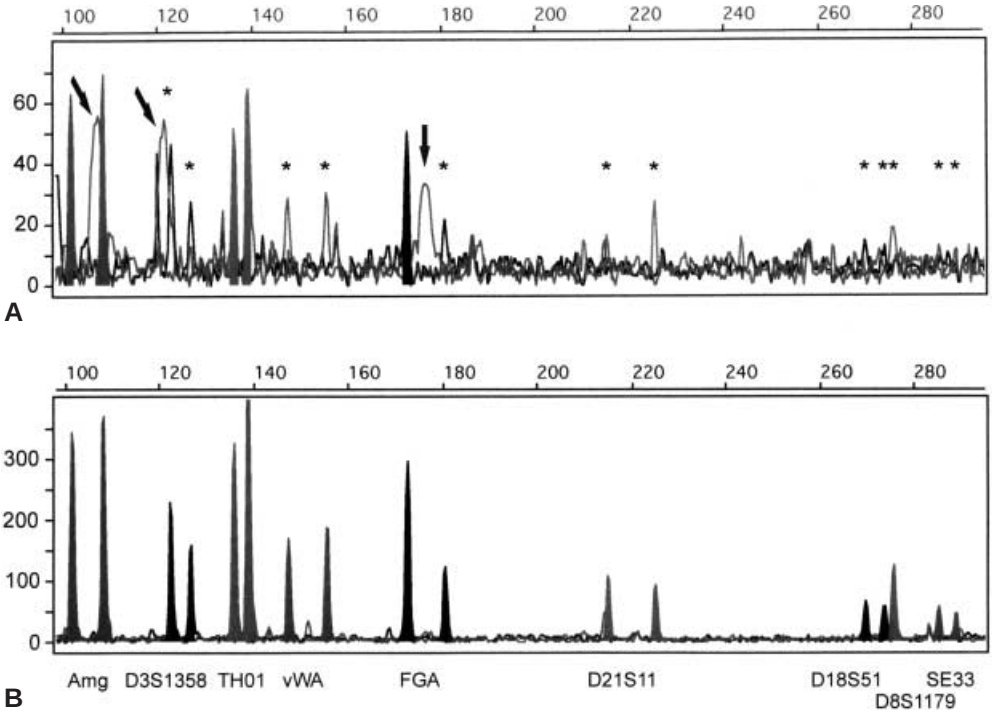

Fig. 2 Electropherograms of two identical blood samples, **A** a non-purified sample and **B** a purified sample. Non-detected alleles (peak height threshold: 50 relative fluorescent units) are marked with an asterisk (*Arrows* depict non-specific signals which may be due to residual fluorescent dye molecules. Runs were performed with a sample volume of 1 µl each and an injection time of 3 s. Amplified loci are indicated at the bottom)

Injection time

In this study, injection times of 1 s–10 s were tested. In this range, a linear dependence between signal intensity and injection time was observed for specific signals as well as for the internal lane standard. This effect occurred independently of sample purification and/or applied sample volume. Nevertheless, injection times longer than 10 s may influence electrophoretic fragment separation and, therefore, can lead to broad signals which may not be sized correctly.

Number of PCR cycles

Theoretically, the addition of three cycles should lead to an 8-fold increase of signal intensity. In practice, factors from up to 10-fold were observed in this study, strongly depending on the total fragment length. While intensities of short fragments (< 200 bp) appeared to be about 3-fold higher (3.0 ± 1.4) after three additional cycles, the factor was only 1.8 ± 0.6 for fragments longer than 200 bp. Signals at the TH01 locus were enhanced by a factor of 7.5 ± 2.0.

In Fig. 3, signals detected at the vWA locus are shown for a highly diluted saliva sample of one person. Whereas the allele signals showed a low signal intensity using the non-purified sample (Fig. 3 A), both alleles of the heterozygous genotype were clearly detectable after sample purification (Fig. 3 B). The heterozygous genotype could also be typed when using 33 PCR cycles (Fig. 3 C). Nevertheless, two additional signals appeared, one of which matched with the allelic ladder and, therefore, may lead to a misinterpretation of this sample.

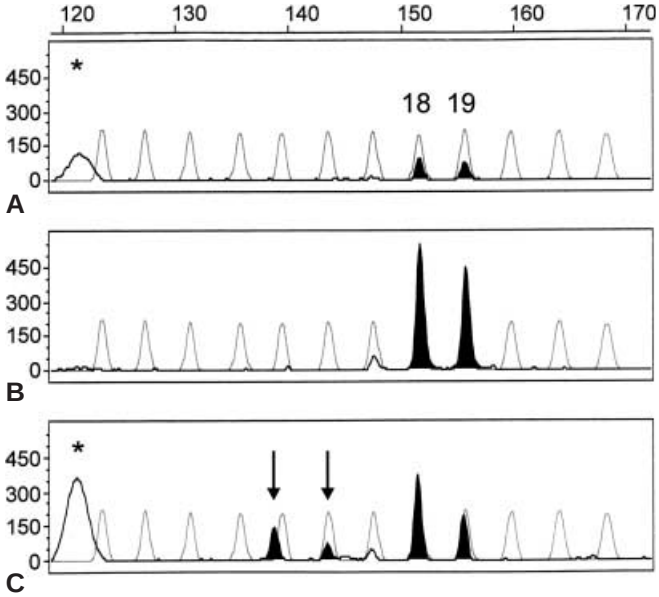

Fig. 3 Amplification products at the vWA locus (genotype 18/19) of DNA extracted from a saliva sample using 30 PCR cycles **A** without and **B** with sample purification and **C** with 33 PCR cycles for amplification (*Arrows* depict non-specific signals, signals representing residual fluorescent dye molecules are marked with an asterisk. Runs were performed with a sample volume of 1 µl each and an injection time of 3 s)

Discussion

The analysis of microspecimens containing limited amounts of potentially degraded DNA often results in non-detection of allelic signals. On the one hand, this effect can be due to non-amplification during PCR (allelic drop-out),

mostly occurring in longer amplicons. This well known effect has recently been addressed by using PCR primers which result in short fragment lengths of the amplified loci [6, 16]. A significant increase in sensitivity has been reported with these systems.

On the other hand, the presented results clearly show that missing signals also can be the consequence of non-detection during electrophoresis. Interestingly, the enhancement of signal intensities by post-PCR purification was not coupled to the size of amplified fragments, indicating another underlying mechanism. In fact, the reason for a lack of detection can mainly be seen in the electrokinetic injection of the amplified fragments, which is greatly influenced by the ionic strength of the samples [3]. Optimal injection efficiency has been reported for a residual anion concentration of 3.5 mM or less [14]. Moreover, small DNA fragments such as non-implemented primer molecules compete with allelic fragments for sample injection due to their low molecular weight. Especially when using multiplex PCR systems, high amounts of residual primer molecules are present in the amplification mix and, therefore, may reduce the overall sensitivity of the assay. In previously published results it has been shown that sample preparation, buffer composition or precipitation methods may influence the efficiency of electrokinetic injection in DNA sequencing [5]. In addition, similar but weaker effects have recently been described for samples amplified with the AmpFlSTR Profiler Plus™ and COfiler™ kits from Applied Biosystems [13].

A positive effect of sample purification has not only been seen for allelic signals but also for the signal-to-noise ratio. Unspecific signals, which have previously been reported to be fluorescent dye molecules split off primer fragments, which may interfere with allelic allocation ([9], see arrows in Fig. 2 A), could not be detected in purified samples.

Whereas significant enhancing effects have been shown for samples purified after the amplification reaction, the studies revealed that in general, the increase of the applied sample volume had only little or, even worse, a negative effect on signal intensities. In the worst case, the application of sample volumes larger than 3 µl may result in a lack of correct standard allocation, as relative fluorescent intensities fall below the detection limit of the system. Therefore, applying larger sample volumes is not an appropriate way to enhance the performance of CE. In addition, the concentration of formamide is decreased with higher sample volumes, possibly resulting in poor denaturation of the DNA strands and, therefore, leading to non-satisfying electrophoretic fragment separation.

For samples containing high amounts of DNA, injection times up to 3 s are recommended to avoid the appearance of off-scale signals (CE310 users manual, Applied Biosystems). However, the analysis of microspecimens often results in low signal intensities, suggesting an enhancing effect of longer injection times, which in fact has been observed in this study. Nevertheless, using injection times longer than 10 s can lead to broad signals. As a consequence, the clear allocation of alleles is no longer guaranteed. Of course, this predominantly concerns allelic differences of 1 bp such as 9/9.3 at the locus TH01 or 18.3/19 at the SE33 locus.

Allelic drop-out is a consequence of non-amplification during the PCR. Therefore, the increase of PCR cycle numbers is a widely used way to enhance signal intensities [7, 12]. Nevertheless, the enhancing effect appeared to be strongly dependent on the amplicon length. A further disadvantage of this procedure might be the occurrence of non-specific amplification products and the accentuation of potential contamination.

Conclusion

The purification of multiplex-amplified samples prior to capillary electrophoresis and concomitant increase of the injection time may enhance the intensity of allelic signals approximately 12-fold (11.9 ± 1.1). Compared to an increase of PCR cycle numbers, this method leads to an at least similar enhancement of the signal intensities, independent of fragment length and without the risk of the appearance of non-specific signals and contamination which may complicate the interpretation of analytical results. Therefore this approach represents a simple possibility to enhance sensitivity without the need of re-amplification and further consumption of sample material.

References

1. Buel E, Schwartz MB, LaFountain MJ (1998) Capillary electrophoresis STR analysis: comparison to gel based systems. Forensic Sci Int 43:164–170
2. Butler JM, Li J, Shaler TA, Monforte JA, Becker CH (1998) Reliable genotyping of short tandem repeat loci without an allelic ladder using time-of-flight mass spectrometry. Int J Legal Med 112:45–49
3. McCord BR, McClure DL, Jung JM (1993) Capillary electrophoresis of polymerase chain reaction-amplified DNA using fluorescence detection with an intercalating dye. J Chromatogr A 652:75–82
4. Deforce DLD, Millecamps REM, Hoofstaat D van, Eeckhout EG van den (1998) Comparison of slab gel electrophoresis and capillary electrophoresis for the detection of the fluorescently labeled polymerase chain reaction products of short tandem repeat fragments. J Chromatogr A 806:149–155
5. Hadd AG, Goard MP, Rank DR, Jovanovich SB (2000) Submicroliter DNA sequencing for capillary array electrophoresis. J Chromatogr A 894:191–201
6. Hellmann A, Rohleder U, Schmitter H, Wittig M (2001) STR typing of human telogen hairs – a new approach. Int J Legal Med 114:269–273
7. Klintschar M, Crevenna R (1996) Validation of the STR system FXIIIB for forensic purposes in an Austrian population sample. Forensic Sci Int 81:35–42
8. Lazaruk K, Walsh PS, Oaks F, Gilbert D, Rosenblum BB, Menchen S, Scheibler D, Wenz HM, Holt C, Wallin J (1998) Genotyping of forensic short tandem repeat (STR) systems based on sizing precision in a capillary electrophoresis instrument. Electrophoresis 19:86–93
9. Lederer T, Seidl S, Graham B, Betz P (2000) A new pentaplex PCR system for forensic casework analysis. Int J Legal Med 114:87–92

10. Mansfield ES, Robertson JM, Vainer M, Isenberg AR, Frazier RR, Ferguson K, Chow ST, Harris DW, Barker DL, Gill PD, Budowle B, McCord BR (1998) Analysis of multiplexed short tandem repeat (STR) systems using capillary array electrophoresis. Electrophoresis 19:101–107
11. Marino MA, Devaney JM, Smith JK, Girard JE (1998) Sequencing using capillary electrophoresis of short tandem repeat alleles separated and purified by high performance liquid chromatography. Electrophoresis 19:108–118
12. Martineau F, Picard FJ, Menard C, Roy PH, Ouellette M, Bergeron MG (2000) Development of a rapid PCR assay specific for *Staphylococcus saprophyticus* and application to direct detection from urine samples. J Clin Microbiol 38:3280–3284
13. Moretti TR, Baumstark AL, Defenbaugh DA, Keys KM, Brown AL, Budowle B (2001) Validation of STR typing by capillary electrophoresis. J Forensic Sci 46:661–676
14. Vainer M, Enad S, Dolnik V, Xu D, Bashkin J, Marsh M, Tu O, Harris DW, Barker DL, Mansfield ES (1997) Short tandem repeat typing by capillary array electrophoresis: comparison of sizing accuracy and precision using different buffer systems. Genomics 41:1–9
15. Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10:506–518
16. Wiegand P, Kleiber M (2001) Less is more – length reduction of STR amplicons using redesigned primers. Int J Legal Med 114: 285–287