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TECHNICAL NOTE

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Low-volume amplification on chemically structured chips using the PowerPlex16 DNA amplification kit

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Abstract In forensic DNA analysis, improvement of DNA typing technologies has always been an issue. It has been shown that DNA amplification in low volumes is a suitable way to enhance the sensitivity and efficiency of amplification. In this study, DNA amplification was performed on a flat, chemically structured glass slide in 1-µl reaction volumes from cell line DNA contents between 1,000 and 4 pg. On-chip DNA amplification reproducibly yielded full allelic profiles from as little as 32 pg of template DNA. Applicability on the simultaneous amplification of 15 short tandem repeats and of a segment of the Amelogenin gene, which are routinely used in forensic DNA analysis, is shown. The results are compared to conventional in-tube amplification carried out in 25-µl reaction volumes.

Keywords STR · On-chip amplification · DNA typing · Low-volume amplification · Fragment length analysis

Introduction

DNA analysis has become an indispensable tool in almost every forensic laboratory and is an integral part of main forensic tasks (i.e., paternity testing, human and nonhuman identification, and examination of forensic stains) [1–6]. The application of polymerase-based amplification of DNA—and especially of autosomal short tandem repeats (STRs)—to forensic molecular genetics has been path-breaking [7–10]. Meanwhile, there is a considerable panel of sufficiently validated STR loci that is available in the form of manufactured kits that can be employed to conveniently process the majority of samples that come up in forensic casework [e.g., 11].

However, there are occasions when conventional analyses of autosomal STRs fail. To deal with an ever-increasing amount of samples containing either small amounts of template or degraded DNA, several alternative approaches have been applied to forensic DNA typing, depending on the problem. Whenever maternal relationship is in question, analysis of mitochondrial (mtDNA) and X-chromosome markers can be of major interest [12, 13]. Due to a higher copy number, analysis of mtDNA is promising in degraded samples and in samples lacking great amounts of nuclear DNA (e.g., compact bone and hair shafts) [14, 15]. Analysis of degraded DNA samples has also been improved by using redesigned primers yielding reduced-size STR amplicons [16]. Y-chromosome STRs are applied to analyses of male/female mixed stains [17].

Other than changing the target of analysis, there is a possibility to enhance the sensitivity of DNA amplification and to improve the detection of amplification products in order to generate reliable allelic information from the smallest amounts of template DNA. This objective brought about many attempts to improve existing methods of DNA analysis or to develop alternative strategies (e.g., based on mass spectrometry, denaturing high-performance liquid chromatography, microfluidic electrophoresis, or microarray technologies) [18–24].

Miniaturization of amplification reactions as well as reduction of reaction volumes have been identified as possible methods to enhance the sensitivity of amplification reactions and to simplify and speed up DNA analyses [24, 25]. Initial developments have been made of lab-on-chip solutions that combine sample preparation, DNA amplification and detection, and determination and visualization of results [26]. Various approaches in (micro)miniaturization of PCR reactions, using microchips, capillaries, and surfaces of microarrays, exist [18, 20, 27–29]. Electronic

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microarrays also integrate the amplification and detection of multiple samples [30]. These methods are aimed at automated high-throughput processing of samples and have been introduced to forensic single nucleotide polymorphism typing, rather than to STR typing [31]. However, many chip-based approaches require considerable expansion or even change of laboratory equipment, especially when analyzing STRs [27, 32, 33].

In this paper, the technical feasibility of efficient DNA amplification in minimal reaction volumes, using a chemically structured chip that can be integrated into existing laboratory environments concerned with detection, is shown.

Materials and methods

DNA

Human female DNA 9947A, supplied with the commercially available PCR amplification kit PowerPlex16 (Promega, Mannheim, Germany), was diluted in sterile water into DNA contents of 1,000, 500, 250, 125, 63, 32, 16, 8, and 4 pg, respectively.

DNA amplification on chemically structured chips

Chips

The chips were supplied by Alopex GmbH (Kulmbach, Germany). The basic format is a standard microscope glass slide, which was originally developed for single cell analysis and quantification of single genome equivalents [34]. Each chip exhibits a flat hydrophobic surface, which contains 60 hydrophilic reaction compartments ("anchor spots," 1.6 mm in diameter) arranged in 12 rows of five spots. Each of these anchor spots can be loaded with a 1-µl amplification reaction volume, which is then covered with oil in order to prevent evaporation and contamination (Fig. 1a). The actual pipetting scheme with positioning of negative controls and arrangement of samples is given in Fig. 1b.

On-chip PCR

In the experiments described here, reagents from the PCR amplification kit PowerPlex16 (Promega) were used. Each PCR reaction contained 0.1 µl of Gold Star 10× buffer, 0.1 µl of PowerPlex16 Primer Pair Mix, 0.5 µl of diluted

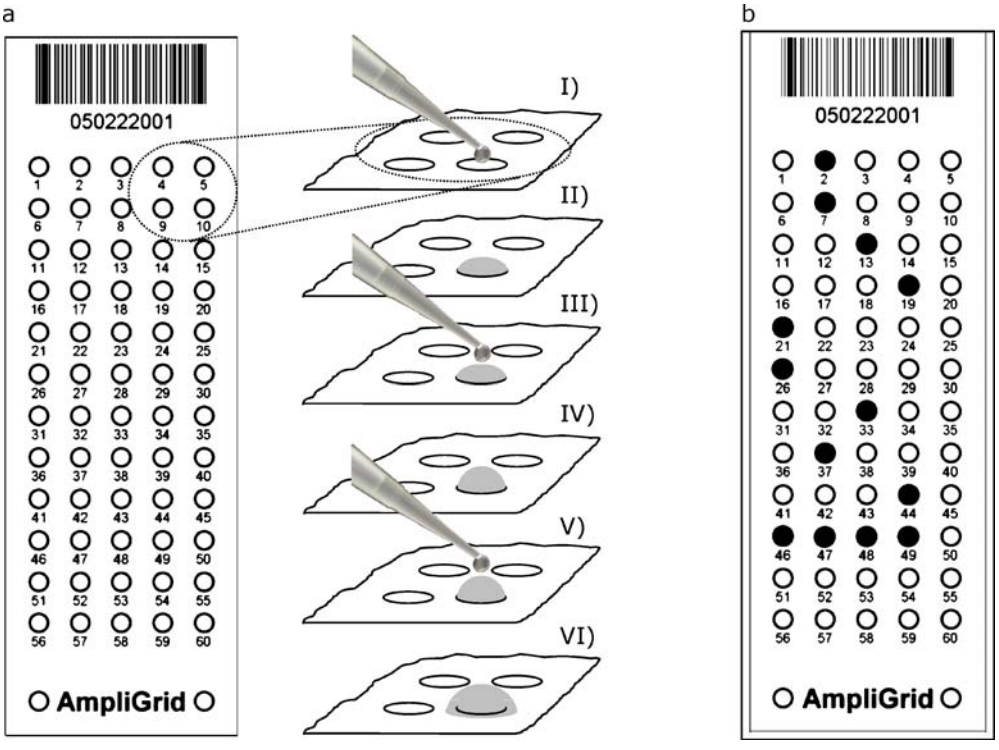

Fig. 1 **a** Preparation of the amplification chip. Application of 0.5 µl of aqueous template solution (*I*), droplet of template solution confined to a hydrophilic reaction compartment (*II*), addition of 0.5 µl of PCR reaction mix (*III*), complete reaction droplet confined to a hydrophilic reaction compartment (*IV*), addition of 5 µl of oil (*V*), finished preparation of one 1-µl amplification reaction covered with 5 µl of oil (*VI*). **b** Example of a pipetting scheme. Amplification chip with randomly distributed negative controls. *Black spots* indicate the positions of the negative controls. Samples were loaded onto 40 of 60 anchor spots and were distributed as follows: *1,000 pg*, reaction compartments 1, 3, and 4; *500 pg*, reaction compartments 6, 8, and 9; *250 pg*, reaction compartments 11, 12, and 14; *125 pg*, reaction compartments 16, 17, and 18; *63 pg*, reaction compartments 22, 23, and 24; *32 pg*, reaction compartments 27, 28, and 29; *16 pg*, reaction compartments 31, 32, and 34; *8 pg*, reaction compartments 36, 38, and 39; *4 pg*, reaction compartments 41, 42, and 43

DNA template, and 0.5 U of Taq DNA Polymerase (Promega), in a total volume of 1 μl. Cycling was performed using an Eppendorf Mastercycler with in situ adapter (Eppendorf AG, Hamburg, Germany). Cycling conditions were set according to the manufacturer's recommendations. Negative controls were performed randomly on different positions on the chip using the same reagent solutions without DNA.

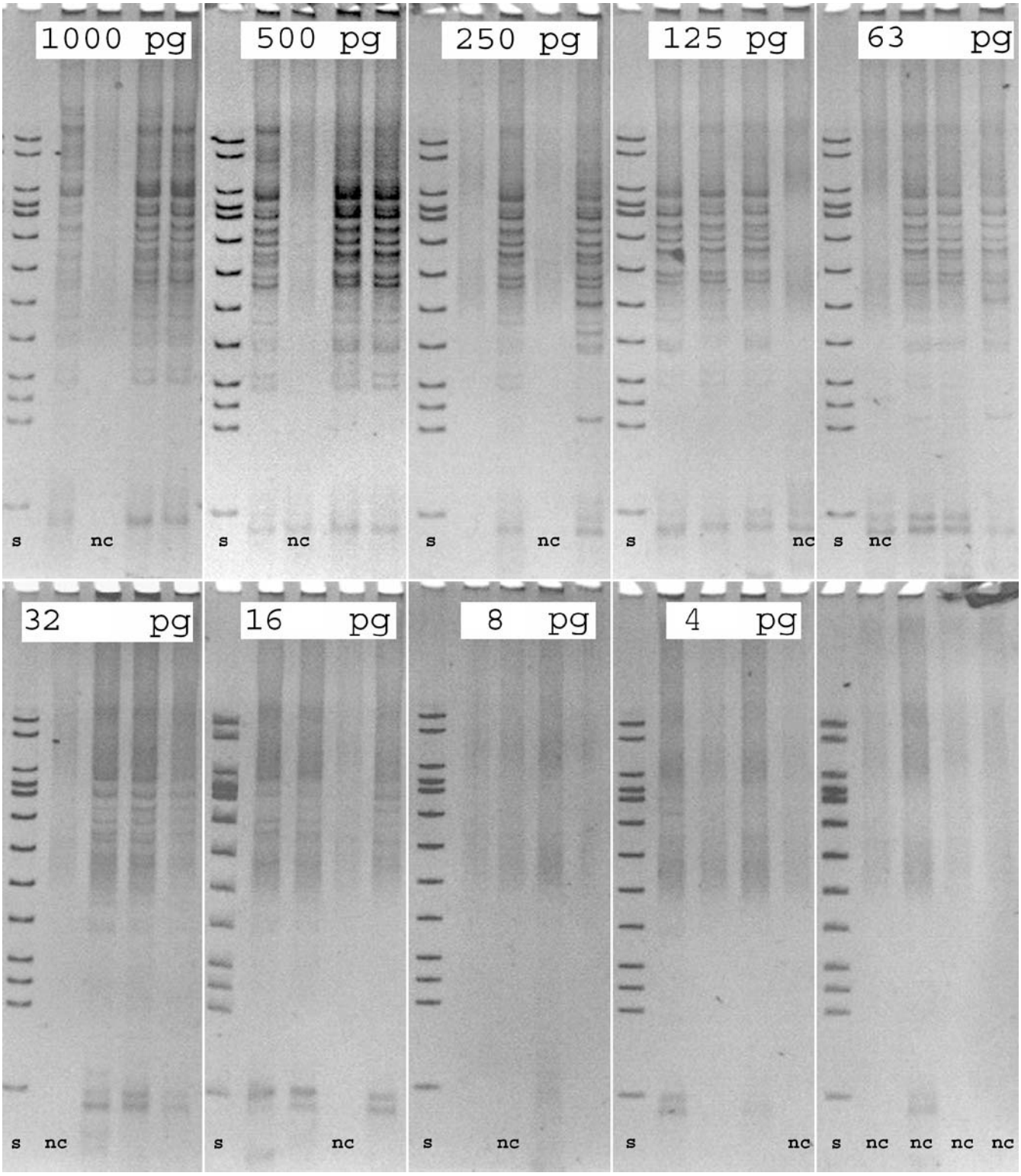

Fig. 2 Polyacrylamide gel electrophoresis analysis of 40 Promega PowerPlex16 amplification products from a single chip. The amount of template is indicated. *nc* Negative control, *s* size standard, DNA molecular weight marker VIII (Roche, Mannheim, Germany), including fragments of (19, 26, 34, 34, and 37 bp), 67, 110, 124, 147, 190, 242, 320, 404, 489, 501, 692, and 900 bp

Table 1 Results of on-chip and in-tube PCR reactions in relation to the amount of DNA template

PCR reactions	Amount of template (pg)								
	1,000	500	250	125	63	32	16	8	4
On-chip PCR									
Complete allelic profile	12	12	10	12	11	5	0	0	0
Allelic dropout	0	0	0	0	0	2	11	6	10
Unsuccessful amplification	0	0	2	0	1	5	1	6	2
Total	12	12	12	12	12	12	12	12	12
In-tube PCR									
Complete allelic profile	6	6	5	5	3	0	0	0	0
Allelic dropout	0	0	1	1	3	6	4	1	0
Unsuccessful amplification	0	0	0	0	0	0	2	5	6
Total	6	6	6	6	6	6	6	6	6

The actual numbers of samples are given

Preparation of the chips

Four chips were prepared for cycling, with each chip containing 27 samples with varying DNA contents and 13 negative controls. Individual anchor spots were supplied with 0.5 μl of DNA template and 0.5 μl of reagent solution. On each chip, 40 of 60 anchor spots (ten rows of four spots) were prepared (see Fig. 1b). Finally, each anchor spot was covered with 5 μl of mineral oil.

Analysis of PCR products

After amplification, all PCR products were transferred into 0.2-ml reaction tubes and mixed with 5 μl of H_2O bidest, and 1 μl of each diluted sample was analyzed on a 6% polyacrylamide (PAA) gel (10 min, 62 W, and 400 mA). Gels were stained with 0.1% $AgNO_3$ and developed with (1:3) 0.3 M NaOH/36.5% formaldehyde solution. The remaining 5 μl was dissolved in 10 μl of formamide. Samples were centrifuged to completely separate the mineral oil from the aqueous sample. Ten microliters of each diluted sample was then analyzed on an ABI Prism 3100 Avant Genetic Analyzer using GeneMapper ID Software Version 3.1 (Applied Biosystems, Darmstadt, Germany).

DNA amplification in PCR reaction tubes

Conventional PCR

Each PCR reaction contained 2.5 μl of Gold Star 10× buffer, 2.5 μl of PowerPlex16 Primer Pair Mix, 1 μl of diluted DNA template, and 4 U of Taq DNA Polymerase (Promega), in a total volume of 25 μl. Cycling was performed using an Eppendorf Mastercycler (Eppendorf AG). Cycling conditions were set according to the manufacturer's recommendations. Negative controls were performed.

Analysis of PCR products

From each amplification product, 5 μl was analyzed on a 2.5% agarose gel prior to capillary gel electrophoresis on an ABI Prism 3100 Avant Genetic Analyzer. For the analysis, GeneMapper ID Software Version 3.1 (Applied Biosystems) was used.

Results

On-chip PCR

Altogether, 108 DNA samples and 52 negative controls were amplified. Figure 2 shows a silver-stained PAA gel of 40 amplification products obtained from one of four chips. The samples include 27 amplification products resulting from the amplification of different contents of template DNA (1,000, 500, 250, 125, 63, 32, 16, 8, and 4 pg) and 13 negative controls.

Capillary gel electrophoresis demonstrated that all 52 negative controls contained no amplification products. A summary of results on the content of DNA template is shown in Table 1. Altogether, 62 complete allelic profiles could be obtained from DNA contents from 1,000 to 32 pg. Figure 3a exemplarily shows an electropherogram of amplification products obtained from 32 pg of template DNA. Unsuccessful amplification occurred in 17 of 108 samples. This phenomenon affected samples with DNA template contents of 63 pg and less, as well as 1 of 12 samples with 250 pg of template. Allelic dropout of one allele (simulating homozygosity) as well as dropout of individual STR systems were found in 29 of 108 PCR products. All of these PCR products had been generated from template contents of 16 pg or less. Figure 3b shows an example of an allelic dropout simulating homozygosity in the STR system Penta E. Allelic dropout was observed in all STR systems.

In-tube PCR

For in-tube PCR, 60 amplification reactions were performed, including six negative controls (Table 1), and 25 samples generated from DNA contents between 1,000 and 63 pg yielded complete allelic profiles. Allelic dropout was observed in samples with template amounts of 250 pg and less. Unsuccessful amplification occurred in samples with DNA contents of 16 pg (two of six) and 8 pg (five of six). The amplification of 4 pg of template failed.

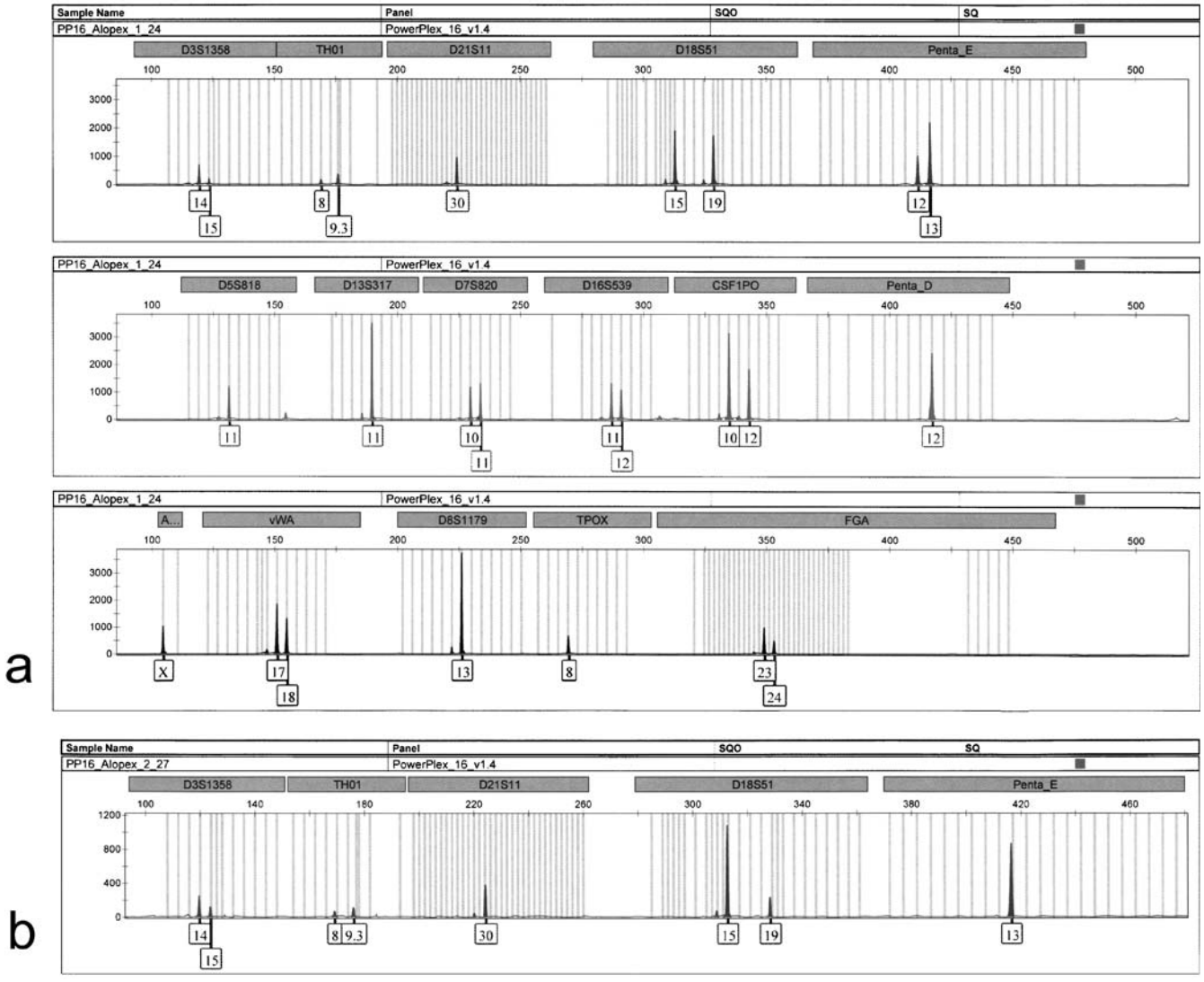

Fig. 3 **a** PowerPlex16 electropherogram of a sample generated from 32 pg of DNA template showing a complete allelic profile. **b** Electropherogram of fluorescein-labeled STRs of PowerPlex16 showing an allelic dropout simulating homozygosity in Penta E. This sample was amplified from 16 pg of DNA template

Discussion

As shown in this preliminary study, low-volume amplification on chemically structured chips has the potential to efficiently amplify even a large panel of forensically used STRs in a very small reaction volume.

Preparation, handling, and manipulation of the chip are very simple due to its chemical structure. Aqueous solutions (reaction mixes and template DNA) are attracted to the defined hydrophilic anchor spots.

Control of PCR yields is required prior to fragment length analysis. However, the small reaction volume of the chip PCR is challenging. Good impression of PCR results could be obtained with a small portion of the diluted samples on a PAA gel, with electrophoresis parameters as described here.

Initial testing showed that the close local arrangement of the anchor spots did not lead to any contamination of negative controls. However, 4 of 108 samples with template concentrations of 32, 16, and 4 pg, respectively, showed additional alleles in 1 of 16 analyzed loci. Most likely, these additional signals were PCR artifacts resulting from a minor activity of the enzyme used during chip preparation, which was performed at room temperature. Allelic dropout was observed in all STR systems. A correlation between allelic dropout and length of missing alleles or size of missing STR systems could not be detected. Allelic dropout, as a consequence of a higher dilution factor, was randomly distributed. Unsuccessful amplification reaction, as observed in 17 samples, was more likely due to the geometry of the droplets than to the inhibition from too much template. Further studies on this topic are necessary.

Allelic dropout simulating homozygosity was observed in all samples from DNA contents of 32 pg and less. A few of these samples did not show any dropout of complete

STR systems. Although the signals detected were not always balanced, the minor DNA content of the original sample was not obvious. Thus, the dropout would not have been discovered in all of these cases (see Fig. 3b). On the other hand, DNA amounts as low as 32 pg produced full allelic profiles, which was an improvement compared to conventional DNA amplification. The potential of a reduced reaction volume in regard to the sensitivity and efficiency of DNA amplification has also been shown for in-tube amplification [25].

The possibility of integrating chip-based DNA amplification into established laboratory equipment is a great advantage. The major detection platform for fragment length analysis of fluorescence-labeled PCR fragments in forensic DNA testing is capillary gel electrophoresis. Supplementary technologies, such as mass spectrometry or microchip devices [23, 27], rely on completely different methodologies and equipment and are unlikely to replace capillary gel electrophoresis. In contrast, use of the chemically structured chip presented in this study requires only slight modifications of pre-PCR processing.

The microscope slide format, in combination with the high-quality glass substrate of the PCR chip, provides the option of an optically—and even microscopically—controlled loading of small amounts of template (e.g., a few or even single epithelial cells). Single cell analysis without previous extraction of DNA has successfully been performed with this technique [34]. A future option might be an application for forensic microstain analysis without an extra DNA extraction step, since loss of DNA is still a problem in pre-PCR processing of microstains. However, omission of DNA extraction might lead to an increased concentration of PCR reaction inhibitors, especially in the very small reaction volume of 1 μl. Furthermore, with on-chip extraction and amplification, a repeated analysis would not be possible.

Last but not the least, the extremely reduced amount of reagent solutions required for a single chip-based amplification reaction makes analyses much more cost-efficient, especially if commercially available PCR amplification kits are utilized.

Low-volume amplifications on chemically structured chips hold great potential for forensic applications. Ongoing studies will further examine potential pitfalls and options of this interesting approach. The field of microstain analysis might especially benefit from this challenging new technique.

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