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TECHNICAL NOTE

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Sequential multiplex amplification (SMA) of genetic loci: a method for recovering template DNA for subsequent analyses of additional loci

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Abstract A method called Sequential Multiplex Amplification (SMA) has been developed whereby a limited amount of DNA extracted from a sample can be reutilized for several single polymerase chain reaction (PCR) amplifications. The method involves recovery of genomic template DNA by microfiltration of PCR-amplified samples. Up to 5 different loci have been typed, each in a single system PCR-based assay, beginning with a test quantity of 5 ng template DNA. Genotypes of the DNA donors were compared with those obtained from individual amplifications and shown to be identical. This could be a useful technique for typing a number of loci from a limited amount of DNa and to recover template DNA from samples previously subjected to PCR. Obviously, when small quantities of template DNA are available, this technique can prove quite useful.

Key words DNA · PCR · Sequential multiplex amplification (SMA) · STR · Forensic

Zusammenfassung Die Methode der sequentiellen multiplex Amplifikation (SMA) ermöglicht die Wiederverwendung einer limitierten Menge genomischer DNA für mehrere aufeinanderfolgende PCR Amplifikationen. Die Isolierung genomischer DNA aus den PCR-amplifizierten Proben erfolgte durch Mikrofiltration. 5 ng genomischer DNA wurden als Ausgangsmenge eingesetzt und bis zu 5 verschiedene Loci in getrennten PCR-Ansätzen nacheinander untersucht. Die Amplifikationsmuster der sequentiellen multiplex Amplifikation wurden mit denen aus individuellen Amplifikationen verglichen und waren identisch. Diese Technik ermöglicht die Untersuchung verschiedener Loci ausgehend von einer begrenzten Menge an genomischer DNA und kann daher besonders Anwendung finden, wenn geringe Mengen an genomischer DNA für die PCR Amplifikation zur Verfügung stehen.

Schlüsselwörter DNA · PCR · sequentielle multiplex Amplifikation (SMA) · STR · Forensik

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Introduction

Typing polymorphic loci at the DNA level has become a routine procedure in the identity testing field. One technique, the polymerase chain reaction (PCR) (Saiki et al. 1985), enables amplification of the DNA molecules in a sample. Thus, target DNA at subanalytical quantities can be increased to levels such that detection and typing is faciliated. DNA samples are usually amplified by PCR and subsequently typed: but the remainder of the untyped PCR is generally not used or discarded.

To obtain more genetic information from a sample several distinct loci can be amplified in one PCR simultaneously by a process known as multiplex PCR (Chamberlain 1989; Edwards et al. 1992). The advantage of multiplex systems is that less sample DNA is consumed than when analyzing each locus independently. Additionally, less reagents are required, and the time needed to perform population studies on several loci is reduced. However, multiplex optimization requires substantial effort to develop a robust system, where all loci in the multiplex amplify well under identical PCR conditions.

We propose here an alternate multiplex approach called Sequential Multiplex Amplification (SMA). SMA enables the typing of several loci using only one DNA sample without requiring al the loci to be amplified under one set of PCR conditions.

Furthermore, when a sample fails to amplify, it could be desirable to recover the template DNA and type it with a more robust analytical system.

Materials and methods

The general procedure for SMA typing is as follows:

1. Extract DNA with phenol:chloroform (e.g. Brinkmann et al. 1991).
2. Determine the quantity of the DNA with a slot blot procedure using a human specific alphoid probe (Waye et al. 1989). This step is used to determine the initial input quantity of DNA for SMA. For research purposes, we have determined the quantity of the recovered DNA by slot blot analysis after each amplification and purification step in the SMA. However, this is not a necessary or practicable step in routine forensic casework.
3. Amplify and type DNA for the D1S80 locus according to the method of Budowle et al. (1991) and Baechtel et al. (1993).
4. Recover the genomic DNA from the remaining D1S80 PCR solution by filtration through a Microcon-100 (Amicon, Beverly, MA). Briefly, add 200 µl of sterile deionized water to the filter side of the device. Add all the remaining PCR solution. Centrifuge the filtration device in a microfuge at 2,000 g for 6 mins. Add an appropriate volume (i.e. 38.5 µl for a final volume at the next amplification of 50 µl) of retentate solution (100 m*M* Tris, 0.1 m*M* EDTA, pH 8.0), gently vortex the content for 5 s to resuspend the DNA, flip the device over and centrifuge at 1.5000 g for 5 mins. Since this volume contains all the recovered genomic DNA, it is important to attempt to use as much as possible of this volume in the next stage of SMA.
5. Amplify and type the recovered DNA for the HUMTH01 locus (Lorente et al. 1994a; Edwards et al. 1991, 1992; Wiegand et al. 1993).
6. Repeat step 4 and amplify and type for HUMVWA (Lorente et al. 1994b; Möller et al. 1994).
7. Repeat step 4 and amplify and type for HLA-DQx (Saiki et al. 1989).
8. Repeat step 4 and amplify mitochondrial DNA using mtDNA-specific primers. We amplified HV1 of human mitochondrial DNA according to be protocol established by Holland et al. (1993) with the following modifications: primers L15997 (5' CAC CAT TAG CAC CCA AAG CT 3') and H16395 (5' TGA TTT CAC GGA GGA TGG TG 3') were used in a 50 µl reaction. Cycling parameters were 95° C for 30 s, followed by 30 cycles of 95° C for 10 s, 60° C for 30 s, 72° C for 30 s. A 15° C soak for 10 mins followed the cycling procedure.

Results and discussion

SMA has easily enabled amplification of 5 loci using 5 ng of genomic DNA. In this study the loci were D1S80, HUMTH01, HUMVWA, HLA-DQalpha, and the D-loop of mitochondrial DNA. Each sample was amplified under it's own optimized PCR conditions, and each sample was typed correctly. There were not differences in typing results when using SMA or a single PCR approach (Fig. 1).

It is imperative to wash the PCR sample via Microcon-100 filtration. This process removes primers and other PCR reagents that could interfere with subsequent amplification of another locus. We have attempted to conduct SMA without an itnervening filtration step, but typing has not been successful.

As a general rule, it is recommended that amplifcation should proceed as follows: length polymorphisms first, in descending order by size, followed by sequence polymorphisms (currently detected by sequence-specific oligonucleotide probes) and mitochondrial DNA sequencing last. The reason for this hierarchy is threefold. First, since residual amplicons from preceding PCRs will be present, it is desirable to amplify the locus with the largest fragments first and with the smallest locus being amplified last. Therefore, length polymorphism loci that do not overlap in size should be employed. Moreover, by typing the largest length polymorphisms initially, heteroduplex bands from smaller loci will not interfere with DNA profile interpretation. Second, sequence-specific oligonucleotide probe detection will not be inhibited by length polymorphism amplicons that will be present in subsequent amplifications. Third, mitochondrial DNA sequencing was performed last because of the increased copy number of mitochondrial DNA compared with nuclear DNA and the high degree of specificity afforded by sequencing.

The efficiency of gemonic DNA recovery was related to the recovery solution volume. Table 1 shows that the efficiency of recovery of genomic DNA can be affected by the volume of retentate in the Microcon-100 device.

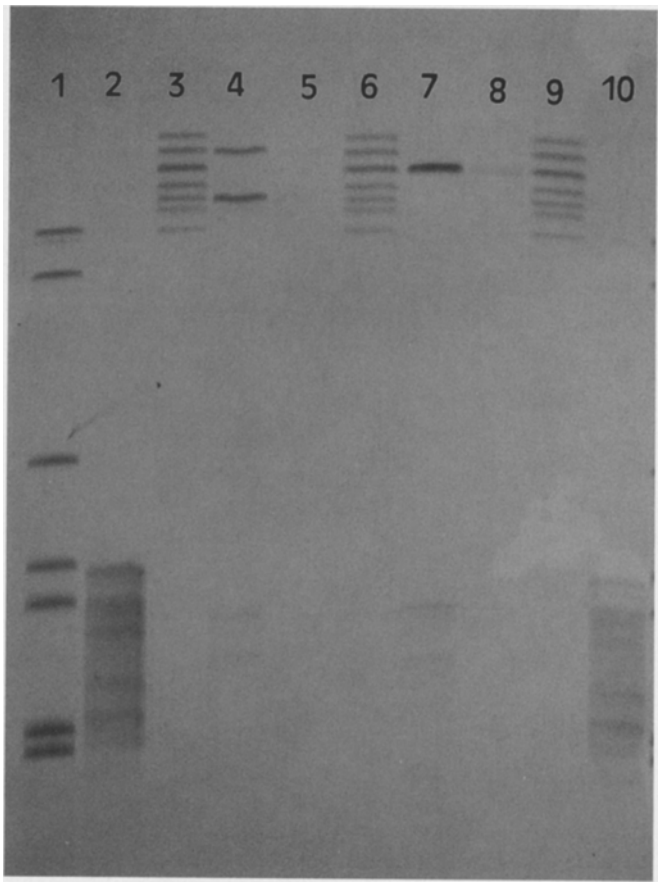

Fig. 1 HUMTH01 amplifications of 2 individuals with SMA. Lane: #1: 1 kb ladder; #2 & #10: D1S80 ladder; #3, 6 & 9: HUMTH01 ladder; #4: D1S80 and SMA-HUMTH01 genotypes of individual *a* (HUMTH01 amplification was performed by SMA after D1S80 amplification) #5: HUMTH01 genotype of individual *a* by single PCR; #7: D1S80 and SMA-HUMTH01 genotypes of individual *b* (HUMTH01 amplification was performed by SMA after D1S80 amplification). #8: HUMTH01 genotype of individual *b* by single PCR (D1S80 ladder and amplified alleles are blurred because they are shown in a HUMTH01 gel; they were previously amplified and typed in a D1S80 specific gel, but the product remains; for more explanations, see Discussion)

Table 1 Average percentage of recovery in the first 3 steps of SMA.

Recovery volume	1st recovery	2nd recovery	3rd recovery
38.5 µl	69%	44%	39%
77.0 µl	89%	61%	52%

The larger the Microcon-100 retenate volume, the higher the percentage of genomic DNA that was recovered. For example, when the recovery volume was 38.5 µl, an average of approximately 69% of the remaining genomic DNA was recovered. However, when the volume was increased to 77 µl, an average of 89% of the remaining genomic DNA could be recovered. The larger retenate volume might enable more effective solubilization of the DNA residing on the Microcon-100 filter membrane. Because some DNA is trapped in the membrane, even after the recovery procedure, the lower the amount of genomic DNA is, the less amount of DNA will be recovered.

The recovery of genomic DNA is enhanced by the presence of amplified DNA. In the course of this study we found that genomic DNA, at times, may be trapped on the Microcon-100 membrane. Since the amplified material is in excess in the sample compared with genomic DNA, amplified DNA, can most likely more readily bind to the Microcon-100 membrane and block the genomic DNA from non-specifically binding to the membrane. Similar results of increased recovery of genomic DNA were found when carrier DNA was added to the sample prior to washing in the Microcon-100 device.

The membrane in the Microcon-100 device should be saved since it may serve as a potential source of recovered genomic DNA. Amplification of trapped gemomic DNA can be achieved as follows: the membrane is removed and placed in a 1.5 ml tube containing 45 µl of TE (10 m*M* Tris, 0.1 m*M* EDTA, pH 8.0) buffer. After incubation at 56° C overnight, the membrane is removed and placed with the DNA side down in a Microcon-100 device which the membrane has been removed. [Note: do not discard the TE buffer solution from the overight incubation]. The device is then centrifuged in microfuge at 8,000 rpm for 10 mins. The overnight incubation solution is then added to the centrifugation product, and this combined solution can serve as a DNA template source for further amplification.

In conclusion, SMA has enabled typing of 5 different loci from as little as 5 ng of genomic DNA. Although only 5 loci were assayed in this study, it is possible to obtain results from more loci, if necessary. A high degree of discrimination can thus be obtained from a small quantity of sample. Additionally, individually optimized PCR conditions can be used for each locus. Since an individual amplification of the SMA can be performed using some multiplex systems, such as AmpliType PM PCR Amplification and Typing Kit (Perkin Elmer Corporation, Norwalk, CT), the discrimination potential can be increased.

In any case, the SMA procedure can be useful for situations where the quantity of template DNA is limited or to amplify additional loci from DNA templates that have been previously used and that were adequately frozen or refrigerated. We are carrying out experiments with frozen evidentiary material from samples that were amplified previously for one or two loci (HLA-DQA1 and/or D1S80) 3 to 4 years ago. It is possible to recover the DNA and amplify by SMA the HUMTH01 and HUMVWA STR loci starting with only 1 ng of template DNA (data not shown). With this procedure it is also possible to recover template DNA from PCR tubes that yielded no amplification product (e.g. DNA from evidentiary samples that might be degraded for a given locus, but that could be amplified for some other loci).

References

Baechtel FS, Smerick JB, Presley KW, Budowle B (1993) Multigenerational amplification of a reference ladder for alleles at locus D1S80. J Forensic Sci 38:1176–1182

Brinkmann B, Rand S, Wiegand P (1991) Population and family data of RFLP's using selected single- and multi-locus systems. Int J Leg Med 104:81–86

Budowle B, Chakraborty R, Giusti AM, Eisenberg AJ, Allen RC (1991) Analysis of the VNTR locus D1S80 by the PCR followed by high-resolution PAGE. Am J Hum Genet 48:137–144

Chamberlain R (1988) Deletion screening of Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 16:11141–11156

Edwards A, Civitello A, Hammond HA; Caskey CT (1991) DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am J Hum Genet 49:746–756

Edwards A, Hammond HA, Jin L, Caskey CT, Chakraborty R (1992) Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 12:241–253

Holland MM, Fisher DL, Mitchell LG, Rodriguez WC, Canik JJ, Merril CR, Weedn VW (1993) Mitochondrial DNA sequence analysis of human skeletal remains: identification of remains from the Vietnam War. J Forensic Sci 38:542–553

Lorente JA, Lorente M, Budowle B, Wilson MR, Vilanueva E (1994a) Analysis of the HUMTH01 allele frequencies in the Spanish population. J Forensic Sci 39: (in press)

Lorente JA, Lorente M, Budowle B, Wilson MR, Villanueva E (1994b) Analysis of the Short Tandem Repeat (STR) HUMVWA in the Spanish population. Forensic Sci Int 65:169–175

Möller A, Wiegand P, Grüschow C, Seuchter SA, Baur MP, Brinkmann B (1994) Population data and forensic efficiency values for the STR systems HumVWA. HumMBP, and HumFABP. Int J Leg Med 106:183–189

Saiki RK, Scharf S, Faloona F, Mullis K, Horn GT, Erlich A (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–1354

Saiki RK, Walsh RH, Levenson CH, Erlich HA (1989) Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc Natl Acad Sci USA 86: 6230–6234

Waye JS, Presley LA, Budowle B, Shutler GS, Fourney RM (1989) A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts. Biotechniques 7: 852–855

Wiegand P, Budowle B, Rand S, Brinkmann B (1993) Forensic validation of the STR systems SE33 and TC11. Int J Leg Med 105:315–320