ESTABLISHMENT OF A HIGHLY DISCRIMINATING PENTAPLEX-PCR-SYSTEM FOR DETECTION OF PCR-FRAGMENTS IN SILVERSTAINED POLYACRYLAMIDE GELS

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Introduction

Multiplex-PCR is defined as the simultaneous amplification of multiple sequences in a single reaction. It was first indroduced by CHAMBERLAIN et al. in 1988 for the amplification of nine segments of the human dystrophin gene [1]. This method was transferred to routine forensic work for simultaneous amplification of VNTR and/or STR-systems [3, 5, 7, 8, 9, 10]. Most of the systems introduced so far are based on automated fluorescent detection. Because the necessary equipment is not easily acquired for many laboratories, different detection methods have to be used. Therefore we use the sensitive silverstain method from BUDOWLE et al. to detect the DNA-fragments after polyacryamide gel electrophoresis, a method which is already employed for singleplex-PCR [4].

We have evaluated a sensitive multiplex-PCR-system with five different STR-regions. The fragments were detected by silverstaining, and the results were highly discriminative. We employed the STR-loci HUMFIBRA, D18S21, D21S11, HUMVWFA31 and HUMTH01 listed in table 1 for our pentaplex-system. These five STR-systems are all of tetrameric nature and are located on different chromosomes. The repeat sequence structure was already described in 1994 by URQUHART et al. [10].

Table 1: Data of the 5 Loci

STR-loci	GenBank Access. No.	Chromosomal localisation	Repeat motif
HUMFIBRA (Fibra)	M64982	4q28	TTCT
D18S51	L18333	18q21.3	AAAG
D21S11	M84567	21	TCT[AvG]
HUMTH01 (TH01)	D00269	11p15-15.5	AATG
HUMVWFA 31 (vWF)	M25858 M25716	12p12-pter	[AvG]TCT

Since a successful multiplex-PCR involves the design of appropriate primers, the primer specifications of the 5 loci are listed in table 2. The primer length, the sequence, the %GC content and the calculated melting temperature for the forward and reverse strand are listed. The fragment range and the alleles of each STR-region of this pentaplex-system, which can be expected after electrophoretic separation are also listed in table 2.

Table 2: Primer sequences, length , % GC-content, melting temperature (Tm) and corresponding fragment length

STR-Locus	Primer sequences	Length [bp]	% GC	Tm [°C]	Fragment Range (Alleles)
HumFIbra	Forward:AAGGCTGCAGGGCATAACATTATC	24	45.8	63.9	455 (32) to
	Reverse:CAGCCACATACTTACCTCCAGTCG	24	54.2	62.5	401(5)
D18S51	Forward:CAAACCCGACTACCAGCAAC	20	55.0	57.9	323(29) to
	Reverse:GAGCCATGTTCATGCCACTG	20	55.0	57.0	275(5)
D21S11	Forward:ATATGTGAGTCAATTCCCCAAG	22	40.9	55.3	249(76) to
	Reverse:TGTATTAGTCAATGTTCTCCAG	22	36.4	49.0	205(54)
HumTH01	Forward:GTGGGCTGAAAAGCTCCCGATTAT	24	50.0	64.8	202(11) to
	Reverse:ATTCAAAGGGTATCTGGGCTCTGG	24	50.0	66.9	179(5)
HumVWFA31	Forward:CCCTAGTGGATGATAAGAATAATCAGTATG	30	36,7	62.9	167(21) to
	Reverse:GGACAGATGATAAATACATAGGATGGATGG	30	40.0	66.4	135(11)

Materials and Methods:

Amplifications were carried out in a 50 µl total reaction volume. 0,5 to 10 ng template-K562 DNA, 1x Perkin Elmer PCR-buffer, 200 µM of each dNTP, 1.25 Units Taq-polymerase and primersets in concentrations from 0,04 to 04 µM were tested. The PCR conditions started with a single denaturation for 5 minutes at 94°C, then a three step PCR of 95, 60 and 72 °C each for 1 min. and a post-PCR extension for 10 minutes at 72 °C. To establish the multiplex-PCR-conditions, different reaction parameters described by CHAMBERLAIN et al. 1994 were tested [2].

Results and Discussion:

Figure 1 shows a native 7.5%T, 5%C-PDA and 60 mM formate polyarylamide gel after variation of primer concentrations and annealing temperature of all 5 loci using K562 template-DNA. In lanes 1, 8 and 15 different visual markers are visible. Lanes 2 to 7 show the result of 0.1 µM final primer concentration and lanes 9 to 14 show the result of 0.2 µM primer concentration, both with K562 DNA-concentrations in descending order from 10 to 0.5 ng. On the left side the result of a 0,1 µM primer concentration for all loci is shown at an annealing temperature of 56 °C. At this temperature many unspecific bands are visible at high and low template concentrations. The vWF bands are more intense and the alleles of D21S11 don't show up. At an annealing temperature of 62°C (figure 2) with the same primer concentrations, there are many artefacts at high DNA-concentrations, too. A three banded pattern of K562 for the locus D21S11 is visible and the signal of vWF is weak again. This 3 banded pattern is a suggestion of the presence of an additional chromosome. Since K562 is derived from a tumor cell line and the DNA is purified from a subculture of human chronic leukemia cell line, this aneuploidy of chromosome 21 can occur [6]. Doubling the total primer concentration from 0.1 to 0.2 µM of the primersets results in a lower signal intensitiy for all loci. This is visible on the right side in figure 1a and 1b. The reaction mix with a final primer concentrations of 0,08 µM for FIBRA, 0,04 µM for D18S51, 0,1 µM for D21S11, TH01 and vWF gave the best results (see figure 2). It is visible that all specific alleles of the cell line K562 are detectable with 4 ng template-DNA (lane 4 in figure 2). Lower amounts of template DNA yielded no interpretable results for vWF.

This may be compared to the quadruplex-system based on silverstaining presented by SCHUMM et al. 1993 [8] as being more sensitive. Based on the Pm values that OLDROYD et al. 1995 [7] determined by typing 50 Caucasians, this pentaplex-system would give a combined Pm value of 3,6 x 10-7, meaning that for a randomly selected sample we would expect to find one match in 2,7 billion individuals. This is approximately equivalent to the power of 4 single locus probes.

Conclusions:

A pentaplex system is established for use in routine work in a forensic laboratory. A prerequisite, therefore was, that all the STR loci should be on different chromosomes, which is the case here. With this system it is possible to detect the alleles of each STR-system in distinct separate areas after silverstaining of polyacrylamide gels. It is possible to detect 4 ng of template DNA. This is very sensitive in comparison to the quadruplex-system presented by SCHUMM et al. [8], who obtained the best results with 5 ng of template DNA. The alleles of the loci are shown with nearly the same signal intensity, and the sensitivity of this system is comparable to the multiplex-PCR-system based on fluorescent detection. The combined Pm-value of this Pentaplex-system of with 3,6 x 10-7 for caucasians is very good. Because there is still space between some STR-regions, an integration of other STR-systems and/or sex-typing system like amelogenin is possible.

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Figure 1a:
Horizontal 7.5 % T, 5%C(PDA), 60 mM formate polyacrylamide gel of PCR-products after pentaplex PCR at an annealing temperature of 56°C. Lanes 1, 8 and 15 visual markers (1 kb, pGEM and 123 bp-ladder). Lanes 2 to 7 and 9 to 14 different yields of K562 template DNA in descending order (10, 5, 4, 2, 1 to 0.05 ng). Lanes 2 to 7 all primers at 0.1 µM primer concentrations. Lanes 9 to 14 all primers at 0.2 µM primer concentrations.

Figure 1a

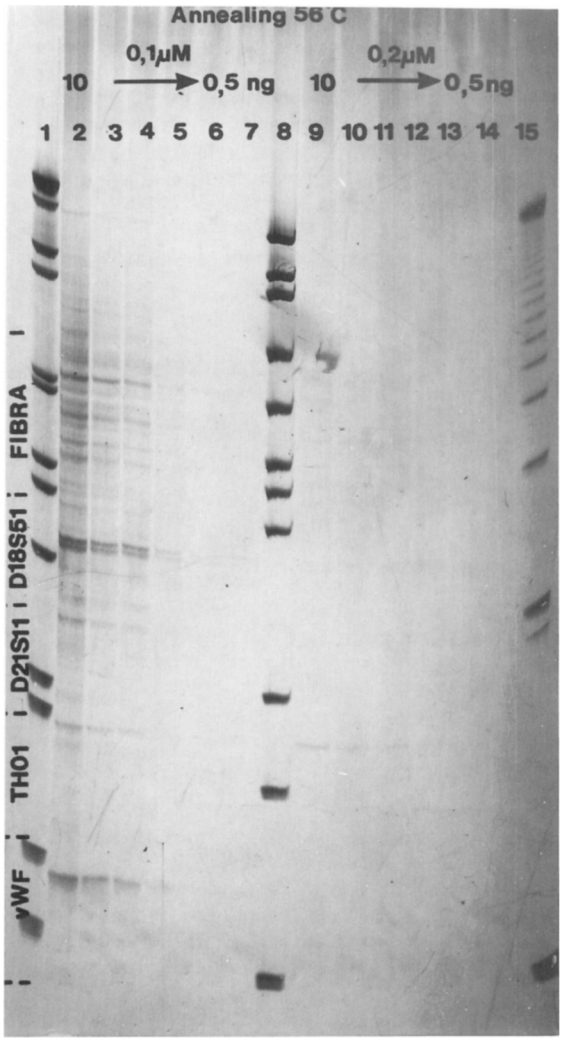

Figure 1b:
Horizontal 7.5 % T, 5%C(PDA), 60 mM formate polyacrylamide gel of PCR-products after pentaplex PCR at an annealing temperature of 62°C. Lanes 1, 8 and 15 visual markers (1 kb, pGEM and 123 bp-ladder). Lanes 2 to 7 and 9 to 14 different yields of K562 template DNA in descending order (10, 5, 4, 2, 1 to 0.05 ng). Lanes 2 to 7 all primers at 0.1 µM primer concentrations. Lanes 9 to 14 all primers at 0.2 µM primer concentrations.

Figure 1b

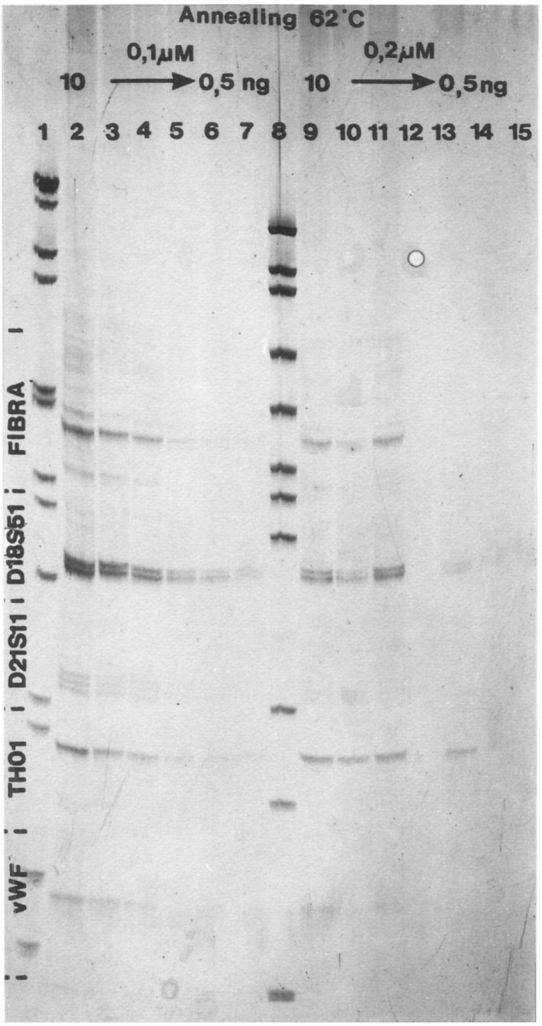

Figure 2:
Horizontal 7.5 % T, 5%C(PDA), 60 mM formate polyacrylamide gel of PCR-products after pentaplex PCR (optimal PCR-conditions see text) at an annealing temperature of 60°C. Lanes 1 and 8 visual markers (1 kb and pGEM). Lanes 2 to 7 different yields of K562 template DNA in descending order (10, 5, 4, 2, 1 to 0.05 ng).

Figure 2