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Long PCR for VNTR Analysis*

REFERENCE: Richie KL, Goldsborough MD, Darfler MM, Benzinger EA, Lovekamp ML, Reeder DJ, O'Connell CD. Long PCR for VNTR analysis. J Forensic Sci 1999;44(6):1176–1185.

ABSTRACT: The Polymerase Chain Reaction (PCR) has revolutionized the analysis of DNA from a variety of sources. With its sensitivity and ability to amplify degraded DNAs and small quantities of samples, coupled with fast turn-around-time, PCR is often the analytical method of choice for DNA profiling in forensic laboratories. RFLP methods, while requiring larger amounts of high molecular weight DNA and needing approximately 6-8 weeks of analytical time, still provide a higher power of discrimination per locus than that achieved using the loci currently available for PCR.

The combination of both RFLP and PCR would be advantageous for some applications. A new technique, Long PCR, allows for the effective amplification of long DNA targets from approximately 0.5 kb to >20 kb of genomic DNA. Currently, several Long PCR systems are commercially available. Using a *Taq/Pyrococcus* DNA polymerase enzyme system and DNA isolated from bloodstains, we have successfully amplified 1-20 ng of Chelex-extracted DNA, an amount commonly used in Amp-FLP technology. The robustness of Long PCR in comparison to RFLP was also examined through the use of partially degraded blood samples. Long PCR was then used to amplify both D2S44 and D5S110 RFLP loci. Although all D2 and D5 alleles were detected, the larger alleles were amplified at significantly lower levels than the smaller alleles.

KEYWORDS: forensic science, RFLP, DNA typing, Long polymerase chain reaction

The discovery of highly polymorphic regions in human DNA by Wyman and colleagues (1) laid the foundation for the use of DNA profiling for human identity. The first application of this technology was reported by Jeffreys and co-workers in their 1985 report entitled "Hypervariable Minisatellite Regions in Human DNA" (2). Over the next several years, a number of these highly polymorphic regions, termed "variable number of tandem repeats," or VNTRs (3–5), were identified. These VNTRs were first characterized by Restriction Fragment Length Polymorphism (RFLP) analysis (1). The VNTR loci that have subsequently been used for DNA Profiling have proven to be extremely informative due to the large number of alleles and high levels of heterozygosity present in human DNA. The VNTRs used for human identity testing yield fragment lengths upon restriction enzyme digestion ranging from 500 basepairs to over 20 kilobases (kb). Identity inclusions based on random match probabilities of one in one million or greater can be achieved with as few as 3 to 4 RFLP loci. Moreover, with the establishment of the Combined DNA Index System (CODIS, 6), a large database of RFLP profiles from convicted sexual offenders and forensic evidence is available for comparison.

More recently, a new family of repeat sequences in the genome, called short tandem repeats (STRs), has been used for identity purposes (7). These are similar to VNTRs in that there are many such repeat sequences in the genome. However, the repeat sequences in STRs are very short (usually less than 10 base pairs (bp)), and the entire repeat locus is usually less than 1 kilobase (kb). Since the size range of the alleles is amenable to polymerase chain reaction (PCR) amplification, analysis can be done in just a few hours, and the amount of DNA input in a PCR reaction is generally 50 to 100 times less than with VNTRs. In addition, samples that are too degraded to yield restriction fragments in the 10 kb range may still contain sufficient intact DNA for amplification of fragments under 1 kb. However, a drawback of the STR technology is that the STRs are by nature much less polymorphic than VNTRs. Many more STR loci need to be analyzed in order to obtain the same level of statistically significant identity inclusion data. Approximately 10–12 STR loci are thought to yield statistically similar profiles as 4–5 VNTR loci (8).

PCR amplification technologies have been continuously improving since the first methods were reported in 1987 (9). Recent advances allow for the amplification of long regions of genomic DNA up to 20 kb in length (10–12). Long PCR uses an enzyme mixture of Taq DNA polymerase and a thermostable proofreading polymerase under optimized conditions for the amplification of these long products. While Long PCR is readily practiced, optimization of several aspects of the PCR reaction is generally necessary to obtain consistent PCR products over several kb in length. One of the inherent drawbacks of PCR amplification where multiple targets are present in the same sample is the preferential amplification of the smaller products over larger ones (13).

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In this study, we sought to determine the feasibility of using Long PCR for the amplification of forensic samples. These experiments were conducted using established sample extraction methods and DNA quantities routinely amplified for STR typing. Using amplification of the single copy tissue plasminogen activator (tPA) gene as a model system to generate PCR products of 4.8 kb and 9.3 kb, we analyzed the efficiency of amplification of DNA extracted from 6-year-old bloodstains. To simulate forensic samples at varying stages of degradation, we treated blood samples with DNase I prior to Long PCR amplification using the tPA primer pairs. Finally, we demonstrated the amplification of PCR products corresponding to the D2S44 and D5S110 VNTR loci used in forensic identification.

Materials and Methods

DNA Samples

We initially used bloodstains kindly provided by the Armed Forces DNA Identification Laboratory, (AFDIL, Rockville, MD). Six-year-old human blood samples were provided by the Southwest Institute of Forensic Sciences and were stored at 4°C. Blood samples for the DNase I studies were obtained from the Illinois State Police Forensic Science Laboratory. Control human genomic DNA was purchased from Boehringer Mannheim Corp., Indianapolis, IN.

DNA Extraction

Genomic DNA was isolated from 3.2 mm (⅛ in.) and 6.4 mm (¼ in.) hole punches from a 200 μL bloodstain spotted on Schleicher & Schuell 903™ filter paper. A Chelex® procedure (14) was used for DNA extraction with modifications incorporated to minimize fragmentation of the DNA. First, vortexing was replaced with gentle inversion where indicated in the protocol. To ensure maximal recovery of the DNA, the extractions of the bloodstains were carried out for 30 min instead of 15 min to compensate for not vortexing. The Chelex extraction was conducted at 56°C for 1 h instead of overnight. The final lysate volume was 200 μL, of which 10 μL was used for each amplification reaction. Gel electrophoresis was used to assess quality of extracted DNAs (15), and the QuantiBlot™ System (PE Biosystems, Foster City, CA) was used to determine the quantity of DNA present.

Amplification Primers

Primers for amplification of the tissue plasminogen activator (tPA) gene were obtained from Boehringer Mannheim. The same forward primer was used to amplify both 9.3 kb and 4.8 kb products: 5′-GGA AGT ACA GCT CAG AGT TCT GCA GCA CCC CTG C-3′. The reverse primer for amplification of the 9.3 kb product was: 5′-CAA AGT CAT GCG GCC ATC GTT CAG ACA CAC C-3′, and for the 4.8 kb product was: 5′-GAT GCG AAA CTG AGG CTG GCT GTA CTG TCT C-3′.

Primers for amplification of the D2S44 (YNH24)(16) locus were selected using the Oligo V primer selection program (National Biosciences, Inc., Plymouth, MN). The forward primer to amplify the D2S44 locus was: 5′-GGT ACA GTG GCC GTG TTC A-3′, and the reverse primer: 5′-GGC CTC TGT TAA TGT GCT TGC-3′. D5S110 primers (17) were selected using Gene Runner© primer design software (Hastings Software, Inc., Hastings, NY). The forward primer to amplify the D5S110 locus was: 5′-GTG TGA GGG CCA TTA GCT TTC-3′, and the reverse primer: 5′-ACA GAC TCC CAC ACA CAT GAG-3′. Three of the four primers contained the *Hae* III restriction enzyme site identified by RFLP analysis (underlined above). The 5′ end of the fourth primer (D5 reverse) is located 3 nucleotides upstream of the *Hae* III site.

Amplification Protocol

A Perkin Elmer 480 Thermal Cycler was used for all amplifications (Perkin Elmer; Norwalk, CT). tPA amplification reactions contained: 2 μL *Taq* and *Pyrococcus* DNA polymerase enzyme mix (Elongase, Life Technologies, Inc., Rockville, MD), 1.75 mmol/L $MgSO_4$, 200 μmol/L each dNTPs, 200 nmol/L each 5′ and 3′ primers, 60 mmol/L Tris-SO_4, pH 9.1, 18 mmol/L $(NH_4)_2SO_4$ in a 50 μL reaction volume. Thermal cycling conditions were as follows: Pre-amplification denaturation: (1 cycle), 94°C for 2 min; amplification: (10 cycles): denaturation, 94°C for 30 s; annealing, 68°C for 1 min; elongation, 68°C for 10 min; amplification with extension: (20 cycles): denaturation, 94°C for 30 s; annealing, 65°C for 1 min; elongation, 68°C for 10 min + 20 s; final elongation: (1 cycle): 68°C for 7 min. A "Hot Start" procedure with Ampliwax PCR Gem 100s™ was used in all amplifications. The amplification with extension protocol was used to allow for amplification of the larger RFLP alleles (11).

With the exception of a lower concentration of $MgSO_4$ being used for amplification of the D2S44 and D5S110 loci (1.3 mmol/L $MgSO_4$ instead of 1.75 mmol/L), PCR conditions were identical to tPA. The thermal cycling conditions for D2 and D5 were also similar to those for tPA with the following modifications: the annealing temperature for D2 and D5 primers was 62°C, and the total number of elongation cycles was changed to 32 (10 cycles without extension, and 22 cycles with a 20 s extension added per cycle).

PCR Product Analysis

Ten to twenty μL of the amplification products were analyzed on 1% agarose (w/v) gels after electrophoresis in 1X TAE Buffer (10X = 400 mmol/L Tris-acetate, 10 mmol/L EDTA, pH 8.3). The 1 Kb DNA Ladder or λDNA/*Hind* III Fragments (Life Technologies, Inc., Rockville, MD) were used for sizing of the PCR products after visualization by ethidium bromide staining. Yields of DNA were estimated by visual comparison of samples with commercial K562 quantitation standards (Life Technologies, Inc.). Sizes of PCR products were determined after gel imaging using the molecular weight calculation function of the Alpha Ease™ software (Alpha Innotech, San Leandro, CA).

Generation of Partially Degraded DNA

DNA was isolated from 700 μL of liquid blood by organic extraction as described previously by Budowle and Baechtel (18). Filter centrifugation using Microcon™ 100 spin filters (Millipore, Beverly, MA) was substituted for ethanol precipitation. DNA concentrations were determined by comparison to standards after gel electrophoresis and staining, as described above.

DNA degradation was simulated by treatment with DNase I for various periods of time. Reaction solutions were prepared containing DNA (5 ng/μL to 40 ng/μL) in 1X *Hae* III buffer (New England Biolabs, Beverly, MA) and DNase I (Boehringer Mannheim Corp., Indianapolis, IN). A minute quantity of DNase I (<500 μg) was dissolved in 1 mL of a buffer consisting of 10 mmol/L Tris, 500 mmol/L KCl and 50% glycerol. DNA was incubated with a 1:1000 dilution of the freshly prepared DNase I solution, and 400 ng aliquots were removed at various time points (10, 15, 25, 35, 45,

55, 65, and 75 min). The reactions were stopped by the addition of 10 μL EDTA (0.5 mol/L) in a Microcon™ 100 filter unit (Amicon, Inc., Beverly, MA). After filter centrifugation, the DNA was recovered in TE buffer (10 mmol/L Tris-HCI, 1 mmol/L EDTA, pH 7.5) and a phenol/chloroform extraction was performed to remove residual DNase I activity. The degree of degradation was then assessed by gel electrophoresis.

Restriction Digestion with Hae *III*

DNA samples were digested with 50 units of *Hae* III (New England Biolabs, Beverly, MA) in the reaction buffer supplied by the manufacturer. After digestion for 1 h at 56°C, the DNA was recovered by filter centrifugation and resolubilized in 18 μL TE. Digestion was verified by gel electrophoresis of 2 μL and the remaining 16 μL were run on an analytical gel.

Electrophoresis and Southern Blotting

The *Hae* III digested DNase-treated samples were subsequently electrophoresed in an 11 × 20 cm 1% LE agarose gel (FMC BioProducts, Rockland, ME) in 1X TAE buffer. Following electrophoresis, the gel was incubated in 1X TAE containing ethidium bromide (1 μg/mL) for 15–30 min for visualization of the DNA. Gels were denatured for 30 min in 400 mMol/L NaOH and transferred to nylon membranes (Pall Biodyne® B, Life Technologies, Inc., Rockville, MD) for 6 h to overnight. Following transfer, the membranes were neutralized in 200 mmol/L Tris pH 7.5.

Hybridization, Autoradiography, and Interpretation

The membranes were probed at D2S44 (19; Promega Corp., Madison, WI) and D5S110 (20; Life Technologies, Inc., Rockville, MD) using ^{32}P detection. Membranes hybridized with ^{32}P-labeled probes were imaged on Kodak XAR film at −80°C for 4–5 days. DNA fragment sizes were determined by band positions using the Local DNA Analysis System (LDAS) of the FBI's Combined DNA Index System (CODIS) (21). For re-hybridization, probe was stripped from membranes by gently rinsing in 400 mmol/L NaOH at room temperature for 30 min followed by neutralization in 200 mmol/L Tris pH 7.5.

Results

To determine whether forensic samples were suitable for Long PCR amplification, a Chelex extraction procedure was used to isolate genomic DNA from recently obtained blood samples (<1 Year Old) spotted on Schleicher & Schuell 903™ filter paper. The Chelex extraction procedure was tested with and without vortexing the bloodstains during extraction to determine the impact of vortexing on DNA breakage. To determine whether the amount of forensic material used for STR analysis was also sufficient to amplify these larger products, DNA was isolated from 3.2 mm (⅛ in.) and 6.4 mm (¼ in.) hole punches of a 200 μL bloodstain. Quantities of isolated DNAs were determined using the Quantiblot™ system (data not shown). The 10 μL of Chelex-extracted sample used for amplification contained approximately 10 ng of DNA (⅛ in. bloodstain) and 20 ng of DNA (¼ in. bloodstain).

Primers specific for the single copy human tPA gene were used for amplification of 4.8 kb and 9.3 kb DNA fragments (Fig. 1). In every case, more PCR product was generated from the 4.8 kb tPA fragment than from the 9.3 kb. Amplification was achieved for both ¼ in. and ⅛ in. samples, with no detectable differences observed between vortexed and unvortexed samples. As expected, the PCR products from the ¼ in. samples (containing approximately twice as much DNA) resulted in more intense amplification than that achieved with the ⅛ in. samples.

We next sought to determine whether these same DNA fragments (4.8 kb and 9.3 kb) could be amplified from 6-year-old blood samples. Bloodstains were freshly prepared from two 6-year-old blood samples as well as from a newly obtained sample (N). Chelex-extracted DNA was then prepared from ⅛ in. hole punches from the bloodstains. The quality of extracted DNA was assessed on a standard yield gel (Fig. 2). DNA obtained from the first old blood sample (O-1), while present in large quantities, showed signs of degradation. The O-2 DNA appeared to be intact but present in low quantities. The QuantiBlot™ system was again used to determine the amount of DNA present in these three samples (data not shown). Lower quantities of DNA were present in these samples than in the original bloodstains examined. These quantities ranged from approximately 0.625 ng/10 μL for sample O-1, to approximately 1.0 ng/10 μL for both O-2 and N. Although the yield for O-2 was greater than for O-1, there appeared to be much less DNA present on the yield gel. We therefore assume that the majority of the O-2 DNA must be degraded.

The same pattern of amplification was seen for the 6-year-old blood samples as for the pristine samples shown in Fig. 1: the 4.8 kb fragments were more robustly amplified than the 9.3 kb fragments (Fig. 3). Although O-1 appeared to contain more DNA than the fresh blood sample, N, amplification was weaker for this sample, probably due to the degradation observed on the yield gel (Fig. 2). Amplification of the 4.8 kb fragment from the O-2 sample was much weaker, consistent with the amount of intact DNA isolated.

The effect of DNA degradation was more pronounced with the amplification of a much larger DNA fragment. A significant reduction in the quantity of PCR product was observed for both O-1 and O-2 with respect to N. Overall, amplification was achievable for single copy (non-repetitive) DNA fragments as large as 9.3 kb, even when samples were present in very low quantities or partially degraded.

To further determine the effect of DNA degradation on Long PCR amplification, a pristine DNA sample was treated with DNase I for increasing lengths of time (Fig. 4A). The pristine DNA sample (0′) appeared to be 15–40 kb in length (data not shown). After 35 min of DNase I digestion, the average size of the DNA was reduced to 8 to 10 kb, and by 75 min, there was a broad size distribution of digestion products ranging from approximately 8.5 kb to 500–750 bp. The bromophenol blue loading dye obscured the detection of any digestion products smaller than 500 bp. Long PCR amplification of the 4.8 kb and 9.3 kb tPA gene fragments was then carried out. PCR products were visible through the 65-min time point for the 4.8 kb DNA fragments (Fig. 4B), but only through the 25-min time point for the 9.3 kb fragments (Fig. 4C).

The DNase I treated samples were then analyzed by Long PCR amplification of two VNTR loci commonly used for human identification, D5S110 and D2S44. The PCR primers designed for these studies were selected to amplify a DNA fragment close in size to the *Hae* III digested DNA fragments detected in RFLP analysis. The D5S110 alleles for the DNA sample analyzed in this study were sized at 2780 bp (2.8 kb) and 2950 bp (3.0 kb), and the D2S44 alleles at 1663 (1.7 kb) and 3338 (3.3 kb) using the Local DNA Analysis System (LDAS) (21). After Long PCR amplification of the D5S110 locus, products were visible for both 2.8 kb and 3.0 kb alleles throughout the digestion, with only the 75-min time point difficult to visualize by ethidium bromide staining (Fig. 5A). The 1.7 kb and 3.3 kb D2S44 alleles were not amplified to the same de-

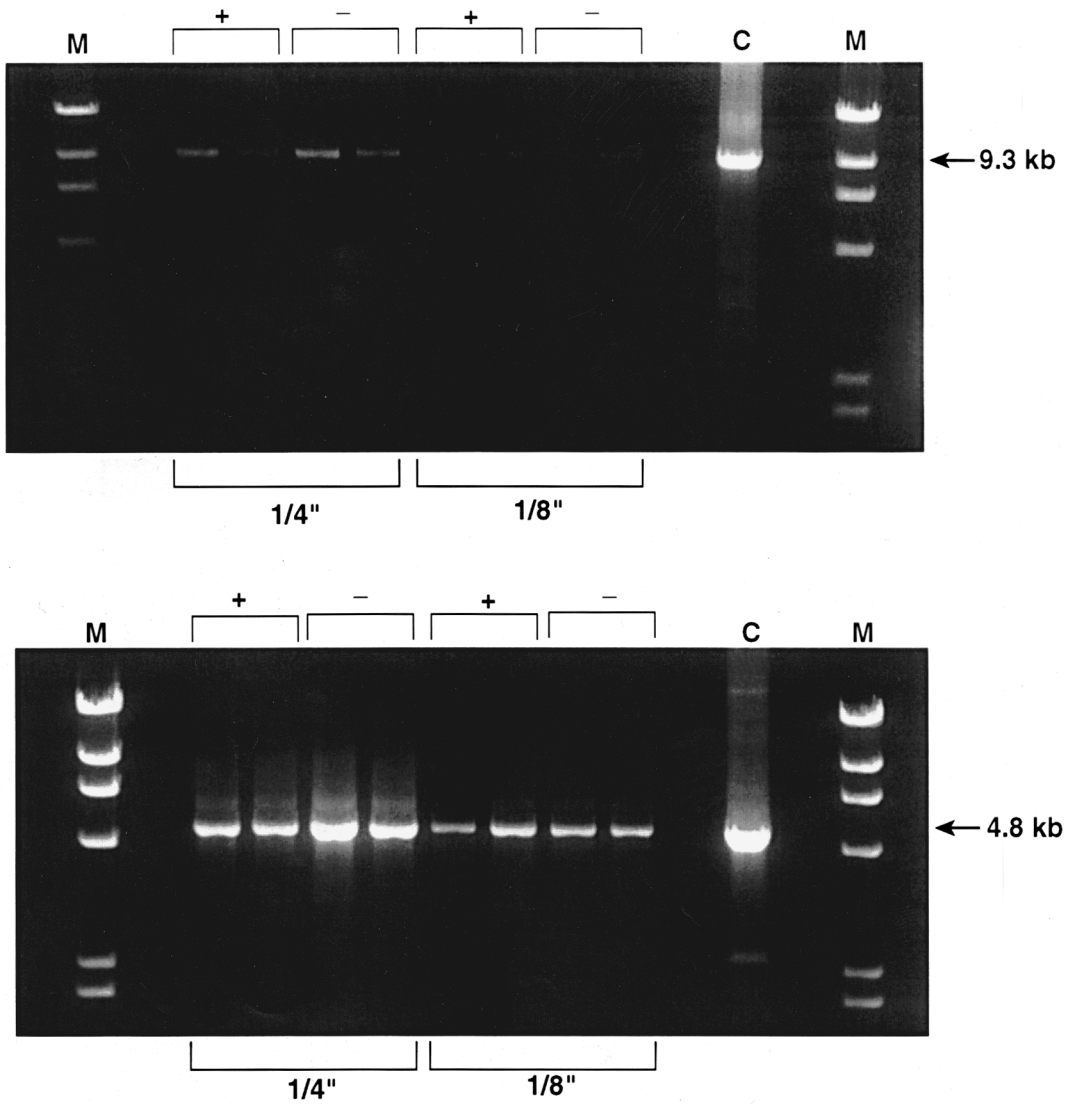

FIG. 1—*Amplification of 9.3 kb and 4.8 kb tPA genomic DNA fragments with* Taq/Pyrococcus *DNA polymerase mix. Arrows indicate positions of the 9.3 kb and 4.8 kb amplification products. M: λDNA/*Hind *III molecular weight marker, +: standard Chelex® isolation, −: Chelex procedure without vortexing, C: 200 ng human genomic DNA control, ¼: bloodstain DNA isolated from a ¼ in. diameter (6.4 mm) hole punch, ⅛: bloodstain DNA isolated from a ⅛ in. diameter (3.2 mm) hole punch. All samples were isolated in duplicate, which are indicated by brackets in the figure.*

gree as the D5S110 alleles (Fig. 5B). While the 1.7 kb DNA allele was amplifiable through the 65-min time point, the 3.3 kb product was only visible up to 35 min of DNase I treatment.

A decrease in DNA band intensity was seen with increasing DNase I treatment for D2S44 PCR products, consistent with the results obtained for amplification of tPA gene fragments (Fig. 4B and 4C). However, the intensity of the D5S110 PCR products remained constant throughout the time course. These 2.8 kb and 3.0 kb D5S110 products were present at similar intensities to the 3.3 kb D2S44 product observed in the early time points (Fig. 5A and 5B).

The DNase I panel was then examined by RFLP analysis of the same VNTR loci. Consistent with this methodology, ten-fold more DNA was used for *Hae* III digestion and Southern transfer than was used for Long PCR amplification. Probes specific for the D2S44 and D5S110 loci were sequentially hybridized to the partially degraded DNA samples bound to the membrane. The results of these studies are shown in Fig. 6. After a 4-5 day autoradiographic exposure of the membrane, allelic bands were seen in the untreated samples (0 time points) for the D5S110 locus, and the upper, 3.3 kb allele for the D2S44 locus. Allelic bands were also seen in the 35-min time point for the D5S110 locus. On longer exposures of the same membranes (12–33 days), both allelic bands were seen in the majority of the samples (data not shown).

We next compared the sizes of the D2S44 and D5S110 Long PCR amplification products to those obtained by RFLP. Sizing data was obtained after gel imaging using the molecular weight calculation function of the Alpha Ease™ software (Alpha Innotech, San Leandro, CA). In Table I, these sizes are shown in comparison to the RFLP allele sizes generated using the Local DNA Analysis System (LDAS) (21). The D2S44 amplification products were larger than the RFLP-derived sizes. The D2S44 alleles differed in size by 30 bp (3.3 kb allele) and 46 bp (1.7 kb allele). Due to the positions of the Long PCR primers with respect to the *Hae* III cleavage sites, the Long PCR products were expected to be 12 bp

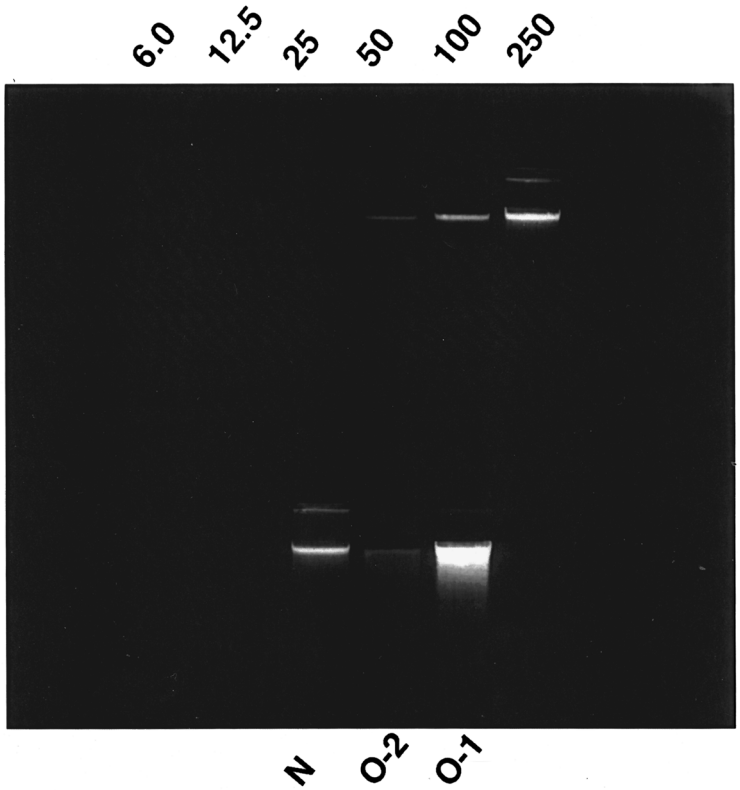

FIG. 2—*Yield gel of 6-year-old blood samples. DNAs were isolated from aged blood samples spotted onto S&S 903™ filter paper and extracted using a modified FBI procedure. A yield gel was run to determine relative intensities of intact DNA. Top row: quantitative standards (6.0 ng to 250 ng, as labeled), bottom row: DNA isolated from a freshly isolated blood (N) and two aged blood samples (O-1 and O-2).*

longer than the RFLP fragments, the size differences are therefore 18 bp (3.3 kb allele) and 34 bp (1.7 kb allele).

The sizes of the D5S110 alleles amplified by Long PCR (Table 1) were slightly smaller than the RFLP-derived alleles by 15 bp (3.0 kb allele) and 21 bp (2.8 kb allele). For D5S110, the Long PCR products were expected to be 5 bp longer than the RFLP fragments, therefore these differences are actually 20 bp (3.0 kb allele) and 26 bp (2.8 kb allele). The small differences in allele size are quite remarkable, given that the Long PCR gel was run under very different conditions than the RFLP gel, and the Long PCR and RFLP sizes were obtained using different molecular weight ladders. As the results reported in this manuscript are based on the data obtained from one gel per experiment, further experiments would have to be conducted with more gels and gels specifically designed for quantitation (i.e., forensic gels) to conclusively demonstrate that the Long PCR sizes are comparable to those obtained from RFLP methodologies.

Discussion

Routine amplification of DNA fragments greater than 2 kb in length has been made possible by the introduction of polymerase enzyme combinations (10–12). Using an enzyme mixture, we have demonstrated that large fragments of DNA isolated by methods routinely used in forensic applications (14) could be amplified from 6-year-old blood. We first successfully amplified DNA fragments of 4.8 kb and 9.3 kb from the single copy tPA gene. This was achieved using an ⅛ in. hole punch from freshly prepared bloodstains, which yields a quantity of DNA typically used for human identification by STR analysis. We then demonstrated that fragments corresponding to VNTR loci commonly used in forensic identification could be amplified.

In recent publications, sequences flanking the VNTR loci have been disclosed (16,17,22,23). In some cases, the authors used Long PCR techniques to amplify the repeat region. Two manuscripts (22,23) discussed a similar goal as that presented in this study: replacing RFLP analysis with Long PCR amplification of the same alleles. However, these investigators did not attempt to amplify the same size fragments as those generated by RFLP analysis. Since there currently exists a well established database (CODIS) with RFLP profiles for tens of thousands of convicted sexual offenders and forensic evidence, the ability to amplify fragments of the same size as the alleles present in the database is desirable. Furthermore, these investigators did not examine the use of DNA isolation techniques for PCR-based forensics (e.g., Chelex extraction). As this simpler DNA extraction procedure is less time-consuming than traditional RFLP extraction techniques, it contributes significantly to the shortened time frame for STR analysis in comparison to RFLP analysis. These other studies did not address whether the amount of forensic material used for STR analysis was also sufficient to amplify these larger products, or the effect of sample degradation on the ability to obtain full-length amplification products.

Over the course of this study, we were able to examine the effect of each modification that was made to the Long PCR protocol. First, the differences between amplification of 3.2 mm (⅛ in.) and 6.4 mm (¼ in.) hole punches were fairly minimal, both amplified the single copy tPA gene fragments well. Moreover, increasing cycle numbers slightly could make up for the differences in quantities of PCR products visualized (data not shown). The effect of vortexing during Chelex extraction was also minimal. We expected this to be true for smaller amplification products, but we also found very little effect on the 9.3 kb PCR products.

Large VNTR alleles are commonly found in several loci used in forensic analysis (D1S7, D4S139, and D5S110). The effect of DNA degradation on the amplification of large fragments was addressed using the same 4.8 kb and 9.3 kb tPA gene fragments. First, we amplified these fragments from 6-year-old blood samples (Fig. 3). Next, using DNase I for increasing periods of time, we created a panel of DNAs that mimicked forensic samples in varying degrees of degradation (Fig. 4). We successfully amplified the 4.8 kb and 9.3 kb tPA fragments in both of these studies.

In the first study (Figs. 2 and 3), old blood samples were used to examine the effect of partial degradation on Long PCR amplification. Although multiple bands were present after amplification using primers specific for both the 4.8 kb and 9.3 kb tPA products, the fragments corresponding to the expected products were more intense than the additional, presumably non-specific, fragments. Additional PCR optimization may eliminate these spurious products.

In the DNase I study (Figs. 4 and 5), both 4.8 kb and 9.3 kb tPA fragments as well as D2S44 and D5S110 alleles were examined. A

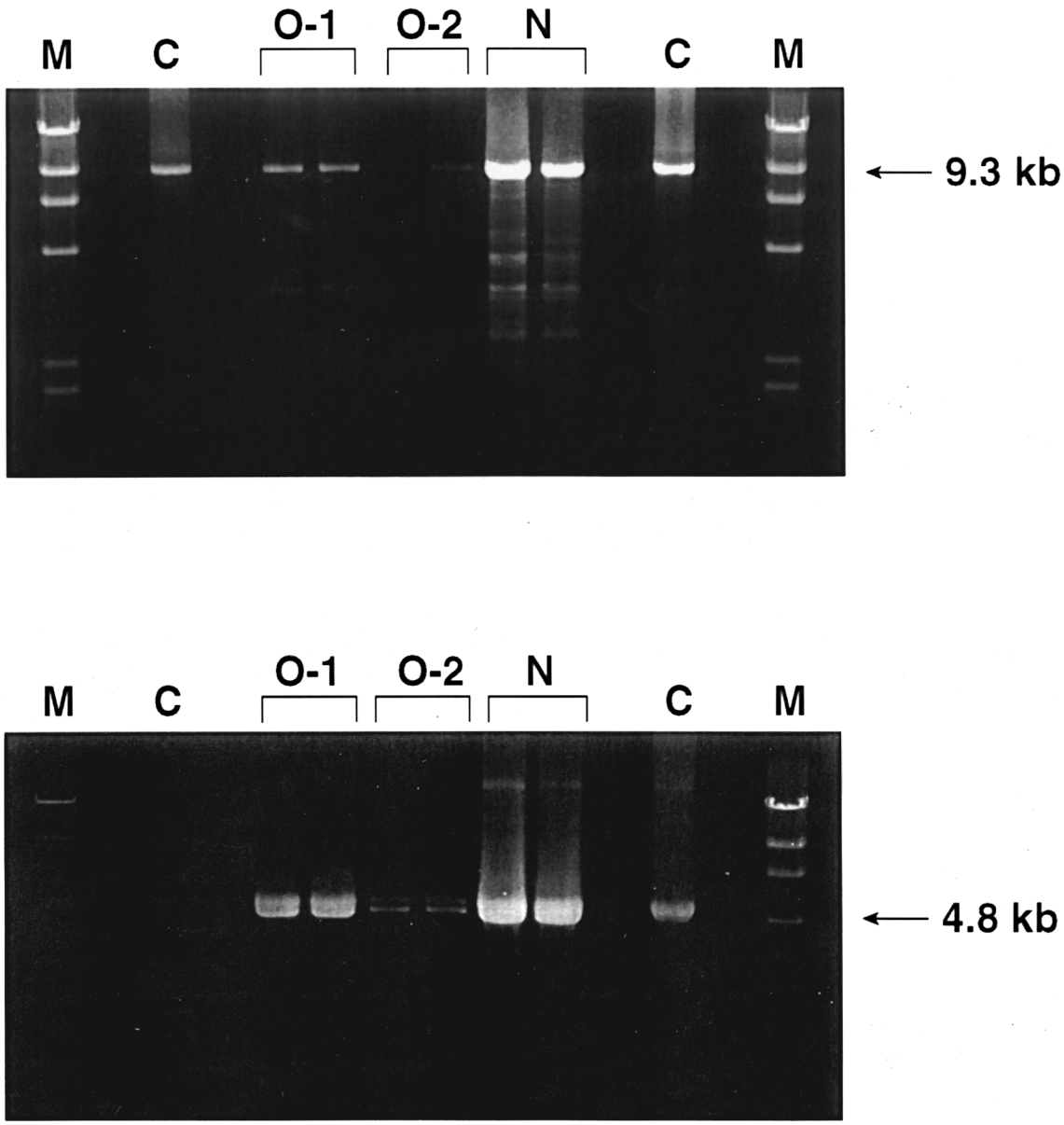

FIG. 3—*Amplification of 6-year-old blood samples. Arrows indicate positions of the 9.3 kb and 4.8 kb amplification products. M: λDNA/*Hind *III molecular weight marker, C: 200 ng human genomic DNA control, O-1 and O-2: 6-year-old blood samples, N: DNA obtained from a freshly prepared blood sample. All samples were isolated in duplicate, indicated by brackets in the figure.*

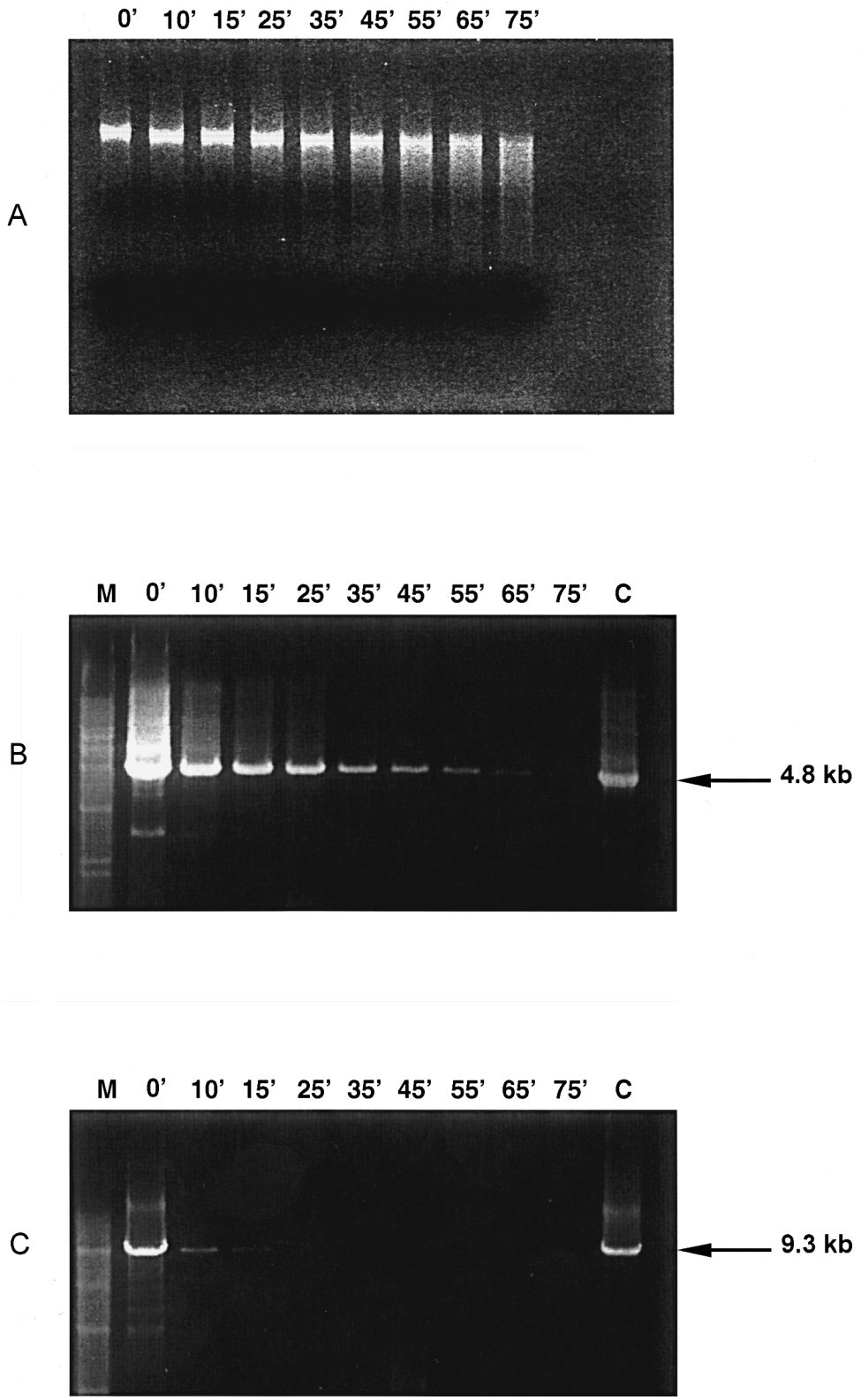

FIG. 4—*Effect of DNase I digestion on Long PCR amplification. Panel A: Yield gel of DNAs after DNase I treatment. The length of DNase I treatments (ranging from 10 to 75 min) are indicated in the figure. B and C: Amplification of two DNase I-treated DNAs. Arrows indicate positions of the 9.3 kb and 4.8 amplification products. M: λDNA/*Hind *III molecular weight marker, C: 200 ng human genomic DNA control.*

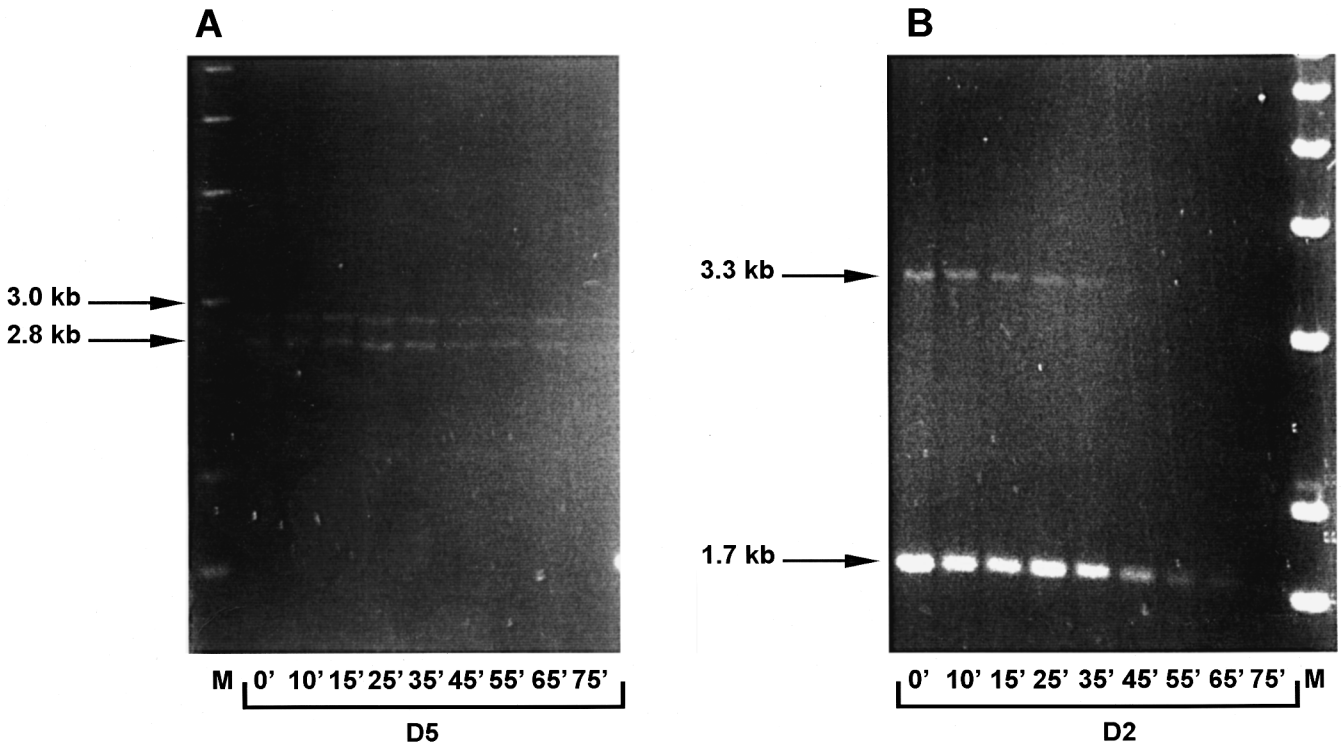

FIG. 5—*Amplification of D2S44 and D5S110 VNTR loci from DNase I treated DNAs. The same panel of DNAs as shown in Fig. 4 was amplified with D5- (Panel A) and D2- (Panel B) specific primers. Arrows indicate positions of the D2 and D5 alleles. M: 1 kb extended molecular weight marker. The length of DNase I treatment ranged from 10 to 75 min, as indicated in Fig. 4.*

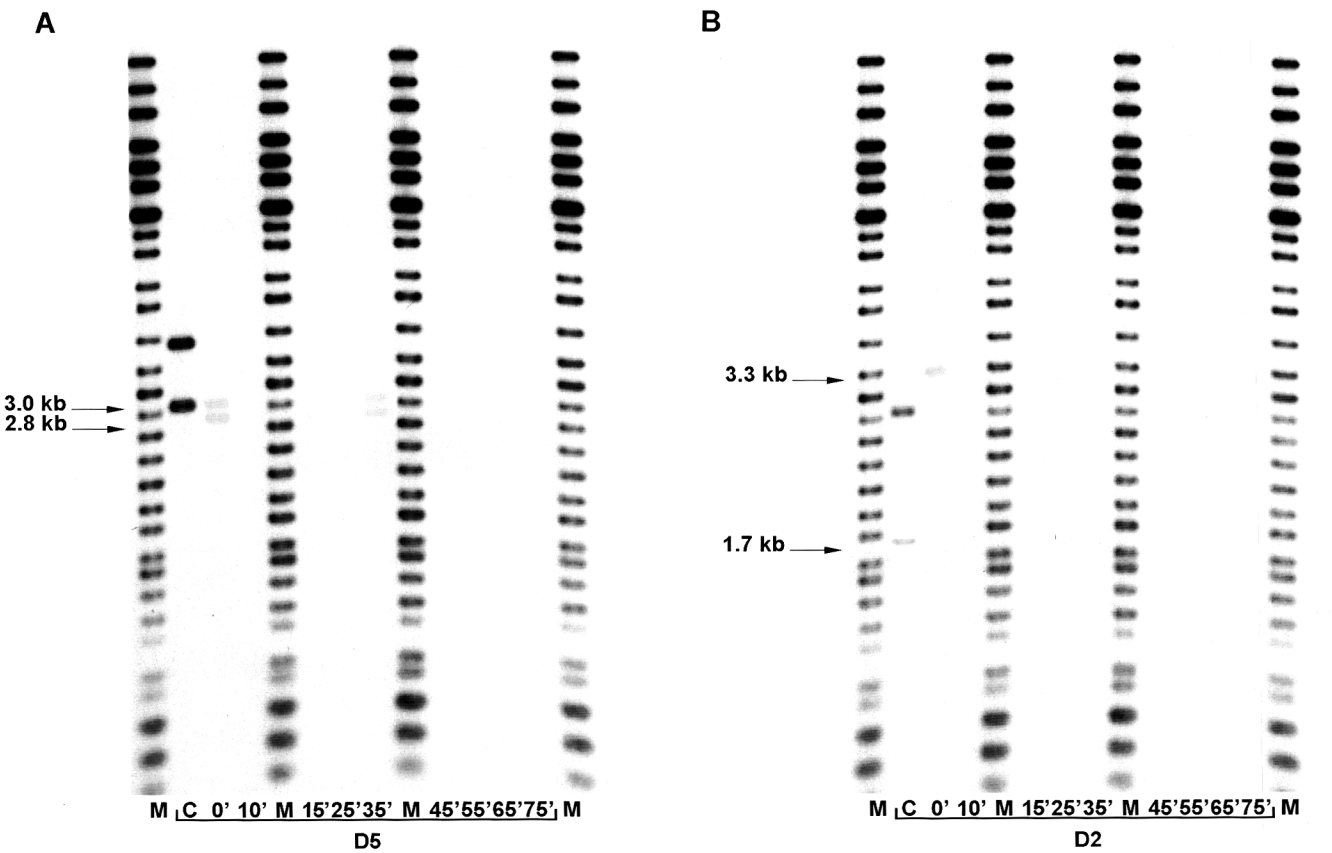

FIG. 6—*RFLP analysis of VNTR loci D2S44 and D5S110. The DNase I treated samples analyzed in Figs. 4 and 5 were digested with* Hae *III and electrophoresed following a standard protocol for RFLP analysis. Arrows indicate positions of the D2 and D5 alleles; lengths of DNase I treatment are as indicated and range from 10 to 75 min. M: DNA analysis marker system, C:* Hae *III digested K562 genomic DNA.*

TABLE 1—*Allelic size differences: RFLP versus Long PCR.*

Human Locus	RFLP Alleles Mean (SD*)	Long PCR Alleles Mean (SD†)	BP Difference: Expected‡	BP Difference: Observed Mean (SD§)
D2S44	3338 (7.9)	3368 (14.7)	12	30 (16.7)
	1663 (3.1)	1709 (06.0)	12	46 (06.8)
D5S110	2950 (3.5)	2935 (17.6)	5	15 (17.9)
	2780 (2.6)	2759 (12.5)	5	21 (12.8)

* $SD/\sqrt{n}$ for RFLP alleles = a.

† $SD/\sqrt{n}$ for Long PCR alleles = b.

‡ Differences based on position of PCR primers in relation to *Hae III* cleavage sites.

§ $\sqrt{a^2 + b^2}$.

decrease in the quantity of Long PCR products was observed with increasing nuclease treatment for the 4.8 kb and 9.3 kb tPA fragments as well as for the 1.7 kb and 3.3 kb D2S44 alleles. The decreases observed were proportional to the amount of intact DNA present. The pattern of sequential decrease in PCR product with increased DNase I treatment was not seen for the two D5S110 alleles. This was puzzling given the sequential decreases observed for all other PCR products.

In general, Long PCR amplification was more robust for the single copy (tPA) gene fragments than for the repetitive VNTR regions. Amplification products at the 65-min time point were only visible for the three smaller DNA fragments (both D5 alleles and the 1.7 kb D2 allele), suggesting that degradation limits the ability to detect the larger fragments. Although the second D2 allele was only 3.3 kb, amplification products were visible only through the 35-min time point; whereas the larger 4.8 kb tPA fragment could be amplified through the 65-min time point. This could be due to better optimization of the tPA primers and amplification conditions in comparison to those used for D2. Further optimization of PCR primers may therefore significantly increase our limits of detection.

Alternatively, the differences in amplification ability may result from competition between the two D2 alleles as seen during amplification of STRs, where shorter alleles are amplified more robustly than much longer ones (13). If so, competition would not be expected between alleles of similar sizes. This was the case for the two D5 alleles present in the blood sample tested (Fig. 5A), both 2.8 kb and 3.0 kb alleles were amplified to the same intensity throughout the time course.

As seen in Figs. 5 and 6 and Table I, the products obtained by Long PCR amplification were similar in size to those obtained by RFLP analysis. The Long PCR-derived D2S44 and D5S110 alleles examined in this study differed from their RFLP counterparts by only 15–46 base pairs, remarkable given the differences in the two protocols used. Three of the four alleles examined in the DNase I study were detectable by Long PCR through the 65-min digestion period, with the fourth allele (3.3 kb D2S44) detectable through the 35-min time point.

The same panel of DNase I treated samples were analyzed in a standard RFLP protocol with a typical 4–5 day exposure using ^{32}P for detection. Three out of four D2S44 and D5S110 alleles were detected in these experiments; however, these alleles were detectable only in the samples that were not treated with DNase I (0 time point). The 1.7 kb D2S44 allele was not detectable. Although DNA loss during sample preparation may have contributed to the low detection levels, a standard forensic protocol from start to finish takes a minimum of 4–6 days. In contrast, when using Long PCR, we were able to isolate genomic DNA, amplify the VNTR alleles, separate them by gel electrophoresis, detect the bands, and call the two alleles with respect to size in under two days. In comparison to RFLP analysis, this is a considerable savings in both time and technical resources.

Our results show that Long PCR can be used for VNTR analysis. Our current studies are focused on expanding the number of VNTR loci that we can amplify by Long PCR, and extending the size range of the alleles that can be amplified using this method. Presently, we are able to amplify alleles in the 6–7 kb range if the two alleles are fairly close in size (data not shown). We are attempting to optimize the Long PCR reaction to extend this capability to include alleles up to 12 kb, as well as those that are further apart in size. With further optimization of the amplification protocols that we have developed for the D2S44 and D5S110 VNTR loci, and extending this technology to other VNTR loci, Long PCR may prove extremely useful to the forensic community.

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