CASE REPORT

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Use of Nested PCR to Identify Charred Human Remains and Minute Amounts of Blood

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ABSTRACT: Reliable single cell PCR requires nested or hemi-nested PCR and careful optimization of conditions. This report describes the successful use of nested PCR for gender identification and reverse paternity testing in a forensic case where the only available materials consisted of charred human remains and a minute quantity of blood that were unsuitable for standard PCR. Use of nested PCR allowed the blood and burned tissue to be identified as human female. Analysis of two PCR length polymorphisms (AMPFLP) was successful on the blood sample and reverse paternity testing yielded a 98% probability that the blood spot was from the victim. The defendant was convicted of murder following a bench trial and the verdict was upheld by the Appellate court.

KEYWORDS: forensic science, nested PCR, DNA typing, minute amounts, polymerase chain reaction, amelogenin, APOB, vWF

DNA typing has been in use in American courtrooms for several years. Initially, testing was done exclusively by Southern Blot analysis using either variable number tandem repeat (VNTR) probes present once per haploid genome (single locus probe testing, RFLP analysis) (1) or using multilocus probes in a patented process called DNA Fingerprinting (2). These techniques usually require at least 100 ng of intact DNA for successful analysis. Unfortunately, less than half of all crime scene specimens yield interpretable results using Southern blot technology (3–5).

More recently, polymerase chain reaction (PCR) based RFLP analysis called AMPFLP analysis has been applied to VNTR loci (3,4). Unlike Southern analysis AMPFLP analysis can be performed on smaller or even partially degraded samples (3–5). We have applied AMPFLP analysis to rapidly perform prenatal paternity testing following chorionic villus sampling (10).

Sajantila et al. reported using AMPFLP analysis to successfully identify fire victims' bodies (4). These burned bodies were not burned to completion as muscles and bones could be identified and were available for analysis.

Our laboratory has participated in developing PCR techniques for single cell genotyping for preimplantation genetic diagnosis. A single cell contains approximately 10 pg of DNA, or 10,000–100,000 fold less DNA than required for RFLP analysis and 100–1000 fold less than standard PCR techniques. Routinely more than 40 cycles of PCR are required to obtain a signal visible by ethidium bromide staining of polyacrylamide gels in single cell genotyping. Initially, we performed single cell genotyping using standard PCR conditions of a single primer set and ''hot start'' methodology. In general 40–45 cycles needed to be run in order to obtain visible bands on ethidium bromide stained gels. This technique was not sufficiently robust, in that often no bands were observed or the presence of artifactual bands obscured analysis.

The advent of nested PCR allowed single cell genotyping to be carried out routinely. In nested PCR, two sets of primers are designed; an outside pair used for the initial 15–25 rounds of PCR and then an inside pair, laying within the sequence amplified by the first pair. Following the initial rounds of PCR a small aliquot (usually 5%–10%) of the reaction mixture is added to fresh PCR buffer containing the inside primers and an additional 20–25 rounds of PCR is performed. The PCR products can then be visualized on ethidium bromide stained gels. Any nonspecific PCR products from the first round of PCR will not amplify in the second round because they will not contain complementary sequences to the new set of primers. Nesting eliminates artifacts of non-specific bands. Only the specific product bands appear on the gel. When primer concentrations are optimized there is no primer or primer-dimer artifact observed either. Hemi-nesting involves the addition of 1 inside primer with the second primer being the same as in the first PCR reaction. Hemi-nesting works equally well as full nesting in most circumstances.

Several laboratories, including our own, can reliably genotype a single diploid or haploid human cell (6–9). A single human diploid cell is expected to have a DNA content of approximately 6 pg (6×10^{-12} g). Dilution experiments using known quantities of DNA demonstrate 100% amplification until a dilution of 100 pg and approximately 50% amplification at dilutions between 100 and 5 pg presumably indicating failure to obtain a DNA molecule in the PCR mixture. We applied nested PCR amplification techniques developed for single cell analysis to successfully analyze samples in a forensic case which were untypable by standard Southern blot RFLP and traditional PCR analysis.

Materials and Methods

DNA Preparation

Approximately 100 mg of a pink flake was taken from a cotton swab using a sterile disposable forceps and subjected to stan-

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dard DNA isolation procedures. Approximately 10 g of presumed charred human flesh was minced with a razor blade and subjected to proteinase K digestion at 50° for 36 hours followed by chloroform/phenol extraction and ethanol precipitation as described elsewhere (11).

PCR

Several additions to standard PCR technique must be made in order to reliably analyze minute quantities of DNA. These have been described in detail elsewhere and include stringent techniques and controls to prevent laboratory contamination by extraneous DNA sequences (7–9). It is now clear that nested or hemi-nested PCR is far better than standard PCR for the analysis of minute quantities of DNA in that it increases the robustness of the reaction while decreasing spurious analyses due to nonspecific priming (8).

We designed nested PCR primer sets for 3 loci in the human genome; the amelogenin locus on the X and Y chromosomes (used for gender determination) (12), and 2 AMPFLP loci, the von Willebrand factor (VWF) locus on chromosome 12 (13) and the Apolipoprotein B locus (Apo-B) on chromosome 2 (14).

The primer sequences are shown in Table 1. Oligonucleotides were synthesized as described elsewhere (9).

Each reaction mixture includes 1–2 positive controls of known DNA containing the largest and smallest observed fragments for each locus. These samples are added to PCR tubes in a separate laboratory after the evidentiary sample preparation has been completed and those tubes have been sealed and placed in the PCR machine. This is to avoid cross contamination of evidentiary reactions with control DNA. Negative controls consisting of water are performed in parallel to the evidentiary preparation. At least 3 negative controls per 10 lanes of sample are used.

The first round of PCR is performed with a reaction volume of 500 mL containing 50 mL of Promega 10× Reaction Buffer with KCl (Promega Corp.), 20 mL of 8 mM dNTP solution (Promega), 30 mL of 25 mM MgCl (Promega), 50 mL of 50% glycerol (Fisher or Sigma), 3 mL Taq polymerase (PE or Promega) 1 unit/mL, and 10 mL of outside primers at a concentration of 10 mcM, and 1–5 mL of sample. The solution is covered with 2 drops of sterile mineral oil and the tubes placed in a Perkin-Elmer model 9600 thermo-cycler. We use a volume of 500 mL because we have found that using lower reaction volumes increases PCR failure, probably because of inhibitors in evidentiary samples copurifying with the DNA. Using thin walled PCR tubes and a PCR machine with a heated lid (Perkin-Elmer Model 9600) we have had no difficulty in obtaining reproducible PCR results and have had no problems with unequal heating.

Hot start PCR is used with magnesium added when the tubes are at 70°. Thermocycling is performed as follows: 95° 7 min (melting), 72° 10 min (hot start), then 10 cycles of: 95° 30 s, 55° 2 min, 72° 20 s, followed by 20 cycles of 95° 30 s, 55° 1 min, 72° 20 s, followed by an elongation step of 72° 10 min.

For the second round, a separate fresh 500 mL master mix is constituted containing 50 mL 10× Reaction Buffer (Promega), 10 mL 8 mM dNTP (Promega), 30 mL of 25 mM MgCl (Promega) and 20 mL of each inside primer. 48 mL of this buffer is placed in each PCR tube and covered with 2 drops of sterile mineral oil. 2 mL of the first round PCR product is added to the appropriate tubes. Cycling is performed as follows, 95° 5 min followed by 25 cycles of 95° 30 s, 55° 30 s, 72° 30 s, and then an elongation step of 72° 7 min.

PCR products were analyzed on gels using 4% metaphor for amelogenin or 8% polyacrylamide for VWF and Apo-B. Gels were stained with ethidium bromide and photographed as described elsewhere (9).

TABLE 1—*Nested primer sequences for PCR analysis.*

AMELOGENIN		Expected Product Size
Outside:	AM-1up: cttcccagtttaagctctg	681 bp (X)
	AM-1dn: ctgaacctgtgagagaagtaag	492 bp (Y)
Inside:	AM-2up: atggttggcctcaagcctg	531 bp (X)
	AM-2dn: ctcatattatacttgacaaagca	352 bp (Y)
VWF		
Outside:	VWF-3: ttagatagattagacagacag	179–211 bp
	VWF-4: tcatctatcctatctctatc	(approximate)
Inside:	VWF-1: agctatatatctatttatcat	98–152 bp
	VWF-2: agatacatacatagatatagg	(approximate)
Apo-B:		
Outside:	ApoB-1: taaaatgttctggcacag	600–900 bp
	ApoB-2: cagacagtcagtgaggaagac	(approximate)
Inside	ApoB-3: atctggggtccgtatt	570–830 bp
	ApoB-4: tgaaagacagtgaaacgag	(approximate)

Statistical Analysis

All data is stored in a proprietary database program called VNTR-db written by the author. This program stores all data and calculates allele frequencies and probability of exclusion as described elsewhere (10). Statistical analyses were performed using the Chi squared test in the computer program Epi-Stat.

Results

A forensic case (People of the State of Illinois vs Garry F. Huff, case number 93 CF136) involved a man accused of murdering his wife and burning her body to completion in a steel drum in his garage. No identifiable human remains were found in the drum, but there were pieces of charred material that could not be identified with certainty as human remains. In addition, a single pink spot was found on the garage wall which could not be identified by microscopic or serologic methods as blood.

Yield gels following DNA isolation revealed no visible ethidium bromide staining material present from the charred tissue, whereas the presumed blood showed a faint smear beginning at approximately 500 base pairs and continuing until the end of the gel. Despite multiple attempts, no PCR amplification products could be obtained using either the charred material or the stain using standard unnested PCR. These attempts included 40 rounds of PCR using a single set of primers for amelogenin, VWF, D1S80, and THO. In addition, we attempted reamplification by taking PCR products from the first attempt, adding them to fresh buffer and amplifying for another 20–30 cycles. In no cases could specific PCR product be observed.

We therefore attempted to amplify these specimens using nested PCR technology. Initial analysis was performed using the amelogenin locus. The functional amelogenin gene is located on the X chromosome. The Y chromosome contains a truncated copy of the same gene. The PCR product from the Y chromosome is therefore smaller (352 bp) than that of the X chromosome (531 bp). Thus, amplification of a sample with a single nested primer set allows reliable gender determination from single cells; females will have a single band at 531 and males will have two bands, one at 531

bp and a second at 352 bp corresponding to the Y chromosome sequence (10). With degraded DNA this PCR product may be too large to amplify successfully and we are in the process of designing new nested primer sets to amplify a shorter region. However, in this cases analysis was successful at these loci using these primers. Under the stringency conditions used, no specific amplification product is observed at 531 bp or 352 bp following amplification of mouse DNA (unpublished data).

Amplification using the nested amelogenin gene system was successful for both the charred remains and presumed blood spot. Figure 1, lane 2 contains amplified product from the father of the victim, lane 3, contains amplified product from the burned tissue, lane 4 contains amplified product from the presumed blood stain, and lane 5 contains amplified product from the victim's mother. The presence of a single band at the appropriate molecular weight confirms that the charred remains and the blood spot are human female. The father shows the expected 2 bands for a human male. In the father's and mother's lanes there are faint additional bands observed at a slightly larger molecular weight. These are PCR products from the outside primers and are often observed in nested PCR experiments using large amounts of template DNA.

The VWF locus lies on chromosome 12. The amplified product ranges in size from approximately 200 bp to 90 bp. Our statistics for 781 unrelated Caucasians demonstrate 11 separate alleles and a probability of exclusion of 0.52288. We use the repeat scoring system described by Peake et al. in which repeat values vary from between 6 and 14 (13). This scoring system is different from the Promega Gene Print kit. We applied nested PCR for the VWF locus to the samples. The results of this analysis are shown in Fig. 2. No amplification was seen in the charred remains (lane 8) or in the negative controls (lanes 1 and 9). However, the blood spot yielded two distinct bands; one at 7 repeats and one at 12 repeats. The father is homozygous with a single band at 7 repeats (lane 4) and the mother is homozygous with a single band at 12 repeats (lane 5). Thus, any child of this couple would have to be genotype 7/12. The genotype of the blood spot is 7/12 as shown in lanes 6 and 7.

The locus Apo-B lies on chromosome 2. Amplified product sizes range from approximately 570 bp to 830 bp. In our data base of 564 unrelated Caucasians there are 23 different alleles and a probability of exclusion of 0.6827. The Apo-B analysis is shown in Fig. 3. Lane 2 is the amplified product of the father, lane 3 represents the amplified product of the blood spot, lane 4 is the product of the mother and lane 5 is a second amplification product of the blood spot. The father is heterozygous with 37 and 45 repeats, the mother is heterozygous with alleles of 37 and 47 repeats. The blood spot is homozygous for 37 repeats. In the gel shown in Fig. 3 the 45 and 47 repeat bands did not separate well, but the size difference was demonstrated on gels run for longer times.

Since both the blood and tissue samples were identified as female, a final observed probability of 1.2% and a predicted probability of 0.36% were presented to the judge.

The suspect was found guilty and the verdict was upheld on appeal to the Appellate Court of the State of Illinois.

Discussion

Using nested PCR we were able to perform analysis on forensic specimens that yielded no results using standard PCR methods. In

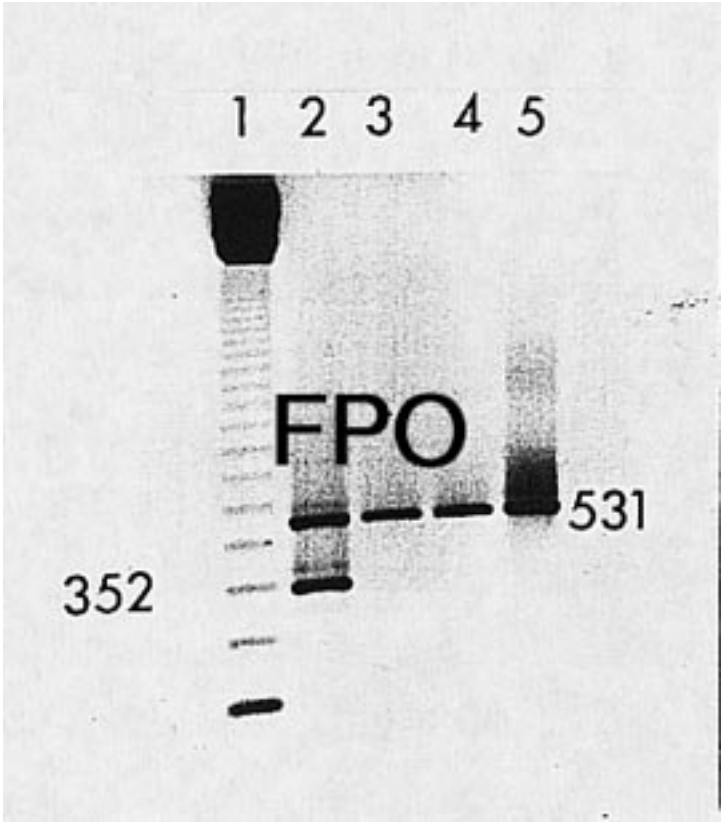

FIG. 1—*Nested amplification of forensic specimens using amelogenin primers. 531 bp = X chromosome band, 352 bp = Y chromosome band. Lane 1: Size standard. Lane 2: Father of victim. Lane 3: Charred tissue. Lane 4: Blood spot. Lane 5: Mother of victim.*

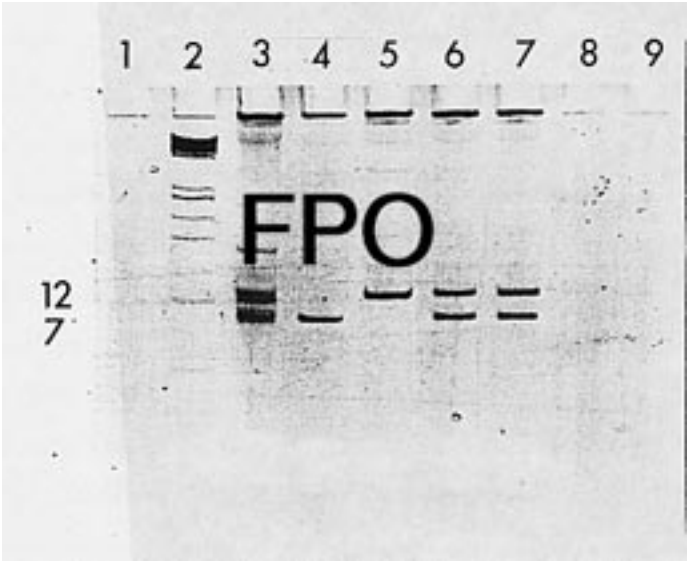

FIG. 2—*Nested amplification of forensic specimens using VWF primers. 7 = 7 repeats, 12 = 12 repeats. Lane 1: Blank control. Lane 2: Size standard. Lane 3: Size standard. Lane 4: Father of victim. Lane 5: Mother of victim. Lane 6: Blood spot. Lane 7: Blood spot. Lane 8: Charred tissue. Lane 9: Blank control.*

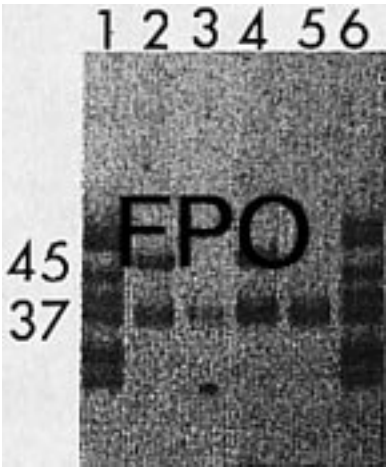

FIG. 3—*Amplification of forensic specimens using Apo-B primers. 37 = 37 repeats, 45 = 45 repeats. Lane 1: Size standard. Lane 2: Father of victim. Lane 3: Blood spot. Lane 4: Mother of victim. Lane 5: blood spot.*

this case, both a burned tissue sample and a minute blood stain showed no PCR product when subjected to standard PCR AMPFLP analysis. In the report by Sajatilla et al. (4) describing successful identification of burned bodies, the bodies were not burned to completion as muscles could be identified. In this case there were only completely charred fragments available for analysis.

It should be noted that other factors can contribute to failure of PCR analysis including technical difficulties, extraction technique, sampling variation, or inadequate specimen volume. At the time of this case we had only 3 nested systems optimized for this use, amelogenin for gender determination and two AMPFLPs, VWF, and Apo-B. We currently have added 3 additional nested systems to use for this purpose. Nested PCR should be considered in cases where standard PCR techniques are unsuccessful.

In our probability calculations, the observed frequencies in our population database were consistently in excess of the predicted frequencies. Although these differences did not reach statistical significance it may indicate a substructure of genotypes within the population. It is our recommendation that until more data is obtained that data be presented to courts regarding both the predicted and observed genotypes within a population.

PCR analysis has several advantages and disadvantages when compared to standard Southern Blot analysis. PCR is faster, can be performed on minute amounts of material, can be successful with degraded material, and a much larger percentage of crime scene specimens will be analyzable.

In addition, in Southern VNTR analysis, the size fragments are in a continuous distribution requiring complicated statistical adjustments to obtain final probability figures. Juries are often confused by arguments about fixed bins and floating bins, means and standard deviations. In contrast, AMPFLPs are discretely distributed by copy numbers so that no such statistical manipulation is necessary (4) and simple, easily understandable probability estimates can be made.

A further benefit of AMPFLP analysis is that a large amount of amplified product is available for analysis allowing mixing experiments with reference PCR products thus eliminating the problems of band shifting that complicate Southern RFLP analysis.

Another major advantage of PCR testing over Southern analysis is that frequently the entire evidentiary specimen is consumed for Southern analysis making it difficult if not impossible for confirmatory testing to be done by other laboratories. Since only a small amount of DNA is required for successful PCR analysis, in most cases a sample of the original evidence can be saved should further testing be required.

The major disadvantage of AMPFLP systems when compared to standard Southern RFLP systems is that they are much less polymorphic than Southern analysis, so that more systems must be used to obtain comparable statistical power in the event of a non-exclusion. As more systems are added, however, this should become less of a problem.

Another disadvantage of PCR DNA typing is the problem of potential contamination from extraneous DNA. Because of the ability of PCR to amplify minute quantities of DNA, improper handling or storage of material can lead to spurious results. These results, however, would almost always result in false exclusion (15) and education of evidence technicians can prevent such problems.

This report demonstrates the feasibility of using nested PCR in forensic cases where standard PCR is incapable of yielding interpretable results. As more nested AMPFLP systems are developed, this technique should allow successful DNA typing in almost every case where a biological sample is present at a crime scene.

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