Catherine Theisen Comey,[1] Ph.D.; Barbara W. Koons,[1] M.S.; Kathryn W. Presley,[1] M.S.; Jill B. Smerick,[1] M.S.; Carl A. Sobieralski,[2] B.S.; Donna M. Stanley,[3] B.S.; and F. Samuel Baechtel,[1] Ph.D.

DNA Extraction Strategies for Amplified Fragment Length Polymorphism Analysis

REFERENCE: Comey, C. T., Koons, B. W., Presley, K. W., Smerick, J. B., Sobieralski, C. A., Stanley, D. M., and Baechtel, F. S., **"DNA Extraction Strategies for Amplified Fragment Length Polymorphism Analysis,"** *Journal of Forensic Sciences,* JFSCA, Vol. 39, No. 5, September 1994, pp. 1254–1269.

ABSTRACT: A polymerase chain reaction-based DNA typing method, amplified fragment length polymorphism (AMP-FLP) analysis, has shown promise as a means of analyzing forensic biological evidence. A variety of DNA extraction methods were evaluated for their suitability for AMP-FLP analysis. Factors that were considered in the evaluation included DNA yield, ability of DNA to be amplified, the presence of DNA fragments other than those expected for the alleles in the sample, and differential amplification of different sized alleles for a sample. An initial screen of eight extraction methods was conducted on bloodstains deposited on cotton sheeting. These methods included Chelex® 100, organic extraction followed by Centricon 100® (Amicon, Inc., Beverly, MA) dialysis and concentration, Geneclean™ (Bio 101, La Jolla, CA), GlassMax™ columns (Gibco BRL, Gaithersburg, MD); GlasPac™ (National Scientific Supply Co., Inc., San Rafael, CA), Qiaex (Qiagen Inc., Chatsworth, CA), Elu-Quik™ (Schleicher and Schuell, Keene, NH), and DNA Capture Reagent (Gibco BRL, Gaithersburg, MD). Then, four methods, Chelex® 100 extraction, organic extraction followed by ethanol precipitation, organic extraction followed by Centricon 100® (Amicon, Inc., Beverly, MA) dialysis and concentration, and Geneclean were evaluated on blood and semen stains. These stains were deposited on a variety of substrates, including cotton sheeting, denim, wallboard, nylon, wood, and carpet. The effect of addition of bovine serum albumin (BSA) to the amplification reaction was also examined. The method judged most suitable for AMP-FLP analysis was organic extraction followed by Centricon 100® dialysis and concentration, with BSA added to the amplification reaction. Additionally, a modification of an existing differential extraction procedure for separating non-sperm from sperm DNA was developed.

KEYWORDS: pathology and biology, DNA extraction, polymerase chain reaction (PCR), amplified fragment length polymorphisms (AMP-FLPs)

Received for publication 25 Oct. 1993; revised manuscript received 31 Jan. 1994; accepted for publication 7 March 1994.

This is publication number 94-01 of the Laboratory Division of the Federal Bureau of Investigation. Names of commercial manufacturers are provided for identification only and their inclusion does not imply endorsement by the Federal Bureau of Investigation.

[1]Research Chemists (Comey and Baechtel) and Physical Scientists (Koons, Presley, Smerick), Forensic Science Research and Training Center, FBI Academy, Quantico, Virginia.

[2]DNA Analyst, Indiana State Police, Indianapolis, Indiana.

[3]Criminalist, Texas Department of Public Safety, Austin, Texas.

A variety of DNA extraction methods has been used for forensic DNA analysis procedures. For example, digestion of body fluid stains using SDS and proteinase K, followed by purification of DNA by extraction with phenol/chloroform and ethanol precipitation, is very successful and is routinely used for forensic samples analyzed by restriction fragment length polymorphism (RFLP) typing. This method, however, was found to have limitations when applied to a polymerase chain reaction (PCR)-based DNA typing method used in forensic analysis, specifically HLA DQα typing. When this method was applied to bloodstains, it was found that, although sufficient DNA was recovered for analysis, it did not always amplify using PCR [*1*]. This failure of amplification is thought to be caused by the presence of hematin in bloodstains, which is an inhibitor of PCR [*2*]. Other DNA extraction methods were more successful in yielding amplifiable DNA, such as an organic extraction described above but followed by dialysis and concentration using Centricon 100® devices (Amicon, Inc., Beverly, MA) rather than ethanol precipitation [*1*], or a Chelex® 100-based extraction method [*3–5*] which also can be followed by further Centricon 100® purification [*4*].

Recently, another PCR-based DNA typing method, used for the analysis of amplified fragment length polymorphisms (AMP-FLPs), has shown promise as a means of analyzing forensic biological evidence [*6–11*]. Before AMP-FLP analysis could be implemented in our forensic laboratory, it was desirable to evaluate a number of DNA extraction methods to determine the most suitable one for AMP-FLP analysis. Factors that should be considered when comparing various methods include DNA yield, suitability of DNA for amplification, presence of DNA fragments on a silver stained gel other than those expected for the alleles in the sample, and differential amplification of alleles of different sizes in a sample. A variety of extraction methods was evaluated for these factors, including organic extraction followed by either ethanol precipitation or Centricon 100® dialysis and concentration, Chelex® 100 extraction, and several commercially available DNA extraction kits. The results of these studies are presented here.

Materials and Methods

In the first part of this study, eight different DNA extraction methods were used on panels of 3 μL bloodstains placed on washed cotton sheeting. In the second part, four extraction methods, including three of the eight methods showing promise in the first part of the study, were evaluated further, by examining their effectiveness on a variety of substrates and by testing the effect of addition of bovine serum albumin (BSA) to the amplifications.

DNA Extraction—Part I

Digestion of Bloodstains—Blood was drawn from 11 different individuals by venipuncture into EDTA Vacutainer® tubes. Blood (3 μL) was placed on washed cotton sheeting and dried. These samples represented the following D1S80 types: 18-17; 24-18 (two samples); 24-20; 28-22; 28-24; 28-25; 29-25; 31-18; 31-29; and 32-29. Thus, over the 11 samples, allele 17 was the smallest represented, and allele 32 the largest. The sample with the largest difference between alleles was the 31-18. Stains were cut from the substrate and placed in sterile 1.5 mL microcentrifuge tubes. Stain extraction buffer (100 μL total volume, containing 10 mM Tris-Cl, pH 8.0, 100 mM NaCl, 10 mM EDTA, 2% SDS, and 0.2 mg Proteinase K) was added to the cuttings and the tubes were incubated overnight at 56°C. Following the incubation, samples were purified by the methods described below.

Organic Extractions with Centricon 100® Purification—Phenol/chloroform/isoamyl alcohol (24:24:1; 100 μL) was added to each tube. Tubes were then vortexed briefly, and spun

in a microcentrifuge for 5 minutes at maximum speed. Following the organic extraction, the aqueous layers were dialyzed and concentrated to approximately 40 μL by using Centricon 100® microconcentrators (Amicon, Inc., Danvers, CT) [*1*]. This method will be referred to here as the "organic/dialysis" method.

Chelex® 100 Extractions—Bloodstains (3 μL, two stains from each of 11 individuals) were extracted using Chelex® 100 resin (BioRad, Richmond, CA) as described [*12*].

GlassMax™, GlasPac™, Geneclean™, and Qiaex Purification—Following digestion of bloodstains, some samples were purified using commercially available DNA purification kits. Samples were purified by using GlassMax™ columns (Gibco BRL, Gaithersburg, MD); suspensions of GlasPac™ (National Scientific Supply Co., Inc., San Rafael, CA); Geneclean™ (Bio 101, La Jolla, CA) glass beads; or Qiaex silica gel suspension (Qiagen Inc., Chatsworth, CA) according to manufacturers' instructions. All of these purification schemes, based on the purification method described by Vogelstein et al. [*13*], include binding of DNA to beads in a NaI solution, washing of beads containing bound DNA, and elution of the DNA in water of TE^{-4} (10 mM Tris-Cl, pH 8.0, 0.1 mM EDTA).

Elu-Quik™ and DNA Capture Reagent Purification—Some bloodstains were digested and their DNA purified according to manufacturers' instructions using the Elu-Quik™ kit (a glass bead-based purification scheme; Schleicher and Schuell, Keene, NH; four stains from each of six individuals were tested) or the DNA Capture Reagent kit [*14*] (the reagent consists of methidium-spermine bound to Sepharose® 4B Gibco BRL, Gaithersburg, MD; four stains from each of six individuals were tested). Additional bloodstains (six for each method) that were subjected to either Elu-Quik™ or Capture Reagent purification were initially subjected to digestion of bloodstains as described above rather than to digestion using reagents provided by the manufacturers. Some DNA samples purified by Capture Reagent were subjected to further purification by ethanol precipitation (six stains) or Centricon 100® dialysis (six stains) and concentration. Some samples (two stains from each of six individuals) were purified by using Capture Reagent which had been washed in 1 N NaOH. Finally, some samples (two stains from each of six individuals) were purified by using 5-fold less Capture Reagent than recommended by the manufacturer.

DNA Extraction—Part II

Blood and semen (5 μL) were placed on a variety of substrates including washed cotton sheeting, denim, painted wallboard, nylon panties (referred to hereafter as nylon), carpet, and wood (see Results for number of stains analyzed and D1S80 types of samples in individual studies). Stains were cut from substrates and placed in sterile 1.5 mL microcentrifuge tubes. Some stains were extracted using Chelex® 100 resin (BioRad, Richmond, CA) as described [*12*]. Other stains were extracted as follows.

Digestion of Bloodstains—Stain extraction buffer (100 or 400 μL total volume, containing 10 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, 39 mM DTT, 2% SDS, and 0.5 mg/mL Proteinase K) was added to the cuttings, which had been placed in Spin-X tubes (Costar Corporation, Cambridge, MA). Tubes were incubated overnight at 56° C. The substrate cuttings were placed in the basket of a Spin-X tube (the membranes in the basket were previously removed and discarded) and the baskets placed into the tubes in which the extractions had been done. The tubes were spun briefly in a microcentrifuge to collect extraction buffer. The baskets and cuttings were discarded. Following the incubation, samples were purified by a variety of methods described below.

Geneclean™ Purification—Some samples were purified by Geneclean™ (Bio 101, La Jolla, CA) according to manufacturer's instructions.

Organic Extraction with Ethanol Precipitation—Some samples were subjected to organic extractions and ethanol precipitation according to Budowle et al. [*15*].

Organic Extractions with Centricon 100® Purification—Some samples were subjected to organic extraction and Centricon 100® dialysis and concentration as described in Part I. Some samples were purified without the butanol extraction, and some samples were purified in Centricon 100® tubes by bringing the sample volume to approximately 500 μL with sterile distilled, deionized water for the first concentration, performing one wash with 200 μL water, and adding 200 μL water to the retentate prior to its collection.

DNA Quantification

Extracted DNA was quantified by hybridization to the human alphoid probe D17Z1 using a slot blot method described by Waye et al. [*16*].

Effect of Bovine Serum Albumin (BSA) on Amplification

To examine the effects of different formulations of BSA on amplification, DNA from 58 bloodstains (5 μL) on cotton sheeting and extracted using the Chelex® 100 method, were amplified at the locus D1S80 without BSA or in the presence of one of four formulations of BSA at a concentration of 160 μg/mL [*7,17*]. Three of the BSA formulations were from Sigma Chemical Co. (St. Louis, MO; cat. nos. 3350, 4503, and 4378), and one from Gibco BRL (Gaithersburg, MD; cat. no. 5561UB). All BSA formulations were Fraction V preparations. The BSA formulations from Sigma, which had been prepared by cold alcohol precipitation, were all supplied as powders from which 50 mg/mL solutions were made. Sigma 3350 (which will be referred to here as BSA pH 7.0) had undergone a pH adjustment by the manufacturer such that a 1% solution would be at pH 7.0, while Sigma 4503 (which will be referred to here as BSA pH 5.2) had been adjusted to pH 5.2. Both were 96–99% albumin. Sigma 4378 (which will be referred to here as BSA recrystallized) was further crystallized and lyophilized by the manufacturer and was 97–99% albumin. The Gibco BRL BSA (which will be referred to here as molecular biology grade BSA) had been acetylated to remove nucleases and proteases, and was supplied as a 50 mg/mL solution. Two types of BSA (BSA pH 7.0 and BSA pH 5.2) were further examined on 77 blood and 49 semen stains deposited on six substrates (cotton sheeting, denim, wallboard, carpet, nylon, and wood) extracted by the Chelex® 100, organic/ethanol, and Geneclean™ methods.

Differential Extraction of Semen-Containing Stains

Thirty-six vaginal swabs (from two donors; the swabs from one of these donors was not semen-free), with semen (from one donor), 5 μL or dilutions equivalent to a total of 1 μL, 0.2 μL, and 0.04 μL semen, were extracted by one of the three following methods (triplicate analysis for each semen amount/method combination; the assignment of one or the other vaginal swab donor for any semen amount/method combination was random): 1) the organic method used for RFLP analysis [*18*] except that the ethanol precipitation step was replaced with Centricon 100® concentration and dialysis steps (this method does not include any washes of the sperm pellet following digestion of non-sperm cells); 2) as in 1) except the sperm pellet was washed five times with wash buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 50 mM NaCl, 2% SDS) following digestion of non-sperm cells, and 3) the Chelex® 100 differential extraction method as described by Comey et al. [*12*].

To determine the number of washes of the sperm pellet necessary, 36 vaginal swabs (from two donors; the swabs from one of these donors was not semen-free), with semen (from one donor), 5 μL or dilutions equivalent to a total volume of 0.04 μL, and 0.008 μL semen, were extracted by method two as mentioned with the following modifications: zero, one, three, or five washes of the sperm pellet were done (each semen amount/wash number was analyzed in triplicate; the assignment of one or the other vaginal swab donor for any semen amount/wash number combination was random).

DNA Amplification and Typing

Extracted DNA (5 ng, or 20 μL of the extract if 5 ng were not available) was amplified at the locus D1S80 [*19*] using conditions according to Baechtel et al. [*20*]. Amplified D1S80 DNA was typed by electrophoresis on ultrathin polyacrylamide gels using a discontinuous buffer system [*21*] under conditions described by Baechtel et al. [*20*] except that for some gels the front was allowed to migrate 10 cm or 14 cm rather than 21 cm. Gels were silver stained according to Budowle et al. [*21*].

Some samples were also amplified at the locus HUMTHO1 [*22*]. Amplifications (50 μL reactions) contained 5 ng DNA, 0.2 mM each dNTP, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.001 % gelatin, 0.25 μM each primer, and 2.5 u *Taq* polymerase (Perkin-Elmer Corp, Norwalk, CT). Amplifications were carried out in the GeneAmp PCR System 9600 (Perkin-Elmer Corp., Norwalk, CT) for 27 cycles using the following parameters: 95°C, 45 s, 60°C, 30 s, 72°C, 30 s. Electrophoresis was carried out using a discontinuous buffer system similar to Baechtel et al. [*20*] except the composition of the gel was 7% T, 5% C, with piperazine diacrylamide as the crosslinker. The front was allowed to migrate 19 cm.

Results

Part I—Screen of Eight Extraction Methods

Organic/dialysis, GlassMax™, GlasPac™, Geneclean™, Qiaex, and Chelex® 100—For each of these six extraction methods, DNA was extracted from 3 μL bloodstains on washed cotton sheeting. Samples from 11 different individuals were analyzed in duplicate (in quadruplicate for Geneclean™). Table 1 shows the average, range, and standard deviation of DNA yield with each method. Average yields range from 60 ng/stain for the Chelex® 100 method to 190 ng/stain for the organic/dialysis method.

Although DNA yield is one important measure of the effectiveness of a particular DNA extraction method, more important are the suitability of the DNA for amplification and the

TABLE 1—*DNA yields and amplification success for six extraction methods.*

Extraction method	Yield (Avg., ng/3 μL stain) (number of stains)	Standard deviation	Range	Amplification success (number successful amplifications/ number attempted)
Organic/dialysis	190 (22)	29.4	100–200	22/22
GlassMax™	160 (22)	49.2	100–200	15/22
Geneclean™	150 (44)	50.6	80–200	42/44
GlasPac™	90 (22)	51.1	30–200	22/22
Qiaex	65 (22)	30.2	40–100	19/22
Chelex® 100	60 (22)	23.1	30–100	22/22

clarity of the typing results. Successful amplifications are defined here as those in which both alleles of heterozygous samples, (or one apparent allele in single band patterns), are clearly visible and differential amplification is not substantial. Typings that demonstrated the presence of DNA fragments other than the specific alleles will be noted. Each DNA sample was subjected to amplification at the locus D1S80 followed by typing of the locus by electrophoresis on ultrathin polyacrylamide gels and silver staining. Table 1 shows the number of successful amplifications and typings for each method. Success ranges from a low of 15/22 for GlassMax™ (this method gave rise to the second highest average DNA yield) to 22/22 for organic/dialysis, Chelex® 100, and GlasPac™.

Elu-Ouik™ and DNA Capture Reagent—The Elu-Quik™ extraction method was evaluated according to manufacturer's instructions on a panel of 24 bloodstains (3 μL; six individuals assayed in quadruplicate). The yields for all samples were low (generally less than 40 ng/sample) and inconsistent in the amount recovered from duplicates. The extracted DNA could not be amplified at the locus D1S80. Amplification could be obtained by using the digestion method used for the other extraction methods described above, rather than the buffer included with the kit. Because this method demonstrated no advantages over other glass bead based methods, evaluation of this method was terminated at this point.

Purification using the DNA Capture Reagent relies on the binding of DNA to an intercalating dye, methidium, which is attached to Sepharose® beads by a spermine linker [*14*]. Although DNA extracted from bloodstains and bound to the Capture Reagent could be eluted in sufficient quantities for amplification, it could not be amplified at the locus D1S80. A number of modifications were made to the method; however, none yielded amplifiable DNA. The presence of inhibition in the eluted DNA was confirmed by mixing DNA obtained by Capture Reagent extraction with DNA obtained by Geneclean™ extraction. In this case, no amplification occurred, while the control DNA extracted using the Geneclean™ method could be amplified as expected.

Part II—Evaluation of Four Extraction Methods on Various Substrates

In the second part of this study, the extraction method used for RFLP analysis, organic extraction followed by ethanol precipitation, was compared with two of the more successful extraction methods described above, the Chelex® 100 and the Geneclean™ methods. DNA was extracted from 5 μL bloodstains from each of two donors (D1S80 types 28-24 and 28-25) or 5 μL semen stains from one donor (D1S80 type 31-29) on each of six substrates (wallboard, cotton sheeting, denim, carpet, nylon panties, and wood). Each method/donor/substrate combination was analyzed in octuplicate, for a total of 432 analyses. After extraction by one of the three methods, samples were amplified at the locus D1S80. Average DNA yields, as well as the range and standard deviation, from blood and semen stains are summarized in Table 2. Although there is a wide range of yields from replicate stains in most categories, all methods on all substrates yielded sufficient DNA for two amplifications and, in most categories sufficient DNA was recovered for at least five amplifications. The average yields from semen stains on carpet were lower than from other substrates. Since it was difficult to visualize the semen stains on this substrate, it could not be easily determined whether or not the entire stain was cut out. Tables 3 and 4 show the results of D1S80 amplification for bloodstain DNA and semen stain DNA, respectively. The Chelex® 100 method was more successful in yielding typing results from DNA from bloodstains than the other two methods, while none of the three methods were as successful in yielding typing results from DNA from semen stains. Many Chelex® 100 extracted samples which amplified (77%) also exhibited extra bands of higher molecular weight than the allelic ladder. When Centricon 100® dialysates of the Chelex® 100 extracts were amplified, the extra bands were not eliminated (data not shown).

TABLE 2—*Average DNA yields (ng) per 5 μL stain.*

		Organic/Ethanol			Geneclean™			Chelex®100		
		Avg.	Standard deviation	Range	Avg.	Standard deviation	Range	Avg.	Standard deviation	Range
Wallboard	B	55	37.6	12.5–100	66	60.2	12.5–200	62	31.6	25–100
	S	100	46.3	50–200	78	41.0	25–100	>200	...	all >200
Denim	B	56	55.4	0–200	51	55.0*	0–200	26	27.6[a]	0–100
	S	38	17.7	12.5–50	12.5	...	all 12.5	188	35.4	100–200
Cotton	B	30	25.0	12.5–100	75	35.4	0–100	88	72.6	25–200
	S	88	23.1	50–100	75	26.7	50–100	>200	...	all >200
Carpet	B	32	35.6[a]	0–100	32	23.3	12.5–100	22	14.1	0–50
	S	12.5	23.1[a]	0–50	25	34.7[a]	0–100	69	87.1[a]	0–200
Nylon	B	48	45.8	25–200	46	30.3	12.5–100	34	41.1[a]	0–100
	S	150	53.5	100–200	125	65.5	50–200	>200	...	all >200
Wood	B	62	51.3	12.5–200	88	41.2	12.5–200	44	28.1	0–100
	S	138	51.8	100–200	112	58.2	50–200	200	...	all 200

NOTE: B: bloodstain.
S: semen stain.
Sixteen bloodstains and eight semen stains were tested for each method/substrate combination.
[a]The presence of both high and low yields contribute to the standard deviations being greater than the means.

TABLE 3—*D1S80 Amplification results—blood.*

	Wallboard	Denim	Cotton	Carpet	Nylon	Wood	Totals
Organic/Ethanol	6/16	0/16	11/16	0/16	16/16	15/16	48/96
Geneclean™	5/16	4/16	9/16	0/16	14/16	15/16	47/96
Chelex® 100	8/16[a]	10/16[a]	16/16[a]	12/16[a]	15/16[a]	16/16[a]	77/96

NOTE: The numbers in each category represent the number of successful amplifications/number of attempted amplifications.

[a]Extraneous bands of higher molecular weight than alleles.

Effect of Addition of BSA to Amplification

Fifty-eight bloodstains (5 μL; D1S80 types 31-18 and 18-17) made on cotton sheeting and extracted using the Chelex® 100 method were amplified at the locus D1S80, either with no BSA or with one of four formulations of BSA (BSA pH 7.0, BSA pH 5.2, BSA recrystallized, and molecular biology grade BSA). Fifty-six of 58 samples amplified in the absence of BSA. Using the four formulations of BSA, 56, 56, 54, and 49 of the 58 samples, respectively, were amplified. However, one of the BSA formulations that led to amplification in 56 samples (molecular biology grade BSA) gave rise to differential amplification in some of the samples with the genotype 31-18 in which the 18 allele was preferentially amplified as compared with the 31 allele.

Two formulations of BSA (BSA pH 7.0 and BSA pH 5.2) were further tested on 77 bloodstains (two donors; D1S80 types 28-24 and 28-25) and 49 semen stains (one donor; D1S80 type 31-29) made on a variety of substrates (cotton sheeting, denim, wallboard, carpet, nylon, and wood) and extracted using the Chelex® 100, organic/ethanol, or Geneclean™ methods. Tables 5 and 6 summarize the results for blood and semen DNA, respectively. DNA from bloodstains made on nylon or wood amplified equally well with or without BSA, while BSA improved the amplification success rate of DNA extracted by all three methods from stains made on cotton, denim, carpet, and, especially, wallboard. Figure 1 shows the effect of BSA addition to amplification of DNA extracted from nine bloodstains deposited on wallboard. In eight of nine extracts which failed to amplify in the absence of BSA, amplification was possible at the locus D1S80 with the addition of BSA. One formulation of BSA (BSA pH 7.0) was somewhat more successful in improving amplification of DNA from bloodstains than the other (BSA pH 5.2; 24% of the bloodstains amplified with no BSA, 86% amplified with BSA pH 7.0, and 77% amplified with BSA pH 5.2). Thus, BSA formulation pH 7.0 was chosen for the remaining studies.

Comparison of Chelex® 100 and Organic/Dialysis Extraction Methods

Although the Chelex® 100 method was relatively successful in producing D1S80 results in the above study, the presence of extra bands, coupled with the low success rate on semen

TABLE 4—*D1S80 amplification results—semen.*

	Wallboard	Denim	Cotton	Carpet	Nylon	Wood	Totals
Organic/Ethanol	4/8	0/8	8/8	0/8	8/8	8/8	28/48
Geneclean™	3/8	0/8	7/8	2/8	8/8	8/8	28/48
Chelex® 100	0/8	0/8	3/8	0/8	7/8[a]	7/8	17/48

NOTE: The numbers in each category represent the number of successful amplifications/number of attempted amplifications.

[a]Extraneous bonds of higher molecular weight than alleles.

TABLE 5—*Effect of BSA addition on amplification of blood DNA.*

	Wallboard			Denim			Cotton			Carpet			Nylon			Wood			Totals		
Extraction method	C[a]	O[b]	G[c]	C	O	G	C	O	G	C	O	G	C	O	G	C	O	G	C	O	G
no BSA	2/4	1/8	0/8	0/3	0/8	0/8	0/0	3/5	2/4	0/2	0/8	0/8	1/1	1/1	2/2	0/0	2/2	5/5	3/10	7/32	9/35
BSA (pH 7.0)	4/4	8/8	7/8	1/3	8/8	6/8	0/0	5/5	4/4	2/2	5/8	5/8	1/1	1/1	2/2	0/0	2/2	5/5	8/10	29/32	29/35
BSA (pH 5.2)	4/4	8/8	7/8	0/3	8/8	2/8	0/0	5/5	4/4	2/2	5/8	3/8	1/1	1/1	2/2	0/0	2/2	5/5	7/10	29/32	23/35

NOTE: The numbers in each category represent the number of successful amplifications/number of attempted amplifications. Samples were extracted by:
[a]Chelex 100® (C).
[b]Organic extraction/ethanol precipitation (O).
[c]Geneclean® (G).

TABLE 6—*Effect of BSA addition on amplification of semen DNA.*

	Wallboard			Denim			Cotton			Carpet			Nylon			Wood			Totals		
Extraction method	C[a]	O[b]	G[c]	C	O	G	C	O	G	C	O	G	C	O	G	C	O	G	C	O	G
no BSA	0/4	3/4	1/4	0/4	0/4	0/4	3/4	1/1	2/3	0/4	0/4	2/4	0/0	0/0	1/1	2/2	1/1	1/1	5/18	5/14	7/17
BSA (pH 7.0)	4/4	4/4	4/4	3/4	1/4	0/4	4/4	1/1	3/3	2/4	2/4	2/4	0/0	0/0	1/1	2/2	1/1	1/1	15/18	9/14	11/17
BSA (pH 5.2)	4/4	4/4	4/4	3/4	1/4	0/4	4/4	1/1	3/3	3/4	2/4	2/4	0/0	0/0	1/1	2/2	1/1	1/1	16/18	9/14	11/17

NOTE: The numbers in each category represent the number of successful amplifications/number of attempted amplifications. Samples were extracted by:
[a]Chelex 100® (C).
[b]Organic extraction/ethanol precipitation (O).
[c]Geneclean® (G).

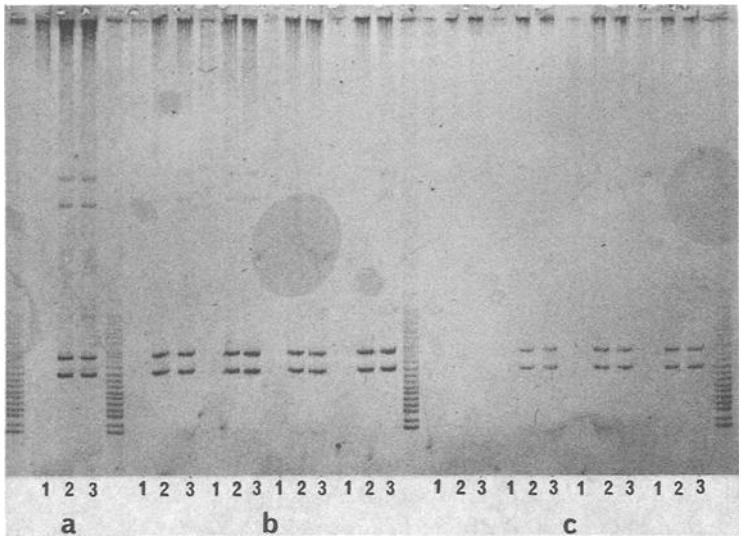

FIG. 1—*Effect of BSA addition on D1S80 amplification. DNA from 5 μL bloodstains deposited on wallboard extracted by Chelex® 100* (a), *organic/ethanol* (b), *or Geneclean™* (c). *Each extract was amplified without BSA (1) or with one of two BSA formulations (2, BSA pH 7.0, and 3, BSA pH 5.2) at a concentration of 160 μg/mL in the amplification reaction. The unmarked lanes contain a D1S80 allelic ladder. The D1S80 type of the samples is 28-25.*

stains, made it not entirely satisfactory. Therefore, the organic/dialysis method, which showed promise in the first part of the study, was examined further. In this study, eight 5 μL blood (one donor; D1S80 type 28-24) or semen samples (one donor; D1S80 type 22-18) were placed on each of three substrates—cotton sheeting, denim, and carpet (denim and carpet were chosen because, in the above studies, they gave rise to lower success rates for amplification than did other substrates). Following treatment in the stain digestion buffer and a single phenol/chloroform/isoamyl alcohol extraction, one of the two following Centricon 100® treatments was used. For half of the samples, the aqueous layer (approximately 350 μL) was placed in the Centricon 100® device and centrifugation was carried out at 3000 × g. After centrifugation, 200 μL of water was added to the retentate, the tube was inverted, and the retentate was collected by centrifugation. For the remaining samples, the aqueous layer was added to a Centricon 100® device along with 200 μL of water (it had been previously determined that butanol extraction following the phenol/chloroform/isoamyl alcohol extraction [*1*] was unnecessary). After centrifugation at 3000 × g, 200 μL of water was added to the retentate and the device was centrifuged again. Then, 200 μL of water was added to the retentate, the device inverted, and the retentate collected by centrifugation. All samples were amplified with and without BSA pH 7.0. Table 7 shows the results of this study. It is apparent that both the addition of the water wash step and the addition of BSA to the amplification reaction are important for a high success rate for amplification.

A comparison was then made of the two extraction methods which had the highest amplification success rates—organic/Cetricon 100® and Chelex® 100, both with BSA pH 7.0 added to the amplification. Sixteen bloodstains (one donor; D1S80 type 32-29) and 16 semen stains (one donor; D1S80 type 28-25) were made on each of three substrates—cotton, denim, and carpet. Eight stains from each category were extracted by one of the two methods (one sample, a bloodstain on cotton extracted by Chelex® 100, was lost during

TABLE 7—*Effects of Centricon 100® wash and addition of BSA on D1S80 amplification.*

		Wash		No Wash	
		− BSA	+ BSA	− BSA	+ BSA
Cotton	Blood	0/2	2/2	1/2	2/2
	Semen	2/2	2/2	0/2	1/2
Denim	Blood	0/2	1/2	0/2	0/2
	Semen	0/2	2/2	1/2	2/2
Carpet	Blood	1/2	2/2	0/2	0/2
	Semen	0/2	2/2	0/2	1/2
Totals		3/12	11/12	2/12	6/12

NOTE: Eight bloodstains and eight semen stains were made on each substrate, DNA was extracted by the organic method and subjected to Centricon 100® concentration, either with or without a wash step. DNA was amplified with or without BSA pH 7.0. The numbers in each category represent the number of successful amplifications/number of attempted amplifications.

processing). DNA yields and amplification results are presented in Tables 8 and 9, respectively. Yields were generally higher using the organic/dialysis method. All samples amplified using the organic/dialysis method, while 43/47 amplified using Chelex® 100. Although both methods produced correct typing results, bloodstain DNA (all eight samples) extracted from denim using Chelex® 100 showed the presence of extra bands of higher molecular weight than the alleles (Fig. 2). These samples were then amplified at the locus HUMTH01 [22] (bloodstain HUMTH01 type 6, semen stain HUMTH01 type 7-6). Again, all samples amplified (48/48) using the organic/dialysis method. Four of eight semen samples on denim demonstrated extra bands. Some of these bands were within the size range defined by the HUMTH01 allelic ladder, while others were considerably larger and well above the ladder. However, the specific allele bands were much more intense than the extra bands. The occurrence of these extra bands, signalled by the presence high molecular weight bands, indicate that caution should be exercised when interpreting potentially mixed samples. The Chelex® 100 method was somewhat less successful (44/47 samples amplified) and a greater number of samples contained extra bands (eight of eight bloodstains on denim and two of five bloodstains on carpet).

The recently available Microcon 100® microconcentrators (Amicon, Inc., Danvers, CT) were a potential replacement for Centricon 100® devices. The volumes used for the digestion, concentration, and dialysis steps in this study could be accommodated by Microcon 100

TABLE 8—*Average DNA yields (ng) per 5 μL stain extracted using organic/dialysis and Chelex® 100 methods.*

		Organic			Chelex® 100		
		Yield (avg.)	Standard deviation	Range	Yield (avg.)	Standard deviation	Range
Cotton	Blood	144	62.3	50–200	86	24.4	50–100
	Semen	>400	—	all >400	62	23.1	50–100
Denim	Blood	138	51.8	100–200	56	17.7	50–100
	Semen	>400	—	all >400	200	—	all 200
Carpet	Blood	75	26.6	50–100	150	53.5	25–400
	Semen	288	124.6	100–400	119	127.3	25–400

NOTE: For each category, eight stains were analyzed, with the exception of cotton/blood/Chelex, where seven stains were analyzed.

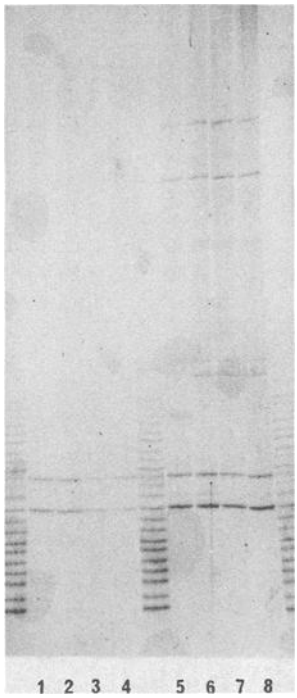

FIG. 2—*Comparison of organic/dialysis with Chelex® 100 methods. DNA was extracted from eight replicate bloodstains deposited on denim by organic/dialysis (1-4) or Chelex® 100 (5-8) and amplified at D1S80. The unmarked lanes contain a D1S80 allelic ladder. The D1S80 type of the samples is 28-25.*

TABLE 9—*Comparison of D1S80 amplification success with organic/dialysis and Chelex® 100 methods.*

		D1S80		HUMTH01	
		Organic	Chelex® 100	Organic	Chelex® 100
Cotton	Blood	8/8	7/7	8/8	7/7
	Semen	8/8	8/8	8/8	8/8
Denim	Blood	8/8	8/8[a]	8/8	8/8[a]
	Semen	8/8	8/8	8/8[b]	8/8
Carpet	Blood	8/8	8/8	8/8	5/8[c]
	Semen	8/8	4/8	8/8	8/8
Totals		48/48	43/47	48/48	44/47

NOTE: The numbers in each category represent the number of successful amplifications/number of attempted amplifications.

[a] Extra bands in 8/8 samples.

[b] Extra bands in 4/8 samples.

[c] Extra bands in 2/5 samples.

devices. To ensure that Microcon 100® devices could replace Centricon 100® devices, and, more importantly, to demonstrate that the organic/dialysis extraction method described above, the most successful method examined here, was effective for amplifying DNA from the sample which contained the largest spread of D1S80 alleles of all samples examined in the mentioned studies (31-18), this method (using Microcon 100® devices) was tested on bloodstains made on cotton sheeting and denim. Eight bloodstains (containing 5 μL of blood) were made on each of the two substrates. The DNA was extracted by the organic/dialysis method, and amplification was carried out in the absence or presence of BSA. Seven of eight samples on cotton amplified in the absence of BSA, while eight of eight amplified with BSA. Seven of eight DNA samples from bloodstains made on denim amplified without or with BSA (the same sample failed to amplify without or with BSA). In all samples which amplified, both alleles were clearly visible and no extra bands were visible. Figure 3 shows the results of this experiment for two of the bloodstains on cotton and two of the bloodstains on denim.

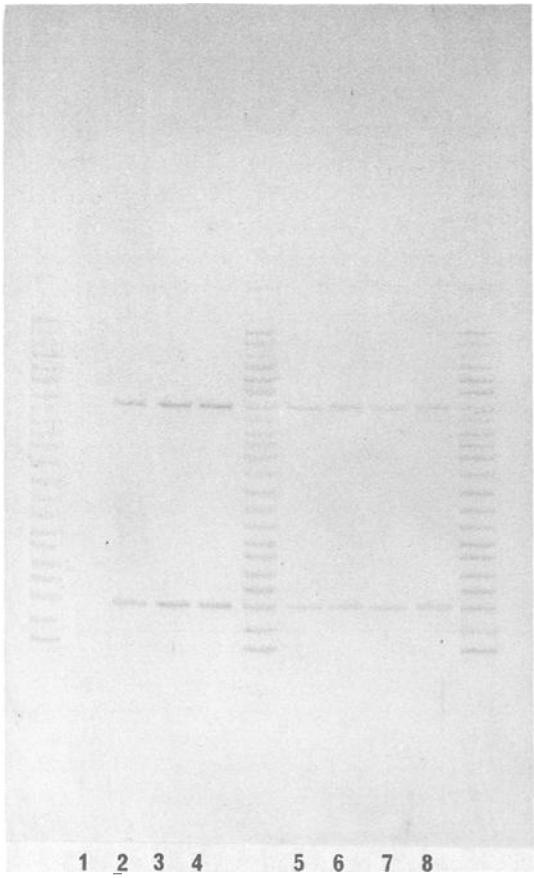

FIG. 3—*Test of organic/dialysis extraction method on sample with D1S80 type 31-18. DNA was extracted by the organic/dialysis method from replicate bloodstains deposited on cotton or denim and amplified at D1S80. Lanes 1, 2, 5, and 6 show the D1S80 types of the samples deposited on cotton, lanes 3, 4, 7, and 8 represent those deposited on denim. Samples 1–4 were amplified without BSA while samples 5–8 were amplified with BSA pH 7.0 at a concentration of 160 μg/mL in the amplification reaction. The unmarked lanes contain a D1S80 allelic ladder.*

Establishment of a Differential Extraction Method

Three extraction methods were examined for their effectiveness on separating sperm and non-sperm DNA from vaginal swabs: 1) the organic method used for RFLP analysis with the ethanol precipitation step replaced by Centricon 100® concentration and dialysis steps (this method does not include any washes of the sperm pellet following digestion of non-sperm cells); 2) as in 1) except the sperm pellet was washed five times following digestion of non-sperm cells (see Materials and Methods), and 3) the Chelex® 100 differential extraction method as described by Comey et al. [*12*]. Twelve swabs were tested for each method, three containing 5 μL semen, three each containing dilutions of semen equivalent to 1, 0.2, and 0.04 μL semen. Samples were amplified and typed for D1S80. The sperm fractions from all twelve samples extracted by the organic method without washes of the sperm pellet showed the alleles from the female donor of the swab as well as the alleles from the semen contributor. The sperm fractions in 7 of 12 samples extracted by the Chelex® 100 also showed D1S80 alleles from the female donor (and at least one sample showed this mixture in each semen amount category). None of the sperm fractions from the twelve samples extracted by the organic/dialysis extraction method with 5 washes of the sperm pellet showed D1S80 alleles from the female donor. Thus, this method was judged most effective of the three. (No non-sperm sample exhibited sperm DNA in any of the 36 samples.)

To determine whether fewer than five washes of the sperm pellet would be sufficient to remove the contribution of DNA from the female donor, zero, one, three, or five washes were done of the sperm pellets arising from swabs containing 5 μL semen or dilutions equivalent to 0.04 or 0.008 μL semen (each semen amount/wash number was analyzed in triplicate for a total of 36 swabs). For swabs containing 5 μL semen, three and five washes of the sperm pellets were equally effective in removing non-sperm DNA (Fig. 4). Note that two of the alleles present in Fig. 4, lane 10, as well as in lanes 1–4, 6, and 8, arise from other sperm DNA present on the vaginal swabs, not from the female donor of the swabs. For swabs containing the equivalent of 0.04 μL semen, trace female DNA was detectable in two of three samples which were washed three times, and none in the samples washed 5 times (the alleles of the semen donor were readily detectable in all six of these

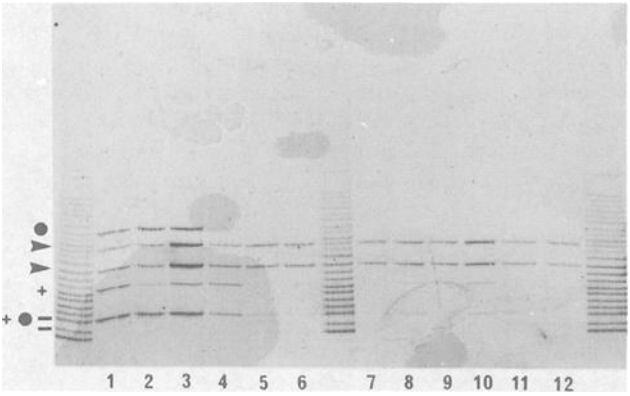

FIG. 4—*Effect of number of sperm pellet washes in the differential extraction method. Five μL of semen (▶, D1S80 type 28-25) was added to each of 12 vaginal swabs from two donors (a (●, 31-18) and b (−, 18-17)); donor a in lanes 1–4, 6, 8, and 10, donor b in lanes 5, 7, 9, 11, and 12. (Note that additional sperm DNA (+, 22-18) is present on swabs from donor a). Sperm pellets were washed with sperm wash buffer 0 (lanes 1–3), 1 (lanes 4–6), 3 (lanes 7–9), or 5 (lanes 10–12) times. The unmarked lanes contain a D1S80 allelic ladder.*

samples). In none of swabs containing the equivalent of 0.008 μL semen were the alleles of the semen donor detectable. With both three and five washes, the female DNA could be detected in one of three samples. Since the goal of a differential DNA recovery from semen-containing vaginal swabs is to reduce the female's contribution to the total DNA of the sperm fraction, some female DNA in the sperm fraction is tolerable as long as it is not excessive and alleles can be detected from both male and female sources. With 0.04 μL semen, three washes of the sperm pellet were necessary to ensure an acceptable ratio of male to female DNA so that alleles from both individuals were detected. Thus, a differential extraction method using organic extraction, three washes of the sperm pellet, and a Centricon 100® concentration step followed by a Centricon 100® wash step (using 200 μL water) for each fraction was judged suitable for amplification at the D1S80 locus.

Discussion

This study evaluated a total of nine DNA extraction methods for their effectiveness in yielding DNA suitable for AMP-FLP analysis. One method, organic extraction followed by Centricon 100® concentration and dialysis, proved to be most effective in yielding sufficient DNA suitable for amplification from blood and semen stains placed on a variety of substrates. In the presence of BSA, DNA could be amplified from DNA extracted from blood and semen stains placed on a variety of substrates (many of these samples failed to amplify without BSA—see Table 7). Differential amplification was not observed, and presence of extra bands was minimal (seen only on 4 of the 96 stains evaluated, these were on denim). Sufficient DNA could be extracted from 3 or 5 μL bloodstains or 5 μL semen stains for analysis at multiple AMP-FLP loci using approximately 5 ng DNA per locus (based on the data presented in Table 8, at least five loci could be amplified). Sufficient DNA for analysis at a few or several loci should be available after extraction from smaller stains. There are additional advantages to this approach. It is technically similar to the method most widely used for RFLP analysis [*15*], making transition to AMP-FLP typing easier for a caseworking laboratory. Studies are underway to establish the effectiveness of the method chosen here for RFLP and DQα analysis.

In an effort to reduce the chances for sample-to-sample contamination, a modification was made in the organic extraction protocol described by Budowle et al. [*15*]. Instead of placing the stain cutting in a punctured microcentrifuge tube lid to recover the digestion buffer, a capped tube with an inserted basket (for example, Spin-X tubes, Costar Corporation, Cambridge, MA) is used for this step. In this way, all tubes and their contents can remain capped for all steps of the procedure. The time necessary for the concentration and dialysis can be reduced by the use of the recently available Microcon 100® tubes in place of Centricon 100® tubes (Amicon, Inc., Danvers, CT). These devices can be accommodated in a microcentrifuge and centrifugation times are less than those required for Centricon 100® tubes. Additionally, use of a microcentrifuge may allow analysis of more samples at one time. The volumes used for the digestion, concentration, and dialysis steps in this study are also accommodated by Microcon 100® devices. The extraction method chosen here is also effective for the differential extraction of semen-containing evidentiary material and should enable users of AMP-FLP analysis to obtain typing results from blood and semen stains deposited on a range of substrates.

References

[*1*] Comey, C. T. and Budowle, B., "Validation Studies on the Analysis of the HLA-DQα Locus Using the Polymerase Chain Reaction," *Journal of Forensic Sciences,* Vol. 36, No. 6, Nov. 1991, pp. 1633–1648.

[*2*] Higuchi, R., "Simple and Rapid Preparation of Samples for PCR," in *PCR Technology: Principles and Applications,* H. E. Erlich, Ed., Stockton Press, New York, 1989, pp. 31–38.

[3] Singer-Sam, J., Tanguay, R. L., Riggs, A., "Use of Chelex to Improve the PCR Signal from a Small Number of Cells," *Amplifications*, Vol. 3, 1989, p. 11.

[4] Jung, J. M., Comey, C. T., Baer, D. B., and Budowle, B., "Extraction Strategy for Obtaining DNA from Bloodstains for PCR Amplification and Typing of the HLA-DQα Gene," *International Journal of Legal Medicine*, Vol. 104, 1991, pp. 145–148.

[5] Walsh, P. S., Metzger, D. A., and Higuchi, R., "Chelex® 100 as a Medium for Simple Extraction of DNA for PCR-based Typing from Forensic Material," *BioTechniques*, Vol. 10, 1991, pp. 506–513.

[6] Hochmeister, M. N., Budowle, B., Borer, U. V., Eggmann, U., Comey, C. T., and Dirnhofer, R., "Typing of DNA Extracted from Compact Bone from Human Remains," *Journal of Forensic Sciences*, Vol. 36, No. 6, Nov. 1991, pp. 1649–1661.

[7] Hochmeister, M. N., Budowle, B., Jung, J., Borer, U. V., Comey, C. T., and Dirnhofer, R., "PCR-Based Typing of DNA Extracted from Cigarette Butts," *International Journal of Legal Medicine*, Vol. 104, 1991, pp. 229–233.

[8] Sajantila, A., Strom, M., Budowle, B., Karhunen, P. J., and Peltonen, L., "The Polymerase Chain Reaction and Post-mortem Forensic Identity Testing: Application of Amplified D1S80 and HLA-DQα Loci to the Identification of Fire Victims," *Forensic Science International*, Vol. 51, 1991, pp. 23–34.

[9] Rand, S., Puers, C., Skowasch, K., Wiegand, P., Budowle, B., and Brinkmann, B., "Population Genetics and Forensic Efficiency Data of Four AMPFLPs," *International Journal of Legal Medicine*, Vol. 104, 1992, pp. 329–333.

[10] Sajantila, A., Budowle, B., Strom, M., Johnsson, V., Lukka, M., Peltonen, L., and Ehnholm, C., "PCR Amplification of Alleles at the D1S80 Locus: Comparison of a Finnish and a North American Caucasian Population Sample, and Forensic Casework Evaluation," *American Journal of Human Genetics*, Vol. 50, 1992, pp. 816–825.

[11] Kloosterman, A. D., Budowle, B., and Daselaar, P., "PCR-Amplification and Detection of the Human D1S80 Locus: Amplification Conditions, Population Genetics, and Application in Forensic Analysis," *International Journal of Legal Medicine*, Vol. 105, 1993, pp. 257–264.

[12] Comey, C. T., Budowle, B., Adams, D. E., Baumstark, A. L., Lindsey, J. A., and Presley, L. A., "PCR Amplification and Typing of the HLA DQα Gene in Forensic Samples," *Journal of Forensic Sciences*, Vol. 38, 1993, pp. 239–249.

[13] Vogelstein, B. and Gillespie, D., "Preparative and Analytical Purification of DNA from Agarose," *Proceedings of the National Academy of Sciences USA*, Vol. 76, 1979, pp. 615–619.

[14] Harding, J. D., Gebeyehu, G., Bebee, R., Simms, D., and Klevan, L., "Rapid Isolation of DNA from Complex Biological Samples Using a Novel Capture Reagent—Methidium-Spermine-Sepharose," *Nucleic Acids Research*, Vol. 17, 1989, pp. 6947–6958.

[15] Budowle, B., Waye, J. S., Shutler, G. G., and Baechtel, F. S., "Hae III—A Suitable Restriction Endonuclease for Restriction Fragment Length Polymorphism Analysis of Biological Evidence Samples," *Journal of Forensic Sciences*, Vol. 35, 1990, pp. 530–536.

[16] Waye, J. S., Presley, L. A., Budowle, B., Shutler, G. G., and Fourney, R. M., "A Simple and Sensitive Method for Quantifying Human Genomic DNA in Forensic Specimen Extracts," *BioTechniques*, Vol. 7, 1989, pp. 852–855.

[17] Pääbo, S., Gifford, J. A., and Wilson, A. C., "Mitochondrial DNA Sequences from a 7000-year Old Brain," *Nucleic Acids Research*, Vol. 16, 1988, pp. 9775–9787.

[18] "Procedures for the Detection of Restriction Fragment Length Polymorphisms in Human DNA," *Protocol of the DNA Analysis Unit*, FBI Laboratory, Washington, DC, 1990.

[19] Kasai, K., Nakamura, Y., and White, R., "Amplification of a Variable Number of Tandem Repeats (VNTR) Locus (pMCT118) by the Polymerase Chain Reaction (PCR) and Its Application to Forensic Science," *Journal of Forensic Sciences*, Vol. 35, 1990, pp. 1196–1200.

[20] Baechtel, F. S., Smerick, J. B., Presley, K. W., and Budowle, B., "Multigenerational Amplification of a Reference Ladder for Alleles at Locus D1S80," *Journal of Forensic Sciences*, Vol. 38, 1993, pp. 1176–1182.

[21] Budowle, B., Chakraborty, R., Giusti, A. M., Eisenberg, A. J., and Allen, R. C., "Analysis of the VNTR Locus D1S80 by the PCR Followed by High-Resolution PAGE," *American Journal of Human Genetics*, Vol. 48, 1991, pp. 137–144.

[22] Edwards, A., Civitello, A., Hammond, H. A., and Caskey, C. T., "DNA Typing and Genetic Mapping with Trimeric and Tetrameric Tandem Repeats," *American Journal of Human Genetics*, Vol. 49, 1991, pp. 746–756.

Address requests for reprints or additional information to
Catherine Theisen Comey, Ph.D.
FSRTC
FBI Academy
Quantico, VA 22135