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TECHNICAL NOTE

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A Simplified Method for Mitochondrial DNA Extraction from Head Hair Shafts

ABSTRACT: DNA isolation from hair shafts can involve a number of steps, each of which adds time to the procedure and increases the risk of contamination. A simple alkaline digestion procedure that directly dissolves hairs was developed and compared with a widely used glass grinding/organic extraction method, using samples collected from 30 volunteers with varying population ancestries, hair colors, and hair treatments. A 203 bp mtDNA product could be amplified from 90% of samples extracted by alkaline digestion and 73% of hairs extracted by glass grinding. DNA obtained from alkaline digested hair generated equal or greater amplification success for virtually all criteria examined, and mtDNA sequences matched buccal control sequences in all cases. The two methods were similar in DNA yield (amplification success at template dilution) and quality of DNA obtained (amplicon length). Alkaline digestion of hair shafts required 6–7 h to complete, compared to 22–24 h for glass grinding, and proved a less laborious yet equally robust method for mtDNA extraction.

KEYWORDS: forensic science, DNA typing, mitochondrial DNA, alkaline digestion, sodium hydroxide, glass grinding

Human hair is one of the most common types of biological material associated with legal investigations, although its analysis and subsequent evidentiary value have been problematic when contrasted with other biological specimens. Microscopic comparison of morphological characteristics between questioned and exemplar hairs has been the primary method for association or exclusion of a suspected source since the early 20th century (1). However, recent post-conviction exonerations in cases where morphological hair comparison played a key role in findings of guilt (2) have raised questions about the reliability of this technique. In this regard, DNA analysis of 170 hairs previously examined microscopically by the FBI showed that 9 of 80 (11%) positive morphological associations were in error (3). Further, while 71 of 170 hairs were unsuitable for microscopic examination or gave inconclusive results, only 9 of the 170 hairs could not be successfully tested genetically. Finally, the ability to utilize population statistics to assign weight to associations of questioned hairs and suspects or victims make DNA-based analysis the preferred method for source identification of forensic hair samples.

The most precise method of DNA testing—STR analysis of nuclear DNA—is possible when the root portion of the hair and/or adhering tissue is present. On the other hand, shed (telogen) hairs, which are often associated with a crime, may harbor no nuclear material. While the nucleus degrades as the hair shaft hardens during keratinization, cellular mitochondria and mitochondrial DNA (mtDNA) remain relatively intact, making mtDNA analysis of hair shafts possible (4). Unfortunately, the protein-rich nature of hair samples requires extra steps to break down the shaft and release DNA, such as fragmentation using a glass grinder followed by organic extraction (5–7), thus exposing the specimen to increased risk of contamination.

A simplified method that expedites DNA extraction from hair shafts, reduces steps and contamination potential, and ideally meets or exceeds results of present methods, would be useful to the forensic community. Previous work with a variety of forensic samples (8), including keratinized material (9), suggested that alkaline digestion may be ideal for DNA extraction from hair shafts. Likewise, alkaline treatment of DNA has been broadly used in forensic laboratories, most often in blotting methods for DNA quantitation or RFLP testing. In the research presented here, a simple alkaline-based DNA extraction technique, developed and tested on human head hair shafts, successfully met the goals listed above.

Materials and Methods

Sample Collection and Preparation

Control buccal swabs, strands of shed head hair, and demographic information including sex, population ancestry, hair color, and hair treatments were collected anonymously from 30 adult volunteers, using a Michigan State University IRB-approved protocol. Buccal DNA was isolated through organic extraction (10). Reagent blanks were included with all isolations.

Hair samples were processed separately from buccal controls, in batches of ten. Reagent blanks were included with all extractions. Approximately ½ cm of each hair's root end was removed, and starting at the root end, the shaft was cut into 1 cm fragments, dividing it equally between two 1.5 mL microcentrifuge tubes in alternating fashion; this practice guarded against artifacts related to time since keratinization. Twenty-seven of thirty hair samples were extracted from 6–7 cm of hair shaft; the remaining three samples were extracted from 3, 4, and <1 cm of hair. Hairs were cleaned

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immediately prior to DNA extraction by successive 5 minute soaks in 1mL of UV-treated 5% Terg-a-zyme (Alconox Inc., White Plains NY), 95% ethanol, and sterile water, while being agitated on a platform rocker.

Standard DNA isolation from hair shafts followed a glass grinding/organic extraction protocol (7) with the following alterations: chloroform extraction replaced 1-butanol extraction; Centricon YM-50 columns (Millipore, Bedford MA) were used in place of YM-30 columns; and DNA was eluted in 25 μL of TE rather than 50 μL. Finally, two grinders in every batch of 10 extractions acted as full reagent blanks, while the other 8 were tested for contamination by thorough rinsing with 200 μL of TE, which was then precipitated and resuspended in 10 μL of TE for PCR amplification.

An extensive series of experiments was performed during development of the alkaline digestion procedure, and a summary protocol is presented at the end of this manuscript. Preliminary experiments included use of different NaOH concentrations and testing of neutralization methods and filtration techniques. While concentrated NaOH has been shown to have minimal impact on forensic DNA analysis (e.g., 9), any potential negative effect on the small amounts of DNA present in hairs was tested by placing 1 ng or 100 pg of purified male DNA (Promega, Madison WI) into 500 μL of 5N NaOH and incubating and processing it as described below. One twentieth of the recovered DNA was used for PCR amplification. The same experiment was run with the addition of bovine serum albumin (BSA) at a mass equal to a 1 cm fragment of hair (ca. 0.13 mg), mimicking the keratin that is hydrolyzed when hair is exposed to NaOH.

For DNA isolation trials detailed hereafter, experiments followed a standardized regimen. After cleaning, 500 μL of freshly prepared 5N NaOH was added to microcentrifuge tubes containing hair fragments. Tubes were agitated on a platform rocker and/or vortexed for 10 seconds hourly until hair fragments were no longer visible, generally 2–5 h. The solution was then neutralized using 400 μL of an equal mixture of concentrated HCl (11.6M) and 2M Tris (pH 8); a final pH of 6–8 was verified by spotting 1 μL of the solution onto pH paper. If an acidic pH was reached samples could be neutralized by addition of more Tris (see standard protocol), although this was not necessary with the hair samples detailed below. This solution was then filtered through a Microcon YM-30 column at 14,000 x g in two spins of 400–500 μL, followed by three washes of 300 μL TE, with a final retentate volume of 25 μL.

MtDNA Amplification and Sequencing

MtDNA was amplified using primers F15989, F16190, F15, F82, R285, and R484 (7), generating amplicon sizes of 203 bp (F82–R285), 469 bp (F15–R484), 664 bp (F16190–R285) 865 bp (F15989–R285), and 1064 bp (F15989–R484; for buccal DNAs). PCR products were observed by separating 5 μL of PCR product on a 2% agarose gel. The ability to successfully amplify mtDNA from hair shafts was examined using cycling conditions described previously (9, 10), with the 203 bp amplicon acting as the standard test. PCR reactions contained 1 μL of DNA in a 20 μL volume, as well as a 1:20 dilution of the same (equivalent to 0.05 μL of DNA in a 20 μL reaction). Positive and negative PCR controls were included with each run, and control (buccal) PCR reactions were always amplified separately from experimental samples. Finally, DNA sequencing of the 30 buccal samples and of all alkaline-digested DNAs that produced PCR product was undertaken to ensure that results from the alkaline technique matched the control buccal material. No attempt was made to sequence the standard glass grinding samples, as the source accuracy of this method had already been established in our and other laboratories. Sequencing products were separated on a CEQ 8000 Genetic Analyzer (Beckman-Coulter, Fullerton CA) utilizing the manufacturer's kits and protocols, and aligned using BioEdit Sequence Alignment Editor (11). Greater detail on the methods employed throughout these trials can be found in (10).

Ten hair shaft samples that generated 203 bp amplicons (using either 1 or 0.05 μL of DNA) for both the glass grinding and alkaline digestion techniques were tested to determine if larger fragments of DNA (469, 664, and 865 bp) could be amplified. Likewise, 1:100 dilutions of the same samples were amplified to see if the alkaline procedure had any obvious negative impact on DNA yields.

Statistical differences in DNA amplification success rates between DNA extraction methods, as well as among demographic and hair treatment variables, were determined using a two-tailed Z test (12). For objectivity, no efforts were made to "optimize" results in side-by-side comparisons of the techniques, or to obtain results from one method (such as diluting samples prior to PCR, incorporating extra cleaning steps, etc.) and not the other.

Results and Discussion

When small amounts (e.g., 100 pg) of purified, total genomic DNA were placed directly in 5N NaOH and processed as described, subsequent mtDNA PCR reactions (representing 5 pg of starting DNA) were often negative. This result was not encountered when hairs were processed, nor when larger amounts of starting DNA (e.g., 100 ng) were used. Because NaOH is not expected to directly damage DNA, it was surmised that the NaOH was having an effect on the polypropylene microcentrifuge tube itself, perhaps making it somewhat 'sticky' and thus binding small amounts of DNA. In contrast, NaOH treatment of hairs hydrolyzes the substantial protein (keratin) present, which could act as a blocking agent, and explain the incongruent results. In parallel experiments, small quantities (1 ng or 100 pg) of purified DNA were exposed to 5N NaOH alone or in the presence of BSA with a mass corresponding to 1 cm of hair. In all instances DNA processed along with hair-equivalent quantities of protein was readily recovered and amplified; there was no discernable damage to the DNA from the NaOH treatment.

A major advantage of the alkaline DNA extraction over the standard DNA preparation was the time required to complete each procedure. For a batch of 10 hair samples, the alkaline digestion method—from hair cleaning through DNA elution from the filtration column—took 6–7 h. The glass grinding/organic extraction method, with the standard overnight incubation, required 22–24 h, including far more "hands on" time. The alkaline protocol entails far fewer transfer steps, an entirely disposable set of supplies (both of which decrease the chance of contamination), and less expensive materials and chemicals, particularly if glass grinders are discarded after use. Finally, pigments, chiefly from dark hairs, were in some cases carried over using both techniques; however, the alkaline method appeared to denature or otherwise affect pigments such that they could be removed via brief centrifugation (i.e., pigments could be pelleted) while those from the grinding technique could not. This may have contributed to the higher PCR success rate obtained with the alkaline method (see below).

MtDNAs from all buccal samples and all alkaline-digested samples that generated PCR product were sequenced to ensure this experimental procedure was producing the correct mtDNA type. Data from the first 15 buccal samples sequenced through HV1 and HV2 indicated that individuals could be genetically differentiated by examination of bases 82–285 in HV2 of the mtDNA control region; thus, this region was used for all subsequent sequence confirmation. Of the 30 individuals examined, four were

found to share a mtDNA sequence with another person within the 203 bp region; however, in all alkaline-digested samples from which DNA sequences were generated, control and experimental mtDNAs clearly matched. In no case was sample contamination of the alkaline-digested material evident from the sequence data, although two hair samples showed evidence of possible heteroplasmy, one at a single nucleotide and the other at two positions.

MtDNA amplification success rates for all variables are presented in Table 1. Region 82–285 was successfully amplified (defined as the visible presence of a correctly sized PCR product on an agarose gel) from 90% (27/30) of hair samples extracted by alkaline digestion. The same region amplified in only 73% (22/30) of the samples processed using the glass grinding/organic extraction protocol, although these figures are not statistically different ($p = 0.0953$). More specifically, at the higher DNA concentration (1 μL) most PCR reactions were inhibited (85% for both techniques). With 1:20 dilution, 3 samples were still inhibited (based on the presence/lack of primer-dimer activity), and 5 were negative using the glass grinding/organic extraction while 1 sample was inhibited and 2 were negative from the alkaline digestion; 25 of these 27 positive samples produced clean DNA sequences on the first attempt. Testing of the shorter hairs was positive in all cases. Given the various criteria examined in Table 1, the alkaline extraction procedure produced equal or better results than the glass grinding/organic extraction method in all categories, including population ancestry (with the sole exception of one Asian sample), hair color, and hair treatments.

TABLE 1—*Comparison of amplification success.*

Sample Type	#	Success Rate (%) Glass Grinding	Success Rate (%) Alkaline Digestion
Total	30	73	90
Sex			
Male	8	100	100
Female	22	64	86
Population ancestry			
Caucasian	16	81	100
African American	6	83	100
Asian	6	67	50[a]
Hispanic	2	50	100
Hair color			
blond	1	100	100
lt brown	8	63	100
dk brown	12	83	100
dk brown/black	9	63	63[b]
Hair treatments			
blow dry daily/often	15	60	93
dyed within 1 year	12	58	92
perm/relaxer within 1 year	5	80	100
treated (total)	21	62	90
Short hairs	3	100	100
Amplicon length			
469bp	10	60	50
664bp[c]	10	70	70
865bp	10	70	40
1:100 template dilution	10	50	60

PCR results for sex, population ancestry, hair color, and hair treatments showing percentage (%) of samples that generated a 203 bp amplicon visible on an agarose gel using either of two template concentrations (straight extract or 1:20 dilution). Data are shown for total "treated" samples, as the sub-categories in this heading were not mutually exclusive. Amplicon length and template dilution data were generated from a subset of 10 samples that successfully produced the 203 bp product:

[a] Statistically different from Caucasian and African American samples extracted by alkaline digestion.

[b] Statistically different from less-pigmented hairs combined.

[c] The increased amplification success of amplicons larger than 469 bp likely results from differences in the robustness of the primers utilized.

When examining the samples processed using alkaline digestion in detail, the three that could not be amplified were from individuals of Asian descent (two of these were treated hairs, and two of the three did not amplify using the grinding method; this could possibly be due to primer site polymorphisms, though pigmentation seems more likely), resulting in a statistical difference between their amplification success and those for Caucasian or African American samples ($p = 0.0023$ and 0.0455, respectively). Likewise, amplification of alkaline-extracted dark brown/black hairs was also statistically different than less-pigmented hairs ($p = 0.0053$), again, due to the three failed Asian samples.

There were no other statistically significant differences between the techniques (at $p < 0.05$) when considering the other variables in Table I, though some factors stood out. The most important was that the alkaline extraction technique was more successful in generating amplifiable DNA from treated hairs—those classified as undergoing dying, permanent, or relaxer treatment within the last year, or experiencing daily or frequent blow drying—which tend to be more difficult to analyze (5). The PCR success rate of treated hair samples extracted by alkaline digestion was 90% (19/21), while the same samples were successful 62% of the time (13/21) using the glass grinding/organic extraction method. The latter value is in line with the 71% previously reported for treated hairs (6).

The length of DNA that could be successfully amplified using each technique, as well as DNA yield, were also assayed (Table I). No statistical difference between the two methods was found; each produced PCR products as large as 865 bp as well as 203 bp products at 100-fold DNA dilution.

Conclusions

The alkaline digestion mtDNA extraction method developed in this study represents a faster and less labor-intensive protocol that equals or exceeds the mtDNA amplification success rate of the standard glass grinding/organic extraction techniques currently used by many forensic laboratories. As important, the reduced number of transfer steps lessens the potential for sample contamination and likely reduces sample loss, an important consideration for trace samples such as single hairs. The method employs reagents, supplies, and equipment readily available in forensic laboratories, and its simplicity and effectiveness should provide incentive for its validation at facilities currently analyzing mtDNA. Likewise, its ease will help in implementation of mtDNA analyses in those laboratories that have yet to undertake forensic mtDNA testing of hairs.

Alkaline digestion protocol for mtDNA extraction from hair shafts:

1. Sterilize and/or UV-treat (~6 J/cm^2) reagents and disposables before use.
2. Cut hair into 1 cm fragments and place in a 1.5 mL tube. Clean hair using successive 1 mL rinses of 5% Terg-a-zyme, 95% EtOH, and sterile H_2O, shaking each for 5 min.
3. Digest hair through addition of 500 μL of fresh 5N NaOH (aged NaOH can make neutralization difficult). Ensure all hair fragments are submerged. Incubate hairs at room temperature on a shaker or rocker, vortexing hourly (optional), until hairs are no longer visible.
4. Neutralize the solution by adding 400 μL of a fresh 1:1 mixture of concentrated HCl (11.6M) and 2M Tris base (pH 8). Immediately test for a pH of 6–8 by spotting 1–2 μL on pH

paper. If the solution becomes acidic, add 2M Tris until a neutral pH is reached (generally 50 μL).

5. Filter and concentrate the solution on a Microcon YM-30 column (similar columns may work as well, although a high centrifuge speed is advantageous). Wash the sample 3x with 300 μL TE (or as appropriate for the chosen filtration device). Elute in 25 μL TE or desired volume.

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