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TECHNICAL NOTE

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An Improved Method for Post-PCR Purification for mtDNA Sequence Analysis*

ABSTRACT: Mitochondrial DNA (mtDNA) analysis of forensic samples typically is performed when the quantity and quality of DNA are insufficient for nuclear DNA analysis or when maternal relatives may be the only reference source. Many of the steps required in the analytical process are both lengthy and labor intensive. Therefore, improvements in the process that reduce labor without compromising the quality of the data are desirable. The current procedure requires purification of the amplicons by centrifugal filtration after PCR and prior to cycle sequencing. Because this method requires several manipulations to perform, alternate cleanup procedures were investigated. These include the use of 1) Qiagen QIAquick PCR Purification columns, 2) Concert Rapid PCR Purification columns, and 3) ExoSAP-IT™ reagent. When the yield of purified amplicons, quality of the sequence profile, and ease of assay were evaluated, the use of ExoSAP-IT™ reagent for post-amplification purification was chosen to replace the filtration method.

KEYWORDS: forensic science, mitochondrial DNA, polymerase chain reaction, DNA sequencing, ExoSAP-IT™

Mitochondrial DNA (mtDNA) typing is a valid and robust method for characterizing forensic biological specimens, particularly hair shafts, old bone, old teeth, and in situations where maternal relatives serve as the reference source (1–3). Removal of unincorporated primers and dNTPs from mtDNA amplicons is necessary to generate good quality sequence with low background noise. The current cleanup procedure utilizes Microcon-100 filters to purify the amplified DNA product by centrifugal filtration (4). Microcon-100 filters retain the amplified DNA product while allowing the unincorporated dNTPs and primers to flow through a membrane filter. Although 95% of the DNA potentially can be recovered, in practice there is often a greater than 5% loss of amplified product during purification with Microcon-100 filters. In addition, the procedure is laborious and time consuming.

In an effort to improve the efficacy of the post-amplification cleanup step, the Microcon washing protocol was compared with alternative mechanisms of amplicon purification. These include purification by the Qiagen QIAquick PCR Purification Kit, Concert Rapid PCR Purification columns, and ExoSAP-IT™ reagent. Both the Qiagen and Concert columns rely on the use of silica for the purification of DNA. These spin columns are somewhat less labor intensive than Microcon filters and report the potential for 95% DNA recovery. The ExoSAP-IT™ reagent employs Exonuclease I and Shrimp Alkaline Phosphatase to purify DNA after PCR amplification. The $3' \rightarrow 5'$ nuclease activity of Exonuclease I degrades single stranded primers while the Shrimp Alkaline Phosphatase catalyzes the removal of $5'$ phosphate groups from unincorporated dNTPs that remain after amplification. Since the ExoSAP-IT™ reagent is added directly to a completed PCR and no centrifugation step is required, the cleanup process is considerably less labor intensive than a spin column approach. Moreover, product loss is minimal because there is no centrifugation or transferring of reagents between tubes.

Materials and Methods

Samples included DNA purified from the HL60 cell line (American Type Culture Collection, Manassas, VA), hair shaft ($N = 8$), whole blood ($N = 6$), and a tooth that had been extracted and stored for at least three years ($N = 1$).

DNA extraction and amplification of hypervariable regions I and II (HVI and HVII) were performed as previously described (4,5). Multiple replicates (i.e., 10–20) from a single DNA source (genomic DNA from HL60 cell line) were amplified. The products from a single source were pooled to provide sufficient consistent material for comparison studies. The concentration of PCR product was determined by capillary electrophoresis (Beckman P/ACE system) as described previously (6).

Microcon-100 filters (Millipore Corporation, Bedford, MA) were used according to standard operating procedures (5). After pooling the amplification reactions, a 20 μL aliquot of the pooled amplification reaction and 400 μL of sterile water were placed into a filter cup and the tube was subjected to centrifugation at 3000 xg

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for 5 min. After discarding the eluate, 400 μL of sterile water were added to the filter cup and the apparatus was subjected to centrifugation at 3000 xg for 5 min. To recover the amplified DNA, 40 μL of sterile water were added to the filter cup, the filter cup was inverted into a clean collection tube, gently vortexed, and subjected to centrifugation at 10 000 xg for 3 min. The volume of eluate recovered was measured for each sample to account for any tube-to-tube variability in volume recovery.

Qiagen QIAquick PCR Purification columns (Qiagen, Valencia, CA) were used according to the manufacturer's recommended protocol. An aliquot of the pooled amplification reaction was mixed with 5 volumes of Buffer PB and applied to the column. The column was subjected to centrifugation at 12 000 xg for 45 s. After discarding the flow through fraction, 750 μL of Buffer PE were added to the column and the column was subjected to centrifugation at 12 000 xg for 45 s. The flow through fraction was discarded and the column was subjected to centrifugation at 12 000 xg for 1 min to ensure that the wash buffer had been removed from the silica matrix. Forty μL of elution buffer (Buffer EB) were added to the column. The column was incubated at room temperature for 2 min then transferred to a clean collection tube and subjected to centrifugation at 12 000 xg for 1 min. The recovered volume of eluate was measured for each sample.

The Concert Rapid PCR Purification columns (Life Technologies, Gaithersburg, MD) were used according to the manufacturer's recommended protocol. An aliquot of the pooled amplification reaction was mixed with 400 μL Binding solution (Buffer H1) and applied to the column. The column was subjected to centrifugation at 12 000 xg for 1 min. After discarding the flow through fraction, 700 μL of Wash buffer (Buffer H2) were added to the column and subjected to centrifugation at 12 000 xg for 1 min. The flow through fraction was discarded and the column was subjected to centrifugation at 12 000 xg for 1 min to ensure that the wash buffer had been removed from the silica matrix. Forty μL of TE buffer (preheated to 60°C) were added to the column and incubated at room temperature for 1 min prior to transfer of the unit to a clean collection tube. The tube was subjected to centrifugation at 12 000 xg for 2 min. The volume of recovered eluate was measured for each sample.

ExoSAP-IT™ reagent (USB Corporation, Cleveland, OH) was used according to the manufacturer's recommended protocol (2 μL reagent per 5 μL amplified DNA product) unless otherwise specified in the text. ExoSAP-IT™ reagent was added to an aliquot of the post-amplification reaction and incubated in a Perkin Elmer 9700 thermal cycler (PE Biosystems, Foster City, CA) for 15 min at 37°C followed by an additional incubation for 15 min at 80°C.

The post purification DNA concentration was determined by capillary electrophoresis (6,7). Based on recovery volume and initial input DNA, the percent recovery was determined.

DNA sequencing was performed as previously described followed by electrophoresis in an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA) (4,5).

Results and Discussion

The current method for post-amplification cleanup is by centrifugal filtration using Microcon-100 filtration devices. Initially, several variations in the use of the Microcon-100 protocol were explored. None of the permutations, including changes in the number of washing or elution steps, substantially altered the recovery of DNA. Therefore, three alternate approaches, the use of Qiagen QIAquick PCR Purification columns, Concert Rapid PCR Purification columns, and ExoSAP-IT™ reagent were evaluated in comparison to the standard operating protocol (SOP) to determine if the efficacy of the purification process could be improved. Three general criteria were considered to evaluate performance. The criteria were 1) recovery of purified post-amplification product; 2) quality of sequencing profiles from the purified PCR product; and 3) degree of labor required to carry out the procedure.

Recovery of Purified Post-Amplification Product

A specified amount of amplified HL60 DNA (i.e., 500–2000 ng in 20 μL) was purified by each of the cleanup approaches. The experiments were performed with specified starting amounts of DNA, the quantity and volume of recovered DNA was determined, and the percent recovery was calculated by dividing the recovered DNA by the starting DNA for each of the purification systems. Figure 1 shows the efficiency of each procedure for recovery of post amplification product. Following the SOP, the average amount of DNA recovered from the Microcon-100 filters was approximately 30% of the input DNA and varied notably across the samples. Higher yields were obtained with the Concert columns, although the range of recovery was greater and more variable than that obtained by the Microcon-100 technique. In contrast, the Qiagen QIAquick PCR Purification Kit provided an average recovery of 75% of the input DNA with substantially less variation in recovery observed among samples. The ExoSAP-IT™ reagent enabled an average 78% recovery of DNA and was similar in performance to that of the Qiagen QIAquick PCR Purification Kit. While some sample sizes are small, the data consistently show a trend of greater DNA recovery by ExoSAP-IT and QIAquick PCR Purification columns than by Microcon-100 filtration. Thus, in terms of recovery of purified DNA product, the Qiagen QIAquick PCR Purification Kit and ExoSAP-IT™ reagent are more effective than either the Microcon-100 filtration devices or Concert columns.

Quality of Sequencing Profiles

Following quantitation, the amplified DNA purified by each of the four methods was subjected to cycle sequencing and the se-

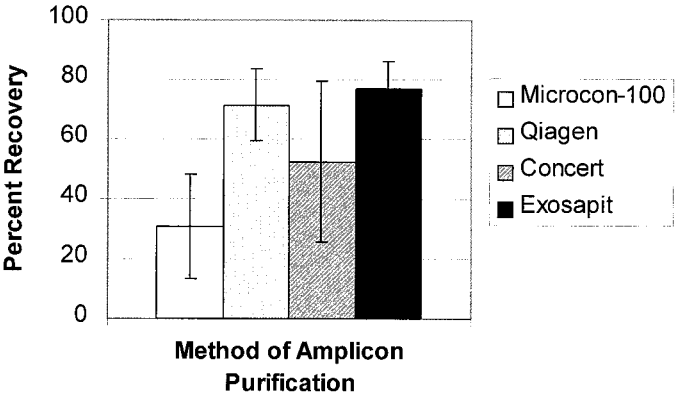

FIG. 1—*Comparison of DNA recovery. PCR product purification methods were compared to evaluate the amount of DNA recovered. The experiments were performed with specified starting amounts of DNA and percent recovery was determined by dividing the recovered DNA by the starting DNA. A predetermined amount of input DNA (500–2000 ng) was purified by each of the systems. The output DNA was quantified using the Beckman P/ACE system and the percent recovery was calculated for each of the purification systems. The percent recovery after purification by Microcon-100 (N = 10), Qiagen QIAquick PCR Purification columns (N = 8), Concert Rapid PCR Purification columns (N = 3), and ExoSAP-IT™ reagent (N = 8) is depicted on the y-axis. Error bars represent the 95% confidence interval.*

quencing products separated and detected using an ABI Prism 377 automated sequencer (PE Biosystems, Foster City, CA). Using the products that were generated for Fig. 1, the DNA sequence was determined for one sample from each method of purification (Fig. 2). Figure 2A shows a representative sequence from a sample that was not subjected to post-amplification purification. While the sequence generated from the unpurified amplicons has a notable level of background noise, all methods of purification tested provided similar quality sequence profiles (Fig. 2*B*). The amplitude of peaks was similar, background noise was negligible, and no differences in typing were observed when samples were purified by Concert Rapid PCR Purification column, ExoSAP-IT™, Microcon-100, or the Qiagen QIAquick PCR Purification column.

Labor Requirements

A comparison of the steps required to carry out each of the post-amplification procedures is shown in Table 1. Clearly, the ExoSAP-IT™ reagent is the easiest to perform. There is no centrifugation required and thus the procedure is more amenable to automation compared with the other purification methods. Although the total time required for the use of ExoSAP-IT™ (30 min) is slightly longer than the time required for purification of a single sample by the other procedures, the ExoSAP-IT™ process is completed without user intervention, i.e., unattended. Furthermore, when samples are processed by any of the three spin column methods, the amount of time required to process samples is often increased substantially with the addition of more samples because of the additional manipulations that are required. Since the only manipulation required for the purification of amplified DNA by the use of ExoSAP-IT™ is the addition of the reagent to the amplified DNA, additional samples do not significantly alter the processing time. At the time these studies were performed, a price comparison was conducted and it was found that while ExoSAP-IT™ reagent, Qiagen QiaQuick PCR Purification columns, and Concert Rapid PCR Purification columns are comparably priced, Microcon-100 filters are priced somewhat higher. Thus, there may be a cost benefit to the use of ExoSAP-IT™ for purification of PCR products when ExoSAP-IT™ is used as recommended in this manuscript.

Optimization and Validation of ExoSAP-IT™

Based on the above criteria, ExoSAP-IT™ was the method of choice for further evaluation. Additional experiments focused on 1) evaluating the effect of active enzymes in the ExoSAP-IT™ reagent on cycle sequencing; 2) determining the effect of varying the amount of ExoSAP-IT™ reagent used for amplicon purification; and 3) analyzing the ability of ExoSAP-IT™ to purify DNA amplicons derived from evidentiary-type samples.

The ExoSAP-IT™ system is based on enzymatic activity and subsequent enzymatic inactivation prior to cycle sequencing. Therefore, the effect on sequence quality was evaluated when the enzymes were not inactivated prior to sequencing. Amplicons were treated with ExoSAP-IT™ reagent at 37°C followed by incubation at either 80°C or 4°C. Following storage overnight at 4°C, the amplicons were quantified and subjected to cycle sequencing. Comparable quality sequence is generated whether or not the ExoSAP-IT™ reagent is heat inactivated (Fig. 3). Therefore, either active ExoSAP-IT™ reagent that is not heat inactivated does not impact significantly on the quality of the sequencing results, the ExoSAP-IT™ reagent is inactivated during the 95°C incubation step of cycle sequencing, or after a certain period of time the enzymatic activity of the ExoSAP-IT™ reagent is reduced. Regardless, it is prudent practice to heat inactivate enzymes.

If too little ExoSAP-IT™ reagent is used, insufficient purification of post amplification product may result. Additionally, too much ExoSAP-IT™ reagent might have an adverse effect on the sequencing reaction, possibly due to glycerol present in the ExoSAP-IT™ reagent storage buffer. Therefore, the amount of ExoSAP-IT™ reagent, ranging from one quarter to two times the manufacturer's recommended amount, was evaluated for its effect on sequence profile quality. Across the range of ExoSAP-IT™ used, no unincorporated primers were detected by capillary electrophoresis (data not shown). Figure 4 shows the sequence electropherograms from amplification reactions treated with varying amounts of ExoSAP-IT™ reagent. The sequence generated after using as little as one quarter (Fig. 4, panel labeled 0.25X) of the manufacturer's recommended amount of ExoSAP-IT™ reagent was comparable to the recommended amount of reagent. As much as a two-fold excess quantity of reagent had no negative impact on sequence quality (Fig. 4, panel labeled 2X). Therefore, one half of the manufacturer's prescribed amount of ExoSAP-IT™ reagent (1 μL reagent per 5 μL amplified DNA product) is sufficient for post-amplification mtDNA product purification.

Forensic samples, particularly hairs and teeth, often contain minute quantities of DNA. Thus, maximum recovery of amplified DNA product is desirable. The ability of ExoSAP-IT™ to purify DNA amplicons derived from evidentiary-type samples was evaluated using samples of whole blood (N = 6), hair (N = 8), and a tooth. After extraction and amplification of each of the samples, the amplicons were purified by using either Microcon-100 filters or ExoSAP-IT™ reagent (1 μL reagent per 5 μL amplified DNA product). Following the purification step, the amplified DNA was quantified and sequenced (Fig. 5 and data not shown). Both ExoSAP-IT™ treatment and Microcon-100 filtration provided a sufficient quantity of clean DNA to achieve good quality sequence from all evidentiary-type samples. For example, Fig. 5 shows electropherograms derived from an amplified hair extract. The use of ExoSAP-IT™ reagent for purification (Fig. 5, Panel A) resulted in sequence profiles of comparable quality to sequences generated from Microcon-100 purified samples (Fig. 5, Panel B). Furthermore, the use of the ExoSAP-IT™ treatment results in efficient post amplification DNA recovery of forensic-type samples. Table 2 shows the amount of PCR product amplified from the evidentiary-type samples. The yield of amplified product varies with the particular primer sets used, therefore this should not be compared across primer sets. Regardless, more DNA was recovered after purification with ExoSAP-IT™ reagent than by using a Microcon-100 filtration device for all the regions of amplification except one.

In conclusion, Microcon-100 filtration devices, Qiagen QIAquick PCR Purification columns, Concert Rapid PCR Purification columns, and ExoSAP-IT™ reagent were evaluated for purification of post-amplification PCR product. Based on several criteria, the ExoSAP-IT™ treatment provided a robust, effective, and prac-

TABLE 1—*Comparison of features of post-amplification purification assays.*

Method	No. Centrifugations	No. Manipulations	Total Time for One Sample
Microcon-100	3	9	20 min
Qiagen	4	10	7–10 min
Concert	4	10	8–10 min
ExoSAP-IT™	0	2	30 min

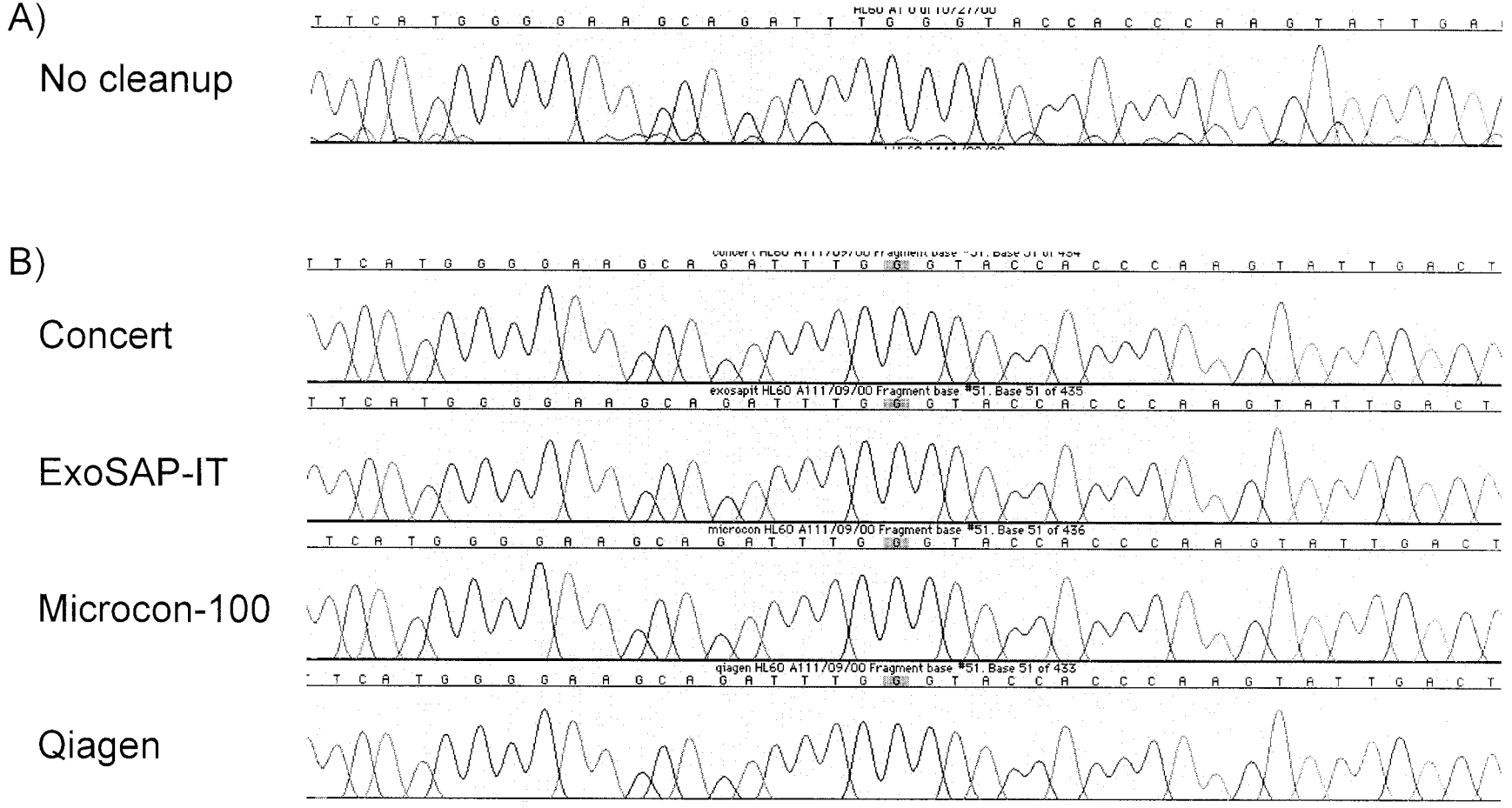

FIG. 2—*Comparison of sequence quality after use of each cleanup method. For each sample, 26 ng of DNA were sequenced. A portion of the sequence electropherogram from (A) one sample that was not subjected to purification or (B) a sample that was purified by the Concert Rapid PCR Purification column, ExoSAP-IT™ reagent, Microcon-100, or the Qiagen QIAquick PCR Purification column is shown. All sequences derived using the A1 primer.*

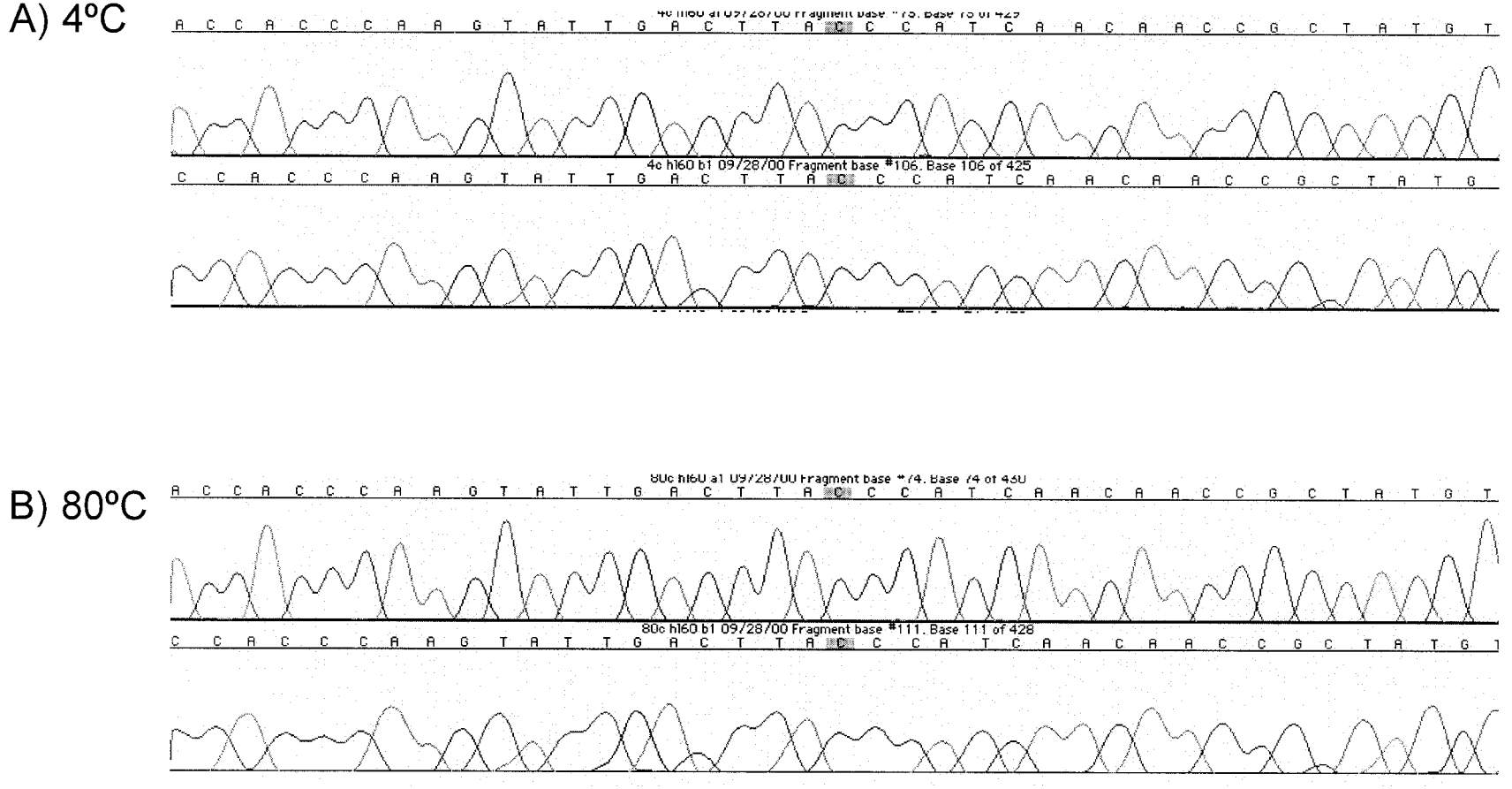

FIG. 3—*Effect of enzyme inactivation on sequence quality. (A) Sequence derived from DNA treated with ExoSAP-IT™ reagent and subsequently incubated at 4°C. The upper panel shows sequence derived using the forward sequencing primer (primer A1); the lower panel shows sequence from the reverse sequencing primer (primer B1). (B) Sequence derived from DNA treated with ExoSAP-IT™ reagent and subsequently incubated at 80°C. The upper panel shows sequence derived using the A1 sequencing primer; the lower panel shows sequence from the B1 sequencing primer.*

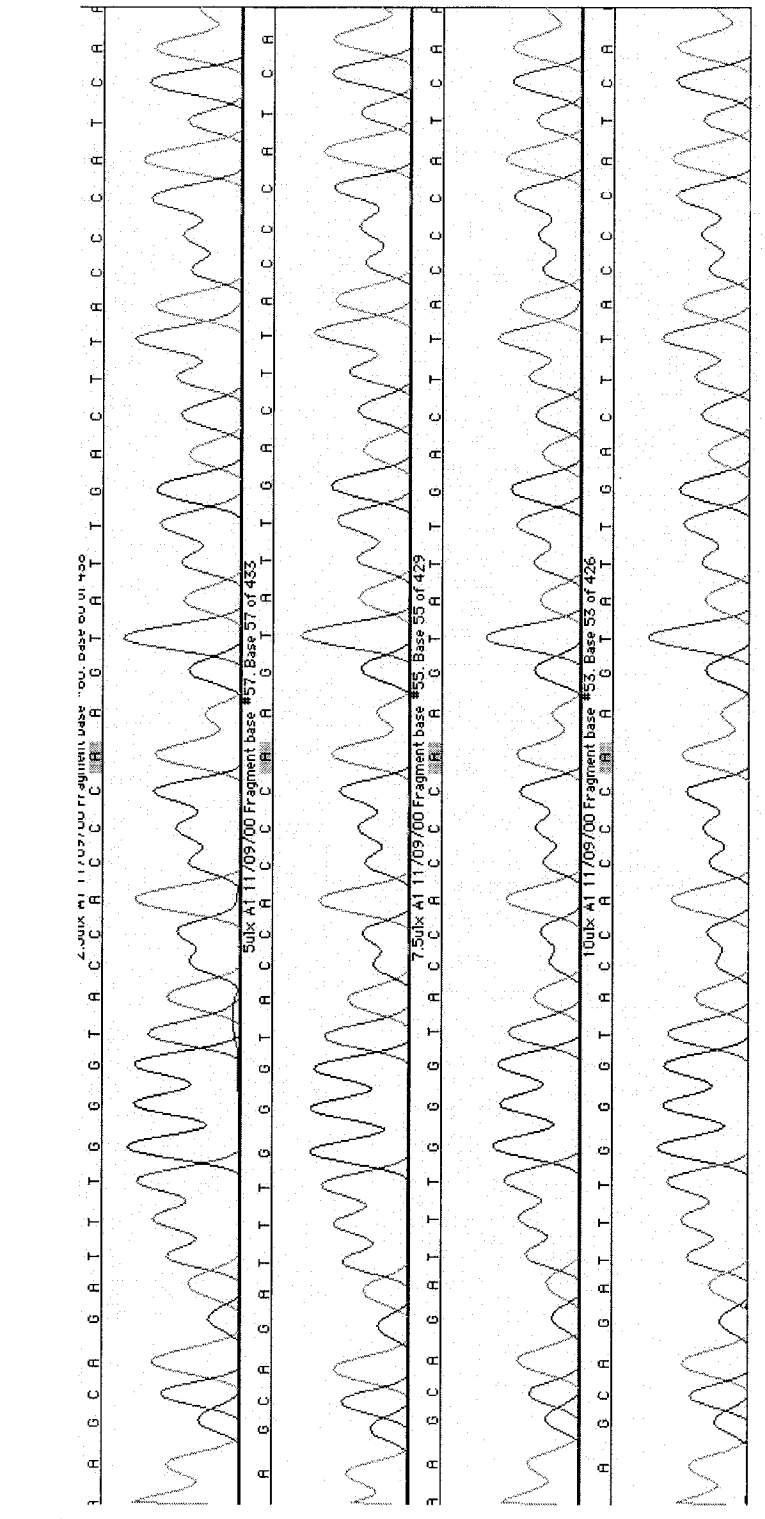

FIG. 4—*Effect of varying amounts of ExoSAP-IT™ reagent on sequence quality. Sequence electropherograms of amplified DNA treated with one quarter (0.25X), one half (0.5X), the recommended amount (1X), and two times (2X) the manufacturer's recommended amount of ExoSAP-IT™ reagent. All sequences derived using the A1 primer.*

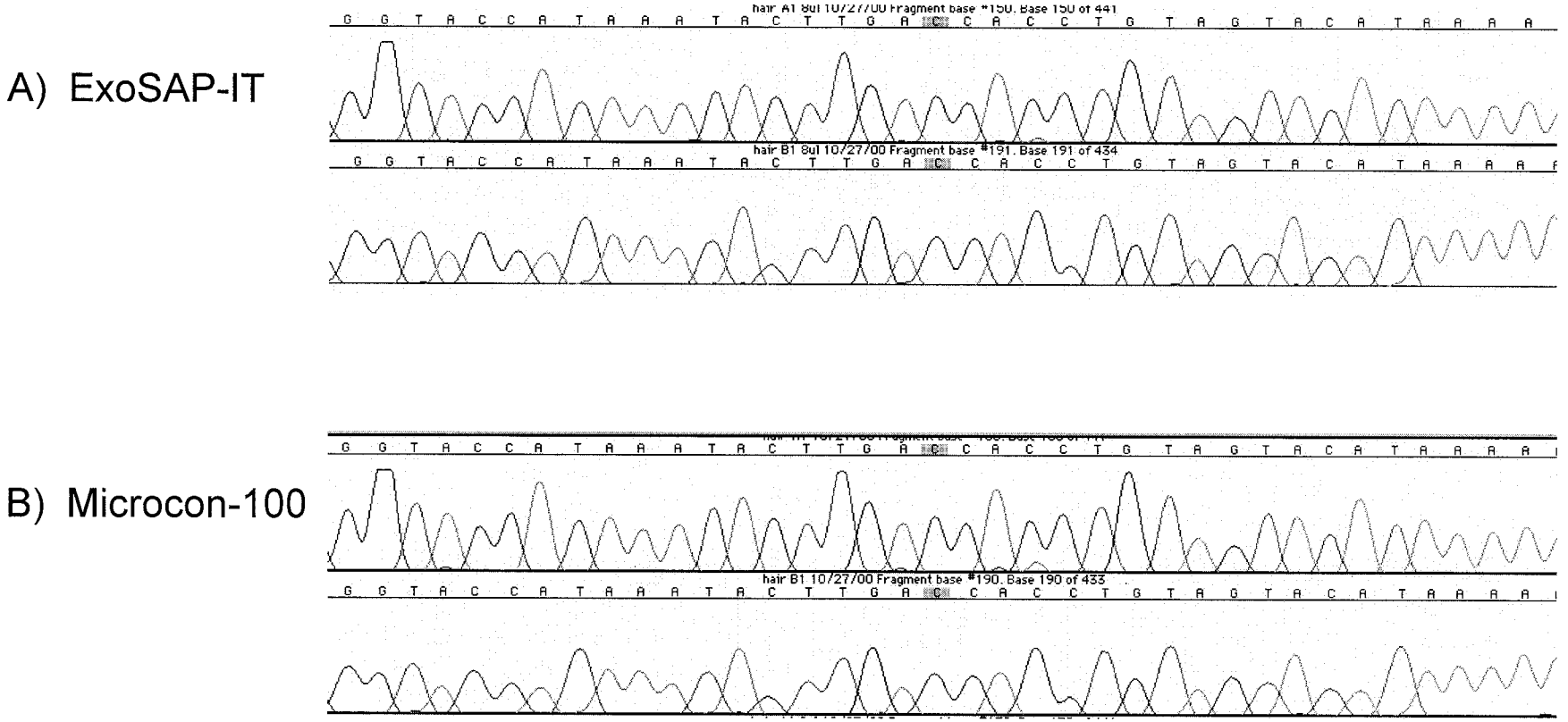

FIG. 5—*Comparison of post amplification purification methods on a hair extract. PCR product derived from a hair extract was purified using (A) ExoSAP-IT™ or (B) Microcon-100 filtration. The upper panel shows sequence derived using the A1 sequencing primer; the lower panel shows sequence from the B1 sequencing primer.*

TABLE 2—*Quantity of post-amplification PCR product recovered from forensic samples.*

	Tooth ($N = 1$)		Hair ($N = 4$)		Whole Blood ($N = 4$)	
Amplified Region*	Microcon	ExoSAP-IT	Microcon	ExoSAP-IT	Microcon	ExoSAP-IT
HVIA	3.2 ng/μL	4.3 ng/μL	nd†	nd	nd	nd
HVIB	5.0 ng/μL	6.4 ng/μL	3.7 ng/μL	15.3 ng/μL	nd	nd
HVIIA	2.1 ng/μL	4.3 ng/μL	3.1 ng/μL	9.4 ng/μL	nd	nd
HVIIB	2.9 ng/μL	2.6 ng/μL	2.1 ng/μL	2.4 ng/μL	nd	nd
HVI	nd	nd	nd	nd	8.7 ng/μL	31.8 ng/μL
HVII	nd	nd	nd	nd	2.7 ng/μL	39.8 ng/μL
Average Recovery‡	3.3 ± 1.2 ng/μL	4.4 ± 1.5 ng/μL	3.0 ± 0.9 ng/μL	9.0 ± 5.1 ng/μL	5.7 ± 4.2 ng/μL	35.8 ± 20.1 ng/μL

* For mtDNA analysis, regions HVI and HVII are amplified for whole blood and regions HVIA, HVIB, HVIIA, and HVIIB are amplified for hair and teeth as dictated by the FBI Standard Operating Protocol for mtDNA sequencing.

† nd: not determined.

‡ Average recovery ± the 95% confidence interval.

tical means of PCR product purification. The use of ExoSAP-IT™ for post-PCR DNA purification provides consistent, efficient recovery of amplified DNA. The amount of DNA recovered exceeds that obtained by use of a Microcon-100 filtration device. Moreover, the quality of subsequent sequencing profiles is comparable for all purification methods evaluated whether the amplified DNA is analyzed immediately or after longer term storage (up to 2 months). Also, ExoSAP-IT™ treatment is amenable for forensic-type samples, as demonstrated on evidentiary-type samples consisting of whole blood, hair, and a tooth.

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