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Improved MtDNA Sequence Analysis of Forensic Remains Using a "Mini-Primer Set" Amplification Strategy*

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ABSTRACT: Mitochondrial DNA (mtDNA) analysis of highly degraded skeletal remains is often used for forensic identification due largely to the high genome copy number per cell. Literature from the "ancient DNA" field has shown that highly degraded samples contain populations of intact DNA molecules that are severely restricted in size (1–4). Hand et al. have demonstrated the targeting and preferential amplification of authentic human DNA sequences with small amplicon products of 150 bp or less (1,2). Given this understanding of ancient DNA preservation and amplification, we report an improved approach to forensic mtDNA analysis of hypervariable regions 1 and 2 (HV1/HV2) in highly degraded specimens. This "mini-primer set" (MPS) amplification strategy consists of four overlapping products that span each of the HV regions and range from 126 to 170 bp, with an average size of 141 bp. For this study, 11 extracts representing a range of sample quality were prepared from nonprobative forensic specimens. We demonstrate a significant increase in MPS amplification success when compared to testing methods using ~250 bp amplicons. Further, 16 of 17 independent amplifications previously "unreported" due to mixed sequences provided potentially reportable sequence data from a single, authentic template with MPS testing.

KEYWORDS: forensic science, DNA typing, mitochondrial DNA, hypervariable regions 1 and 2, mini-primer sets, ancient DNA, polymerase chain reaction

Analysis of mitochondrial DNA sequence information has become a powerful tool in forensic identity cases (reviewed in 5). The high copy number of mtDNA per cell routinely permits successful testing in highly degraded samples when too little nuclear DNA persists to allow PCR amplification. For the same reason, mtDNA has been a predominant target in academic studies involving "ancient DNA" samples (reviewed in 6). The development of mtDNA as a locus for forensics followed the extensive knowledge gained from ancient DNA studies, and therefore forensic mtDNA testing of degraded samples may properly be considered an applied field of ancient DNA analysis. Considerations of contamination avoidance, criteria for determination of data authenticity, and issues concerning fragmented and damaged DNA templates are similar in the two endeavors and can be addressed accordingly (6).

In the seminal paper in the field of ancient DNA, Pääbo demonstrated that DNA from ancient sources is fragmented into short elements, such that PCR amplicons greater than ~150 bp in length could not be recovered from ancient tissue specimens (4). Numerous subsequent studies investigating ancient DNA preservation supported Pääbo's original findings of decreased fragment lengths in severely degraded samples (3,6–8). Two detailed analyses of human mtDNA sequences recovered from considerably aged mummified tissue document a population of authentic template molecules that is highly skewed toward smaller (~150 bp) fragments (1,2). In these particular cases, PCR amplicons of greater lengths were sometimes obtained, but the resulting sequence data exhibited the presence of low level modern contaminating human DNA rather than the authentic sequences obtained when shorter amplicons were targeted. The use of shorter amplicons effectively targeted the abundant size class of authentic template molecules, permitting these fragments to predominate the reaction.

Forensic mtDNA testing generally targets two hypervariable regions (HV1 and HV2) of the mtDNA control region. In highly degraded remains, amplification of entire HV regions (300–400 bp) is frequently unattainable due to the high degree of DNA fragmentation. Recognizing the potential for reduced fragment sizes in aged samples, forensic laboratories commonly target amplicons ~250 bp in length with a total of four overlapping primer sets for HV1 and HV2 (9). While these primer sets produce longer fragments than those typically used in ancient DNA analysis, robust testing can be achieved on a wide range of forensic samples with this approach (5). Shed hairs from crime scenes or even significantly aged dried bone samples often harbor enough intact mtDNA for reliable, successful testing using 250 bp products (9–11). However, our laboratory routinely processes bone specimens exposed for decades to acidic soils in conditions of high heat and humidity, and in the most difficult cases (~15%) testing is unsuccessful. This can be a result of failed amplification attempts due to the small

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number of intact template molecules and/or the presence of PCR inhibitors, or to the recovery of multiple sequences, presumably resulting from coamplification of low level contaminating DNA and authentic template. In an attempt to increase our success in these extreme cases, we investigated the utility of primer pairs that produce shorter fragments, similar to those employed by the ancient DNA field.

In this paper, we report the design, testing, and validation of an amplification strategy that uses a battery of eight "mini-primer sets" (MPSs). These overlapping primer sets completely span both HV1 and HV2, with amplicon sizes ranging from 126 to 170 bp. MPS testing of ten nonprobative forensic specimens produced greater amplification success of authentic sequences when compared to previous testing with ~250 bp amplicons, and greatly decreased the incidence of sequences that were unreportable due to mixture. The application of this MPS amplification strategy therefore expands the range of degraded samples available for forensic mtDNA analysis.

Materials and Methods

MPS Design and Amplification

For HV1 trials, primer pairs were selected from previously published ancient DNA studies (1) with slight modifications. HV2 primer pairs were designed using either Lasergene PrimerSelect Version 4.0 (DNASTAR, Inc., Madison, WI) or Oligo Version 5.0 (National Biosciences Inc., Plymouth, MN) software programs to determine suitable primer characteristics and "optimal" annealing temperatures. Primer pairs were then tested for appropriate sensitivity and specificity with high quality genomic DNA at levels of 100, 10, and 1 pg. Template DNA was extracted from whole blood by organic methods, quantitated by measuring the OD_{260}, and diluted to the appropriate working concentrations. PCR reactions were carried out in 50 μL volumes using 1X AmpliTaq polymerase buffer with 1.5 mM $MgCl_2$ final concentration (PE Applied Biosystems, Foster City, CA), 200 μM each dNTP (Boehringer Manheim, Indianapolis, IN), 0.025 mg/mL bovine serum albumen (Life Technologies, Gaithersburg, MD), 0.4 μM each forward and reverse amplification primer (synthesized in-house with ABI 392 DNA Synthesizer), and 5 units of AmpliTaqGold polymerase (PE Applied Biosystems). All reactions were performed using a 10 min activation step at 96.0°C with 20 s strand separation and primer annealing steps, followed by a 30 s extension step at 72.0°C for a total of 38 or 42 cycles. The minimum desired sensitivity was robust amplification at the 1 pg level, consistent with that of the standard primer sets in our laboratory. For each MPS, three annealing temperatures were compared, centered on the "optimal" temperature as predicted by primer design software or previously published annealing temperatures, and three degrees above and below this value. To ensure that the optimization results were not being influenced by contamination, two PCR negative controls were included with each group of amplifications. Additionally, a separate set of five PCR negatives using elevated polymerase levels (12.5 units) was performed to increase detection of contaminants present in the primer stocks, and demonstrated that the primers were free of systematic intrinsic contamination.

DNA Extraction

Following optimization and selection of suitable primer pairs, DNA was extracted from dried skeletal remains using standard AFDIL protocols. Initially, 15 bone samples were extracted for amplification with MPS1A to test the utility of this approach. These samples (S1–S15) were chosen to represent the most extreme cases of degradation seen in our laboratory. When it was determined that MPS amplification was suitable for the recovery of mtDNA fragments from degraded remains, we then tested the remaining MPSs on a second set of nonprobative specimens, chosen to represent a wide range of sample quality. Eight bone samples (01A–08A) were used for this testing with all MPS primer pairs except MPS1A. The initial weights of the bone specimens for extraction ranged from 1.2 to 7.8 g. The outer and inner surfaces were sanded thoroughly to remove spongy bone material, and the resulting fragments were measured at 0.4 to 4.4 g prior to pulverization. Samples 01A, 02A, and 03A were found to be suitable for two independent extractions (designated 01A1, 01A2, etc. . .) whereas samples 04A–08A were sufficient for only a single extraction. The fragments used for the second extraction ranged from 1.2 to 2.0 g. For each separate set of extractions, one "reagent blank" (a mock extraction without bone sample) was carried through all of the extraction steps simultaneously with the test specimens. Each bone fragment was pulverized and incubated overnight at 56°C in the presence of extraction buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 50 mM EDTA (pH 8.0), 0.5% SDS + 0.1 mL of 20 mg/mL proteinase K) and mixing. Phenol:chloroform:isoamyl alcohol (25:24:1) extraction was performed with an equal volume, and separation of aqueous and organic layers was achieved by centrifugation. The upper aqueous layer was recovered and the extraction repeated twice. An *n*-butanol extraction was performed, and the lower aqueous layer was recovered, added to a Centricon-100™ (Amicon® Inc., Beverly, MA) concentrator, and centrifuged. The filtrate was discarded, and two Tris-EDTA washes were performed prior to sample recovery.

Testing with Nonprobative Extracts

For validation of MPS1A, 15 samples (S1–S15) were amplified using the above conditions and similar amounts of extract (2.0 μL) and AmpliTaqGold polymerase (12.5 units) as were previously employed with ~250 bp product amplifications. A second and separate set of 11 nonprobative extracts (from eight bone samples, 01A–08A, three of which were extracted twice) was amplified with the remaining MPSs using the same conditions. Extract from sample 08A was tested only with MPS2B and MPS4A, and was used to replace testing on sample 04A, all of which was consumed prior to the complete set of MPS testing. For each group of samples, a positive control amplification with 100 pg of high quality genomic DNA of known mtDNA sequence was included along with two PCR negative control amplifications to test for contamination in the amplification reagents. To test for contamination in the extraction reagents, 2.0 μL of the extraction reagent blank was processed simultaneously with the test samples throughout all amplifications. In each case, 10% of the amplification reaction was analyzed by agarose gel electrophoresis stained with ethidium bromide to note DNA band intensity and resolution. Amplicon product from nonprobative specimens, along with the corresponding PCR negative controls and reagent blank controls that showed visible product, were then purified using Centricon-30™ concentrators (Amicon, Inc.), and the recovery was dried in a speed vacuum. Samples were resuspended in deionized water so that DNA concentrations were roughly normalized prior to sequencing.

Cycle Sequencing

Five to 20 ng of Centricon-purified product was used for cycle sequencing with BigDye Terminator chemistry (PE Applied

Biosystems). Cycle sequencing was done in 20.0 μL volumes with 8.0 μL of BigDye Terminator mix and 0.5 μM of either the forward or reverse primer as used for amplification. Cycle sequencing was performed as follows: 96.0°C for 15 s, 50.0°C for 5 s, 60.0°C for 4 min for 25 cycles. The 100 pg positive control products of known mtDNA sequence were carried throughout the analysis to verify confirmation of sequence data. One positive sequence control was performed for each group of samples with pGEM plasmid and M13 primer. Sequencing reactions were purified with Centriflex AGTC Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD), and the recovery was dried in a speed vacuum. Samples were resuspended in 4.0 μL of loading buffer (deionized formamide + 50 mg/mL blue dextran in 25 mM EDTA in a 5:1 ratio), and a standard 4.0% polyacrylamide gel (19:1) was run on the ABI 377 DNA Sequencer. Data collection and analysis were performed with Sequence Navigator software (Applied Biosystems).

Results

For the initial design of the MPS amplification strategy, desired primer selection characteristics included product overlaps of at least several bases, amplification with suitable sensitivity and specificity, and to the extent possible, avoidance of highly polymorphic sites within primer binding regions. Figure 1 and Table 1 outline the primer pairs chosen from sensitivity experiments, along with their sequences and suitable amplification conditions. For initial testing on nonprobative extracts, MPS1A (170 bp) was used only for amplification on 15 specimens chosen to represent the most difficult cases processed in our laboratory, 14 of which provided no reportable sequence data with standard (~250 bp) primer set testing. Following a single amplification attempt with MPS1A, 9 of the 15 samples provided positive amplification products and corresponding sequence data (Table 2*A*). Following this MPS1A testing, we then selected 11 different nonprobative bone extracts (01A1, 01A2, 02A1, 02A2, 03A1, 03A2, 04A-08A) for testing the remaining MPSs. This was done so that a significant amount of previously reported DNA sequence information could be replicated with the MPS testing method (due to the limited amount of available extract, MPS1A was validated separately on the set of 15 highly degraded samples mentioned above). Of the 11 extracts, seven represented "good" quality extracts that provided robust amplification after a single attempt with standard primer sets, and four extracts represented "moderate to poor" quality, which either required multiple amplifications to provide sufficient product for direct sequencing or were unsuccessful in all attempts. For comparison purposes, we used the same reaction component concentrations and template amounts as had been employed in previous testing. Although four of the samples tested required multiple amplifications with standard primer sets, only a single MPS amplification attempt was performed for each extract. This was due to both the lim-

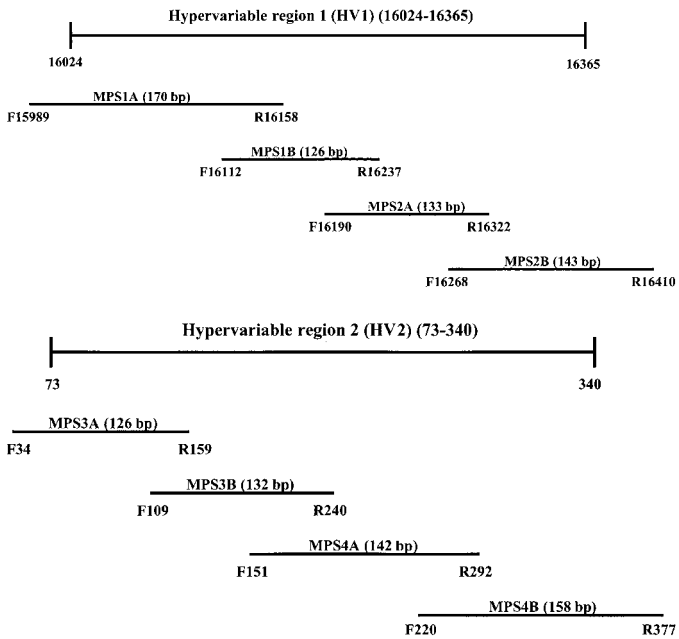

FIG. 1—*Layout of the mini-primer set amplification strategy for HV1 and HV2. Primer designations are labeled according to the 5′-end of each primer as it relates to the Anderson reference sequence (12).*

TABLE 1—*Mini-primer set names and designations.*

MPS	Ampicon Size (bp)		Primer Sequence	Range of DNA Sequence Info.	Annealing Temperature	Cycle Number
MPS1A	170	F15989	5'-CCC AAA GCT AAG ATT CTA AT-3'	16009-16138*	50°	38
		R16158	5'-TAC TAC AGG TGG TCA AGT AT-3'			
MPS1B	126	F16112	5'-CAC CAT GAA TAT TGT ACG GT-3'	16132-16217	50°	42
		R16237	5'-TGT GTG ATA GTT GAG GGT TG-3'			
MPS2A	133	F16190	5'-CCC CAT GCT TAC AAG CAA GT-3'	16210-16302	46°	38
		R16322	5'-TGG CTT TAT GTA CTA TGT AC-3'			
MPS2B	143	F16268	5'-CAC TAG GAT ACC AAC AAA CC-3'	16288–16390	48°	42
		R16410	5'-GAG GAT GGT GGT CAA GGG AC-3'			
MPS3A	126	F34	5'-GGG AGC TCT CCA TGC ATT TGG TA-3'	57–135	54°	38
		R159	5'-AAA TAA TAG GAT GAG GCA GGA ATC-3'			
MPS3B	132	F109	5'-GCA CCC TAT GTC GCA GTA TCT GTC-3'	133–219	46°	42
		R240	5'-TAT TAT TAT GTC CTA CAA GCA-3'			
MPS4A	142	F151	5'-CTA TTA TTT ATC GCA CCT-3'	169–273	46°	42
		R292	5'-ATT TTT TGT TAT GAT GTC T-3'			
MPS4B	158	F220	5'-TGC TTG TAG GAC ATA ATA AT-3'	240–357	47°	42
		R377	5'-GTG TTA GGG TTC TTT GTT TT-3'			

* NOTE: the control region begins at position 16024. List of primer sequences, suitable amplification conditions, and range of sequence information obtained from each amplicon. The parameters listed were found to be sensitive to 1 pg of high quality genomic DNA.

TABLE 2A—*Comparison of amplification success of most highly degraded remains.*

	Primer Set	Mini-Primer Set
Sample	PS1	MPS1A
S1	−	−
S2	+	−
S3	−	−
S4	−	+
S5	−	+
S6	−	+
S7	−	+
S8	−	−
S9	−	+
S10	−	+
S11	−	−
S12	−	−
S13	−	+
S14	−	+
S15	−	+

NOTE: Comparison of results obtained with standard primer set 1 (PS1) and mini-primer set 1A (MPS1A) testing. "+" indicates positive amplification and generation of reportable sequence data, and "−" indicates unsuccessful amplification. The PS1 and MPS1A amplicon sizes are 280 bp and 170 bp, respectively.

ited amount of the extracts and also the success obtained with the MPS amplifications. For three nonprobative bone samples, two independent extractions (01A1/01A2, 02A1/02A2, and 03A1/03A2) were performed to test reproducibility of this method as there was sufficient starting material for reextraction. The remaining samples (04A1-07A1) were sufficient for only a single extraction (sample extract 04A1 was consumed during testing and 08A1 was used as a replacement in two instances: MPS2B and MPS4A). Figure 2 shows the enhanced MPS amplification success and the general decrease in nonspecific artifacts and amplicon smearing that occurred with the standard ~250 bp primer sets. This figure is representative of numerous results obtained with various MPSs.

Sequence data generated from MPS amplifications were consistent with those obtained from standard primer set testing in all cases that provided reportable data. Thus, the utility of MPS amplification on degraded DNA extracts was demonstrated by the replication of 1704 bp of confirmed DNA sequence. The MPS amplifications also yielded a large amount of additional potentially reportable results in cases where previous testing gave no positive amplification or consistently yielded mixed sequences (Table 2*B*). This corresponds to an additional 1340 bp of confirmed sequence information obtained with the MPS approach.

Figure 3 demonstrates the ability of MPS amplification to target authentic template molecules to provide a single, clean sequence from extracts previously determined to contain mixed sequences following standard primer set testing. In this example, sequence data generated from two independent standard primer set amplifications from the same sample extract show a mixture in base calling at position 16249 (Anderson reference sequence) (12). In panels A and B, cytosine (C) prevails as the major base with a mixture of thymine (T), and in panels C and D, "T" is the predominant base with a mixture of "C." The MPS amplification shows an unambiguous "T" at 16249 with no underlying peaks. Similar results were seen throughout testing with various MPSs. In this study, there were 12 instances of mixed sequences following amplification with standard primer sets. This corresponds to 24 MPS amplifications as each standard primer set amplification spans a region roughly twice the size of each MPS. Of these 24 amplifications, 16 provided potentially reportable sequence data free from mixtures, but one also showed the presence of mixed sequences (02A) found with primer set testing. For the remaining seven cases, four samples produced no amplification product after a single attempt, and three samples were untested because no extract remained.

Throughout MPS testing contamination levels were closely monitored to ensure data authenticity. For the initial sensitivity testing on high quality template DNA (5 units of AmpliTaqGold polymerase per reaction), 6 of 58 (~10%) PCR negative controls showed visible product by ethidium bromide staining of agarose gels. Since these samples were used only to test sensitivity and did not relate to the nonprobative bone testing, these PCR negative controls were not sequenced. For the contamination portion of the study (5 PCR negatives for each amplification set), which used elevated AmpliTaqGold levels (12.5 units per reaction), 5 of 70 (~7%) PCR negatives showed positive amplification. For the nonprobative bone specimens, 1 of 18 (~5.5%) PCR negative control amplifications showed visible product whereas 5 of 26 (~19%) reagent blank controls (reagents used for DNA extraction) yielded positive results. For the nonprobative testing portion of the study, attempts were made to sequence all PCR negatives and reagent blank controls that showed visible product by stained agarose gels. These products were often too faint to provide interpretable sequence data, and those that did provide data were determined to contain sporadic low level contaminants unrelated to the other sample extracts or to the DNA sequence of the operator performing the extraction and amplification setup.

Discussion

The data presented for the MPS amplification strategy show the benefit of targeting smaller amplicons in highly degraded forensic specimens. MPS amplifications of 126 to 170 bp products exhibit an increase in product yield and specificity on a range of highly degraded samples. Successful targeting of shorter molecules that are present in greater abundance enables the increased production of authentic DNA sequences, and a corresponding decrease in the amount of nonspecific artifact bands. Further, replication of a substantial amount of DNA sequence information, obtained previously with standard primer sets, demonstrates the reliability of MPS testing. The ability of MPS amplification to provide additional sequence information in cases that failed to provide reportable data with standard primer sets further shows the benefit of this strategy with extremely degraded samples.

The production of mixed sequences due to low level modern DNA contamination occasionally appears in ancient DNA remains analysis despite exhaustive efforts to minimize its effects. These mixtures presumably result from the coamplification of authentic template and a low level human mtDNA contaminant present either in the reagents or intrinsic to the samples. Our study demonstrates that with MPS testing the incidence of this type of mixture is decreased, as extracts that gave uninterpretable sequences due to mixture were analyzed successfully upon reamplification with MPSs. Twelve instances showed the presence of mixed sequences when tested with standard primer sets. For the corresponding MPS amplification products that provided sequence information, all but one instance provided data from a single template. This corresponds to 16 of 17 cases where MPS amplification provided clean, poten-

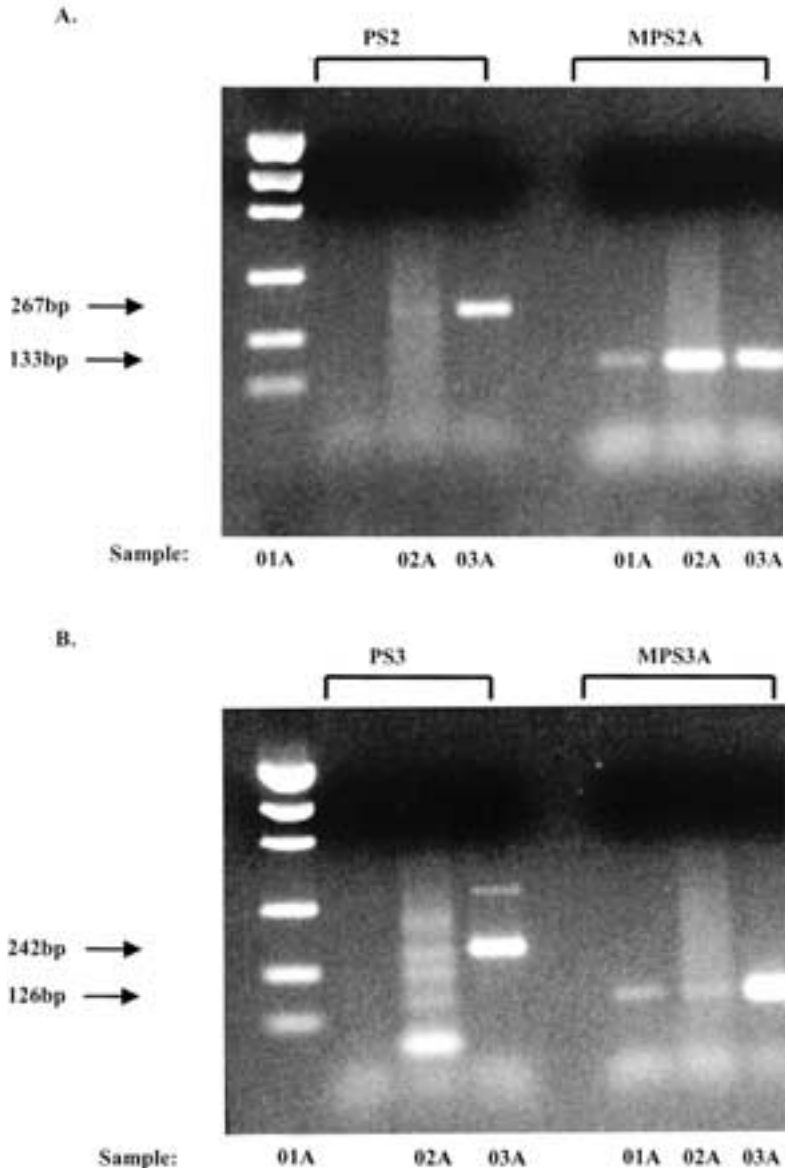

FIG. 2—*Agarose gel picture showing side by side amplification of sample extracts with standard primer sets and corresponding mini-primer sets.* (a) *Standard primer set 2 (PS2) and mini-primer set 2B (MPS2B) and* (b) *Standard primer set 3 (PS3) and mini-primer set 3A (MPS3A). Results indicate increases in band intensity and resolution as well as a corresponding decrease in nonspecific artifacts and amplicon smearing with MPS amplification. Data are representative of numerous trials throughout testing.*

tially reportable sequence data when previous primer set data was inconclusive due to mixture. This observation can be attributed to greater accessibility to the highly abundant population of intact DNA molecules present in degraded remains. Successful targeting of this population enables greater production of authentic human DNA relative to less degraded modern contaminants and therefore an increased contribution to the total number of DNA amplicons.

Large amounts of bacterial and/or fungal DNA relative to authentic DNA sequences challenge PCR specificity greatly and occasionally result in the production of either minor or major nonspecific artifacts and smearing of amplicons. Post-PCR purification methods based on molecular weight cutoffs often retain these undesired DNA products and sequence quality is greatly diminished. One significant drawback of the MPS strategy is the susceptibility to high sequence background levels that can result from smearing or faint nonspecific artifact bands present in PCR products. In our experience, the use of internal sequencing primers is an effective means to decrease background levels with lesser quality templates. However, due to the small amplicon size of the short primer sets, internal sequencing primers decrease the total available sequence information that can be obtained with each run. This strategy then lessens the benefit of MPS amplification and therefore is not a feasible option for complete sequence reporting. Therefore, alternative purification methods are desirable to lessen overall background effects and prevent sequence read "late starts" to provide confirmation between overlaps. Preliminary data in our laboratory demonstrate the ability of ion-pairing reverse-phase high performance liquid chromatography (IPRP-HPLC) to preparatively isolate desired products from nonspecific amplicons to provide clean, high quality sequence data (data not shown).

TABLE 2B—*Comparison of reported primer set and mini-primer set sequence information.*

	Primer Set				Mini-Primer Set							Reportable Sequence (%)	
Sample	PS1	PS2	PS3	PS4	MPS1B	MPS2A	MPS2B	MPS3A	MPS3B	MPS4A	MPS4B	PS	MPS
01A1/01A2	+	+	+	+	+	+	+	+	+	+	+	99.8	99.4
02A1/02A2	+	−	+	+	+	MIX	+	+	+	+	+	55.7	84.7
03A1/03A2	+	+	+	+	+	+	+	+	+	+	+	99.5	96.4
04A1	−	−	−	−	−	+	NA	+	−	NA	−	0.0	31.9
05A1	+	−	−	+	+	+	+	−	+	+	+	49.9	86.5
06A1	−	−	−	−	+	+	+	+	+	+	+	0.0	96.4
07A1	−	−	−	−	+	−	+	−	−	−	−	0.0	30.0
08A1	+	+	+	+	NA	NA	+	NA	NA	+	NA	100	100

NOTE: Comparison of reported primer set and potentially reportable mini-primer set data on a range of extracts from degraded bone specimens (MPS1A was tested on a separate set of extracts, see Table 2A). Specimens 01A, 02A, and 03A each had two extracts, for which results are pooled in this table (as would be done in case work replicate testing). Testing was performed with each MPS pair on all the duplicate extracts, with matching results for all but MPS2A, which gave mixed results with extract 02A1. All sequence information was confirmed by unambiguous base calling on both strands at each position; % reportable sequence indicates the number of confirmed bases obtained divided by the total number of bases in the targeted regions (x100). "+" indicates positive amplification and interpretable results, "−" indicates no amplification or unreportable mixtures, and "NA" denotes samples not tested (sample 04A1 was consumed during testing and therefore 08A1 was substituted for testing with MPS2B and MPS4A). For primer set testing, the following samples gave no amplification product despite repeated attempts: 07A1 with PS1, PS2, PS3, and PS4. For all other instances of nonreporting with standard primer sets, a faint band was obtained after multiple amplification attempts, but proved to yield mixed sequences. The sole instance of mixed sequences obtained with MPS testing is indicated as 'MIX.' Standard primer set data were obtained with one (01A1/A2, 02A1/A2, 03A1/A2) or multiple (04A-07A) amplification attempts whereas MPS data were obtained with a single amplfication attempt.

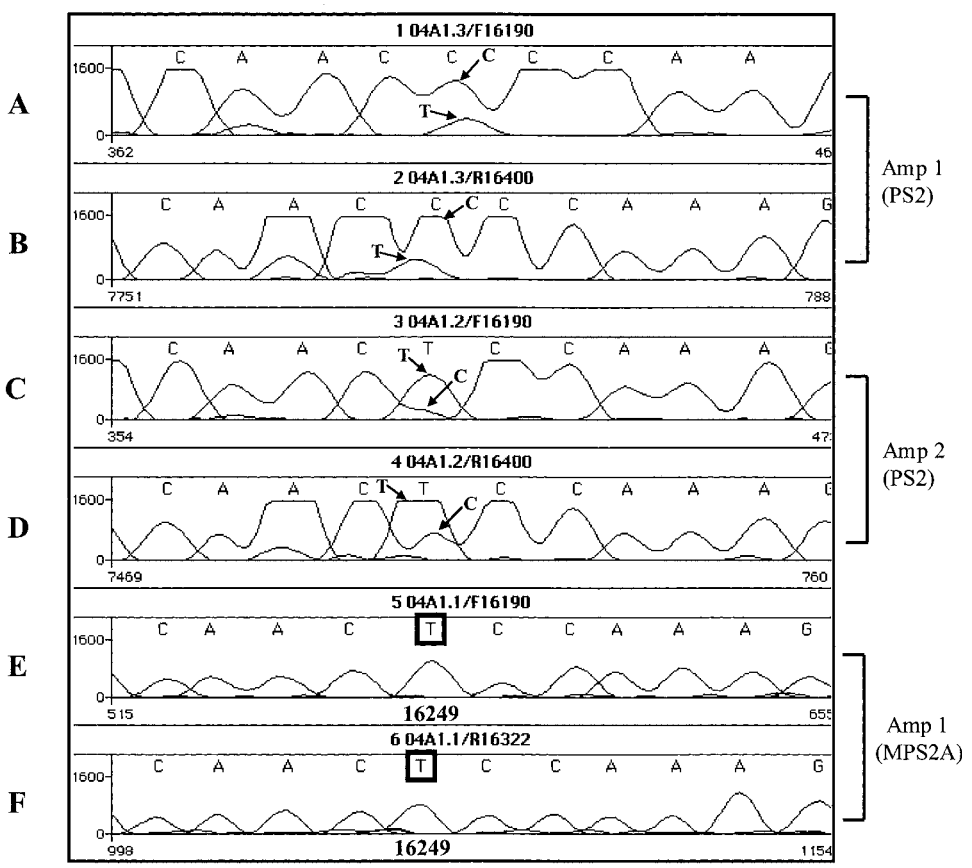

FIG. 3—*Sequence electropherograms demonstrating the decreased effects due to low level contamination with MPS testing. Panels A–D show a discrepancy in base calling at position 16249 with standard primer set testing. Panels A and B show sequence data from the first amplification with PS1 (280 bp amplicon), demonstrating a C/T mixture at this position with "C" as the predominant base. Panels C and D show data from the second amplification that suggest "T" as the predominant base with underlying "C." Panels E and F show sequence data generated from MPS2A amplification exhibiting a clean "T" at position 16249 with no evidence of mixture. This observation is representative of numerous results seen throughout testing.*

Primer-template mismatches tend to cause a decrease in amplification, and since many primer binding regions are used for MPS amplification, widespread polymorphic locations create the potential for mispairing. One candidate MPS required 45 cycles to obtain sensitivity at 1 pg starting template, which was apparently due to the presence of primer-template mismatches at positions 182, 185, 189, and 195 relative to the Anderson sequence. When this MPS was applied to a template that provided full complementarity, sensitivity was restored at 38 cycles. From this observation, we examined the frequency of potential primer-template mismatches with the MPS approach. In a database of roughly 1300 individuals representing various racial origins and geographic locations, the frequency of two or more base mismatches underlying a single primer binding region is not substantial, with the greatest occurrence of $<12\%$ (AFDIL, unpublished data). The positive control template DNA used in the study contained this two base mismatch ($C \rightarrow T$ transition at positions 16270 and 16278) in the binding region of forward primer F16268 (MPS2B). Despite the presence of these mismatches, amplification of 1 pg template was successful at 42 total cycles (data not shown). From this assessment, we determined that the overall success of the MPS amplification strategy will be largely unaffected by primer-template mismatches. Keeping this in mind, however, forensic mtDNA laboratories must maintain the flexibility to employ modified or alternative testing methods when desired results are not readily attainable. Design of degenerate primers at common polymorphic locations or alternate primer sets could be used to alleviate the potential loss of sensitivity due to primer-template mispairing. Further, whenever unexpected amplification failures occur with particular MPSs, the sequences of the primer binding sites should be examined in the data obtained from adjacent MPS amplicons. If polymorphisms in the binding sites are preventing amplification, modifications in the PCR conditions may overcome the amplification failure.

The ability to extract sufficient amounts of DNA, while minimizing the recovery of PCR inhibitors, is an important factor in the analysis of highly degraded specimens. For this study, bone sample extracts were prepared using Centricon-100 devices. However, since the MPS strategy targets molecules that fall near the molecular weight limit of the Centricon-100 filter, a loss of desired target molecules can occur. Smaller cutoffs offered by Centricon-30 preparations have the ability to retain a greater number of short DNA elements, thereby increasing the potential for successful MPS amplification. We performed a small set of preliminary experiments with samples extracted by organic methods, split into equal fractions, and subjected to either Centricon-30 or Centricon-100 purification. Results show that amplification success increases with the Centricon-30 preparations (data not shown). The use of Centricon-30 devices then may further enhance the benefit of the MPS method for analysis of highly degraded remains. However, experience shows that lower molecular weight cutoffs may retain more PCR inhibitors. At present, we have not fully investigated Centricon-30 preparation in this regard.

The data presented for the MPS approach demonstrate the usefulness of the strategy to provide relevant data in cases where current mtDNA testing methods fail. However, as forensic mtDNA analysis expands its capabilities to reach extreme cases of degradation, potential pitfalls associated with amplification from few and/or damaged template molecules must be monitored. Amplification of damaged DNA base sites in early PCR cycles can lead to the production of a majority of amplicons that contain one or several misincorporated bases. The resulting sequence data, then, may provide clean but erroneous sequence information that could be overlooked by an examiner. Further the phenomenon of "jumping PCR" between neighboring templates can produce recombinant molecules that consist of portions of different DNA molecules (13). For the highly degraded remains routinely processed in our laboratory, experience shows that accurate data confirmation can be obtained from multiple amplifications on sample extracts to provide consistent, interpretable sequence results. In extreme cases, however, forensic scientists must be cognizant of anomalies that can occur throughout the analysis of highly degraded remains and maintain the flexibility to address these issues when they arise.

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