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Evaluation of a Multicapillary Electrophoresis Instrument for Mitochondrial DNA Typing*

ABSTRACT: Laser-induced detection of fluorescent labeled PCR products and multi-wavelength detection (i.e., multicolor analysis) enables rapid generation of mtDNA sequencing profiles. Traditionally, polyacrylamide slab gels have been used as the electrophoretic medium for mtDNA sequencing in forensic analyses. Replacement of slab gel electrophoresis with capillary electrophoresis (CE) can facilitate automation of the analytical process. Automation and high throughput can be further enhanced by using multicapillary electrophoretic systems. The use of the ABI Prism® 3100 Genetic Analyzer (ABI 3100, Applied Biosystems, Foster City, CA) as well as the ABI Prism™ 310 Genetic Analyzer (ABI 310, Applied Biosystems, Foster City, CA) were evaluated for mtDNA sequencing capabilities and compared with sequencing results obtained on the platform currently in use in the FBI Laboratory (the ABI Prism™ 377 DNA Sequencer, ABI 377, Applied Biosystems, Foster City, CA). Various studies were performed to assess the utility of the ABI 3100, as well as the ABI 310 for mtDNA sequencing. The tests included: comparisons of results obtained among the ABI 3100, the ABI 310 and the ABI 377 instruments; comparisons of results obtained within and between capillary arrays; evaluation of capillary length; evaluation of sample injection time; evaluation of the resolution of mixtures/heteroplasmic samples; and evaluation of the sensitivity of detection of a minor component with reduced template on the ABI 3100. In addition, other studies were performed to improve sample preparation; these included: comparison of template suppression reagent (TSR, Applied Biosystems, Foster City, CA) versus formamide; the use of Performa™ DTR Gel Filtration Cartridges (Edge BioSystems™ Inc., Gaithersburg, MD) versus Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ) for product purification after cycle sequencing; and sample stability after denaturation. The data support that valid and reliable results can be obtained using either capillary electrophoresis instrument, and the quality of sequencing results are comparable to or better than those obtained from the ABI 377 instrument.

KEYWORDS: forensic science, mitochondrial DNA sequencing, polymerase chain reaction, capillary electrophoresis, validation, mtDNA

The use of electrophoresis to separate sequencing reaction fragments is integral to the analytical process of mitochondrial DNA (mtDNA) typing. Although vertical polyacrylamide slab gel electrophoresis is used for many high throughput applications, it can be time-consuming, tedious, laborious, and does not lend itself to automation. As an alternative technique for electrophoresis of DNA fragments, capillary electrophoresis (CE) has been used successfully with high reproducibility and efficiency (1,2).

The use of single capillary instruments is not new to mtDNA sequencing analyses. Use of single capillary instruments for research and forensic casework has been demonstrated (3,4). However, one disadvantage to this technology is the length of time required for a single capillary instrument to complete electrophoretic separations on an individual sample for mtDNA sequencing of the two hypervariable regions of the mtDNA control region. Consider the DNA from a single hair that is amplified for four mtDNA regions (HV1A, HV1B, HV2A and HV2B) (5). For each of the four regions there will be eight sequencing reactions, because both the light and heavy strands are sequenced for all amplicons. Also included is a set of eight positive control sequencing reactions of a known DNA standard, eight sequencing reactions for a reagent blank and eight sequencing reactions for a negative control. Thus, for a typical forensic hair sample there are a total of 32 sequencing reactions that are then subjected to electrophoresis. The ABI Prism™ 377 DNA Sequencer (ABI 377, Applied Biosystems, Foster City, CA) can run 36 to 96 samples per gel. Each ABI 377 gel run lasts four and one half hours. The ABI Prism™ 310 Genetic Analyzer (ABI 310, Applied Biosystems, Foster City, CA), a single capillary instrument, would take at least 19 h to run the minimum 32 reactions (36 min per sample).

Recently, multicapillary electrophoresis instruments have become commercially available that have 16 or 96 capillary arrays. The ABI Prism® 3700 Genetic Analyzer contains 96 capillaries and was used almost exclusively for sequencing the human genome (6). However, a 96 capillary instrument may have too high a throughput for routine forensic analysis. The ABI Prism® 3100 Genetic Analyzer (ABI 3100, Applied Biosystems, Foster City, CA), provides a means of semi-automated sequencing of DNA samples by using an array with 16 capillaries that operate in parallel. On the ABI 3100, fluorescently-tagged PCR-amplified fragments from a dRhodamine Dye termination cycle sequencing reaction are detected by laser-induced fluorescence as the molecules pass through a detection window near the anodal end of the capillary array. Fluorescent signals from 525–680 nm are captured by a charged-coupled device camera detection system. Computer-assisted data analysis is performed to designate the fluorescent dye color related

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to the terminal nucleotide of each fragment. Moreover, a robotic mechanism allows for the consecutive injection of up to two sample trays, each tray with a 96 sample capacity, in a hands-free, computer-assisted, and user-defined manner. Thus, manual loading of samples is eliminated. Because amplified fragments are separated within an aqueous sieving medium, at the conclusion of each sample separation set, the polymer is automatically pumped out of the capillaries and replenished prior to the injection of the next set of samples. Thus, carryover contamination from previous electrophoretic runs is eliminated.

The ABI 3100 offers several advantages over the use of the ABI 377 for mtDNA sequencing. In order to replace the ABI 377 with the ABI 3100, the results obtained with the ABI 3100 should be of the same or higher quality to those typically observed on the ABI 377. As a means of defining operational parameters and limits for the analysis of mtDNA using CE, several studies were performed using the ABI 3100 to determine if consistent and reliable mtDNA typing results can be generated. The studies were: (1) comparison of results obtained from samples run on the ABI 377 and/or the ABI 310 and with those obtained on the ABI 3100; (2) comparison of results obtained from the same samples run multiple times on a single ABI 3100 instrument; (3) comparison of results obtained from samples run on two different ABI 3100 instruments; (4) comparison of results obtained within and between capillaries in an array; (5) evaluation of capillary length; (6) evaluation of sample injection time on the ABI 310; (7) evaluation of mixtures and heteroplasmic samples; and (8) evaluation of sensitivity of a minor component with reduced template on the ABI 3100. In addition, other studies were performed to improve sample preparation; these included: (1) comparison of template suppression reagent (TSR) with formamide; (2) comparison of Performa™ DTR Gel Filtration Cartridges (Edge BioSystems™ Inc., Gaithersburg, MD) with Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ) after cycle sequencing; and (3) sample stability after denaturation. This work demonstrates that the ABI 3100, as well as the ABI 310, can be used to obtain reliable mtDNA sequencing results in forensic casework and research. The quality of results obtained from the ABI 310 and 3100 are comparable or better than those produced by an ABI 377.

Materials and Methods

DNA Samples

DNA was extracted from 18 bloodstains, two hairs, one buccal swab and one bone (femur) by an organic phenol/chloroform method as previously described (5,7,8,9). All bloodstains, hairs, buccal swab and bone DNA were previously sequenced and the mtDNA profiles were known. Commercially available DNA from human cell lines 9947A (Applied Biosystems, Foster City, CA) and HL60 (American Type Culture Collection, Manassas, VA) also were used. In accordance with the FBI Laboratory's DNA Analysis Unit II Mitochondrial DNA Analysis Protocol, most analyses were run with corresponding negative controls or reagent blanks.

Amplification and Post-amplification Quantification

DNA was amplified by PCR according to previously described methods (5,7,8,9). Blood, cell lines and oral swabs were amplified for regions HV1 and HV2. Bone and hair DNA was amplified for regions HV1A, HV1B, HV2A, and HV2B, unless otherwise indicated in the text. Extracted DNA was amplified in 25 μL reaction volumes using the Gene Amp® PCR System 9700 (Applied Biosystems, Foster City, CA). Post-amplification quantification was performed on the Beckman MDQ capillary electrophoresis system (Beckman, Fullerton, CA) according to the manufacturer's recommendations and the FBI Laboratory's DNA Analysis Unit II Mitochondrial DNA Analysis Protocol.

Sample Preparation for Electrophoresis

For the purpose of this study, one cycle sequencing reaction is considered to be a single sample for analysis. As an example, four amplicons were generated from one bone preparation, resulting in eight cycle sequencing reactions; thus eight sequencing samples were generated for validation.

PCR products were subjected to cycle sequencing according to the manufacturer's instructions for ABI Prism® dRhodamine Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems, Foster City, CA). Gel filtration was used to remove unincorporated fluorescent terminators from completed cycle sequencing reactions. The cycle sequencing reactions were dried by lyophilization and prepared for electrophoresis.

Sequencing reactions were prepared for the ABI 3100 by adding 10 μL of Hi-Di formamide (Applied Biosystems, Foster City, CA). Initially for the ABI 310, 20 μL of TSR (Template Suppression Reagent, Applied Biosystems, Foster City, CA) were used to prepare samples. These preparations were heated at 95°C for 2 min and cooled at 4°C for a minimum of 3 min. Some sequence reactions subjected to electrophoresis on the ABI 310 were prepared using Hi-Di formamide as prescribed for ABI 3100 electrophoresis.

Electrophoresis and Analysis Using the ABI 377, ABI 310 and ABI 3100 Genetic Analyzers

Samples analyzed on the ABI 377 were run according to previously described methods (5,7,8,9). Electrophoresis conditions for the ABI 310 were as follows (unless otherwise specified): usage of an uncoated capillary (47 cm long, 50 μm interior diameter; Applied Biosystems, Foster City, CA) and the separation medium Performance Optimized Polymer 6 (POP™ 6, Applied Biosystems, Foster City, CA), a 30 sec electrokinetic injection at 2 kV and separation of amplified products at 15 kV and 50°C. Data were collected using ABI Prism® 310 Collection software for the ABI 310 (version 1.2.2, Applied Biosystems, Foster City, CA) with virtual filter E. Data were analyzed with Sequencing Analysis software (version 3.4.1, Applied Biosystems, Foster City, CA).

Electrophoresis conditions for the ABI 3100 were as follows: usage of uncoated capillaries (36 or 50 cm long, 50 μm interior diameter, 16 in each array, Applied Biosystems, Foster City, CA); separation medium POP™ 6; a 10 sec injection at 1.5 kV and separation at 12.2 kV and 50°C. Data were collected using ABI Prism® 3100 Genetic Analyzer Collection software for the ABI 3100 (versions 1.β 3 and version 1.0.1, Applied Biosystems, Foster City, CA) with virtual filter E. The data were analyzed with Sequencing Analysis software (version 3.7, Applied Biosystems, Foster City, CA). The results from all instruments were analyzed using Sequence Navigator (version 1.0.1; Applied Biosystems, Foster City, CA).

Platform Comparison

Mitochondrial DNA cycle sequencing fragments from 1 hair (8 sequence reactions and 8 reagent blanks run on all three platforms), 5 bloodstains (18 bloodstain sequence reactions run on all three

platforms) and human cell line HL60 preparations (68 HL60 sequence reactions and 60 negative controls run on all three platforms) were used for comparison of sequencing results on different electrophoretic platforms. Split samples were run on the ABI 377, ABI 310 and two separate ABI 3100 instruments. There were 94 sequencing reactions that were analyzed on three different platforms as well as an additional ABI 3100 instrument for a total of 376 sample sequences and 272 negative/reagent blanks evaluated.

Within and Between Capillary Performance on the ABI 3100

Mitochondrial DNA cycle sequencing fragments from 2 bloodstains with reagent blanks (176 bloodstain sequence reactions, 176 reagent blank sequence reactions) were injected into the ABI 3100. An additional 176 bloodstain sequence reactions and 176 reagent blank sequence reactions were injected into a second ABI 3100 instrument. Subsequently, sample plates were switched between the two instruments and re-injected. Thus a total of 720 bloodstain sequences, and 704 reagent blank sequences were evaluated (aborted runs were not included in the totals). The same experimental format was used for the human cell line HL60 with negative controls (256 HL60 sequence reactions, 256 negative control sequence reactions for a total of 576 HL60 sequences, and 560 negative control sequences were evaluated) and human cell line 9947A with negative controls (160 9947A sequence reactions, 160 negative control sequence reactions, for a total of 384 9947A sequences, and 320 negative control sequences were evaluated) were used for comparison of sequencing results obtained within and between capillaries in array(s) on two ABI 3100 instruments in the first phase of this study.

In the second phase, a similar approach to the above experiments was used, except variation between arrays was tested by exchanging arrays between the two ABI 3100 instruments and running the same samples on each array. Mitochondrial DNA cycle sequencing fragments from human cell line HL60 with negative controls (64 HL60 sequence reactions, 4 negative control sequence reactions, for a total of 192 HL60 sequences, and 12 negative control sequences were evaluated for each ABI 3100 instrument) were subjected to electrophoresis on a capillary array on each of the two ABI 3100 instruments. The original array was exchanged for a new array and the same prepared samples were subjected to electrophoresis on the same ABI 3100 instrument. The original array was returned to the original instrument and the same prepared samples were subjected to electrophoresis on the same ABI 3100 instrument for a third time. The samples in each plate were run a total of three times.

Capillary Length

Mitochondrial DNA cycle sequencing fragments were used for comparison of sequencing results on both 50 cm and 36 cm capillary length arrays on the ABI 3100 instrument. Preparations in duplicate were from 7 bloodstains (56 bloodstain sequence reactions and 28 reagent blanks), 1 bone (16 bone sequence reactions and 8 reagent blanks) and 1 buccal swab (12 buccal swab sequence reactions and 6 reagent blanks). The buccal swab was a HV2 length heteroplasmy C-stretch sample. Therefore, a total of six sequencing reactions were undertaken for the buccal swab. In addition, DNA from human cell line HL60 was prepared in duplicate, but was analyzed for four separate amplicons (16 HL60 sequence reactions and 8 negative controls). There were 200 sample sequences and 100 negative control/reagent blanks evaluated.

Sample Injection Time

Mitochondrial DNA cycle sequencing fragments from three bloodstains, one hair (8 hair sequence reactions) and human cell line HL60 and 9947A preparations (24 control sequence reactions) were used for comparison of sequencing results with different times for electrokinetically injecting the sample into a capillary. Of the three bloodstains, one of the bloodstains was analyzed in quadruplicate, one was analyzed in triplicate and one was analyzed in duplicate (32 bloodstain sequence reactions). The manufacturer's recommended injection time for samples on the ABI 310 is 30 sec. The manufacturer set a default injection time of 10 sec for the ABI 3100 instrument, and it was not changed for this study. Initially, injection times on the ABI 310 instrument were varied from 5 to 30 sec in 5 sec increments. There were 124 sample sequences evaluated (aborted runs were not included in the totals).

Resolution of Mixtures and Heteroplasmic Samples

For the study of the resolution of mixtures, samples were mixed after amplification. Samples consisted of a mixture of HL60 and bloodstain 1 prepared in duplicate for HV1/HV2 (112 mixture sequence reactions and 16 reagent blank sequence reactions), an HV1 mixture of bloodstain 2 and bloodstain 3 prepared in duplicate (52 mixture sequence reactions and 8 reagent blank sequence reactions), and an HV2 mixture of bloodstain 4 and bloodstain 5 prepared in duplicate (52 mixture sequence reactions and 8 reagent blank sequencing reactions). Two sets of dilutions were performed on each mixture so that each sample in the set would serve as the major contributor. DNA from each of the two samples was mixed in ratios of 12:1, 10:1, 8:1, 6:1, 4:1, 2:1, and 1:1 and then each mixture was subjected to cycle sequencing. These samples were prepared and run on an ABI 3100 instrument. There were 216 mixture sequences and 32 reagent blank sequences evaluated.

Three bloodstain samples and reagent blanks (24 bloodstain sequence reactions and 24 reagent blanks) that exhibited HV1 length heteroplasmy C-stretches (homopolymeric regions containing cytosine) and two samples (one hair with reagent blank and one buccal swab with reagent blank for a total of eight hair sequence reactions, 6 buccal swab sequence reactions, and 14 reagent blanks) that exhibited HV2 length heteroplasmy C-stretches were subjected to electrophoresis on the ABI 3100 instrument. The quality of these sequences was compared to previous runs on the ABI 377 instrument. For the ABI 377 data, only the heteroplasmic region and corresponding reagent blank (18 blood sequence reactions, four hair sequence reactions, four buccal swab sequencing reactions, and 26 reagent blanks) were used for comparison. There were 64 sample sequences and 64 reagent blank sequences evaluated.

Sensitivity of Detection of a Minor Component with Reduced Template

The purpose of this part of the evaluation was to determine the minimal concentration of mtDNA required to obtain a quality sequence from the ABI 3100 and to determine at what point a minor component in a mixture is not detectable. A bloodstain with reagent blanks (only region HV2 was prepared) and human cell line HL60 with negative controls (only region HV2 was prepared) were analyzed separately and then mixed at the following ratios: 8:1, 4:1 and 2:1 (bloodstain:HL60). All preparations were diluted to 20 ng, 10 ng, 7 ng, 3 ng, 2 ng and 1 ng. Samples were analyzed on one ABI 3100 instrument.

In Phase 1 of the study, the mixtures and dilutions were prepared after cycle sequencing, and only the D2 primer was used. All preparations were made in duplicate, except for the following: 7 ng, 3 ng, 2 ng and 1 ng bloodstain, 20 ng HL60 and mixtures at 20 ng (for a total of 52 bloodstain/HL60/mixture sequence reactions and two negative control sequence reactions).

In Phase 2 of this study, mixtures and dilutions were prepared prior to cycle sequencing and C1 and D2 primers were used. All preparations were made in duplicate except for the following: 20 ng and 10 ng bloodstain, 3 ng and 1 ng HL60 (for a total of 114 bloodstain/HL60/mixture sequence reactions and four negative control sequence reactions).

Template Suppression Reagent (TSR) versus Hi-Di Formamide on the ABI 310

Mitochondrial DNA cycle sequencing fragments from two bloodstains prepared in duplicate and one bloodstain prepared in quadruplicate (32 bloodstain sequence reactions), a hair prepared in duplicate (16 hair sequence reactions), and six different human cell line HL60 preparations (48 HL60 sequence reactions) were tested for quality of sequencing results on the ABI 310 instrument using Template Suppression Reagent (TSR, Applied Biosystems, Foster City, CA) versus formamide. Initially, 20 μL of TSR were used to prepare cycle sequencing products for ABI 310 electrophoresis. Preparations were heated at 95°C for 2 min and cooled at 4°C for a minimum of 3 min when using the TSR. Samples also were prepared using Hi-Di formamide (Applied Biosystems, Foster City, CA) as prescribed for sample preparation for ABI 310 electrophoresis. There were 92 sample sequences evaluated (aborted runs were not included in the total).

Performa™ DTR Gel Filtration Cartridges versus Centri-Sep Spin Columns for Sample Clean-up after Cycle Sequencing

Mitochondrial DNA cycle sequencing fragments from HL60 human cell line preparations (48 HL60 sequence reactions and 16 negative control sequencing reactions) were cycle sequenced using ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kits and ABI Prism™ Big Dye Terminator Cycle Sequencing Ready Reaction Kits. Duplicate sequencing reactions were combined and then divided, in order for one set to be filtered through Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ) and the other set was filtered using the Performa™ DTR Gel Filtration Cartridges (Edge BioSystems™ Inc., Gaithersburg, MD) according to the manufacturer's recommendations. The experiment was repeated with HL60 human cell line preparations (40 HL60 sequence reactions) and two bloodstains prepared in duplicate (16 bloodstain sequence reactions). The samples were cycle sequenced using the ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kits. The samples were run on the ABI 310. There were 94 sample sequences and 16 negative control sequences evaluated (aborted runs were not included in the total).

Sample Stability after Denaturation

One hair (8 hair sequence reactions and 8 reagent blank sequencing reactions), two bloodstains and HL60 human cell line preparations were evaluated (16 sequencing reactions). Following the addition of Hi Di Formamide, the samples were analyzed, then stored at −20°C for approximately one month and then re-run. Additionally, HL60 human cell line preparations (40 HL60 sequencing reactions and 32 negative control sequencing reactions) were stored in Hi Di Formamide at −20°C for 16–20 days and then run on the ABI 3100. These HL60 samples were stored for an additional month and tested again on the ABI 3100. There were 128 sample sequences and 80 negative control/reagent blank sequences evaluated.

Results and Discussion

The primary purpose of this study was to evaluate the use and performance of the ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), a 16 capillary instrument, for mtDNA sequencing. The quality of results obtained were compared with the slab gel-based platform currently in use in our laboratory (the ABI 377 Prism™ DNA Sequencer, Applied Biosystems, Foster City, CA). To replace the currently used instrument, the mtDNA sequencing results obtained using a multicapillary ABI 3100 instrument will have to be at a minimum, comparable with the results obtained from the ABI 377 instrument. The data in this study support the use of the ABI 3100 for mtDNA sequencing. In addition, the use of the ABI 310 for forensic mtDNA analysis has been on going since 1998. However, since some laboratories may choose to use the ABI 310 instrument, either as a back-up for the ABI 3100 or as a primary instrument, some of the experiments were performed using the ABI 310 instrument for evaluation.

The results of performance testing and manipulation of analytical conditions are presented to assist the forensic laboratory in implementing and using the ABI 3100 instrument for forensic casework. The total number of sequencing reactions described in the following studies does not include negative controls or reagent blanks. Even though negative controls and reagent blanks were not counted in the results section, they were evaluated to determine how the machines were functioning. The negative controls and reagent blanks are important for determining the overall evaluation of sequencing quality.

Platform Comparison

A total of 376 sequences generated by the three electrophoretic platforms were evaluated. The same sequencing results were obtained on each platform. However, peak definition for the ABI 3100 overall was better defined than that observed on the ABI 377 and similar to that obtained on the ABI 310 (Fig. 1).

Within and Between Capillary Performance on the ABI 3100

Among 7 arrays on one ABI 3100 instrument and 5 arrays on a second ABI 3100 instrument, the quality of the sequences for identical samples from each capillary within an array was comparable for most runs. In those few instances where differences were observed in sequence quality within or among capillary runs, the differences were usually due to background noise, low signal strength, anomalies (e.g., spikes or bubbles), capillary degradation and/or poor spacing of the initial or final bases from a run (the latter usually addressed effectively with manual editing). All of the sequences were typed correctly.

For phase one of the study, the quality of sequences for identical samples obtained from different arrays and different ABI 3100 instruments was comparable. A total of 1680 sequences were evaluated between the instruments. Sporadic anomalies (e.g., spikes) observed in one run on one array were not reproduced on different arrays, as expected. Background noise between arrays was generally consistent. The signal strength of sequences at the end of the capillary life (approximately 100 runs) was lower as expected, cre-

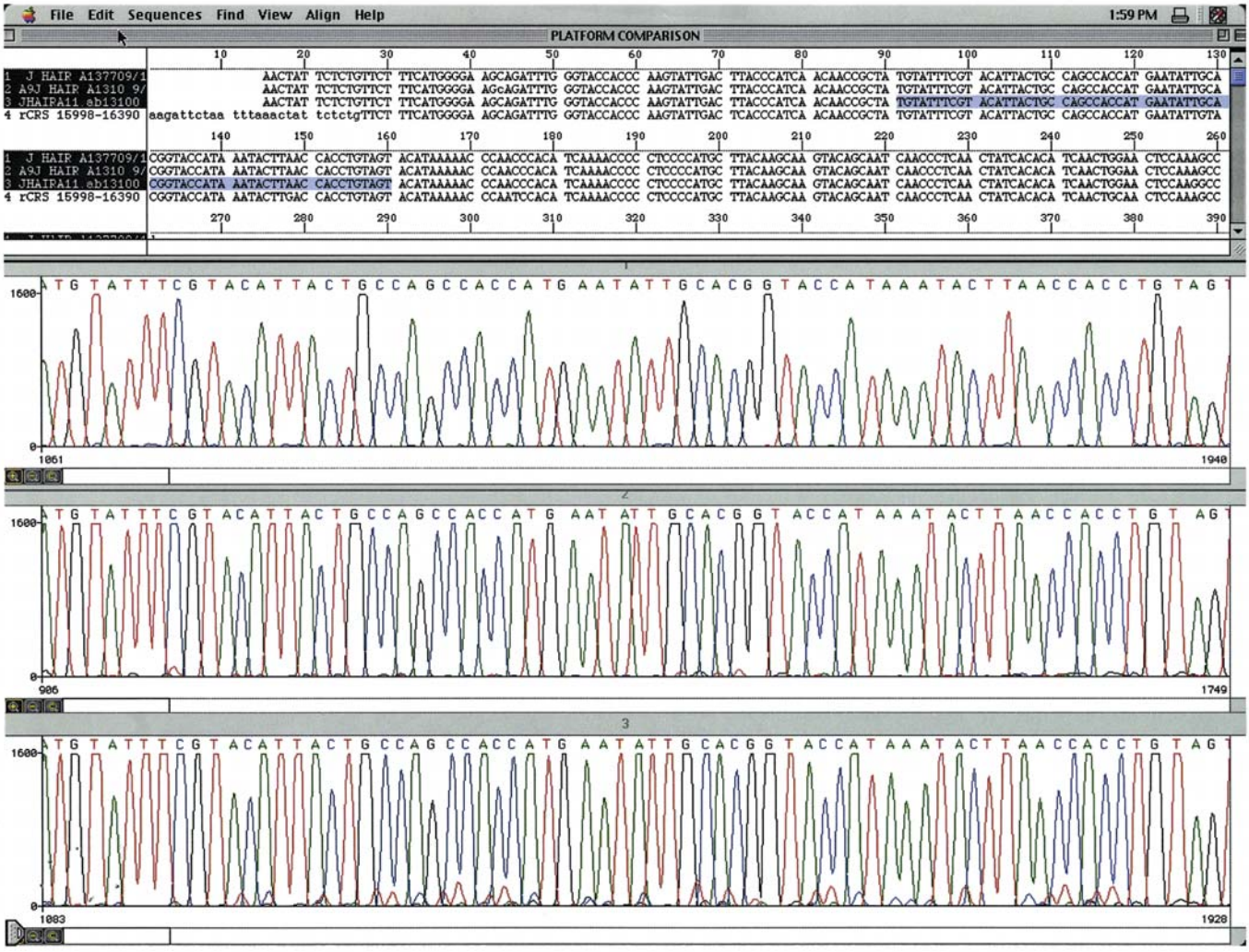

FIG. 1—*Comparison of sequencing results on different sequencing platforms. Top panel—ABI 377, Middle panel—ABI 310 and Bottom panel—ABI 3100.*

ating an increase in background noise; however, all sequences gave typeable results and typed correctly. There was some variation in peak height that was observed between different ABI 3100 machines (with different arrays on each machine); this was probably due to variations between apparatus default settings, laser alignment and output and CCD camera performance. The variation between the ABI 3100 instruments did not affect the ability to determine the base composition of the sequences typed.

For phase two of the study, the quality of sequences for identical samples obtained from different arrays on the same ABI 3100 instruments was comparable. A total of 192 sequences were evaluated. Slight differences in peak height were seen between array runs. This is expected with electrokinetic injections, since less template is present for each re-injection. All sequences typed correctly.

Capillary Length

Both 36 and 50 cm arrays were tested for sequence quality and to determine if the fragments that are used in mtDNA sequencing could be run on a shorter capillary. A total of 200 sequences were analyzed on both the 50 cm and 36 cm array. The arrays produced comparable results. Amplified sequences of 200–340 bp, the length of the sequences typically analyzed in our laboratory, were readable on both capillary lengths (Fig. 2). The separation time of the 50 cm array was 1 h and 7 min for 16 samples and the separation time for the 36 cm array was 40 min for 16 samples. All samples typed correctly.

Sample Injection Time for the ABI 310

The sample injection time for the ABI 3100 was set at 10 sec by the manufacturer and at the time of the study, could not be readily changed. However, the sample injection time for the ABI 310 was tested. A total of 124 sequences were generated for both injection time intervals. Although one would expect a decrease in sequence signal by lowering injection times from 30 sec to 10 sec, the quality of the sequences was sufficient for typing within the range of injection times. However, at a 5 sec injection, the signal strength decreased notably and the sequences were difficult to read.

Mixtures/Heteroplasmy

Six samples were mixed in various ratios to determine the levels at which a minor contributor could be detected. Reagent blanks were included in this study. A total of 216 sample sequences were evaluated. At the 12:1, 10:1, 8:1 and 6:1 mixture levels, the minor

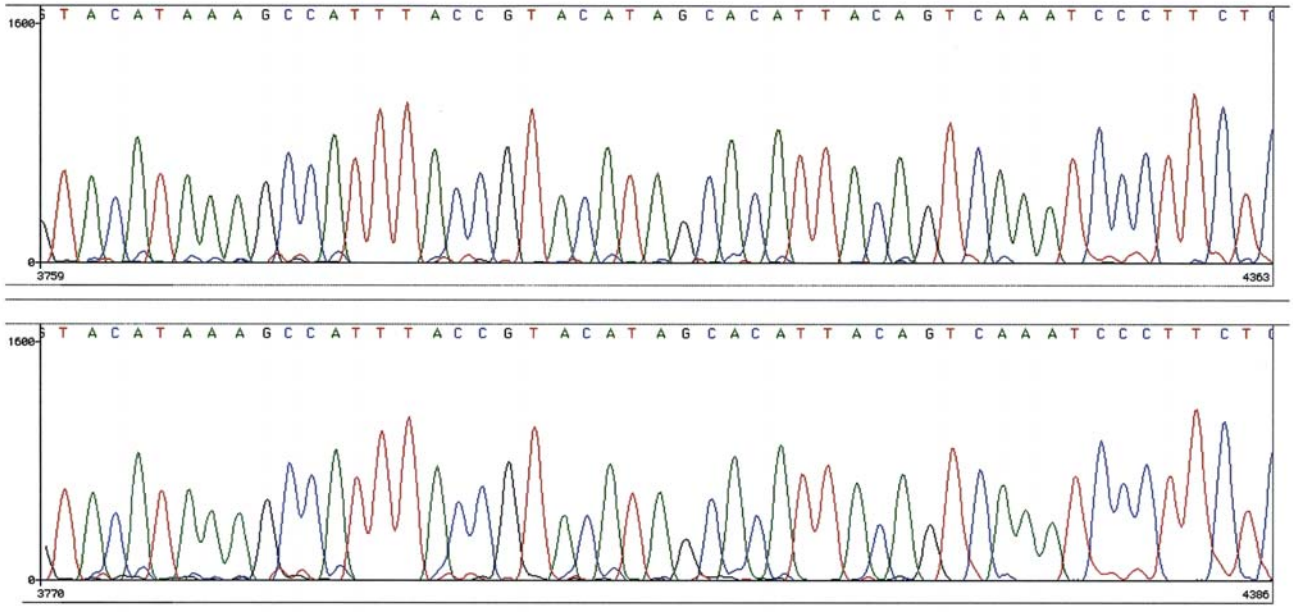

FIG. 2—*Comparison of sequencing results using different capillary lengths. Top panel—50 cm capillary length and bottom panel—36 cm capillary length.*

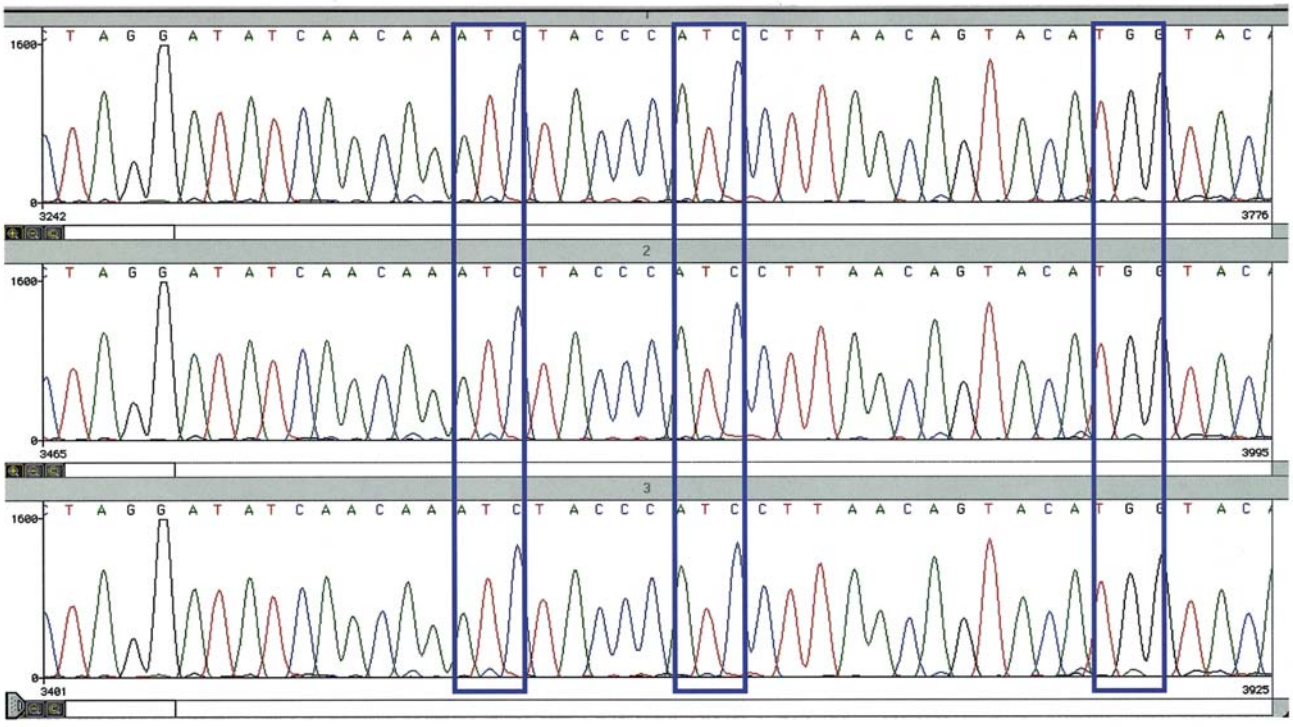

FIG. 3—*Comparison of sequencing results with different ratios of major and minor components. Boxes indicate the sites that differ between the two samples of the mixture. Top panel is a 12:1 mixture, middle panel is a 10:1 mixture and the bottom panel is a 8:1 mixture.*

component of the mixture was at or near background levels and all base calls were correct for the major contributor. At the 4:1 mixture level, the minor component was slightly above the background level, but the base calling was not affected for the interpretation of the major contributor. At the 2:1 level, the minor component was evident and above the background noise. All mixture results on the ABI 3100 platform were comparable to those observed on the ABI 377 platform. These results are comparable to those obtained in previous validation studies (5,8) and is independent of the three platforms evaluated in the current study (Figs. 3 and 4).

This study was predicated upon and the findings are consistent with the FBI Laboratory DNA Analysis Unit II Mitochondrial DNA Analysis Protocol's 10 to 1 ratio, currently used to assess potential background contamination (5,8). Our use of quantitative CE can detect DNA at a minimum level of 100 picograms. This is fifty times more sensitive than traditional agarose yield gels. The

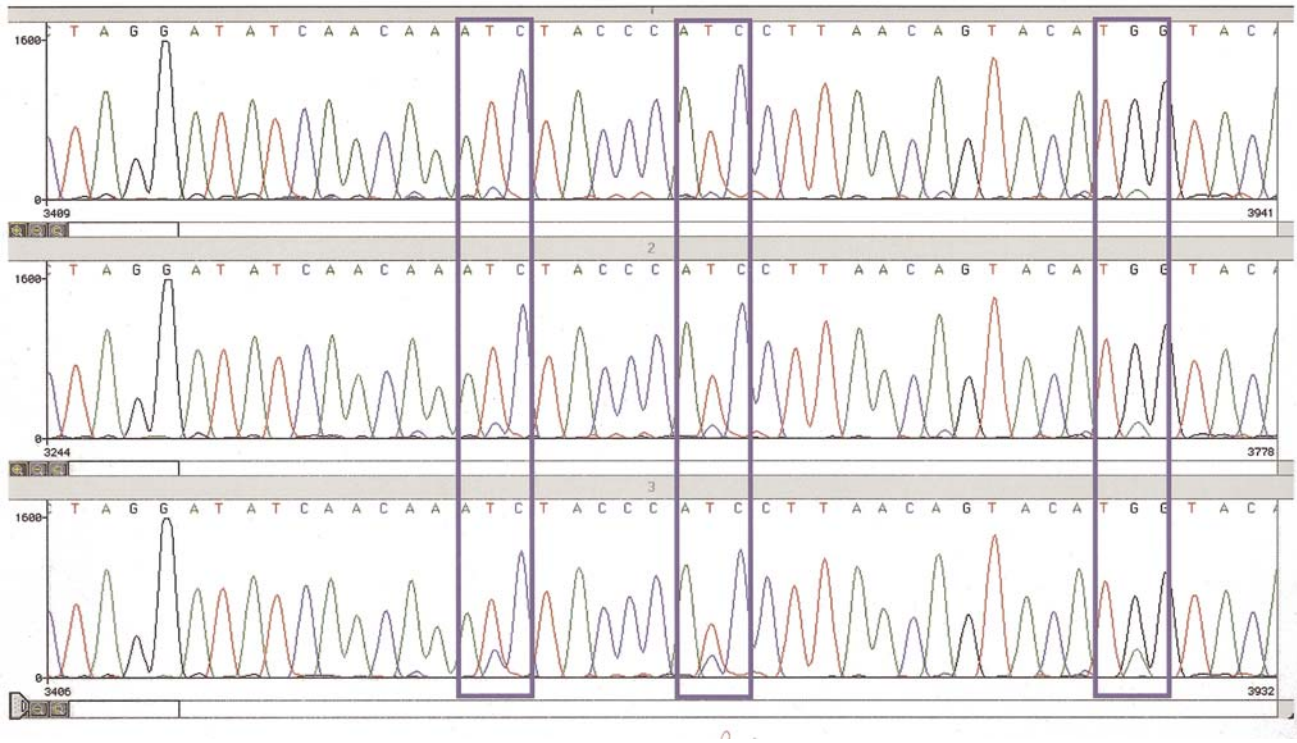

FIG. 4—*Comparison of sequencing results with different ratios of major and minor components. Boxes indicate the sites that differ between the two samples of the mixture. Top panel is a 6:1 mixture, middle panel is a 4:1 mixture and the bottom panel is a 2:1 mixture.*

amount of mtDNA in the reagent blank and negative controls is quantified and compared to the amount of mtDNA in the amplified product of the evidence and reference samples. If the negative control samples contain one-tenth or less mtDNA than the reference and evidence samples, sample processing continues. Thus, when adhering to the 10 to 1 ratio application, low level background contamination will not affect sequence interpretation (5). The current mixture study demonstrates that the 10 to 1 ratio is a conservative method in evaluating the affect of low level contamination on sequencing results.

Length heteroplasmy samples included 3 bloodstains (HV1 only), 1 buccal swab (HV2) and 1 hair (HV2). There were 64 total sequences evaluated. All of the samples had been previously sequenced using the ABI 377. Length heteroplasmy sample results from the ABI 3100 were consistent to those seen on the ABI 377.

Sensitivity of Detection of a Minor Component with Reduced Template

According to the FBI Laboratory's DNA Analysis Unit II Mitochondrial DNA Analysis Protocol, a minimum of 7 ng of mtDNA template are generally used for each cycle sequencing reaction. In this study it was determined that a good quality sequence can be obtained for the major component from as little as 2–3 ng of template DNA. In fact, Phase 1 of this study demonstrated that as little as 1 ng of template DNA after cycle sequencing could generate interpretable sequences on the ABI 3100. At the 8:1 ratio, the minor component was at or below background levels for all quantities of template DNA. A good quality sequence with the presence of the minor component was consistently obtained from as little as 3 ng of template DNA for the 4:1 and 2:1 ratios. For Phase 1, a total of 52 sequence reactions were evaluated. Phase 2 of this study demonstrated that good quality sequences were obtained on the ABI 3100 from most samples containing 2 ng and 3 ng of template DNA. Detection of the minor component was similar to the results obtained in phase 1. For all samples, as the concentration of the template decreased, the amount of background noise increased. For phase 2, a total of 114 sequence reactions were evaluated. While 7 ng of template mtDNA are generally used in the FBI protocol, this study demonstrates that reliable results can be obtained by using less than 7 ng of template mtDNA.

In addition to the general sensitivity studies, a comparison was made on the sensitivity of detection between ABI 3100 instruments. It can be expected that different ABI 3100 instruments may have slightly different sensitivities due to differences in charged coupled device cameras, laser effectiveness and alignment and/or cleanliness of optical components. The sensitivity differences among capillary electrophoresis instruments did not impact on the accuracy or quality of mtDNA typing with the ABI 3100.

Template Suppression Reagent (TSR) vs. Hi-Di Formamide on the ABI 310

The Applied Biosystems protocol for electrophoresis of a sample on the ABI 310 recommends that samples should be prepared by the addition of TSR. Samples run on an ABI 3100 are prepared by adding Hi-Di formamide instead of TSR. If there is a need for individual samples from an ABI 3100 analysis to be re-run, it may be desirable to perform the analysis on an ABI 310. Samples that are prepared for the ABI 3100 need a compatible method of sample preparation with that of an ABI 310 protocol. A total of 92 sample sequences were generated on the ABI 310 using Hi-Di formamide and TSR for preparation. The ABI 310 results from samples prepared with HI-Di formamide and TSR were comparable (Fig. 5). All samples were typed correctly. Thus, samples can

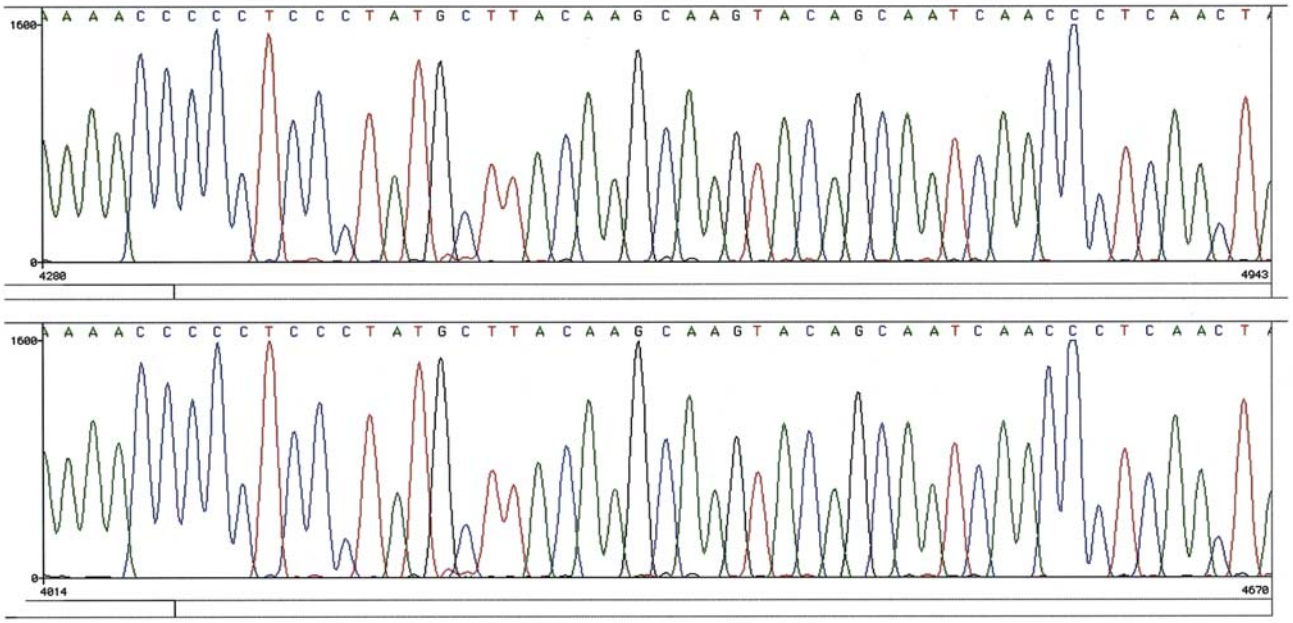

FIG. 5—*Comparison of sequencing results using TSR (top panel) and HiDi Formamide (bottom panel).*

be prepared in Hi-Di formamide and electrophoresed on both ABI 3100 and the ABI 310 instruments without a decrease in quality.

Performa™ DTR Gel Filtration Cartridges versus Centri-Sep Spin Columns for Sample Clean-up after Cycle Sequencing

After cycle sequencing, unincorporated labeled dideoxynucleotides (ddNTP's) are removed prior to electrophoresis of the samples. Typically, this method of cleaning up samples is by size exclusion column chromatography, which traps the unincorporated ddNTP's while the product is recovered by elution. The current FBI Laboratory DNA Analysis Unit II Mitochondrial DNA Analysis Protocol uses Centri-Sep Spin Columns for post-cycle sequencing sample clean-up.

A total of 94 sample sequences were evaluated. In the comparison of the Performa™ DTR Gel Filtration Cartridges with the Centri-Sep Spin Columns, both methods of sample clean-up gave comparable results, and all samples typed correctly. However, for the first 15 bases of a sequence there was more background noise with the Centri-Sep Spin Columns. Additionally, Performa™ DTR Gel Filtration Cartridges require less time than Centri-Sep Spin Columns for sample clean-up after cycle sequencing.

Sample Stability after Denaturation

In some instances, samples may need to be re-run due to a poor quality result obtained due to sample injection failure, capillary degradation or failure, sporadic anomalies, and/or other factors. Under such circumstances, if the sample remains stable during storage in Hi Di Formamide for a length of time, then re-injection of the sample would require no other manipulations. However, if the sample does not remain stable, then a second cycle sequencing reaction must be performed, purified, denatured and injected. In an attempt to minimize the need for additional cycle sequencing reactions, it was important to determine the amount of time denatured samples can be stored at -20°C and continue to yield quality sequencing results was determined.

A total of 128 sequences were evaluated for the stability study. Sample stability from 0 to 16 days exhibited only minor decreases in peak height and definition. All samples were correctly typed. Samples stored for 30 days gave a marked loss of peak definition, and samples stored for 47 days or more yielded difficult to read electropherograms. Thus, samples can be readily stored up to two weeks and be re-injected without additional sample manipulation (Figs. 6 and 7).

Conclusion

Mitochondrial DNA sequencing is a useful tool for genetically characterizing biological samples that contain minute quantities of nuclear DNA or that have been severely degraded. All three platforms, ABI 3100, ABI 310, and ABI 377 can yield reliable results for separating and detecting mtDNA sequence fragments. However, CE instruments, and particularly a multicapillary electrophoretic instrument, eliminate gel preparation and manual loading. Both 50 cm and 36 cm length capillary arrays can be used to obtain reliable mtDNA sequencing results. Run time is approximately 67 minutes for a capillary with a 50 cm length, and less separation time is required for a 36 cm length capillary. As many as 192 samples can be loaded and injected automatically on the ABI 3100. The typing was successful for all three platforms regardless of sample source (i.e., blood, saliva, bone, hair) or region amplified. The data support that valid typing results can be obtained using either CE instrument. The quality of mtDNA sequence results obtained with either CE instrument is equal to or better than those obtained from the ABI 377 DNA Sequencer.

The higher throughput of the ABI 3100 lends itself as a good choice for laboratories that are interested in sequencing mtDNA. However, an ABI 310 is applicable for lower throughput demand laboratories. The ABI 310 also can be used as a backup instrument for individual samples that need to be re-run from an initial ABI 3100 analysis; this approach can be a more efficient method for re-injections than running a few samples on the multicapillary platform. The data also show that results obtained on the ABI 310 and

3100 are comparable. Therefore, the results from experiments performed on one instrument platform can be extrapolated to the other CE platform.

After completing the validation study and after the date of the submission of this article, more than 15 forensic bone cases (over 500 sequencing reactions) have been analyzed for mtDNA on the ABI 3100 (Fig. 8). The sequencing results were comparable to those obtained in the described validation study. The data in total support the use of CE instruments for mtDNA sequence analysis.

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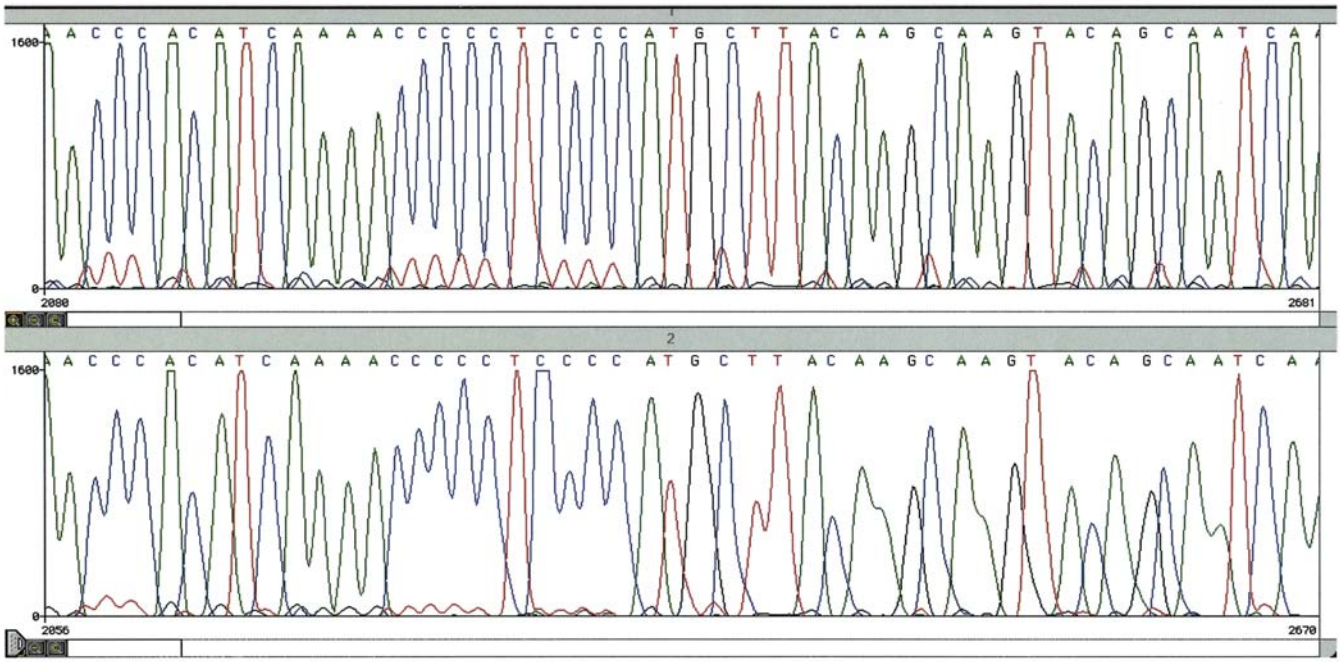

FIG. 6—*Comparison of sequencing results at 0 (top panel) and 30 (bottom panel) days storage.*

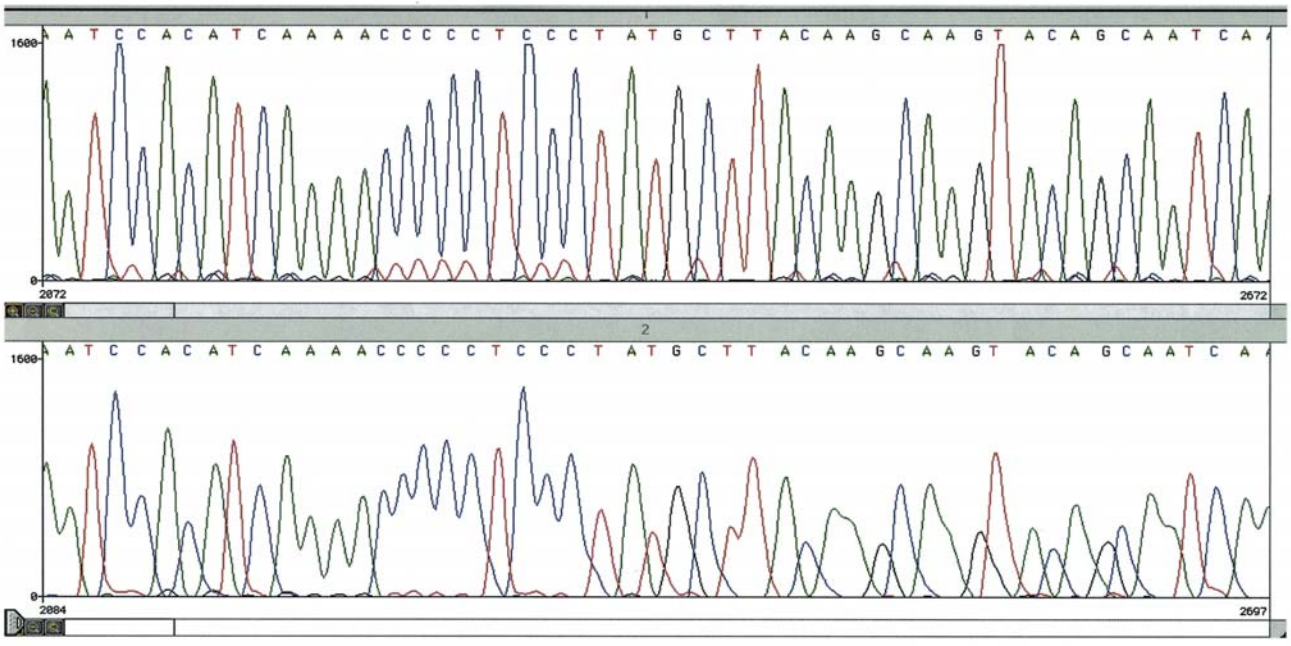

FIG. 7—*Comparison of sequencing results at 17 (top panel) and 48 (bottom panel) days storage.*

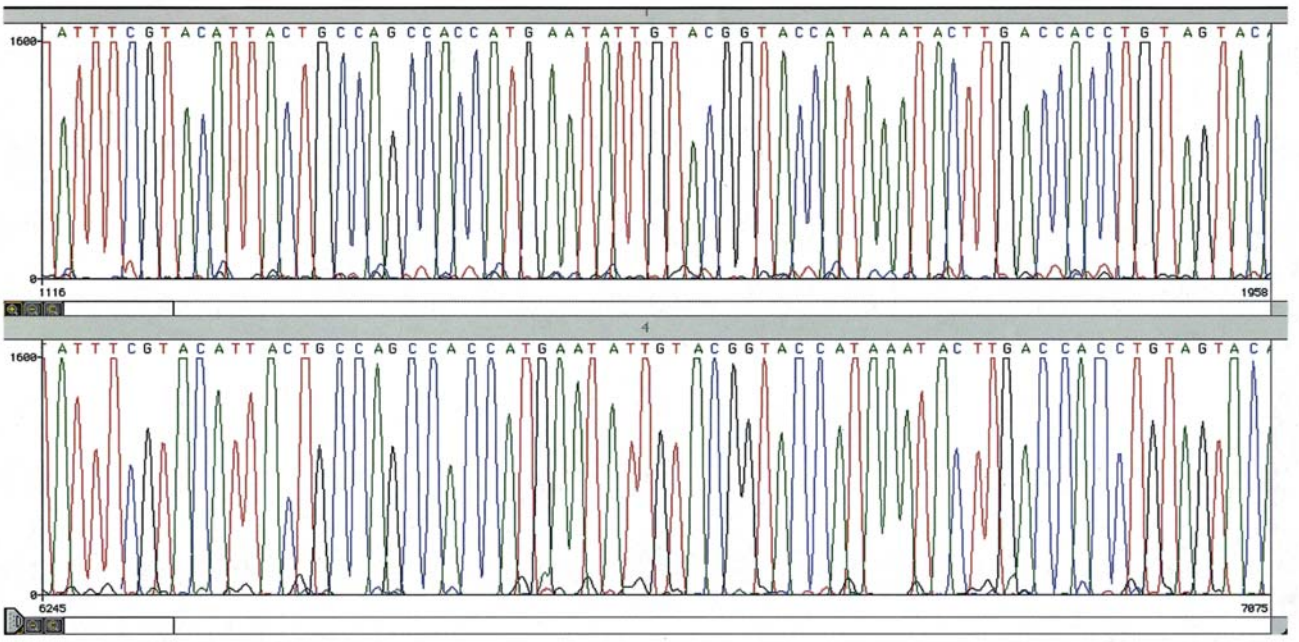

FIG. 8—*Example of a bone sample from casework ran on the ABI 3100.*

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