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TECHNICAL NOTE

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Extraction and Analysis of Human Nuclear and Mitochondrial DNA from Electron Beam Irradiated Envelopes*

ABSTRACT: The United States Postal Service is considering methods such as electron beam irradiation to neutralize biological agents sent through the mail. While this is proven to reduce/eliminate pathogenic organisms, it may also degrade human genomic DNA and therefore hinder the ability to garner forensically informative genetic profiles. To determine the effects of electron beam irradiation on DNA typing, 16 white, standard letter-sized envelopes were licked. Half of the envelopes served as nonirradiated controls while the other half underwent irradiation at dosages sufficient to kill anthrax spores (29.3 and 51.6 kGy). Total cellular DNA was extracted from all envelopes; nuclear short tandem repeat loci, as well as the hypervariable region I from mitochondrial DNA, were amplified by means of the polymerase chain reaction. Short tandem repeat profiles and mitochondrial DNA sequence haplotypes were acquired on an ABI Prism® 310 Genetic Analyzer platform. Analysis of data from irradiated samples revealed evidence of DNA degradation; however, the ability to construct full genetic profiles from both nuclear and mitochondrial DNA remained largely unaffected. The use of the polymerase chain reaction, coupled with florescent fragment analysis and mitochondrial DNA sequencing, should be considered to profile biological material from evidence enduring irradiation to inactivate infectious agents.

KEYWORDS: forensic science, bioterrorism, nuclear DNA analysis, mitochondrial DNA analysis, electron beam irradiation, DNA extraction, saliva, envelope flaps, mail decontamination

When biological agents are used as weapons, government agencies along with a multitude of microbiological and epidemiological experts are called upon to investigate the crime(s) and to ensure public safety. Recent cases involving the distribution of anthrax through the United States Postal Service garnered national attention. These events required the implementation of postal service protocols ensuring the safety of the intended recipients and postal workers. Mail confirmed to contain a pathogenic agent may undergo irradiation with electron beams, X-rays or gamma rays (1). With the primary concern of public safety attained, the next avenue warranting consideration is the identification of the persons responsible for these acts of bioterrorism. Evidence contaminated with infectious agents must be processed using methods preventing the spread of the agent to the examiners both on scene and in the laboratory, while maintaining the integrity of the evidence.

With the advent of the polymerase chain reaction (PCR) and automated sample processing, the ability to acquire intact nuclear and mitochondrial DNA profiles from low copy number evidence is now possible. DNA is routinely isolated and typed from evidence such as bodily fluids, fingerprints, dandruff, cigarette butts and licked envelopes (2–12). However, it has not yet been determined whether DNA can be isolated and typed from forensic evidence that has been sterilized by means of electron beam irradiation.

Our laboratory sought to obtain DNA profiles from licked envelopes exposed to electron beam irradiation dosages consistent with those necessary to inactivate anthrax spores, approximately 10 to 50 kiloGrays (kGy) (13). Inactivation of the spores due to electron beam irradiation is a result of chemical bond breakage and DNA damage (1,14–17). It is, therefore, reasonable to assume that human DNA trapped in the glue matrix of the envelopes would also sustain damage as a result of irradiation. It was the scope of this study to determine if the ability to obtain a genetic profile would be compromised by electron beam irradiation induced DNA damage. Data from short tandem repeat (STR) analysis of human nuclear DNA and sequence analysis of mitochondrial DNA are presented and demonstrate the clear ability to profile samples that have undergone electron beam irradiation.

Materials and Methods

Four laboratory employees (AZW, TF, KB, and RAD) licked and sealed four white, standard letter-sized envelopes each. Two envelopes for each person were sent to Titan Scan Technologies located in Lima, Ohio, where they received electron beam irradia-

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tion at two different dosages; envelopes designated AZW and TF received 29.3 kGy and envelopes designated KB and RAD received 51.6 kGy. Two envelopes per person were retained as non-irradiated controls.

Prior to processing, the envelopes were divided into two sets (Ext 1 and Ext 2), providing the opportunity to reproduce the study in a second experiment. Each set consisted of one irradiated envelope and one nonirradiated envelope from each person. The first set of envelopes was extracted 40 days after irradiation and underwent quantitation, amplification and complete analysis. Processing for the second set was initiated once data analysis was completed on the first set of envelopes (a total of 56 days post-irradiation).

All envelopes were steamed open using a commercial steamer and a set of sterile forceps. The epithelial cells trapped in the glue matrix of the envelope were transferred to a sterile swab moistened with sterile, deionized water. The swab was rubbed across the gummed envelope flap until all the visible glue was absorbed. This process was repeated using a second swab on the opposite face of the envelope, in order to ensure retrieval of the entire amount of cellular DNA. Each swab was cut and the tip was inserted into a sterile 2.0 mL microcentrifuge tube. It is important to note that the entire tip from each swab, theoretically containing all biological material from the glue matrix of each envelope, was inserted into the microcentrifuge tube. Had these envelopes been processed for criminal casework, only a portion of each envelope would have been swabbed to allow for reproducibility of the results.

Both sets of envelopes were extracted using the FBI's protocol for the organic extraction of DNA from envelopes (18). The swab tips were incubated in 300 μL stain extraction buffer (10 mM TRIS- 100 mM NaCl- 39 mM DTT–10 mM EDTA–2% SDS) and 4 μL 10 mM/mL proteinase K at 56°C for 24 h. The dithiothreitol (DTT) was inadvertently omitted from the stain extraction buffer in the incubation step of the first extraction set (Ext 1). The liquid eluted from each swab was then subjected to organic extraction by adding 300 μL Phenol: Chloroform: IsoAmyl Alcohol (PCI) to each tube. The extracted DNA from each sample tube was added to a Micron™ 100 concentrator unit and washed one time by the addition of 200 μL TE^{-4} buffer (10 mM TRIS-HCl–0.1 mM EDTA) and spinning in a microcentrifuge for 10 min at 2500 X g. The purified DNA extract was then eluted from the membrane within the Micron™ 100 concentrator units by adding a volume of 30 μL TE^{-4} buffer, inverting the unit upside down into a new, sterile 2 mL microcentrifuge tube, and spinning in a microcentrifuge for 5 min at 2500 X g. The DNA from both swabs was combined into one tube at this elution step resulting in a total elution volume of 60 μL per envelope.

An aliquot of 5 μL from each extract was quantitated using the colorimetric application (Chromagen: TMB) of the Applied Biosystems® QuantiBlot® kit protocol (19).

Nuclear DNA Amplification and Analysis

The quantitated extracts were amplified using a variation of the protocol outlined in the Perkin Elmer® AmpFℓSTR Profiler Plus PCR Amplification Kit User's Manual (7,20–22). Briefly: Two separate reactions were prepared for each extracted DNA sample, one using the Perkin Elmer® AmpFℓSTR Profiler Plus Kit and the other using the Perkin Elmer® AmpFℓSTR Cofiler Kit. The samples were prepared in a half reaction volume, each containing; 10 μL sample (composed of 1 ng DNA and ddH_2O), 10.5 μL AmpFℓSTR PCR Reaction Mix, 5.5 μL AmpFℓSTR Profiler Plus or Cofiler Primer Set, and 0.5 μL AmpliTaq Gold DNA Polymerase. The samples were then placed on a Perkin Elmer® 9700 Thermal Cycler, and amplified using cycling parameters outlined in the Perkin Elmer® AmpFℓSTR Profiler Plus PCR Amplification Kit User's Manual (20).

A master mix was prepared by combining 1 μL internal size standard (ROX GS 500) and 24 μL deionized formamide per sample to be analyzed. Samples were set up for capillary electrophoresis by combining 1.5 μL of each amplified product to 25 μL of the prepared master mix. The samples were denatured at 95°C for 3 min and chilled on an ice block for 3 min. The denatured samples were then analyzed on an ABI Prism® 310 Genetic Analyzer® utilizing the GS STR POP4 (1 mL) F run module. Raw data were assigned fragment sizes in base pairs with reference to the internal size standard, using the Local Southern Method of GeneScan® Analysis Software v 3.1. The analysis parameters were set with the peak height amplitude threshold at 75 rfus. Allele calls were made by reference to allelic ladders using Genotyper® v 2.5 software.

Mitochondrial DNA Amplification and Analysis

A known quantity of the cellular DNA extracted from the first set of TF and RAD envelopes (both irradiated and nonirradiated) was submitted for mitochondrial analysis. The four samples were amplified using a variation of the Armed Forces Institute of Pathology DNA Identification Laboratory's (AFDIL) protocol for the amplification of human hypervariable region I from mitochondrial DNA (22–24). Using a DNA amplification kit (Applied Biosystems #403445), a master mix was prepared containing 5 μL 10x PCR Buffer; 5 μL 25 mM $MgCl_2$; 4 μL dNTP Mix (200 μM each); 2 μL 10 μM HVI forward primer (5′-CCCAAAGCTAAGATTCTAAT-3′); 10 μM HVI reverse primer (5′-GAGGATGGTGGT-CAAGGGAC-3′) (Primers synthesized by Marshall University's Core Facility); 0.25 μL Taq Gold DNA Polymerase (5U/μL); and 11.75 μL ddH_2O. Thirty μL of the master mix was pipetted into sample tubes. An aliquot of 20 μL of sample (composed of 1 ng total genomic DNA and ddH_2O) was added to each tube and placed on a thermal cycler (MJ Research). The cycling parameters used for amplification were: 95°C for 10 min followed by 35 cycles of 95°C 30 s; 54°C 20 s; 60°C 45 s. The samples were held at 4°C until processed.

A 15 μL aliquot of PCR product was loaded and run on a 2% agarose gel to confirm the presence of an amplicon. Samples were electrophoresed for 2 h at 50 v, stained with ethidium bromide and viewed on a gel box (Fig. 5). PCR products were cleaned using the Qiagen® QiaQuick PCR amplification kit protocol (25). Purified DNA products were subjected to cycle sequencing using Big Dye Terminator ready reaction kit with Taq FS polymerase (Applied Biosystems #4303152) (23,24). Briefly: 2 μL of the purified PCR product was added to 8 μL of a master mix containing 2 μL of the ready reaction mix (containing Taq, ddNTPs, dNTPs, MgCl and buffer), 0.5 μL of a 10 μM unidirectional sequencing primer (either forward or reverse primer listed above) and 6.5 μLddH_2O. The reaction mix was subjected to cycle sequencing using optimized sequencing parameters (30 cycles: 95°C for 30 s, 50°C for 20 s, 60°C for 4 min; then a hold at 4°C).

The sequencing reaction was cleaned using a Centri-sep 100 spin column (Princeton Separations) following provided protocol with minor alterations (26). Briefly: the columns were soaked in ddH_2O for 2 h and spun twice at 750 × g for 2 min discarding the flow through between spins. The entire 10 μL sample was then added to the center of the column and spun at 750 × g for 2 min. The flow through was collected and added to 25 μL of a template suppression reagent. The samples were denatured at 95°C for 4 min and loaded onto a 310 Genetic Analyzer (Applied Biosystems) for sequence analysis.

Samples were processed using standard analysis parameters provided in the Seq POP6 Rapid (1 mL) E module Sequence Analysis software v3.3 and analyzed using the CE-1 base-calling algorithm. Computer analyzed base calls were manually edited for accuracy and the sequences obtained were compared using Sequence Navigator v1.0.1.

Results and Discussion

Nuclear DNA Analysis

Capillary electrophoresis of the samples yielded complete 13 loci (D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820, D16S539, TH01, TPOX, and CSF1PO) genetic profiles from *every* envelope (Table 1). However, a difference in the quantity of DNA recovered from the irradiated versus the nonirradiated envelopes was observed. Cellular DNA was retrieved from the electron beam irradiated and nonirradiated envelopes in the ranges of 3.6 ng to 36 ng and 3.6 ng to 90 ng, respectively (Table 1). A direct comparison of the quantitation results revealed that for all samples (except AZW_{Ext2}), the nonirradiated envelopes yielded more DNA than their irradiated counterpart (Fig. 1). While a direct correlation between irradiation and lower extraction yields is inferred by the data, the possibility exists that the envelopes retained as controls (except the AZW_{Ext2}) simply had more biological material deposited on them when compared to their irradiated counterparts prior to irradiation.

It is also interesting to note that the first extraction set yielded more DNA from the irradiated and nonirradiated envelopes than the second extraction set (Fig. 1). There are three differing variables between the two sets of samples: (1) samples in Ext 1 were processed 40 days after irradiation whereas processing for Ext 2 was not begun until 56 days post-irradiation, (2) samples in Ext 1 contained no DTT in the incubation step, Ext 2 did, and (3) again, perhaps the envelopes in Ext 1 coincidentally contained more DNA than the envelopes in Ext 2. It is highly unlikely that by chance all envelopes containing greater amounts of genetic material would be grouped into one particular extraction. While the exact cause for differing extraction amounts cannot be elicited by the scope of this study, the possibility that irradiated sample DNA was continuing to degrade even after the irradiation event is intriguing and should be explored further.

The GeneScan analyzed data suggested that there was also a difference in DNA quality between electron beam irradiated envelopes and their nonirradiated counterparts (Fig. 2). When typing DNA using the Perkin Elmer AmpFℓSTR® Profiler Plus™ and Cofiler™ kits, differential amplification between the smaller and larger loci may be observed as an indicator of template quality.

TABLE 1—*Analysis of nuclear DNA obtained from both extraction sets (Ext 1 and Ext 2) of electron beam-irradiated and nonirradiated envelopes.*

Envelope/ Sample	Radiation Dosage (kGy)	Total Amount of DNA Retrieved (ng)	Profiler Plus Typing Result	Cofiler Typing Result
$AZW_{Ext\ 1}$	29.3	6	full profile	full profile
$AZW_{Ext\ 1}$	0	12	full profile	full profile
$AZW_{Ext\ 2}$	29.3	6	full profile	full profile
$AZW_{Ext\ 2}$	0	3.6	full profile	full profile
$TF_{Ext\ 1}$	29.3	36	full profile	full profile
$TF_{Ext\ 1}$	0	90	full profile	full profile
$TF_{Ext\ 2}$	29.3	7.5	full profile	full profile
$TF_{Ext\ 2}$	0	42	full profile	full profile
$KB_{Ext\ 1}$	51.6	6	full profile	full profile
$KB_{Ext\ 1}$	0	36	full profile	full profile
$KB_{Ext\ 2}$	51.6	3.6	full profile	full profile
$KB_{Ext\ 2}$	0	15	full profile	full profile
$RAD_{Ext\ 1}$	51.6	30	full profile	full profile
$RAD_{Ext\ 1}$	0	60	full profile	full profile
$RAD_{Ext\ 2}$	51.6	7.5	full profile	full profile
$RAD_{Ext\ 2}$	0	30	full profile	full profile

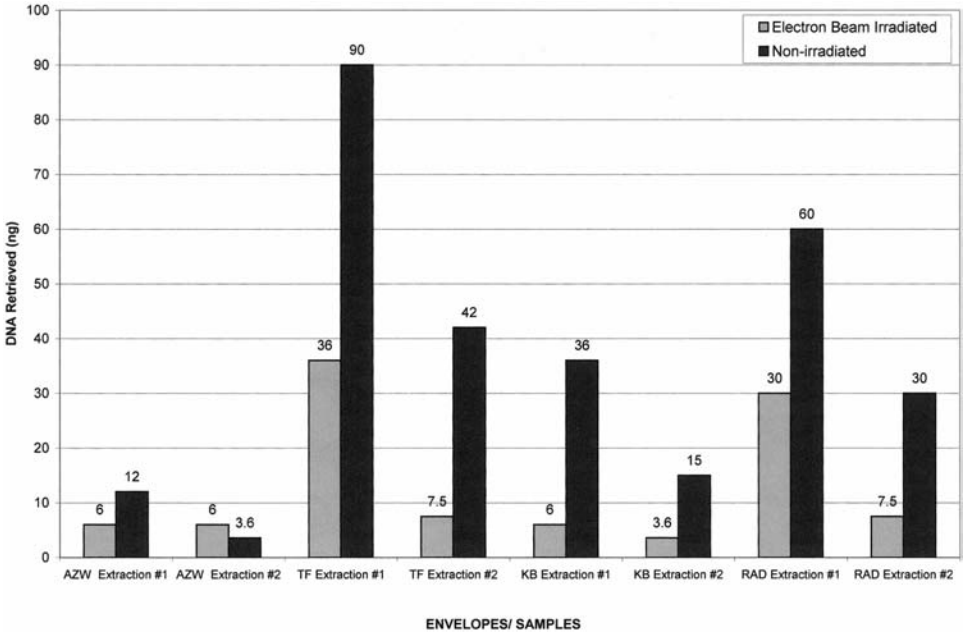
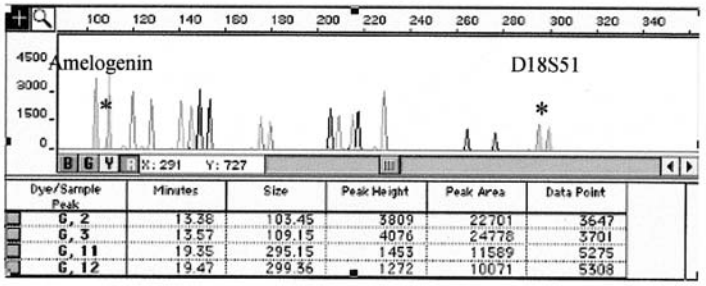


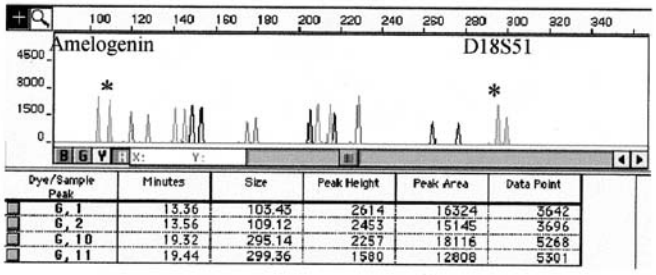
FIG. 1—*Results of Quantiblot analysis, estimating the amount of total genomic DNA in nanograms (ng) retrieved from each electron beam irradiated and nonirradiated envelope.*

A



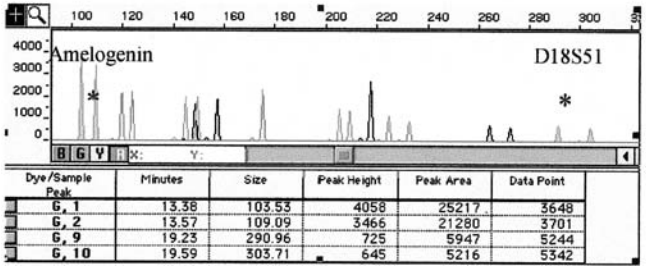
Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
G, 2	13.38	103.45	3809	22701	3647
G, 3	13.57	109.15	4076	24778	3701
G, 11	19.35	295.15	1453	11589	5275
G, 12	19.47	299.36	1272	10071	5308

B



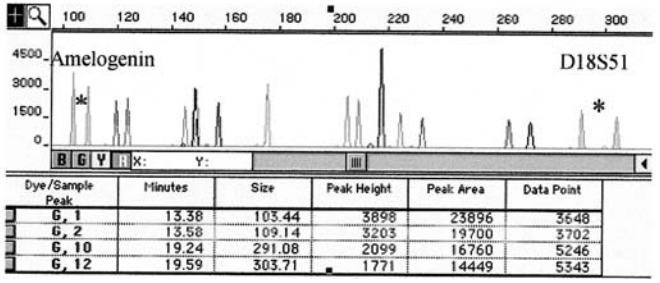
Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
G, 1	13.36	103.45	2614	16324	3642
G, 2	13.56	109.12	2453	15145	3696
G, 10	19.32	295.14	2257	18116	5268
G, 11	19.44	299.36	1580	12608	5301

C



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
G, 1	13.38	103.53	4058	25217	3648
G, 2	13.57	109.09	3466	21280	3701
G, 9	19.23	290.96	725	5947	5244
G, 10	19.59	303.71	645	5216	5342

D



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
G, 1	13.38	103.44	3898	23896	3648
G, 2	13.58	109.14	3203	19700	3702
G, 10	19.24	291.08	2099	16760	5246
G, 12	19.59	303.71	1771	14449	5343

FIG. 2—*GeneScan analyzed data of DNA isolated from electron beam irradiated and nonirradiated envelopes and amplified using Perkin Elmer AmpF*ℓ*STR Profiler Plus® kit. Analyzed data show an apparent difference in the quality of DNA obtained from electron beam irradiated versus nonirradiated envelopes. Data from the irradiated* TF_{ext1} *and* RAD_{ext1} *envelopes (A and C, respectively) exhibit a greater difference in peak height between the largest (D18) and the smallest (Amelogenin) locus in comparison to their nonirradiated counterparts (B and D, respectively).*

Studies show that when DNA is exposed to various environmental insults, loci fail to amplify in the order of decreasing size as the extent of degradation increases (7,20). Upon comparing the largest loci amplified with the AmpFℓSTR® Profiler Plus™ (either D18S51 or D7S820, depending on the individual profile) and Cofiler™ (CSF1PO or D7S820) kits to the smallest loci (Amelogenin) of each sample, we observed that differential amplification was prominent in data obtained from electron beam irradiated samples (Figs. 3 and 4). Nonirradiated samples exhibited balance between loci indicating less DNA degradation.

Mitochondrial DNA Analysis

A subset of extracted irradiated and nonirradiated total genomic DNA was provided for mtDNA sequence analysis. The mtDNA from samples TF_{ext1} and RAD_{ext1}, receiving 29.3 kGy

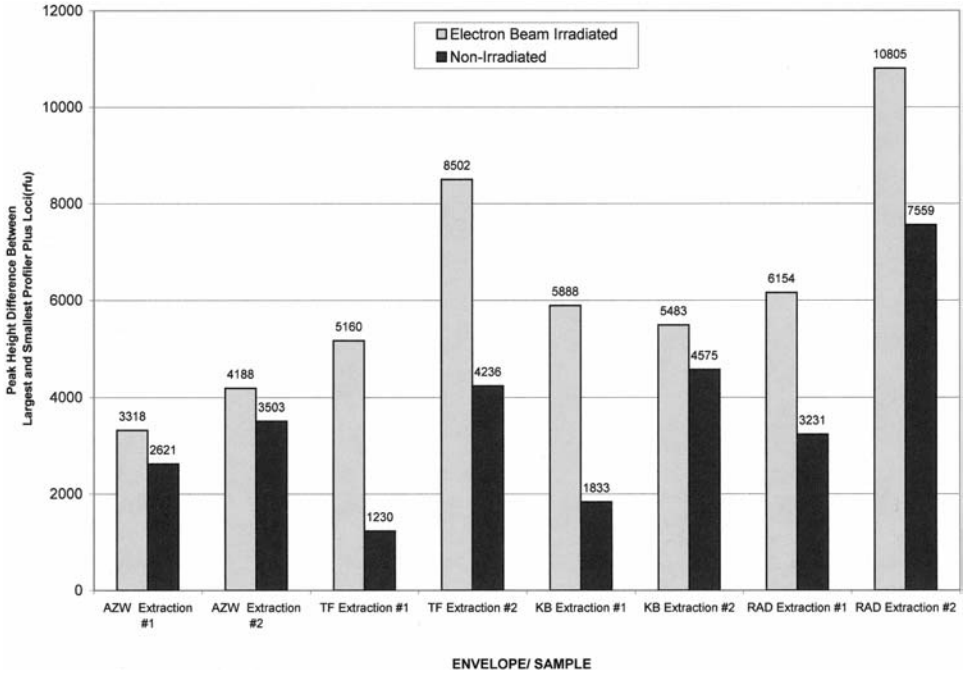


FIG. 3—*Difference in observed allelic peak height between the largest (D18S51) and the smallest (Amelogenin) Profiler Plus loci for electron beam irradiated and nonirradiated envelopes measured in relative fluorescence units (rfu).*

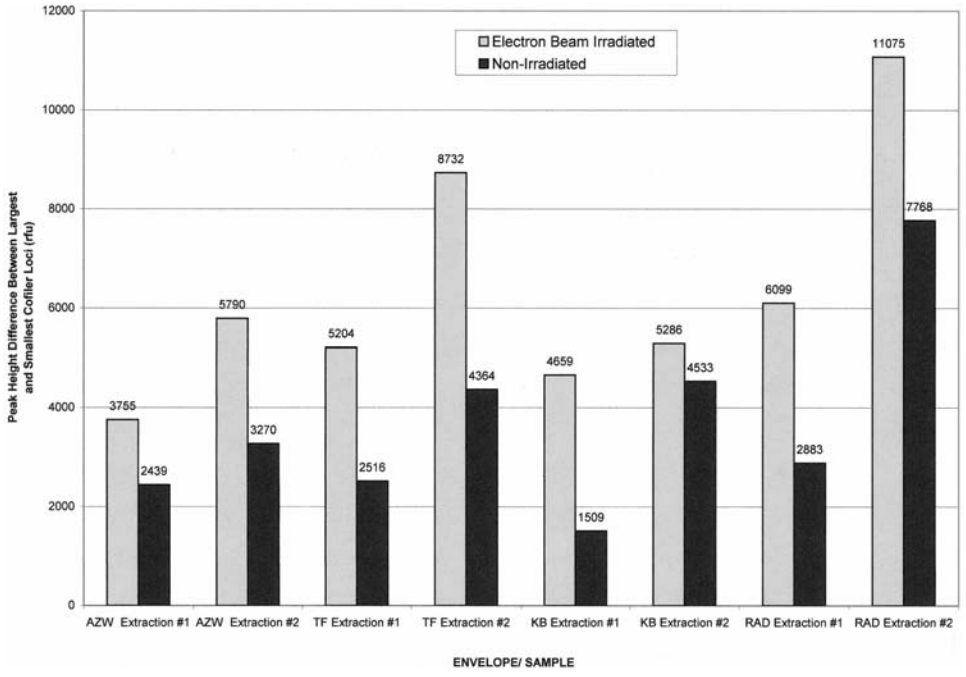


FIG. 4—*Difference in observed allelic peak height between the largest (CSF1PO) and the smallest (Amelogenin) Cofiler loci for electron beam irradiated and nonirradiated envelopes measured in relative fluorescence units (rfu).*

and 51.6 kGy, respectively, were successfully amplified using primers flanking the mitochondrial hypervariable region I (HVI). The clear bands on the product gel, approximately 400 bp in size, are consistent with the PCR product size of HVI amplification (Fig. 5). Note: while end point PCR product analysis and visual estimation of band intensity are not accurate methods for quantitative comparison there is an apparent diminution in product quantity between the electron beam irradiated (lanes 2 and 4) and their respective nonirradiated controls (lanes 3 and 5). This would be expected in samples enduring electron beam irradiation; due in part to DNA strand breakage and oxidative DNA modification (27,28).

The PCR products were subjected to dye terminator cycle sequencing. A successful mtDNA haplotype for HVI was obtained for both irradiation dosages. These sequences matched their respective nonirradiated control haplotypes (Fig. 6). TF_{ext1} differed from the Anderson reference sequence at a single position, 16294, where a transition C to T has occurred. RAD_{ext1} differed from Anderson (29) with base transitions occurring at positions 16126 (T to C), 16163 (A to G), 16186 (C to T), 16189 (T to C), and 16294 (C to T) (Table 2).

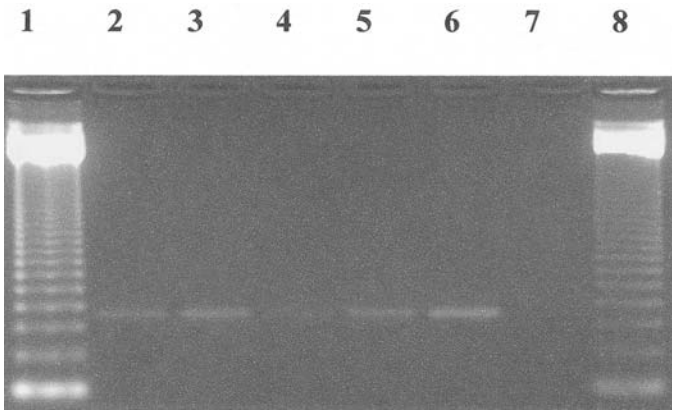


FIG. 5—*Mitochondrial DNA amplification product gel from electron beam irradiated and nonirradiated samples: lanes 1 and 8, 123 bp DNA mass ladder; lanes 2 and 3, 29.4 kGy TF_{ext1} respectively; lanes 4 and 5, 51.7 kGy RAD_{ext1} and 0 kGy RAD_{ext1} respectively; lanes 6 and 7, (+) and (−) amplification controls, respectively.*

TABLE 2—*mtDNA Hypervariable Region I haplotypes from a subset of electron beam irradiated envelopes.*

Sample Name	Radiation Dosage (kGy)	Sequence Polymorphism
TF_{ext1}	29.3	C16294T
TF_{ext1}	0	C16294T
RAD_{ext1}	51.6	T16126C
…	…	A16163G
…	…	C16186T
…	…	T16189C
…	…	C16294T
RAD_{ext1}	0	T16126C
…	…	A16163G
…	…	C16186T
…	…	T16189C
…	…	C16294T

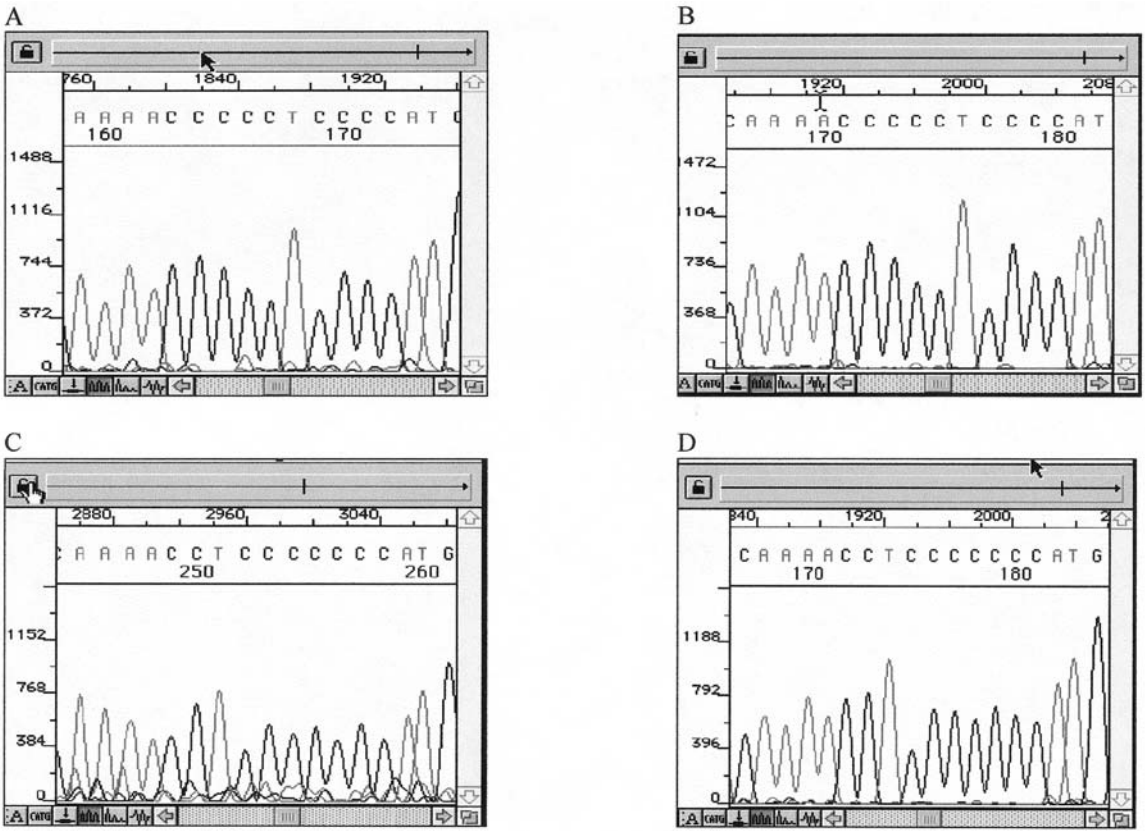


FIG. 6—*Human Hypervariable Region I mitochondrial DNA sequence data (#16769 to 16196) from electron beam irradiated and nonirradiated controls: 29.3 kGy TF_{ext1} (A), 0 kGy TF_{ext1} (B), 51.6 kGy RAD_{ext1} (C), and 0 kGy RAD_{ext1} (D).*

Conclusions

Due to advancements in technology coupled with increasing concern for public safety, the legal community is undergoing constant evolution. No discipline feels this continuous change greater than forensic science. In cases where evidence may be contaminated with a pathogen, the safety of forensic scientists must be ensured. Therefore, evidence must be decontaminated before it is processed in a forensic laboratory. In today's court system, this extra sterilization measure has the potential to add a degree of uncertainty to the validity of acquired data. After evidence undergoes sanitization, the question becomes "Have decontamination procedures compromised the reliability of information gained from forensic samples?" All facets of forensic science, from firearm examination to forensic biology, must take measures to determine differences between treated and untreated evidence and ensure that protocols maintain the integrity of the evidence in question (6,9,10,13,16,17).

This study provides confirmation that DNA profiles can be generated from evidence that has undergone measures to inactivate potential biological agents. While electron beam irradiation of licked envelopes at dosages of 29.3 kGy and 51.6 kGy potentially affected both the quality and quantity of human cellular DNA, the ability to obtain full nuclear STR profiles and mitochondrial HVI sequence haplotypes remained largely unaffected. Given these findings, nuclear and/or mitochondrial DNA analysis should be considered as a means to identify individuals responsible for distributing biological agents even in the event the evidence in question has undergone irradiation prior to sample processing.

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