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Recovery and STR Amplification of DNA from RFLP Membranes*

ABSTRACT: Restriction fragment length polymorphism (RFLP) techniques were utilized in the forensic DNA community until the mid 1990s when less labor-intensive polymerase chain reaction short tandem repeat (PCR STR) techniques became available. During the transition from RFLP technology to PCR-based STR platforms, a method for comparing RFLP profiles to STR profiles was not developed. While the preferred approach for applying new technology to old cases would be to analyze the original biological stain, this is not always possible. For unsolved cases that previously underwent RFLP analysis, the only DNA remaining may be restriction cut and bound to nylon membranes. These studies investigate several methods for obtaining STR profiles from membrane bound DNA, including removal of bound DNA with bases, acids, detergents, various chemicals, and conventional cell extraction solutions. Direct multiplex STR amplification of template in the membrane-bound state was also explored. A partial STR profile was obtained from DNA that was recovered from an archived membrane using conventional extraction buffer components, indicating promise for recovering useful STR information from RFLP membranes that have been maintained in long-term frozen storage.

KEYWORDS: forensic science, DNA typing, polymerase chain reaction, restriction fragment length polymorphism, membrane, amplification

Rapid advances in the field of forensic DNA analysis have allowed laboratories to obtain an exceptional amount of information from samples of limited quantity and quality (1–4). In fact, low copy number DNA analysis is routinely employed in many forensic labs with the application of multiplex polymerase chain reaction short tandem repeat (PCR STR) analysis (5). This application of PCR to forensic biology marked a great advance because prior methodology involving restriction fragment length polymorphism (RFLP) analysis required comparatively large quantities of high molecular weight (HMW) DNA (6,7). Furthermore, analysis performed with PCR technology can be conducted in a matter of days and is capable of generating multiplex typing results from < 100 pg of template or samples that have suffered degradation (8–10). RFLP technology, on the other hand, required 50–500 ng of HMW DNA and took days or weeks to produce multi-locus profiles (11,12). Because PCR analysis is faster, more sensitive, and potentially as discriminatory, PCR STR typing systems virtually replaced RFLP technology and evolved as the predominant analytical method by the late 1990s employed by forensic biology laboratories throughout the world.

During the transition from RFLP technology to PCR technologies, a method for comparing RFLP profiles to that of STR profiles could not be developed because the two systems interrogate different regions of the DNA sequence and bear fundamental differences in analytical platforms. Since the shift in technology from RFLP to STR-PCR, nearly all laboratories, including the Federal Bureau of Investigation (FBI) and Forensic Science Service (FSS), have discontinued RFLP analysis. Moreover, commercially obtained probes and reagents used to carry out validated RFLP procedures are becoming a scarce commodity, which further restricts the forensic community's chances of maintaining the technique even in the private laboratories where RFLP may be performed for specialized reasons. Because results cannot be compared between systems, and RFLP analysis has become an uncommon method, it becomes more and more challenging to continue investigations where the primary method employed was RFLP. This is especially true in cases where DNA from evidentiary items was consumed to generate a profile, since RFLP technology required large quantities of DNA and evidentiary samples were often restriction enzyme-cut and bound to a membrane in their entirety in an effort to obtain a profile for comparison. However, in instances of consumption, it is possible that DNA left from the exhibit in an RFLP case would exist in a restriction-cut form bound to a nylon membrane in laboratories where the casework membranes were preserved following analysis. Where post-analysis membranes have been archived, an opportunity exists for the sample DNA to be recovered from the membrane and used as a template for STR-PCR analysis.

The proposition of recovering membrane-bound DNA requires a fundamental understanding of the process by which it is bound. In 1975, Southern reported methods for hybridization of sequence-specific probes membrane-bound DNA fragments (13); however, attachment of DNA to a two-dimensional matrix was first described in 1963 when Nygaard and Hall reported techniques based upon the binding of DNA and RNA to nitrocellulose (14). These reports were foundational in molecular biology techniques involving immobilization of nucleic acids on static

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supports and are the basis for Southern/Northern transfer, dot and slot blotting techniques, and a variety of other applications that have provided a wealth of qualitative and quantitative information for the past 50 years. It is known that molecular weight, nucleic acid conformation, and ionic forces all play an important role in the binding of macromolecules to membrane supports, but the exact underlying mechanism of adsorption remains somewhat ambiguous (15). Therefore, while recovery of DNA from a membrane support may seem inherently simple, approaches for reversing this interaction are difficult to determine given that the binding processes are not well described and/or have been optimized under proprietary endeavors (16,17). Researchers affiliated with one membrane manufacturer, Pall Corporation, propose a binding model based primarily on hydrophobic interactions, where the surface chemistry (positively or negatively charged groups) plays a much smaller role in the binding process (18). Like proteins coming in close contact with the membrane, nucleic acids maintain hydration associated with secondary structure. These layers of hydration are forced out upon contact with the membrane, allowing the biomolecules to flatten out and providing a stable system of increased entropy to drive the interaction (19,20). Because nitrocellulose membranes are electrostatic, brittle, and less conducive to repetitive probing, the use of nylon membranes composed of 6–6 polymer were selected by most labs conducting RFLP testing because of their superior performance in hybridization-based assays (16). While nylon polymer is mostly non-polar with terminal amino and carboxyl groups, the hydrophobic regions fold away from the surface when cast into a membrane so that terminal polar groups are exposed for interactions with biomolecules (18). Furthermore, many commercially available nylon membranes are surface-modified so that charged groups present on the membrane can interact with the phosphate backbone of the nucleic acid; the linking chemistries most often employed are based on amine, carbonyl, carboxyl, or thiol and serve to enhance hybridization by affecting the orientation in which molecules bind (16).

While interactions between nucleic acids and surface modification molecules are important to consider, also critical are the modifications known to prevent DNA replication, especially changes imparted by chemical insult and ultra-violet irradiation (21–23). While direct PCR amplification from DNA template covalently bound to an uncharged membrane has been reported (24), it is unknown if small quantities of restriction digested template can successfully be amplified using a multilocus STR typing system. Furthermore, aside from damage imparted upon the DNA during the membrane fixation and sequential probing and stripping, one must also consider template damage resulting from initial restriction digestion carried out during the early steps of the RFLP process. Finally, detrimental changes to the DNA from the archival procedure employed by the laboratory are also of concern. Different storage conditions affect the ability to re-probe successfully the membrane (25), and may also induce variability in the success rate for DNA recovery. These considerations are critical for determining a reasonable analytical approach and designing meaningful studies that might ultimately result in achieving the recovery of DNA from archived membrane supports.

The studies herein were undertaken in an effort to develop a method of DNA recovery from membrane supports and the generation of an STR PCR profile from the recovered template. In addition to addressing successful recovery, issues regarding the use of current STR PCR technology to capture information from challenging DNA specimens handled under conditions much more conducive to the introduction of contaminants will also be considered.

Methods and Materials

DNA Extraction, Quantitation, Amplification, and Analysis

For preliminary studies, purified HMW K562 or 9947A DNA were obtained commercially (Promega, Corporation, Madison, WI) at a manufacturer's concentration of 10 ng/μL. Alternatively, dried blood stains from a female volunteer (FV), whose STR profile had been previously established, underwent standard organic extraction (11) using phenol:chloroform:isoamyl alcohol (PCI) followed by Microcon®-100 purification (Millipore Corporation, Billerica, MA) (26) and was brought to a final volume of 10–20 μL of TE^{-4} buffer (10 mm Tris, 0.1 mM EDTA, pH 7.5) or nuclease free water (NFW). These DNA samples and others prepared for membrane recovery assays were quantified using agarose yield gel analysis and/or the QuantiBlot® Human DNA Quantitation kit (Applied Biosystems, Foster City, CA) and then stored frozen until use. Samples analyzed on a flatbed platform were amplified using the PowerPlex® 16 BIO and/or PowerPlex® 2.1 Identification System (Promega Corporation) for 32 cycles according to the manufacturer's recommended protocol (27,28). They were then separated on a 6% polyacrylamide gel (at 52 W for 2.0–2.5 h), and detected using the Hitachi FMBIO® II Fluorescent Imaging Device (MiraiBio, Inc., Alameda, CA). Flatbed gel analysis was performed using the FMBIO® analysis software version 8.0 (MiraiBio), and allelic designations were made using STaR Call software version 3.0 (MiraiBio). Alternatively, samples analyzed using capillary electrophoresis were amplified using the PowerPlex® 16 Identification System (Promega Corporation) for 32 cycles according to manufacturer's recommended protocol (29). The product fragments were then separated and detected by capillary electrophoresis on an ABI PRISM® 3100-*Avant* genetic analyzer (Applied Biosystems), and data files analyzed using GeneMapper™ ID software version 3.1/3.2 (Applied Biosystems). PowerPlex® 16 BIO/PowerPlex® 2.1 versus PowerPlex® 16 chemistry usages are specified for each assay in Table 1 and are detected on the Hitachi FMBIO® II or ABI PRISM® 3100-*Avant* platforms, respectively. Unless otherwise noted, a 50 RFU threshold value was employed for electropherogram interpretation in an effort to detect very minor peaks.

STR Amplification of HaeIII-restricted DNA and Amplicon Sequence Analysis

High molecular weight DNA from FV was incorporated into restriction enzyme reactions to produce digested DNA for subsequent binding/cross-linking to nylon membrane for simulated RFLP membrane recovery tests. Approximately, 100 ng of HMW DNA was incorporated into 20 μL reaction volumes and restriction was carried out for 2–16 h at 37°C in the presence of 5 units of *Hae*III enzyme and 1X Multicore buffer (Promega Corporation). Reactions were checked for digest completion by visualization on agarose gels. Restriction enzyme reactions were also prepared to produce digested DNA for direct amplification using the same method (approximately 100 ng or 3 ng of HMW DNA was incorporated into 20 μL reaction volumes). For STR amplification of restricted DNA, 15 μL of the 3 ng digest reaction (~2.3 ng) was amplified using the PowerPlex® 16 BIO PCR amplification system (Promega Corporation) with a 100 RFU threshold for peak detection; 1 μL of the 100 ng digest reaction (~1 ng) was amplified using the PowerPlex® 16 BIO amplification system.

TABLE 1—*Summary of recovery assays and results.*

Recovery Method	Sample Description	Quantitation Result (ng/μL)	STR Result Summary	STR Chemistry
Heat/alkaline strip	50 ng bound K562 DNA	No signal	3/15 alleles detected	PowerPlex® 2.1
Heat/alkaline strip	50 ng aqueous K562 DNA	1.0	Full profile	PowerPlex® 2.1
Heat/alkaline strip	50 ng aqueous K562 DNA + membrane cuttings	0.50	Full profile	PowerPlex® 2.1
0.75 M acid	50 ng bound K562 DNA	No signal	No profile	PowerPlex® 2.1
0.38 M acid	50 ng bound K562 DNA	No signal	No profile	PowerPlex® 2.1
0.19 M acid	50 ng bound K562 DNA	No signal	No profile	PowerPlex® 2.1
0.09 M acid	50 ng bound K562 DNA	No signal	No profile	PowerPlex® 2.1
0.75 M acid	50 ng aqueous K562 DNA	0.15	No profile	PowerPlex® 2.1
0.38 M acid	50 ng aqueous K562 DNA	0.30	Full profile	PowerPlex® 2.1
0.19 M acid	50 ng aqueous K562 DNA	1.0	Full profile	PowerPlex® 2.1
0.09 M acid	50 ng aqueous K562 DNA	1.5	Full profile	PowerPlex® 2.1
Ethanol, methanol, acetone, DMSO, chloroform, phenol, PCI, formamide	50 ng bound K562 DNA	No signal	Amplification not attempted	QuantiBlot®
Stain extraction buffer (SEB)	50 ng bound K562 DNA 30 ng bound K562 DNA 10 ng bound K562 DNA	0.30 0.15 No signal	Full profile from each	PowerPlex® 16 BIO
Modified sperm lysis buffer	100 ng RD bound DNA	Not performed	10/30 alleles detected	PowerPlex® 16
Modified SEB	100 ng RD bound DNA	Not performed	20/30 alleles detected	PowerPlex® 16
Modified Differex™ lysis buffer	100 ng RD bound DNA	Not performed	21/30 alleles detected	PowerPlex® 16
Modified Differex™ lysis buffer	100 ng RD bound DNA, scraped and diced	Not performed	19/30 alleles detected	PowerPlex® 16
Direct STR amplification from membrane, AmpliTaq Gold® polymerase	100 ng RD bound DNA	N/A	0/30 alleles detected	PowerPlex® 16
Direct STR amplification from membrane, Restorase™ polymerase	100 ng RD bound DNA	N/A	0/30 alleles detected	PowerPlex® 16
Modified SEB, amp with AmpliTaq Gold® polymerase	Lane of archived RFLP membrane from PTC	Not performed	13/25 alleles detected (+4 non-source alleles)*	PowerPlex® 16
modified SEB, amp with Restorase™ polymerase	Lane of archived RFLP membrane from PTC	Not performed	11/27 alleles detected (+2 non-source alleles)*	PowerPlex® 16
Pre-wash, modified SEB recovery and amp with AmpliTaq Gold® polymerase	Lane of archived RFLP membrane from PTC	Not performed	15/27 alleles detected*	PowerPlex® 16

A brief description of recovery methodology and the sample type to which each was applied are listed in the first two columns; a synopsis of the amount of DNA recovered as determined by slot blot quantitation, and STR PCR results obtained are reported in the columns thereafter. The far right column defines the amplification chemistry used for each test. *Indicates results specific to PTC membrane lanes, SCRFSC membrane lanes did not yield profiles.

Binding DNA to Nylon Membrane

Defined quantities of HMW DNA (ranging from 10–50 ng) or restriction digested (RD) aliquots (containing approximately 100 ng) were prepared for membrane binding by denaturation in 250 mL 0.5 M NaCl/0.5 M NaOH solution. MagnaGraph (GE Osmonics) nylon membrane, comprised of only nylon 6–6 polymer with optimized pore structure and no other surface treatment was employed for these studies. The membrane was pre-soaked in 2X SSC (300 mM sodium chloride, 30 mM sodium citrate at pH 7.0) for 5 min; samples were then transferred to the membrane using the Convertible® Filtration Manifold System (Life Technologies, Gaithersburg, MD) in defined areas (slots sized 0.75×7.5 mm) that could be excised for subsequent extraction from the static matrix. With DNA binding boundaries marked, the membranes were placed in a volume 2X SSC-Tris–HCl (2X SSC, 2 M Tris at pH 8.0) that covered the membrane. After a 5 min soak, the membrane was drained, placed between filter paper, and baked at 80°C for 30 min. The DNA was then UV cross-linked with a UVC-515 (Ultra-Lum, Inc., Carson, CA) at an energy setting of 1200 (120 000 μJ/cm^2) on each side. Binding efficiency using this process was verified using the QuantiBlot® Human DNA Quantitation kit. Membranes were then stored frozen (−20°C) and areas of bound DNA excised such that membrane slots could be used for extraction/recovery assays.

Recovery of HMW Membrane-bound DNA with Heat and Alkaline Solution

Three samples were prepared for a heat and alkaline strip assay. The first consisted of 50 ng HMW DNA (K562) bound to nylon membrane. The second consisted of 50 ng HMW DNA (K562) in a 5 μL aqueous volume. Finally, in an effort to ascertain if membrane actions alone interfere with DNA recovery, a 50 ng sample of HMW DNA (K562) was incubated in a 5 μL aqueous volume with a small piece of dry, unused nylon membrane.

These three samples were treated with a 0.4 M NaOH solution (100 μL) for 20 min in a boiling water bath; the wash was then removed and reserved. The samples were then next treated with a 0.1XSSC/0.1% SDS solution (100 μL), soaked at room temperature (25°C) for 10 min and subjected to a boiling water bath for 5 min. The second wash was then removed and combined with the reserved step 1 wash. Washes were brought to a volume of 400 μL with TE^{-4} and DNA recovered by standard ethanol precipitation. Samples underwent slot blot quantitation and then amplified using PowerPlex® 2.1 chemistry. DNA recovered from the bound sample was amplified in full since no signal was detected upon slot blot quantification (6 μL remaining); the free aqueous sample and aqueous sample with membrane fragments were amplified based on slot blot quantification (1 and 2 μL incorporated into the amplification reactions, respectively).

Recovery of HMW membrane-bound DNA Using Acid Treatment

Concentrations of HCl were prepared in the following molarities: 0.75, 0.38, 0.19, and 0.09 M. Single membrane cuttings bound to 50 ng HMW DNA (K562) were treated with the acid solutions (one membrane fragment was placed into 100 μL of each of the four different concentrations). After 15 min, the acid solution was drawn off and placed into tubes containing 300 μL/TE^4. To this, 500 μL of PCI was added. Samples were vortexed briefly and spun at 21,000×g for 2 min. Each aqueous phase was purified using ethanol precipitation and brought to a final volume of 10 μL using TE^{-4}. Sample tubes containing 50 ng HMW DNA (K562) in aqueous solution were subjected to each of the acid treatments and processed as described above to serve as control samples.

Following resuspension, aqueous control samples were evaluated using a 2% (w/v) agarose gel. All samples underwent slot blot quantitation; recovered extracts from bound samples were amplified in full since no signal was detected upon slot blot quantification (6 μL) and recovered extracts from aqueous controls were amplified according to slot blot quantification (0.75, 1.0, 6.0, and 6.0 μL of the unbound extracts were amplified from the 0.09, 0.19, 0.38, and 0.75 M solution recoveries, respectively).

Recovery of HMW Membrane-bound DNA Using Organic Chemicals and Casework Stain Extraction Buffer

Single membrane cuttings bound to 50 ng HMW DNA (K562) were treated with 100 μL of ethanol, methanol, acetone, DMSO, chloroform, phenol, phenol/chloroform/isoamyl alcohol, or formamide and incubated for 15 min at 56°C. Membrane fragments were intact following the incubation and were left in the reaction tubes for subsequent extraction. To each reaction, 400 μL of TE^{-4} was added. Each sample was then treated with 400 μL PCI, vortexed briefly, and centrifuged for 5 min at 21,000×g. The aqueous phase from each then underwent ethanol precipitation and pellets were resolublized in 10 μL NFW.

For the first casework stain extraction buffer assay, single membrane slots bound to 50, 30, and 10 ng HMW DNA (K562) were treated with 400 μL forensic casework stain extraction buffer (SEB) comprised of 10 mM Tris pH 8.0, 100 mM NaCl, 50 mM EDTA pH 8.0, and 0.5% SDS to which 10 μL of 20 mg/mL proteinase K had been added; tubes were incubated overnight at 56°C. Membrane fragments were left in the reaction tubes for subsequent extraction, which was completed with PCI/Microcon® purification and concentration. The samples were brought to a final volume of 16 μL in NFW.

Amplification was not attempted for samples treated with ethanol, methanol, acetone, DMSO, chloroform, phenol, phenol/chloroform/isoamyl alcohol, or formamide since no signal was noted on the slot blot when these recovery extracts underwent quantitation. The samples recovered with SEB underwent amplification; 2 μL of the 50 ng extract, 4 μL of the 30 ng extract, and 15 μL of the 10 ng extract were incorporated into the respective reactions.

Recovery of RD Membrane-bound DNA Using Modified Casework Stain Extraction Buffers

Three lysis buffers prepared with excess dithiothreitol (DTT) and proteinase K were tested on membrane slots bound with ~100 ng RD DNA. The first, *modified sperm lysis buffer*, consisted of a 400 μL solution, 150 μL of which was TNE buffer (10 mM Tris; 100 mM NaCl; 1 mM EDTA; pH 8.0), the remainder of volume was prepared to achieve final concentrations of 50 μg/μL sarkosyl, 12 μg/μL DTT, and 0.5 μg/μL proteinase K in NFW. The second, *modified SEB*, was prepared as described previously, with higher final concentrations of proteinase K and DTT (1 μg/μL and 12 μg/μL concentrations, respectively). The last, *modified Differex™ lysis buffer,* was prepared in a 400 μL volume, 300 μL of which was Differex™ lysis buffer (Promega Corporation, proprietary extraction buffer supplied with the Differex™ differential extraction system), the remainder of volume was prepared to achieve final concentrations of 1 μg/μL proteinase K and 12 μg/μL DTT in NFW.

Extractions were performed on membrane slots bound with RD DNA. One membrane slot was also scraped and diced prior to treatment with the modified Differex™ lysis buffer (referred to as scraping + Differex™ lysis buffer). All were incubated overnight at 56°C. Extractions were completed with PCI/Microcon® purification and concentration, brought to a final volume of 15 μL in NFW, and amplified in full.

Direct STR Amplification of RD and Membrane-bound DNA Using AmpliTaq Gold® and Restorase™ DNA Polymerase

Membrane slots bound with RD DNA were diced and presoaked in 20 μL of 10X Gold ST*R reaction buffer (Promega Corporation) overnight at room temperature on an orbital shaker in an effort to prevent subsequent amplification components from interacting with free charges left on the membrane. Following the buffer soak, the membrane slots were rinsed three times with 100 μL NFW. Multiplex STR PCR reaction components were then added and amplification carried out using AmpliTaq Gold® DNA polymerase. For tests with the Restorase™ enzyme system study, a buffer-blocked membrane slot bound to RD DNA was treated with 19.2 μL NFW, 2.5 μL Gold ST*R 10X buffer, and 0.8 μL Restorase™ DNA polymerase. The samples were incubated at 37°C for 10 min, followed by a secondary incubation at 72°C for 5 min. A volume of 2.5 μL primer mix (PowerPlex® 16) was added following the incubations. The 25 μL reaction then underwent 32 cycles of PCR as is normally employed for the PowerPlex® 16 system; however, the 11 min hot start was omitted to accommodate the Restorase™ system recommended protocol. Template control reactions were performed using AmpliTaq Gold® to amplify 1 ng 9947a in aqueous solution, 1 ng 9947a in the presence of buffer-blocked nylon membrane fragments, 1 ng 9947a in the presence of unblocked nylon membrane fragments; no template control amplifications were performed in the presence and absence of buffer-blocked nylon membrane fragments. The Restorase™ system amplification procedure was also verified on positive and negative template controls (no additional blocked/unblocked membrane controls were tested with the Restorase™ amplification procedure).

Recovery and Amplification of DNA from Archived RFLP Membranes

One archived RFLP membrane was obtained from Paternity Testing Corporation (PTC), Columbia, MO. The membrane was prepared on June 4, 2003 following *Hae*III restriction digestion and had been stored frozen in plastic wrap following the completion of six multi-locus probings. The PTC procedure relies upon fixation by way of using Biodyne® B nylon membrane (6–6 polymeric membrane, 0.45 μm, with positively charged quaternary amine surface chemistry from Pall Corporation, East Hills, NY) with baking; UV cross-linking was not employed. The membrane was accompanied by a reproduction of the film used to capture the

chemiluminescent banding patterns (also referred to as an autolume) for orientation purposes, on which ten strong profile lanes were apparent. The other archived RFLP membrane was obtained from Sedgwick County Regional Forensic Science Center (SCRFSC), Wichita, KS. The membrane was prepared on January 21, 2000 and had been stored frozen between blotting pads following the completion of four multi-locus probings. The SCRFSC procedure relies upon fixation by way of using Biodyne® A nylon membrane (Pall Corporation) with baking and UV cross-linking (12,30). The membrane was also accompanied by an autolume. These exhibits were considered typical of archived RFLP casework and were used for the archived membrane recovery tests herein. Digests generally contained 500 ng of DNA per lane for applications in both laboratories.

For initial studies, two lanes with visible profiles present on the autolume were excised. This was performed by printing a reproduction of the autolume onto a transparency which was then exactly positioned over the membrane to identify lane boundaries. A razorblade was wiped with bleach, ethanol, and flamed prior to excision, which was performed just inside the lane boundary to avoid the inclusion of neighboring lanes. From the PTC membrane, lanes excised were approximately 5×150 mm in size, while SCRFSC lane cuttings were approximately 5×180 mm in size. Cuttings were diced, placed in microcentrifuge tubes, and extracted with the modified SEB as previously described. A portion of the cuttings was placed into a filter basket and centrifuged to dryness (5 min at 21,000×g) prior to purification and concentration with PCI/Microcon®. Samples were brought to a final volume of 15 μL in NFW, and amplified in full. One amplification reaction employed AmpliTaq Gold® polymerase (in accordance with the general protocol for PowerPlex® systems) and one reaction employed Restorase™ DNA polymerase. For the latter, the following were added to the purified DNA: 19.2 μL NFW, 2.5 μL Gold ST*R 10X buffer, and 0.8 μL Restorase™ DNA polymerase. The sample was incubated at 37°C for 10 min, then at 72°C for 5 min after which a volume of 2.5 μL primer mix (PowerPlex® 16) was added following the incubations. The 25 μL reaction then underwent 32 cycles of PCR using a modified version of the cycling parameters as previously described for Restorase™ DNA polymerase amplifications. Appropriate positive and negative amplification controls (9947A and NFW) were analyzed along side the amplification set. The second assay included a pre-processing wash step in an attempt to remove exogenous DNA associated with the membrane. For this study, one lane was excised from each of the PTC and SCRFSC membranes. Extraction was carried out as described above; however, prior to dicing the membrane lane, the membrane lane strips each underwent three washes (5 min each) with TRIS-0.2M: SSC-2X, followed by two NFW washes. Lanes were then dried at room temperature, processed for extraction as described above, and amplified using the PowerPlex® 16 with AmpliTaq Gold® DNA polymerase.

Results

Results presented here are derived from a progression of analytical studies designed to identify the applicability of STR typing of *Hae*III-digested DNA, stepwise identification and refinement of a recovery method, and finally, application of optimal recovery procedures to archived RFLP membranes.

STR amplification of the *Hae*III-digested DNA from FV verified that the digestion did not interfere with the ability to obtain the correct genotype of the source sample (data not shown). However, due to restriction cut sites within Penta E, TH01, and TPOX amplicon sequences, these loci either failed to produce STR results at these loci (3 ng restriction digest reaction amp product), or had extremely reduced band intensity (100 ng restriction digest reaction amp product) at these loci. The flatbed scanning system does not employ an interpretative cut off analogous to that for the capillary system; however, faint bands were noted at TPOX and TH01. No bands were detected at Penta E (data not shown). This is likely due to the fact that a larger quantity of DNA was incorporated into the restriction digest and that incompletely digested DNA subsequently was employed into STR PCR amplification as template. Nevertheless, results obtained from these amplifications verified predicted RD-induced drop-out determined from a sequence search of primer binding sites, STR repeat units, and the intervening sequences of amplicons produced by the PowerPlex® 16 BIO system (personal communication with Promega Corporation).

Initial removal assays tested for this project were based on mechanisms that caused failure when attempting to produce good RFLP results (practices avoided during standard RFLP because of the loss of bound molecules). Primary attempts were based on the stripping mechanisms that were used during the multi-probing process that often times seemed to cause progressive signal reduction. An acid-based removal system was considered since nylon supports are incompatible with acids (personal communication with Pall Corporation), and focused on identifying an acid strength that could disrupt membrane interactions without imparting significant hydrolytic damage to the target DNA. DNA-compatible chemicals and extraction buffers were explored for their ability to disrupt macromolecular interactions or dissolve the nylon such that the DNA would remain in an unbound and relatively unaffected state for subsequent recovery. Physical disruptions, in the form of surface scraping and dicing, were also considered when attempting recovery. Finally, techniques reported in the field of medical diagnostics indicate successful direct amplification of large quantities of DNA covalently bound to nylon membranes (24); therefore, a variety of direct amplification attempts were also made. Initial studies focused on membrane slots with HMW DNA bound and progressed to assays involving membrane slots with RD DNA bound. These tests were the basis for determining a recovery method that would ultimately be applied to archived RFLP membrane lanes.

Results from the recovery attempts are described in Table 1; this table is an overview of single recovery attempts and the progression of test procedures explored prior to extraction of archived membrane lanes. Studies involving heat/alkaline strip, acid, ethanol, methanol, acetone, DMSO, chloroform, phenol, PCI, and formamide did not result in appreciable recovery. However, SEB did result in the recovery of amplifiable DNA when tested on HMW membrane slots. Based on this success, a series of modified extraction buffers similar to SEB were tested on RD membrane slots that did exhibit success in recovery of amplifiable template. The success of each buffering system was measured by the system's ability to produce amplifiable DNA and measured by the number of alleles detected following STR amplification of the recovered sample. Comparison data is summarized in Table 2 and indicates that modified SEB and Differex™ extractions were successful recovering amplifiable DNA such that detection of 60–70% of template alleles occurred with subsequent STR typing. All methods performed fairly consistently (including that which employed scraping and dicing of the membrane prior to extraction) with the exception of the modified sperm lysis buffer, which resulted in substantially less profile information. It was noted that weak alleles were detected at the TH01 locus, even though the restriction sequence exists within the primer sequence. This probably arises as a result of incomplete

TABLE 2—*PowerPlex® 16 profiles obtained from membrane slots bound with restriction digested DNA and subjected to extraction with modified casework extraction buffers.*

Locus	Modified Sperm Lysis Buffer	Modified SEB	Modified Differex™ Lysis Buffer	Scraping + Modified Differex™ Lysis Buffer	Source DNA Profile
D3S1358	14,18	14,18	14,18	14,18	14,18
TH01		9	7,9	7,9	7,9
D21S11	30,31.2	30,31.2	30,31.2	30,31.2	30,31.2
D18S51					14,18
Penta E					12,23
D5S818	12	11,12	11,12	11,12	11,12
D13S317		11,12	11,12	11,12	11,12
D7S820		10	10,12	10,12	10,12
D16S539		9,13	9,13	9	9,13
CSF1PO	12	10,12	10,12	10,12	10,12
Penta D		10			10,11
Amelo	X	X	X	X	X
vWA	17	17	17	17	17
D8S1179	12,13	12,13	12,13	12,13	12,13
TPOX					8,11
FGA		24	20		20,24
Alleles detected	10	20	21	19	30
% Alleles detected	33.3%	66.7%	70%	63.3%	100%

Profiles recovered from membrane slots extracted using modified sperm lysis buffer, modified SEB, modified Differex™ lysis buffer, and scraping+Differex™ lysis buffer are reported here. The right column lists full profile information for the DNA source and the bottom two rows indicate total alleles detected and percent total alleles detected for each extraction system. Positive and negative amplification controls performed as expected.

TABLE 3—*PowerPlex® 16 STR results of amplified products obtained from archived RFLP membrane without pre-rinse prior to modified SEB recovery.*

	AmpliTaq® Gold	Restorase®	AmpliTaq® Gold Expected	Restorase® Expected
D3S1358	16	18	16	18
TH01	[7,9]		9.3	9,9.3
D21S11	29,30,31	[25.2, 26.2] wk28.2, 29.2*	29,30,31	29,30
D18S51		15,16	15,16	15,16
Penta E			5,14	11,14
D5S818	11,12	10 or 11*	11,12	11
D13S317	8	11,15	8	11,15
D7S820	9,11	9,10	9,11	9,10
D16S539	12		11,12	9,11
CSF1PO			9,10	10,11
Penta D			9,13	9,12
Amelo	X		X	Not reported
vWA	16 [wk17,18]	16	16	16
D8S1179	12	11,14	12	11,14
TPOX			8,9	8
FGA			21,24	21,25
Total correct alleles detected	13	11	(27)	(26)
Percent alleles detected	52%	42.3%	(100%)	(100%)

Alleles detected for each sample are indicated at each locus, with alleles not attributable to the lane DNA source bracketed. Notation of ''wk'' indicates an allele pair of weaker intensity. The total number of alleles expected for each template are tabulated in the shaded columns, as are the number of detected alleles for each WGA sample. The bottom row indicates the percent overall profile detected for each WGA sample out of the 100% possible for each template. Known genotypes provided by Paternity Testing Corporation; amelogenin results were not provided for the lane that underwent the Restorase® assay. *Indicates peaks could not be definitively sized because of the electrophoretic shift caused by polymerase/detection incompatibility.

restriction digestion of DNA prior to membrane binding. Had partial digestion occurred, these results may be expected, as smaller DNA sequences may more readily dissociate from the nylon, explaining why TH01 alleles were detected (shorter STR PCR fragments) while TPOX alleles were not (larger STR PCR fragments).

Anticipating structural changes in the DNA during membrane binding (interactions with surface moieties and/or damage induced by UV irradiation of the membrane which could impart nicks, gaps, abasic sites, deaminated cytosines, oxidized guanines, thymine dimers, etc.), Restorase™ (Sigma–Aldrich), a novel polymerase blend designed to amplify highly degraded samples otherwise unable to be amplified by conventional methods (31), was considered for amplifying post-RFLP DNA samples. The Restorase™ enzyme mixture is designed to increase the ability to amplify templates compromised by exposure to acid, alkylating agents, heat, and/or light since these block the progression of polymerase replication (31). Another process tested was pre-amplification of bound or recovered template using whole genome amplification (WGA). The GenomiPhi™ Kit (Amersham Biosciences) for WGA employs Phi29 (ϕ29) DNA polymerase and was developed to copy linear genomic DNA exponentially by strand displacement amplification initiated by random hexamer priming (32,33). This kit was selected for testing in an effort to explore the ϕ29 DNA polymerase's ability to amplify bound or otherwise structurally modified templates. However, because of the high incidence of artifacts observed following STR amplification of WGA products, results were difficult to interpret and determined to be inconclusive overall. This is not surprising since the system has since been reported to have limited forensic application (34,35) and is not marketed for amplification of highly degraded samples (32). For these reasons, WGA was disregarded as the optimal application for generating profiles from recovered template. Direct amplification attempts, using AmpliTaq Gold® (general method for PowerPlex® 16 systems) and Restorase™ DNA polymerase were also not successful in achieving any profile information, as no profile was detected from either assay. However, results from the control samples served to offer some insight regarding the challenges of direct amplification. Bound sample fragments underwent a pre-blocking process because preliminary data (unreported) indicated that the presence of membrane reduced amplification efficiency. AmpliTaq Gold® controls run alongside these samples illustrated this phenomenon in that the addition of unblocked membrane completely inhibited amplification of aqueous template (no profile detected). While the reaction containing the blocked membrane fragments yielded a full STR profile for the 9947a, it was noted that the overall average allele intensity was approximately 25% that of the standard positive control (absent any membrane fragments). Average allele intensity for the standard positive control was 814 RFU, while the average allele intensity for the positive control with blocked fragments added was only 220 RFU. Both AmpliTaq Gold® negative controls performed to expectation, as did the Restorase™ DNA polymerase procedure controls.

Based on these results, it was determined that attempts to recover DNA from archived RFLP membrane lanes would be made with modified SEB followed by STR amplification using AmpliTaq Gold®. The results of the initial studies where pre-washing was not employed are indicated in Table 3; bracketed allele values in this table indicate weak alleles not attributable to the true source. Because such data were obtained from both lanes tested, a pre-wash step was added for the final assay, the results of which are documented in Fig. 1. Data obtained at this injection duration

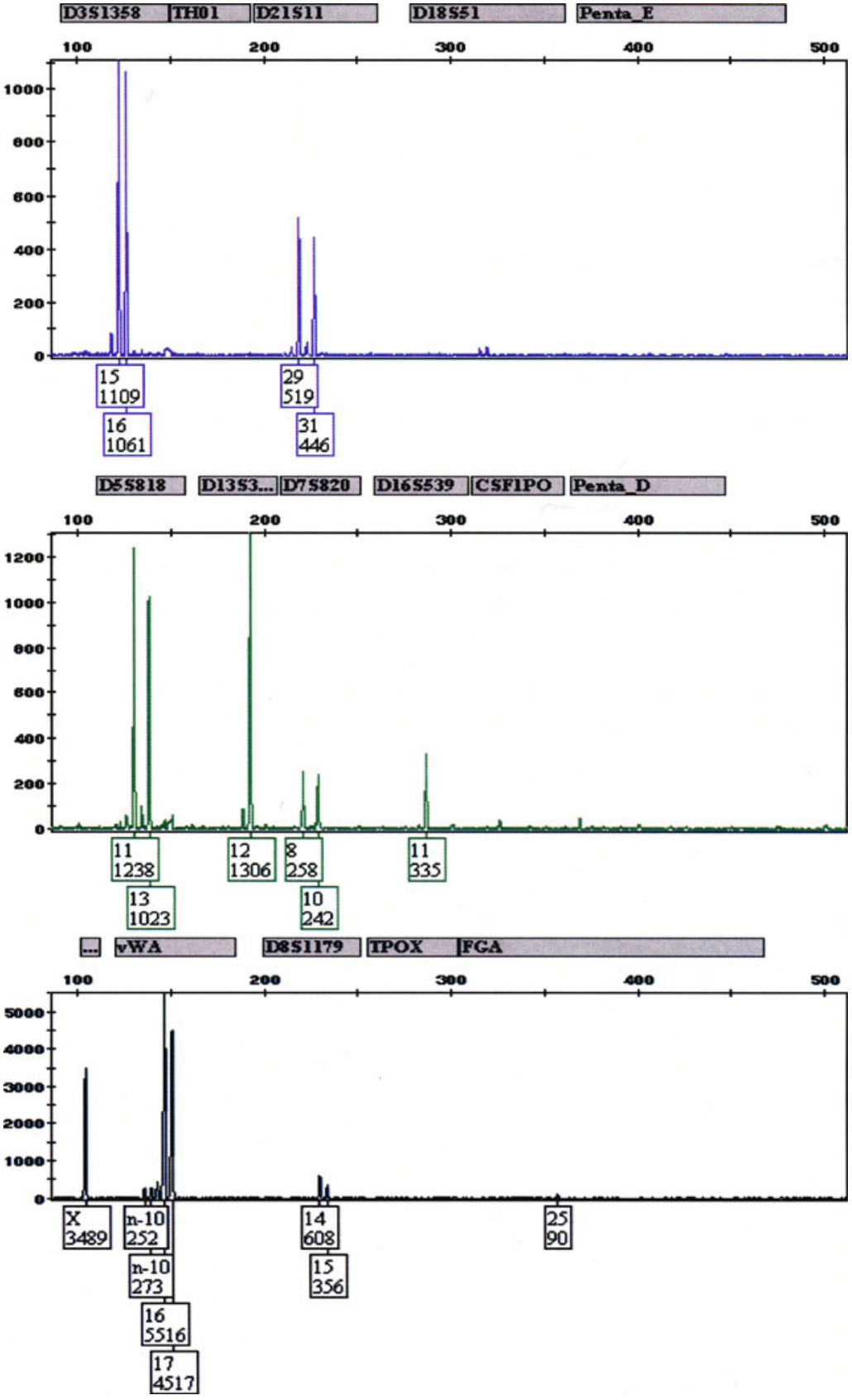

FIG. 1—*Electropherogram data obtained from archived RFLP membrane using pre-rinse prior to modified SEB recovery. Included are loci where peaks were detected (Penta D and Penta E not included), with allele values and RFU for the major peaks at each. Additionally, possible true template peaks were noted at FGA (25) and D18S51 (16,17). A threshold of 100 RFUs was employed, n-10 artifacts common at the vWA locus are denoted as such *1–37.*

(5 sec) resulted in profile information at 9 loci. Fig. 1 does not include data Penta E, TH01, or TPOX as would be expected because of the presence of restriction enzyme cut sites. Peaks visible within the figure that did not exceed the 50 RFU threshold at D18S51 (alleles 16,17), CSF1PO (allele 8), or Penta D (allele 2.2) were verified to be attributable to the source.

Discussion

Processing with heat and alkaline methodology did not result in recovery of an appreciable amount of amplifiable DNA from the sample bound to the membrane slot The aqueous controls did result in profiles, indicating that the method itself is not detrimental to the recovery process. However, based on quantification data it was noted that membrane surface interactions alone may interfere with recovery of free DNA, as was accounted for by addition of more template volume for amplification of sample C (with membrane cuttings) than that for B (without membrane cuttings). While three alleles were detected across D8S1179, vWA, and D3S1358, these are short amplicon length loci within the PowerPlex® 2.1 system, and it was determined that alternative methods would be explored prior to any attempts of applying heat/alkaline recovery to samples that had been restriction digested prior to membrane binding.

Tests conducted using acid recovery methodology did not result in any amplifiable product from any of the membrane slots. With respect to controls, the three least concentrated acid solution assays yielded STR profiles; however, the most concentrated acid environment was not conducive to recovery of amplifiable DNA. These results indicate that acid is not capable of disrupting membrane binding properties to an extent which allows DNA recovery, and that increased concentrations of acid will most likely be detrimental to any free DNA that becomes present in the reaction. As expected, positive control samples exhibited an indirect relationship between acid concentration and DNA recovery; this trend was the basis for addition of greater template volumes to the amplification reactions from samples recovered from progressively higher molarity solutions.

Treatment of test slots with various chemicals (ethanol, methanol, acetone, DMSO, chloroform, phenol, phenol/chloroform/isoamyl alcohol, or formamide did not result in the recovery of a quantifiable amount of DNA. While it is unknown whether any of these systems may have produced amplifiable template, this was not pursued further in light of tests run concurrently using SEB. Initial studies with this as a recovery solution proved promising, as amplifiable DNA was obtained from membrane slots containing as little as 10 ng DNA. Because of these results, it was determined that key components of this solution were the proteinase K and DTT, and that similar extraction buffers would undergo testing on membrane slots bound with RD DNA.

Modified extraction buffers consisted of those commonly employed on casework stains, but with excessive amounts of proteinase K and DTT. This approach was taken since these chemicals are generally added to extractions to induce dissociations between DNA and proteins (i.e., interactions between membrane surface moieties and bound macromolecules). It is speculated that DTT may serve to release DNA from protamine-like interactions with the membrane surface chemistry, while the proteinase K serves to digest protamines. Three different solutions were tested, and the commercially available Differex™ extraction buffer was also tested on a scraped and diced fragment to determine if physical disruption of surface interactions would result in greater recovery. Results suggested that all solutions were capable of recovering amplifiable template. While the modified Differex™ extraction buffer did result in the detection of one more allele than did the modified SEB, this was not considered a relevant difference in product. Of the two tests conducted using the modified Differex™ extraction buffer, the one subjected to scraping resulted in slightly less allelic information than did the intact membrane slot; however, the difference consisted of only two alleles and without conducting the test with replicate samples it is unknown if the difference is due to product loss through manipulation, or just expected sample-to-sample variation. The least amount of profile information came from the sample extracted in modified sperm cell lysis buffer, and yielded about half the number of alleles obtained from the other lysis buffer solutions. Based on these studies, it was determined that the modified SEB or Differex™ solutions performed best for recovery of bound RD DNA. Furthermore, recovery with these solutions was appreciable, resulting in profiles across 12–13 loci, meaning that little allelic information was lost beyond those loci expected not to amplify due to the presence of restriction cut site sequences.

Direct amplification attempts, regardless of the polymerase employed, did not result in STR profiles. This was true for reactions employing AmpliTaq Gold® and Restorase™ amplification systems. It was determined based on previous studies and controls for the direct amplification assays that the membrane itself competes for free DNA (and likely would compete for other charged molecules). For this reason, the membrane slots that underwent direct amplification were first blocked by treatment with high concentrations of amplification buffer components in an effort to reduce this inhibition. This practice was not successful in generating profiles from membrane-bound template and it was determined that DNA conformation (in the bound/cross-linked state) was likely the cause for inability to amplify the template. Attempts made to amplify DNA recovered from archived membrane lanes were moderately successful in initial attempts in that both AmpliTaq Gold® and Restorase™ DNA polymerase were able to generate DNA profiles. However, the profile generated with the Restorase™ DNA polymerase exhibited shifting at all loci (approximately *n*-2). While major peaks could be estimated at most loci, loci where microvariants are prominent (D18S51 and D21S11) are problematic because it is unclear where major peaks would truly bin. While there are definite compatibility issues surrounding the use of the alternate polymerase in a reaction buffer optimized for 16 loci and another polymerase, the size correspondence to ladder is probably because of the incomplete polyadenylation that must occur for the PowerPlex® 16 system to size correctly against kit ladders (29). While this compatibility issue could potentially be addressed and successfully resolved, the Restorase™ amplification system did not seem to give results superior to those achieved with AmpliTaq Gold® polymerase. In fact, the latter did yield a higher percentage of expected alleles overall. Since the repair mechanisms inherent to the Restorase™ system were not evident on this type of template, the system was not further explored for postrecovery typing.

While both the AmpliTaq Gold® and Restorase™ amplifications yielded profiles consistent with the RFLP lane source, there were a number of alleles detected in each profile that were not attributable to the source. True template for these alleles may have been introduced during manipulation of the membranes, or during the recovery and amplification processes. Alternatively, the alleles may have arisen from DNA deposited on the membrane during RFLP processing. PCR methodology requires far more care when manipulating samples than does RFLP because the level of sensitivity is thousands of times greater (5). While the handling of samples decades ago may not have been under as stringent a technique, it would be unlikely that the recovery

approaches tested herein would result in capture of an appreciable amount of minor contributor DNA from any given RFLP lane. The exact template amount could not be estimated with blot quantification technology, but it can be assumed that very limited amounts of DNA were recovered based on the overall appearance of the profile. So unless a minor contributor introduced by a contamination event comprised a substantial portion of the bound sample, it would be very unlikely to detect contamination from the original extraction and analysis by methods described herein. Furthermore, exogenous DNA placed on the membrane after the original analysis (via manipulation), could be reasonably addressed by pre-washing the lanes. This was the case for this study, where during final assays, the lane strips underwent pre-washes in Tris/SSC. Following recovery and amplification of these lanes, minor contributions were not detected, indicating that this wash, or perhaps more stringent washes, may be useful for removing exogenous template present on these membranes.

While these studies hold promise for recovery of profiles from archived membranes, it is noted that the membrane from the Sedgwick County archive failed to yield amplifiable DNA under any assay conditions. While many aspects of the RFLP processing method were conserved between the PTC and SCRFSC membranes, two very notable differences exist. First, PTC employs Biodyne® B membrane, which does not require UV cross-linking prior to repeated probing/stripping. It is possible that this cross-linking imparts damage to the DNA that inhibits the ability of polymerase to interact with and/or assemble the complimentary DNA strands (10). While repair systems may successfully be marketed in the future to overcome damage because of the depurination, repair systems must also include correction for conformational changes in the double helix caused by thymine dimers, either due to DNA-to-DNA interactions or bonds formed during this process between the DNA and compounds present on modified membrane surfaces.

Another difference between the two membranes tested here are the conditions of storage. The PTC membrane had been wrapped airtight in plastic wrap prior to frozen storage. The SCRFSC membrane was dried and stored between blotting paper prior to long-term storage during which the temperature fluctuated over the course of 6 years.

Because of these differences, studies regarding the applicability of recovery procedures must be conducted by each lab and may need to be tailored depending upon how the archived membranes were stored. While studies have been conducted indicating the benefits of freezing membranes for storage if later probings are to be performed (25), DNA recovery from frozen membranes has not been addressed.

In summary, these studies raise the possibility of recovering DNA from archived membranes and generating STR profiles that could be compared directly to suspect/victim standards or possibly even entered into CODIS for database comparison. While modified versions of casework stain extraction buffer may be sufficient for recovering amplifiable template, more vigorous methods may be needed depending on the materials and protocols employed for the RFLP analysis and the nature of the storage conditions thereafter. The studies proved encouraging for the prospect of generating multi-locus STR profiles from archived membranes.

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