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Optimization of a Simple, Automatable Extraction Method to Recover Sufficient DNA from Low Copy Number DNA Samples for Generation of Short Tandem Repeat Profiles

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Aim To develop an automated, high throughput extraction protocol in order to produce database eligible profiles from fingerprints and other low copy number (LCN) DNA sources.

Methods Extraction of either purified control DNA or buccal cells, for example, with commercial kits was compared to extraction with a simple digestion buffer and a subsequent concentration and purification. Results were evaluated based on the amount of DNA recovered and the completeness of the DNA profiles produced.

Results Simple procedures with fewer steps were superior to commercial kits, such as DNA IQ™ (Promega, Madison, WI, USA) and QiaAmp (Qiagen, Valencia, CA, USA), and other protocols with many manipulations. The optimized protocol included a thirty-minute incubation with 0.01% SDS and proteinase K at 56°C, followed by an incubation at 100°C for 10 minutes. Concentration of the extract and removal of the SDS was accomplished with a Microcon® 100 (Millipore, Bedford, MA, USA), which can be assembled into a 96 well plate, the Microcon-96 Retentate Assembly Plate (Millipore) for automation. The addition of 1 ng Poly A RNA to the Microcon significantly improved DNA recovery.

Conclusion A one-step sample digestion followed by sample concentration/purification minimized sample loss and maximized amplification input. Moreover, this methodology can be easily adapted for automation. Implementation of this protocol, due to the numerous potential sources of LCN DNA samples, will enhance the recovery of biological evidence from crime scenes and may be a source of database profiles.

Humans shed approximately 400,000 skin cells daily. In addition, sweat and sebaceous oil collect cells as they pass through the ducts and pores, and subsequently these cells are brought to the surface of the skin. These sloughed off skin cells can then adhere to handled objects. If these objects are involved in crimes, the collection, and interpretation of the DNA from the deposited cells may prove to be a valuable forensic tool.

Investigators have used transfer DNA in their casework. For example, an armed robber entered a bank and wrote a hold-up note using the pen provided by the bank. The pen was swabbed for epithelial cells and the offender's profile was recovered, despite the large number of prior users. In another case, a pair of gloves was left at a crime scene (1). The insides of the gloves were swabbed and the profile of the wearer was obtained (1). These cases demonstrate the role that trace DNA can play in an investigation where more typical DNA sources, such as blood and semen, are absent.

Despite the success of the cases above and many others like them, only 30% to 50% of the potential sources that were swabbed in Wickenheiser's study (1) actually produced a usable type. According to Alexandrini (2), although some fingerprints produce between 40 to 200 pg of DNA, DNA was not detected in a significant number of fingerprints tested. The amount of DNA deposited in a fingerprint depends upon a person's propensity to shed cells. In addition, large variations among digits within an individual exist (3).

These studies indicate that often there is not sufficient DNA present on an item for analysis with standard short tandem repeat (STR) DNA amplified by polymerase chain reaction typing protocols. However, there may be enough DNA if alterations are made to these standard protocols. For example, altering the amplification process by adjusting the primer concentration and increasing the number of cycles to 34, can appreciably improve these results. Using these altered PCR conditions, Findley et al (4) obtained a genetic profile from a single cell from a buccal source with micromanipulation. The application of this technique results in a powerful forensic possibility that objects handled during the commission of a crime may supply the offender's DNA profile, even if the cellular yield is low.

In order to further enhance DNA profiling, the efficiency of the extraction process should be improved. With each manipulation in extraction, for example changing tubes and washing, some DNA is lost. Multiple manipulations would significantly decrease the yield for low-level samples. In fact, for these samples, picogram losses could translate into the loss of the entire sample. It is therefore advantageous to implement a simple DNA extraction procedure.

Simple extraction procedures are currently used in a variety of scientific procedures, not limited to forensic applications. Researchers, trying to isolate DNA from the bacteria that contaminate pure cultures and wastewater, compared six commercially available kits (Puregene, High Pure PCR Template Preparation Kit, InstaGene, QIAamp Tissue Kit, DNAzol, and Elu-Quick) to four standard methods (lysis buffer with Proteinase K, phenol/chloroform/isoamyl alcohol, microwave treatment, and heat treatment) in terms of sensitivity in a subsequent PCR. They found that the lysis buffer with Proteinase K was superior to all other methods tested and that the commercial kits were not suited for the isolation of DNA from samples containing only a few cells. The other non-commercial methods often involved many handling steps such as transferring to new tubes or adsorption of DNA to matrices, which may have lead to a loss in DNA. Additionally, they surmised that the shorter protocol of the lysis buffer extraction meant fewer opportunities for contamination (5).

Another example of low level DNA extraction is the isolation of fungal DNA from soil. Because many fungi are extremely slow growing, there is a need for a protocol that could isolate DNA from minute quantities of fungal material. To achieve this, Manain et al (6), also utilized a simple detergent lysis buffer. They found this method to be better than the previously used Chelex®-100 (Bio-Rad, Hercules, CA, USA) method for extraction (6). Therefore, the application of these simple extraction methods to low level samples may improve DNA recovery and thus produce a more complete profile.

Materials and Methods

Sample Sources

A DNA stock solution, made from the buccal swabs donated by study participants, was used. DNA was extracted from the swab using Qiagen buccal swab extraction method as per manufacturer's directions (7). Multiple swabs from the same donor were then combined and quantified. Otherwise, AmpFLSTR® control DNA 9947A (Applied Biosystems, Foster City, CA, USA) was used. For experiments examining enzymatic digestion, human embryonic kidney (HEK) cells, counted with a hemocytometer, as well as controlled punches from saliva stains, were used.

DNA Quantification

Quantitation was performed by the Quantiblot slot-blot method (Applied Biosystems) according to the manufacturer's protocol on neat, 1/10 and 1/100 dilutions in triplicate. These values were then averaged to find the approximate DNA concentration of the stock solution. Alternatively, samples were quantitated with the Rotorgene 3000 (Corbett Research) and an ALU based real time PCR assay, according to the method described by Nicklas et al (8) with the exception of the addition of 28,500 X SYBR green I (Molecular

Probes) and 0.525 mg/mL BSA, a 25 µL reaction volume with a 2 µL sample addition, and a standard curve ranging from 0.78 pg/µL to 3200 pg/µL.

DNA Extraction

DNA samples were diluted to the following amounts: 200 pg, 100 pg, 50 pg, 25 pg, and 12.5 pg. These samples were then incubated on a 56°C shaker for two hours with Proteinase K (1.8 mg/mL) and 198 µL of one of the following: 0.01% SDS, 5% Chelex-100 (Bio-Rad, Hercules, CA, USA) beads, and water. This was followed by a ten-minute incubation at 100°C and centrifugation. The same DNA amounts were also extracted using DNA IQ™ (Promega) and QiaAmp (Qiagen) extraction protocols as per their manufacturers' directions (7,9). To test additional detergents, uniform punches from saliva stain cards were incubated with Proteinase K and 0.01% SDS, 0.01% Triton X-100, 0.5% Tween 20, 0.05% Tween 20, or 0.01% NP40, and treated as described above. Sufficient HEK cells to yield 50pg of DNA were extracted in 0.01% SDS with varying amounts of Proteinase K and varying times.

DNA Purification/Concentration

Following extraction, samples were concentrated with the addition of 1 ng of Poly A RNA (Sigma, Saint Louis, MO, USA) or Salmon Sperm DNA using either Microcon®-100, Microcon®-50, or Microcon®-30 (Millipore, Bedford, MA, USA) devices according to the manufacturer's recommendations. Samples were eluted in either 20 µL or 43.5 µL of water as specified, by centrifugation at 3,500 rpm (1,000×g) for 3 minutes. For high throughput, Microcon-100s were affixed in the Microcon-96 Retentate Assembly Plate (Millipore), and were centrifuged as indicated in the results section.

DNA Amplification

For the initial experiments, samples were amplified in Profiler® Plus (Applied Biosystems) for 34 cycles at 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute, followed by an incubation at 60°C for 45 minutes. Samples were also amplified with the AmpFlSTR IdentifilerTM (Applied Biosystems) kit for 31 cycles as follows: 94°C for 1 minute, 59°C for 2 minutes, and 72°C for 2 minutes. Amplification was followed by an incubation at 60°C for 60 minutes (10).

STR Analysis

On an ABI Prism®-3100 Genetic Analyzer with an injection of 3 kV and 20 seconds, 2 µL of the PCR products were analyzed. The minimum relative fluorescence unit (RFU) threshold was set to 75. Through the use of a 10% global filter, the highest peak of each locus was determined and peaks that were less than 10% of this height were not assigned allelic values. Data are presented as the percentage of known alleles determined.

Results

Extraction Procedure

The initial reasoning was that simple extraction procedures with fewer steps are likely to recover more DNA than protocols with many manipulations. This was confirmed by the comparison of two commercial kits, DNA IQ and QiaAmp to the Chelex-100 procedure with Microcon-100 concentration and to the 0.01% SDS lysis protocol with Microcon-100 concentration. After amplification with the AmpFlSTR Profiler Plus kit, the Chelex-100 and the 0.01% SDS methods produced at least three times as many allele calls for 25 pg samples than the QiaAmp and the DNA IQ methods (Fig. 1). Moreover, no alleles were detected with 12.5 pg samples with the commercial kits, whereas partial profiles were generated with both the Chelex and 0.01% SDS extraction methods. For samples larger than 100 pg, however, few differences were discernable among the protocols

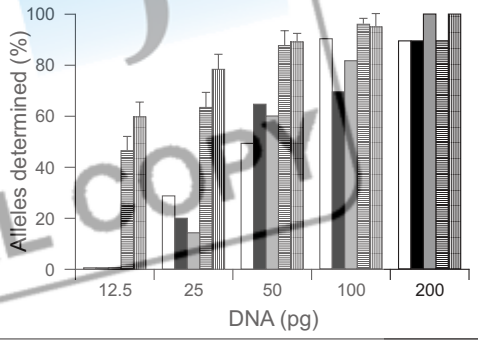

Figure 1. Amplification success for different extraction methods. Open bars – water; closed bars – DNA IQ; grey bars – QiaAmp; horizontally slashed bars – Chelex; and vertically slashed bars – 0.01% SDS. Different amounts of DNA were extracted as described and amplified with Profiler Plus (ABI) reagents for 34 cycles. The 5% Chelex and 0.01% SDS protocols performed best for low amounts of DNA. Values are expressed as a percentage of alleles determined, and are means±standard error of the mean of 4 experiments, n=3.

tested. The Chelex and the 0.01% SDS procedures gave comparable results but the presence of Chelex beads posed problems for automated liquid handling systems and was not pursued for the planned high through put method.

To optimize the results, the 0.01% SDS solution was compared to other detergents such as Triton X-100, Tween 20, and NP40, which are known PCR enhancers (11). Quantitation assays indicated that extracting with 0.01% SDS recovered the most DNA, followed by Triton X-100, Tween 20, and NP40 (data not shown). Nevertheless, the use of these detergents may produce more allelic determinations, since SDS can inhibit PCR. To ascertain the amplification effects of detergents remaining in the extracts following purification, 200µl of 0.01% SDS, 0.005% Triton X-100 or 0.01% Triton X-100 were purified and concentrated to a 25 µ/L volume using Microcon-100 columns. Then, 50, 25, and 12.5 pg of DNA were dissolved in these purified reagents or water and amplified with the AmpFlSTR IdentifilerTM kit (Fig. 2). The percent of assigned alleles was comparable for all of the detergents tested when one accounts for error. Since the use of 0.01% SDS extracts the most DNA and it is sufficiently removed by the Microcon-100, it proved to be the optimal detergent for extraction.

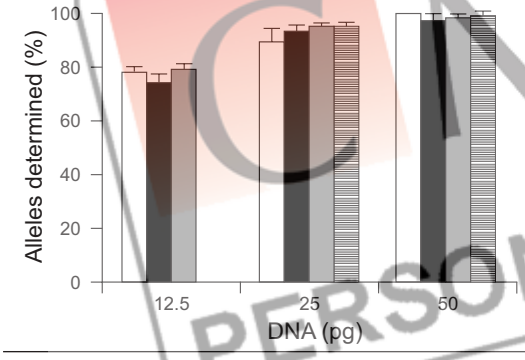

Figure 2. Amplification success for detergents following purification. Open bars – water; closed bars – 0.01% SDS; grey bars – 0.005% Triton X-100; and horizontally slashed bars – 0.01% Triton X-100. SDS and 0.01% Triton X-100 solutions were purified and concentrated to 25 µL of water with Microcon-100 columns and added to known amounts of DNA. The samples were amplified with AmpFlSTR Identifiler™ (ABI) for 31 cycles. Values are expressed as a percentage of alleles determined, and are means±standard error of the mean of 4 experiments, n=2. The 12.5 pg amount was not tested for 0.01% Triton X-100. The data do not indicate PCR inhibition or enhancement of detergents not subjected to purification.

DNA Preservation

As the SDS digests the nuclear membrane, nucleases are released which could potentially destroy DNA. Since Proteinase K inactivates these enzymes, increasing the amount of Proteinase K may improve DNA recovery, a premise that was tested using a defined number of HEK cells. As shown in Figure 3, 0.72 mg/mL of Proteinase K, four times the recommended amount for Chelex digestion with epithelial cells, recovered the most DNA.

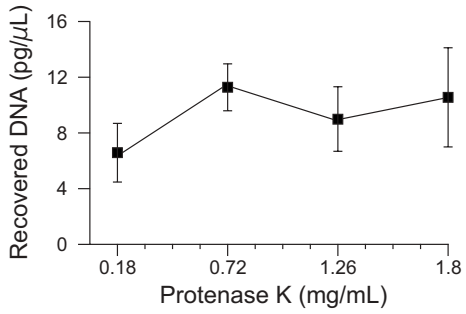

Figure 3. Effect of Proteinase K concentration on DNA yield. The amount of DNA recovered from defined amounts of HEK cells was measured with an ALU-based real time PCR assay. Increasing the concentration of Proteinase K in the digestion buffer to 0.72 mg/mL enhanced DNA recovery. Values from a representative experiment of 2 experiments are expressed as the concentration of DNA in pg/µL, and are means±standard deviation, n=3.

In addition to the Proteinase K concentration, HEK cells were also used to test the effect of the incubation time. A prolonged digestion time may hamper DNA recovery due to degradation of the DNA by nucleases. Alternatively, a short time period may not sufficiently digest the cells. A comparison of 0.5, 1, 2, or 4 hours yielded similar results for all periods tested (data not shown). In order to expedite the process, a thirty-minute increment was selected for the final protocol.

Another possibility for nuclease inactivation are chelating agents, such as Chelex-100 beads or EDTA. However, samples extracted with a 0.01% SDS/14mM EDTA buffer, resulted in profiles that had fewer than 40% of their alleles identified (Fig. 4). It is plausible that the EDTA was not sufficiently removed by the Microcon concentration/purification step and thus inhibited the amplification. To improve the removal of the EDTA, the extracts were treated with an additional 200 µL of water wash step prior to elution. Although wash-

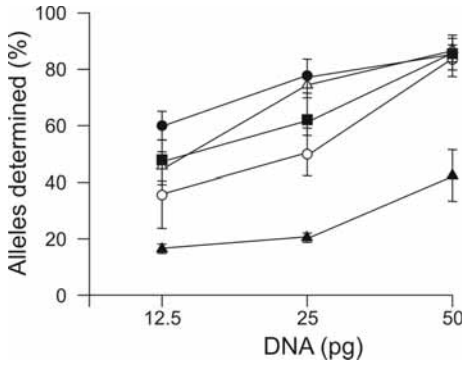

Figure 4. Purification requirements for the SDS/EDTA extraction buffer for productive amplification. Closed circles - SDS; open circles - SDS wash; closed triangles - SDS/EDTA; open triangles - SDS/EDTA wash; and squares - Chelex. Using SDS/EDTA, SDS, and Chelex, 50 pg, 25 pg, and 12.5 pg of DNA were extracted, as indicated, and then concentrated using Microcon-100s. Half of the SDS and SDS/EDTA samples were treated with an additional wash step of 200 µL of water. Samples were amplified with AmpFISTR-IdentifilerTM (ABI) reagents for 31 cycles. A single purification concentration step did not effectively remove EDTA. Values are expressed as a percentage of alleles determined, and are means±standard error of the mean of 4 experiments, n=3.

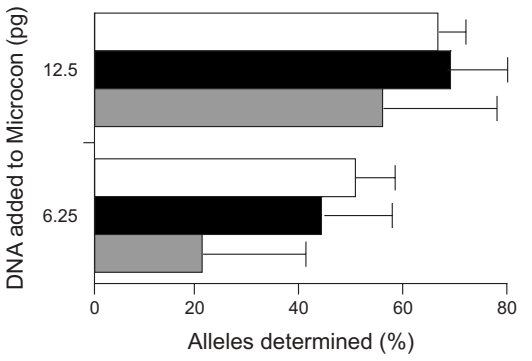

Figure 5. The generation of DNA profiles from low amounts of DNA purified with carrier DNA. Open bars: 1 ng Poly A RNA; closed bars – 1 ng of SS DNA; and gray bars – water. To Microcon-100 (Millipore) membranes pretreated with either 1 ng of salmon sperm DNA or Poly A RNA 6.25 pg, or 12.5 pg of purified DNA were added. Control samples contained only water and DNA. All samples were amplified using AmpFISTR-IdentifilerTM (ABI) reagents for 31 cycles. The addition of Poly A RNA or Salmon Sperm DNA to the Microcon membrane improved LCN DNA Recovery. Values are expressed as a percentage of alleles determined, and are means± standard error of the mean of at least 2 experiments, n=3.

ing recovered the loss of DNA that was generated through use of the agent, the profiles produced were not superior to those generated by 0.01% SDS alone.

Minimizing Sample Loss during Concentration/Purification

To prevent sample loss during the concentration/purification steps, 1 ng of Poly A RNA or 1 ng of salmon sperm DNA were added to the Microcon. Figure 5 shows that both Poly A RNA and salmon sperm DNA improved the DNA allele calls over water alone, but Poly A RNA was slightly more effective at lower DNA amounts. Additionally, the high variability in DNA recovery seen with the water control (indicated by the larger error bar) is reduced when the carriers are used. The carriers therefore make DNA recovery more reproducible. Different amounts of PolyA RNA were tested from 1 ng to 10 ng; no difference in DNA recovery was detected (data not shown).

In order to further improve recovery of DNA, especially of degraded or small pieces of DNA, the Microcon-50 with a smaller pore size was compared to the Microcon-100. Samples purified with the Microcon-50 recovered the same amount of DNA as those purified with the Microcon-100 (Fig. 6). However, samples purified with

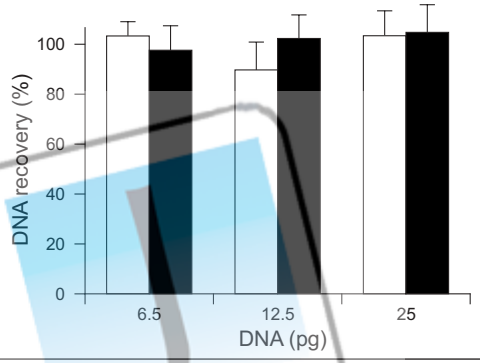

Figure 6. DNA recovery following purification with the Microcon-50 and the Microcon-100 membranes. Open bars – Microcon-100; and closed bars – Microcon-50. Three different DNA amounts were purified with Microcon-50 or Microcon-100 devices pretreated with 1 ng of Poly A RNA as indicated. Samples were concentrated to 25 µL and measured with ALU based real time PCR. The Microcon-50 and the Microcon-100 recovered similar amounts of LCN DNA. Values are expressed as the percentage DNA recovery and are a means±standard deviation, n=3.

the Microcon-30, with even smaller pores, produced at least 50% fewer determined alleles than those purified with the Microcon-100 (data not shown). Adding an additional wash step did improve this result, which indicates that the normal procedure using the Microcon-30 did not remove 0.01% SDS sufficiently (data not shown).

Elution Volume

The final aspect of the modified use of the individual Microcon-50 or -100s was their elution volume, and thus the concentration of the LCN DNA extract to be amplified. A comparison of elution volumes demonstrated that increasing the elution volume, even by 10 microliters from 20 μL to 30 μL, significantly dilutes the extract, resulting in fewer alleles assigned (data not shown).

In order to determine the lowest elution volume that would not compromise DNA recovery from a Microcon membrane, 6.25 pg, 12.5 pg, and 25 pg of DNA were concentrated in triplicate with a Microcon-100 pretreated with Poly A RNA, and eluted in varying volumes from 10 μL to 25 μL. For very low amounts of DNA, such as 12.5 pg, 33% less DNA was recovered with an elution volume of 15 μL as compared to one of 25 μL (Fig. 7). An attempt was made to increase the DNA yield from a small elution volume through shaking the Microcon prior to elution. However, this did not prove successful (data not shown).

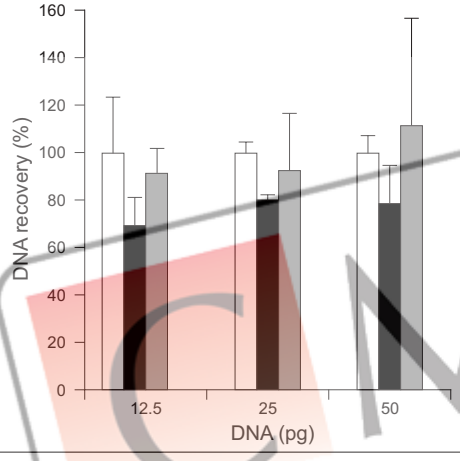

Figure 7. DNA yields from different elution volumes from the Microcon. Open bars – standard; closed bars – 15 μL elution volume; and gray bars – 25 μL elution volume. Three different DNA amounts were concentrated with a Microcon 100 device to either 25 μL or 15 μL, as indicated. Samples and controls were measured with ALU based real time PCR. A 25 μL elution volume recovered more LCN DNA from a Microcon-100 membrane than an elution volume of 15 μL. Values from a representative experiment of two experiments are expressed as the percentage of DNA recovery, and are means± standard deviation, n=3.

High-throughput Purification

Similar to the extraction procedure, the concentration protocol must also be compatible with an automated high throughput system. The Microcon-96 Retentate Assembly plate accommodates 96 Microcon tubes and can be implemented in a robotic system. The protocol for the retentate plate is the same as that for the individual Microcons with a few exceptions. First, because the filtrate plate can only hold 250 μL, the total volume must be divided into two applications. Due to the differences in centrifugal force across the plates, the spin time and speed must be adjusted to produce samples with consistent elution volumes. With the individual Microcons, samples were checked manually after elution to ensure that their volumes were near 20 μL. This is not feasible for the retentate plates.

The speed and time recommended by Millipore resulted in a low DNA recovery, with only an average of 36% correctly identified alleles, and when 20 μL was placed on the Microcon membrane, the resulting elution volume was, on average, only 11 μL. To remedy this problem, the centrifugation speed was reduced and the time adjusted. With each reduction in either centrifugation time or speed, there was an improvement in the percentage of correctly identified alleles and the elution volumes (Fig. 8). Centrifugation conditions of 1,000 rpm (203×G) for 13 minutes, restored the percentage of assigned alleles to 81.53% with an average elution volume of 20.94 μL. This combination of speed and times rendered the Microcon as effective for low amounts of DNA in the retentate plate, as when it was used individually.

Following optimization of the centrifugation conditions, the consistency in DNA recovery throughout every position of the retentate plate and the potential for contamination were examined. In a 96-well retentate plate that had been pretreated with 1 ng of Poly A RNA in 50 μL of water, 50 pg of DNA in 200 μL of 0.01% SDS were purified with Microcon-100s. The columns were covered with parafilm to prevent contamination during filtration. Following centrifugation at 1,000 rpm for 13 minutes in a swing bucket rotor, 250 μL of water was added to each Microcon column and the device was centrifuged for an additional 13 minutes and then 3 more minutes at 1,000 rpm, but was rotated 180°. DNA was eluted with 25 μL of water with centrifugation at 3,500 rpm for 3 minutes. The DNA yield from purification of 96 samples of 50 pg of DNA showed less than 23% variation from the expected value from row to row

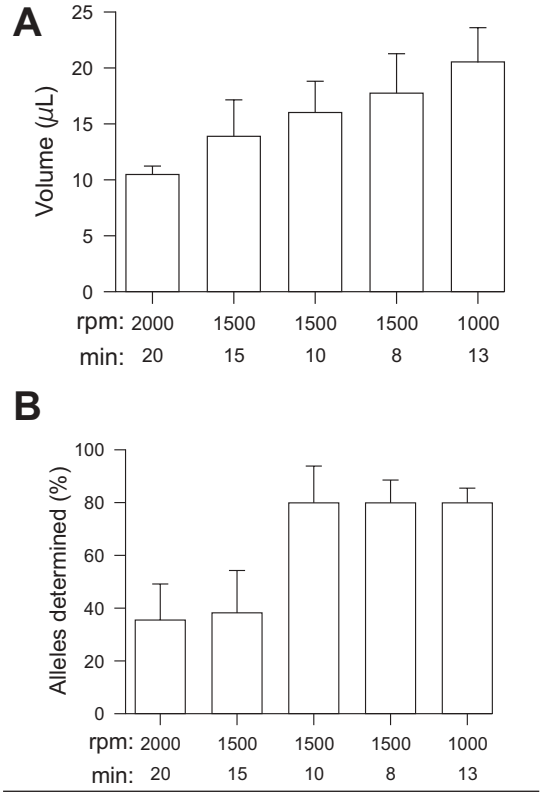

Figure 8. The effects of centrifugation conditions with the Microcon Retentate Plate on the elution volume and amplification success Microcon-100s were placed into a 96-well retentate plate and 50 μL of water was added to each membrane. Following the addition of 25 pg of DNA in 200 μL of water, plates were centrifuged under various conditions. Samples were eluted with 20 μL of water for 3 min at 3,500 rpm and amplified using AmpFlSTR-Identifiler™ for 31 cycles. Centrifugation at 1,000 rpm for 13 min increased the volume recovered and the profiles generated. Effects on (**A**) volume of the elutant; and (**B**) the percentage of alleles determined; both are means±SD, n is at least 9.

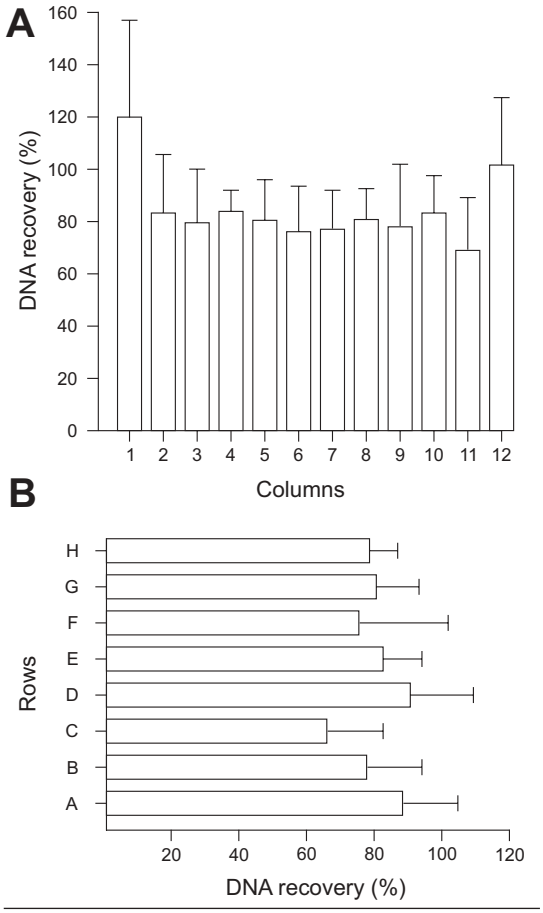

Figure 9. DNA recovery yield for the retentate plate by row and by column. 50 pg of DNA in 200 μL of 0.01% SDS were purified with Microcon-100s in a 96-well retentate plate that was centrifuged twice at 203×G for 13 min, rotated 180° and centrifuged an additional 13 min and then 3 more min. Elutant volumes were recorded and DNA quantity was measured with ALU-based real time PCR. Microcon-100s positioned in a Microcon Retentate Plate in columns 3-10 only recovered consistent yields of LCN DNA. Values from a representative experiment of two experiments are expressed as the percentage difference from the expected value, and are means±SD. Recivery yields from (**A**) Microcons in each column of the plate; and (**B**) Microcons in columns 3-10 on each row.

or column to column, excluding the values from wells in columns 1-2 and 11-12 (Fig. 9). Columns 1-2 and 11-2 deviated 25-31% from the expected value.

In order to ascertain whether DNA migrates to adjacent wells during the elution step, 1 ng of DNA was placed in each microcon arranged in a checkerboard pattern in columns 3, 5, 7, and 9 only. Water was added to the remaining empty wells prior to elution and the DNA in these elutants was measured. All values were below the negative threshold of the ALU quantitation assay (<0.1 pg/μL) and no indication of contamination was found.

Discussion

The production of reliable profiles from low copy number DNA samples can only be successful when each component of the procedure is optimized. DNA must be sufficiently extracted and subsequently supplied to the amplification. Each step must not only minimize DNA loss, but must also maximize the sensitivity.

Several extraction methods were compared for their ability to extract DNA while mini-

mizing DNA loss. The method that involved the fewest manipulations and thus the least potential for sample loss was a simple detergent lysis buffer containing 0.01% SDS with Proteinase K or Chelex-100, which produced superior results than procedures requiring multiple washing steps, tube transfers, or adherence to matrices. For example, some DNA may irreversibly bind to a matrix and although this loss is inconsequential for high copy number DNA samples, for Low Copy Number DNA samples, this loss, albeit minute, is crucial. Regarding robotics, the detergent method is preferable since avoiding automated aspiration of the Chelex-100 resin, coupled with complete recovery of the sample digest, proved problematic.

The SDS extraction method's success is likely due to its simplicity, not the choice of detergent. SDS is a strong detergent that may not be necessary for low copy number samples. Moreover, although at concentrations of 0.01%, SDS does not inhibit amplification (Fig. 3), other detergents exist that are known PCR enhancers (12). Therefore, several such detergents were tested. However, since the amount of detergent that reaches the amplification after purification is diminutive, no enhancements were demonstrated with the tested detergents.

To circumvent any potential loss of DNA in the digestion process, several parameters were optimized to inactivate nucleases that degrade DNA. For example, increasing the amount of Proteinase K enhanced DNA recovery although the relationship was not linear. Furthermore, SDS increases the efficacy of proteinase K and therefore, the plateau may be reached at an earlier concentration with our digestion buffer. Perhaps the proteinase K is functioning optimally at 0.72 mg/mL and therefore, another agent may be required to increase recovery. Alternatively, the ten minute incubation at 99°C may not be sufficient to inactivate the proteinase K itself which could subsequently hamper amplification. The finding that a prolonged digestion period does not reduce DNA recovery suggests that our 0.01% SDS/Proteinase K buffer sufficiently preserves DNA. Since a LCN DNA sample is inherently small, thirty minutes is adequate for digestion.

In order to mimic the role of Chelex-100 beads as a chelating agent, EDTA was added to the digest buffer. The poor profiles produced suggest that the EDTA, an inhibitor of amplification, was not completely removed with the purification step. Although an additional wash step improved the amplification results, the resultant number of determined alleles was not greater than that produced with 0.01% SDS alone or with Chelex, and thus the addition of EDTA was not advantageous. Interestingly, increasing the number of wash steps compromised the generation of profiles for samples digested with 0.01%SDS. This is likely due to an inherent loss of DNA with the concentration process; DNA may become irreversibly trapped within the pores of the Microcon membrane.

Therefore, in order to enhance DNA recovery from the Microcon, Poly A RNA or salmon sperm (ss) DNA were added. It is plausible that the surplus of Poly A RNA or ss DNA in relation to extracted DNA, preferentially occupied this space in the membrane, thus improving DNA recovery. These agents could potentially have served to precipitate the DNA as well (Robert Beaver, the BODE Technology Group, Inc, personal communication). Although both produced similar results, Poly A RNA was preferred since ss DNA could potentially amplify spurious alleles.

Very degraded DNA may be smaller than the threshold of the Microcon 100 membrane pore size. Therefore, Microcon-50s and Microcon-30s were explored. The reduction in the amount of alleles generated with the Microcon-30 attests to the possibility that the SDS was not adequately removed, and thus inhibited the amplification. Although an additional wash step restores a robust amplification, this step is not worth the advantage of recovering very small pieces of DNA because fragments this short are beyond the scope of the tandem repeats utilized in our testing.

Reducing the final sample volume serves to increase the DNA concentration and thus amplify downstream results. However, the amount of water that is applied to the Microcon membrane for elution proved to be a limiting factor. Volumes below 20 µL do not completely cover the Microcon membrane. DNA in these areas of the membrane that are not saturated with water is likely not eluted, diminishing the yield. Although shaking the Microcon devices would seemingly distribute the water, it may not have been a sufficient action to overcome the decreased volume.

To implement this concentration and purification process in a robotic system, the Microcon-96 Retentate Assembly plate was em-

ployed. Although the device utilizes the same Microcon membrane columns, the orientation of the columns in the centrifuge rotor influences the centrifugation speed and time. The consequent reduction in DNA recovery and elutant volume may be attributed to over-drying of the Microcon membrane due to an excessive spin time and speed. If the membrane is too dry, it may absorb the elution buffer (water), thus reducing the final elution volumes. Additionally, the over-dried DNA pellet may be difficult to resuspend. Hence, fewer alleles were produced (Peter Rapicko, Millipore, 2002; personal communication). It follows that decreasing the centrifugation speed while increasing the time removed the filtrate without compromising DNA recovery or the elutant volume.

Since the position of the plate during centrifugation is vertical to the center of the rotor, the periphery of the plate, columns 1-1 and 11-12, reside at the maximum angle. Accordingly, these wells displayed the most variability in DNA yields. Therefore, the use of these columns should be avoided. Excluding these wells, the average DNA yield was within 82 ± 13% of the expected value, representing a slight decrease from the absolute amount of DNA recovered from individual Microcon columns. This decrease can be attributed to the elution step. Centrifugation with a swing bucket rotor requires more time to reach speeds and therefore it is plausible that the samples must be centrifuged for a longer period in order to maximize recovery.

Regarding contamination, the amount of DNA in the blank wells surrounding the positive wells was below the background threshold of detection. Nevertheless, particularly when working with LCN DNA samples, it is prudent to be extra careful and not place any DNA samples directly adjacent to one another in the retentate plate. Positioning samples in a checkerboard pattern only in columns 3, 5, 7, and 9 ensures the integrity of LCN DNA samples while processing 64 samples in four plates, which are accommodated in one centrifuge.

Overall, extraction of LCN DNA samples with 0.01% SDS and Proteinase K followed by purification and concentration with Microcon-100s in a Microcon-96 Retentate Assembly Plate recovers the most DNA and is adaptable to robotics, compared to the methods tested. Collectively, these procedures facilitate DNA typing of small quantities of DNA including fingerprints or handled objects.

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