



## Forensic Population Genetics – Original Research

## Assessment and mitigation of DNA loss utilizing centrifugal filtration devices

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## ABSTRACT

Maximizing DNA recovery during its isolation can be vital in forensic casework, particularly when DNA yields are expected to be low, such as from touch samples. Many forensic laboratories utilize centrifugal filtration devices to purify and concentrate the DNA; however, DNA loss has been reported when using them. In this study, all centrifugal filtration devices tested caused substantial DNA loss, affecting low molecular weight DNA (PCR product) somewhat more than high molecular weight DNA. Strategies for mitigating DNA loss were then examined, including pre-treatment with glucose, glycogen, silicone (RainX<sup>®</sup>), bovine serum albumin, yeast RNA, or high molecular weight DNA. The length of pre-treatment and UV irradiation of pre-treatment reagents were also investigated. Pre-treatments with glucose and glycogen resulted in little or no improvement in DNA recovery, and most or all DNA was lost after silicone pre-treatment. Devices pre-treated with BSA produced irregular and uninterpretable quantitative PCR amplification curves for the DNA and internal PCR control. On the other hand, nucleic acid pre-treatments greatly improved recovery of all DNAs. Pre-treatment time and its UV irradiation did not influence DNA recovery. Overall, the results show that centrifugal filtration devices trap DNA, yet their proper pre-treatment can circumvent that loss, which is critical in the case of low copy forensic DNA samples.

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## 1. Introduction

DNA extraction is a vital step in genetic analysis of forensic evidence, and maximizing DNA recovery is critical, particularly when only trace amounts are present [1,2]. Many extraction methods utilize filters designed to remove unwanted materials and concentrate the DNA [3,4]. These come in various configurations based on nominal molecular weight limit (NMWL), typically measured in kilodaltons (kD), wherein upon centrifugation a (e.g.) 10 kD filter retains molecules larger than 10 kD, while smaller molecules flow through. For forensic purposes filtration often follows a phenol/chloroform based DNA extraction, and it is the retentate, which includes the DNA, that is preserved. The retentate can subsequently be washed with a buffer or water to further cleanse the DNA. An important function of these filters is removal

of salts and PCR inhibitors such as indigo dye [5], heme from blood [6], calcium and collagen from bone [6,7], organic acids in soil [6–9], etc. Given their functionality and ease of use, forensic DNA laboratories have incorporated such filters into their standard operating procedures for some time [e.g., 10,11].

There are multiple manufacturers of centrifugal filtration devices, including Pall, Vivaproducts, and Millipore. The actual makeup of the devices, along with the volume of liquid that can be placed into them, differ among products, as noted in their accompanying literature. As examples, Pall's Microsep<sup>™</sup> Advance filters are made from polyethersulfone, while the filter housing is polyethylene. Vivaproducts' Vivacon<sup>®</sup> 500 and Vivaspin<sup>®</sup> 2 membranes are made of polyethersulfone, cellulose triacetate, or Hydrosart<sup>®</sup>, which is similar to regenerated cellulose, and housings are polypropylene or polycarbonate. Millipore has, and does, manufacture a variety of centrifugal filter devices, including Centricon<sup>®</sup>, Microcon<sup>®</sup>, and Amicon<sup>®</sup>. Amicon<sup>®</sup> devices include Ultrafree-MC, Ultrafree-CL, Ultra 15, Ultra 0.5, and others, whose volumes range from 0.5 to 15 ml. The filters generally utilized for forensic purposes are regenerated cellulose, with the exception of Amicon<sup>®</sup> Ultrafree devices, which have membranes made of

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hydrophilic polyvinylidene fluoride or hydrophilic polytetrafluoroethylene. The housings for Centricon<sup>®</sup> and Microcon<sup>®</sup> devices are made of polycarbonate, Amicon<sup>®</sup> Ultrafree devices have housings made of polypropylene, and Amicon<sup>®</sup> Ultras have housings made of copolymer styrene/butadiene.

Norén et al. [4] examined DNA loss from Microsep<sup>™</sup> and Amicon<sup>®</sup> filter devices using both purified DNA and mock forensic samples consisting of blood, saliva, semen, and hair. They found that 68–86% of the DNA was lost using Microsep<sup>™</sup> filters, and 30–38% was lost using Amicon<sup>®</sup> filters. No conjecture was made about why or where this loss occurred, nor was there any suggestions for preventing DNA loss. Garvin and Fritsch [3] also reported DNA loss when using Microcon<sup>®</sup> and Amicon<sup>®</sup> devices. Mock forensic samples (buccal cells, blood, and semen) were tested, and DNA loss from Amicon<sup>®</sup> devices was lessened using centrifugation speeds substantially slower than the manufacturer recommends, and thus centrifugation times much longer than the manufacturer promotes. The authors suggested that DNA was being trapped within the membrane.

During recent studies at the Michigan State University Forensic Biology Laboratory, DNA loss using centrifugal filtration devices was noticed. Therefore, an in-depth study was performed on Amicon<sup>®</sup> filters with various molecular weight cutoffs, as they are widely used by forensic laboratories, including ours. The first goal of this research was to determine if, and to what extent, DNA was being lost. Then, the loss of both high molecular weight DNA and a 302 bp PCR product were assayed across a variety of filter NMWLs. Finally, multiple strategies for alleviating DNA loss through filter pre-treatment prior to DNA filtration were tested.

## 2. Materials and methods

### 2.1. Sample preparation

Human K562 DNA (Promega, USA) acted as the high molecular weight DNA for this study. Low molecular weight DNA was represented through a 302 bp segment of the bovine (*Bos primigenius taurus*) mitochondrial ATPase 8 gene (National Center for Biotechnology Information BLAST Accession NC\_006853), for which PCR primers (forward 5'-GTG ACA TGC CGC AAC TAG AC-3' and reverse 5'-GGA GGG TTA CAA AGC GAT TG-3') were designed using Primer3 [12]. Primers and probes were produced by Integrated DNA Technologies, Inc. (USA), Sigma–Aldrich (USA), or Applied Biosystems (USA). The ATPase 8 gene segment was amplified from a 1:100 dilution of bovine total DNA [13]. The PCR product was purified using a DNA Clean & Concentrator<sup>™</sup>-5 kit, per the manufacturer's instructions (Zymo Research Corporation, USA). A 1:100,000 dilution of the PCR product was used for subsequent experiments, which resulted in Ct values between 25 and 30 cycles, similar to those obtained for the starting concentration in the human DNA quantification assay (below).

Amicon<sup>®</sup> Ultra-0.5 30K centrifugal filter devices (Millipore, USA) were used in preliminary experiments. A DNA aliquot of 28  $\mu$ l (the approximate column retention volume) was made containing 14 ng of high molecular weight human DNA in low TE (10 mM Tris, pH 7.5; 0.1 mM EDTA). A 1.2  $\mu$ l aliquot was removed, representing the starting DNA concentration (0.5 ng/ $\mu$ l; subsequent DNA quantifications considered previous DNA removal). Three hundred microliters of low TE was added to the remaining DNA, and the filter was centrifuged for 10 min at 14,000  $\times$  g, after which 1.2  $\mu$ l of retentate was removed. Three hundred microliters of low TE was again added to the filter and the process was repeated, for a total of three washes. This procedure was replicated for low molecular weight DNA, beginning with 1  $\mu$ l of the diluted 302 bp product added to 27  $\mu$ l of low TE.

### 2.2. DNA quantification

DNAs were quantified on an iCycler<sup>™</sup> Thermal Cycler and iQ<sup>™</sup>5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA). Data were analyzed using iQ<sup>™</sup>5 Optical System Software. High molecular weight DNA was quantified using a Quantifiler<sup>®</sup> Human DNA Quantification kit (Applied Biosystems) following the manufacturer's protocol. An 88 bp segment of the low molecular weight product was quantified using 7.5  $\mu$ l of iQ<sup>™</sup> Supermix (Bio-Rad), 0.9  $\mu$ l of 10  $\mu$ M forward (5'-CAA AAC ACC CCT TGA GAA ACA-3') and reverse (5'-AGG GTT ACG AGA GGG AGA CC-3') ATPase 8 primers, 0.25  $\mu$ l of 15  $\mu$ M ATPase 8 probe (5'-6FAM-CCT CTT TTA TTA CCC CTG TAA TTT T-BHQ1-3'), 0.125  $\mu$ l Taq polymerase (Syzygy Biotech, USA), and 1.375  $\mu$ l distilled water. Also included was a 77 bp internal PCR control (IPC) [15], for which 0.75  $\mu$ l of 20  $\mu$ M forward and reverse IPC primers, 0.25  $\mu$ l of 15  $\mu$ M probe, and 1  $\mu$ l of a working concentration IPC template (1:1 billion dilution of a 100  $\mu$ M solution of the IPC template) were added. This master mix (13.8  $\mu$ l) and 1.2  $\mu$ l of the template DNA were combined for a reaction volume of 15  $\mu$ l.

### 2.3. Filter pre-treatments

Amicon<sup>®</sup> 30K filters were pre-treated with glucose (Sigma–Aldrich), glycogen (Fisher Scientific, USA), RainX<sup>®</sup> (a hydrophobic silicone polymer; ITW Global Brands, USA), bovine serum albumin (BSA) (Fisher Scientific), ribonucleic acid (RNA) from Baker's yeast (*Saccharomyces cerevisiae*; Alfa Aesar<sup>®</sup>, USA), or salmonid (*Oncorhynchus mykiss*) high molecular weight DNA (produced in house). Solutions were made in low TE at the concentrations noted in Table 1. One microliter of the solution was added to 499  $\mu$ l of low TE, which was placed on the filter for 10 min or centrifuged immediately (0 min). Filters were centrifuged at 14,000  $\times$  g for 10 min, after which high or low molecular weight DNA was added and processed as above. Based on the results, 3K, 10K, 50K, and 100K Amicon<sup>®</sup> filters were then tested, with and without pre-treatment using 1  $\mu$ l of 10  $\mu$ g/ $\mu$ l yeast RNA in 499  $\mu$ l low TE (0 min). High and low molecular weight DNA recovery was assayed as detailed above, using 10 min centrifugation times, except for the 3K (30 min) and 10K (15 min) devices, resulting in approximately 28  $\mu$ l of retentate in all cases. The effects of ultra-violet irradiation of the yeast RNA prior to filter pre-treatment was examined by irradiating RNA solutions at 1  $\mu$ g/ $\mu$ l or a much lower 0.04  $\mu$ g/ $\mu$ l for 5 or 10 min (approximately 2.5 or 5.0 J/cm<sup>2</sup> respectively), after which 1  $\mu$ l of each concentration was added to 499  $\mu$ l of low TE and placed on separate filters. Following centrifugation DNA was added, filtered, and aliquots removed and quantified as described above.

## 3. Results

### 3.1. DNA loss using Amicon<sup>®</sup> filter devices

As noted, we have observed DNA loss when using various brands of DNA filter devices. Surprisingly however, no DNA was

**Table 1**  
Summary of filter pre-treatment methods.<sup>a</sup>

Treatment	Concentrations
Glucose	0.02, 0.2, and 20 mg/ml
Glycogen	0.0002, 0.02, 0.2, and 20 mg/ml
RainX <sup>®</sup> <sup>b</sup>	Neat
BSA	1 $\mu$ g/ml
Yeast RNA	1, 2, 5, 8, 10, 40, 50, 100, and 200 $\mu$ g/ml
Salmonid DNA	1 mg/ml

<sup>a</sup> One microliter of each solution was added to 499  $\mu$ l of low TE and placed on the device. Total  $n = 48$ .

<sup>b</sup> Five hundred microliters of RainX<sup>®</sup> was added to the device.

**Table 2**  
Percent DNA loss using Amicon<sup>®</sup> filter devices without pre-treatment.<sup>a</sup>

Molecular weight cutoff of filter device	Average (%) high molecular weight DNA loss <sup>b</sup>	Average (%) low molecular weight DNA loss <sup>c</sup>
3K	52	81
10K	23	76
30K	69	90
50K	85	86
100K	79	58
Average	62	78

<sup>a</sup> Tests were run in duplicate.<sup>b</sup> Initial concentrations for high molecular weight DNA were 0.5 ng/μl (in 28 μl).<sup>c</sup> Low molecular weight amplicon was 302 bp.**Table 3**  
Percent of DNA loss using Amicon<sup>®</sup> filter devices following pre-treatment with 10 μg of RNA.<sup>a</sup>

Molecular weight cutoff of filter device	Average (%) high molecular weight DNA loss <sup>b</sup>	Average (%) low molecular weight DNA loss <sup>c</sup>
3K	42	40
10K	28	0
30K	2	0
50K	0	0
100K	0	0
Average	14	8

<sup>a</sup> Tests were run in duplicate.<sup>b</sup> Initial concentration of high molecular weight DNA was 0.5 ng/μl (in 28 μl).<sup>c</sup> Low molecular weight amplicon was 302 bp.

detected in the flow-through fraction of the devices (data not shown), indicating that the DNA was being trapped on the device itself. Further testing demonstrated that Amicon<sup>®</sup> filter devices of all molecular weight cutoffs resulted in poor DNA recovery (Table 2), with, on average, a greater percentage of low molecular weight DNA trapped than high molecular weight DNA.

### 3.2. DNA loss following pre-treatment of filter devices

Tests using Amicon<sup>®</sup> 30K devices showed that glucose and glycogen pre-treatment resulted in little or no improvement in DNA recovery respectively, regardless of their starting concentrations (displayed in Table 1). Even worse results were obtained from silicone pre-treated devices, where most or all DNA was lost. BSA pre-treatment resulted in highly irregular amplification curves, including the IPC. In contrast to these, both yeast RNA and salmonid DNA pre-treatment resulted in greatly improved DNA recovery and good amplification curves. Increasing the amount of RNA in pre-treatments did not show any trend in DNA recovery (data not shown), thus 10 μg of RNA was used to pre-treat the filters in subsequent experiments. There was no difference in DNA loss between 0 or 10 min pre-treatments.

RNA pre-treatment of different NMWL devices resulted in substantially less DNA loss (Table 3), where typically all DNA applied to the filter device was recovered. The notable exception was high molecular weight DNA from the 3K and 10K devices, which showed little improvement. Further testing of UV irradiated yeast RNA indicated that the process had no deleterious effect on subsequent DNA recovery (data not shown).

## 4. Discussion

The goals of this research were first to determine if, and to what extent, DNA is lost when utilizing filtration devices for DNA concentration or purification, and to then find a way to reduce its

loss when using such filters. Manufacturers of these centrifugal devices often report 90% or higher DNA recovery either in general [16] or for PCR products [14]. However, if a large amount of starting DNA is applied to the centrifugal filtration device, the percentage loss may be small even though it represents a substantial quantity of DNA from a forensic standpoint. For instance, if the same quantity of DNA is lost from a buccal and a touch sample, the percentage loss could be far different, with the latter representing most or all of the DNA available.

Some DNA loss using centrifugal filtration devices has also been noted by others [e.g. 2,3,17]. One of these [3] specifically examined Amicon<sup>®</sup> and Microcon<sup>®</sup> devices, and found that at the manufacturer's recommended centrifugation of 14,000 × g, approximately 60–75% of commercially prepared human DNA placed on the filters was lost, even though wash steps were not undertaken as would typically be done. Substantially lower g forces improved DNA yields, however centrifugation times increased accordingly. Testing of mock forensic samples (blood, semen, and saliva) showed an average of 57% DNA loss even when using the much reduced g force. All of those tests were performed on high molecular weight DNA, which in the work presented here suffered less loss than did low molecular weight DNA, which is often associated with forensic casework.

In the current study, filtration devices were pre-treated to examine if this might prevent nucleic acid from adhering to the membranes or membrane housings. Silicone treatment has long been used to prevent DNA from sticking to plastics [e.g., 18], however this apparently damaged the devices, as no DNA was recovered. Glucose pre-treatment had no substantial impact on DNA loss, while glycogen pre-treatment showed inconsistent results, sometimes diminishing DNA loss but other times not. Further optimization of glycogen pre-treatment may be warranted, however it was not pursued in this study. BSA pre-treatment resulted in irregular quantitative PCR amplification curves, both for the added DNA and the IPC. The reason for this is unknown, although inaccurate quantitation results are clearly not acceptable. Given the variety of undesirable outcomes using the above pre-treatments, their use was not pursued further.

The two nucleic acids tested—yeast RNA and salmonid DNA—both substantially reduced DNA loss on the devices. However, animal DNA has the potential to cross react with PCR primers designed for other species, while RNA cannot act as a template for PCR. Also, our laboratory regularly undertakes species identification using universal mitochondrial DNA primers that amplify all animal species, including salmonids; thus, introduction of this DNA could cause misleading or erroneous results. Further, yeast RNA is inexpensive, is available at high purity, and we found it can be UV irradiated without reducing its effectiveness. Therefore, for all of these reasons its use is favored.

In the end, pre-treating filtration devices such as those tested here seems to be critical when low copy number DNAs are being purified, which regularly occurs in forensic laboratories. In our laboratory we have incorporated RNA pre-treatment for samples that are expected to harbor low amounts of DNA, particularly touch samples. Equally critical is pre-treating filters when a DNA sample needs to be concentrated, as the DNA level is presumably already very low, all of which could be lost on the device. In contrast, we do not pre-treat filters from rich DNA sources such as buccal swabs, where the DNA is in sufficient quantity that some loss does not impair results. Including this extra step is not prohibitively time consuming, and it would be possible to have a stock solution of pre-treatment RNA available that could be added to filters as needed (in the current study this was not done as different RNA concentrations were examined). It would be preferable that manufacturers themselves pre-treated these devices or developed low-binding plastics or filters, and it seems likely there is ample

demand for them in forensic laboratories. In the meantime, the level of low copy DNA loss on these filtration devices is unacceptable for forensic laboratories, and remedying it through pre-treatment seems a necessary step when DNA quantities are limited.

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