Application Note

Fast flow, high recovery and robust PCR using Microcon® PCR Grade filters

Abstract

Maximum recovery of pure intact genomic DNA from crude biological samples is the critical first step in any DNA analysis workflow. Typically, the process involves cell disruption, chemical or ion exchange-based extraction of the nucleic acid fraction and concentration of the purified DNA sample. While ethanol precipitation is commonly used for DNA concentration in research settings, an effective alternative is ultrafiltration via small spin columns such as the Microcon® DNA Fast Flow device. However, one overriding concern in the field of genetic testing is consumable product contamination with exogenous DNA during their assembly and packaging. To minimize this potential, a dual cycle ethylene oxide (EtO) treatment is employed to disrupt or fragment amplifiable DNA, thereby reducing the risk of false positives or uninterpretable results. To meet this concern, Merck Millipore has released the Microcon® DNA Fast Flow PCR Grade filter, an EtO-treated form of the parent device that has been shown here to match the original Microcon® device in performance.

Figure 1. (left to right): Concentrate tube, Microcon® filter device in filtrate tube, Microcon® filter device

Introduction

For forensic and genetic testing laboratories, it is paramount that recovery and potentially long-term storage of purified samples occur in the complete absence of amplifiable DNA contaminants. While the advent of more robust DNA recovery techniques and analytical platforms have greatly increased assay sensitivity (< 200 pg), it has also resulted in a significant rise in the incidence of exogenous DNA detection[1, 2]. Although the consumables used in such applications are prepared in cleanroom environments, this process cannot completely eliminate the risk of DNA contamination during manufacture and packaging[3]. Unfortunately, most treatment methods rely on high heat, pressure, or the use of abrasive chemicals and are thus not compatible with devices containing membranes, rubber, or plastics. In contrast, EtO-based treatment is suitable for use with a wide range of materials. EtO is a powerful alkylating agent; alkylation results in DNA fragmentation, rendering these molecules incapable of being amplified, thus eliminating the potential for contaminating interference in downstream sample analysis[4, 5, 6].

Once the genomic DNA is extracted from the crude sample, it is often necessary to concentrate and exchange the buffer of the sample before it can be analyzed. While there are numerous methods for concentration, ultrafiltration (UF) devices, such as Microcon® filters, use centrifugal force and semi permeable membranes to fractionate biomolecules based upon their size and shape. The low nonspecific binding of the Ultracel® membrane and other device components, coupled with an inverted recovery spin design, ensure high sample recovery rates, with fast and effective sample processing. DNA samples processed by UF can be routinely concentrated in minutes, with high retentate recovery and without the use of co-precipitants. UF devices can also be used to exchange buffer of samples by diafiltration. In this process, the sample is concentrated, then diluted to the original volume with the desired buffer and concentrated again, thus "washing out" the original solvent.

Here, genomic DNA was processed with a simulated forensic buffer or in TE buffer, and the data were used to compare the performance of the Microcon® DNA Fast Flow device with and without prior EtO exposure (dual cycle treatment, 2x EtO). The results demonstrate that dual EtO treatment does not impact the device's flow properties or genomic DNA recovery. Further evidence demonstrates that quantitative Polymerase Chain Reaction (qPCR) reactions are not inhibited by the dual cycle EtO process used during the manufacturing of Microcon® DNA Fast Flow PCR grade devices.

Materials and Methods

Flow Testing

Human Genomic DNA (0.2 µg/mL Promega Cat. No. G304A) was added to a simulated forensic buffer (10 mM Tris-HCl, pH 8; 10 mM EDTA; 100 mM NaCl; 2% sodium dodecyl sulfate (SDS)). The DNA solution was phase-separated by phenol/chloroform/isoamyl alcohol (PCI; Merck Millipore, Cat. No. 6805, 25:24:1) extraction to reduce the SDS detergent concentration. The final genomic DNA-extracted sample (0.5 mL) was added to pre-weighed empty Microcon® DNA Fast Flow devices (Merck Millipore Cat. No. MCRF0R100 and Cat. No. MCRF0R100ET (PCR Grade, 2x EtO-treated)), and concentrated by centrifugation at 500 x g at 2 minute increments. For each time point, the retentate (concentrate) volume was determined by weight (for dilute solutions, 1 g = 1 mL).

Human Genomic DNA Challenge and Recovery:

Genomic DNA was prepared and extracted as noted in "Flow Testing" above. The PCI-extracted genomic DNA sample (100 ng [0.5 mL of 0.2 µg/mL]) was added to Microcon® DNA Fast Flow and Microcon® DNA Fast Flow PCR Grade devices and centrifuged at 500 x g until retentate volumes were less than 50 µL (>10x concentration, "initial" spin). For some units, device performance was evaluated after one or more washes (or buffer exchange). In these cases, a fresh filtrate tube was attached and the retentate was diluted in Tris-EDTA (TE) buffer, pH 8 to the initial starting volume (0.5 mL). The above cycle was repeated for up to three wash steps. An inverted spin of the Microcon® device into a fresh concentrate recovery tube (1000 x g for 3 minutes) was used to collect the final concentrated genomic DNA sample. All of the devices' recovered retentate volumes were rediluted in TE buffer, pH 8 to the initial starting volume (0.5 mL), and measured against standard curves (0.2 µg/mL genomic DNA in TE buffer or in 10 mM Tris-HCl, pH 8; 10 mM EDTA; 100 mM NaCl) using a SYBR® Green I assay (10,000 x, Life Technologies, Cat. No. S7567, 1:6000 in TE buffer). Generally, the SYBR® Green I stain binds to double stranded DNA and forms a dye complex which can then be measured by fluorescent detection (BioTek Synergy™ H1plate reader using 485 nm excitation/528 nm emission).

Quantitative Polymerase Chain Reaction (qPCR):

A 2.0 Kb fragment of *Hind* III digested phage was cloned into the *Hind* III site of pUC19, resulting in a 4.7 kb pLH2 plasmid, and this template was used for the PCR reaction described. A master PCR mix was prepared (without the pLH2 DNA template) using a *Taq* Polymerase kit (Qiagen Cat. No. 201207), dNTPs (GE Healthcare Biosciences Cat. No. 28-4065-52), SYBR® Green I (10,000x), and primers M13F and ET-7 (Oligos, Etc.). An 1159 bp DNA fragment is expected to be amplified using these primers and templates. This final mix contained 0.03 U/µL *Taq* DNA Polymerase, 1x Qiagen PCR Buffer, 0.25x Q-Solution, 2.5 mM $MgCl_2$, 200 µM dNTP's, 1x SYBR® Green I, 0.2 µM M13F and 0.2 µM ET-7 primers, and diluted into Milli-Q® water. A total of 75 µL of the master PCR mix and template ("Test concentration" 0.02 ng/µL pLH2 DNA) was added to the Microcon® DNA Fast Flow and Microcon® DNA Fast Flow PCR Grade devices, mixed, and incubated (no filtration) for 15 minutes inside the filter unit. After incubation, 50 µL was removed from each device and placed into PCR tube strips (Bio-Rad Cat. No. TLS0801). Unprocessed control PCR reactions (50 µL) were set up with multiple concentrations of pLH2 template DNA (Control "no" template 0 ng/µL, "Test concentration" 0.02 ng/µL or "High control concentration" control 0.2 ng/µL) added directly to PCR tubes strips. All qPCR samples were processed with a thermocycler (DNA Engine Opticon® 2 system, MJ Research Inc.) using the following amplification protocol: 94° C 2 min., 94° C 30 sec., 55° C 30 sec., read plate, 72° C 3 min., read plate, repeat step two 39 times, incubate 72° C 5 min., and hold 4° C. The plate reads generated threshold cycle (Ct) output results from the SYBR® Green I DNA binding dye fluorescent signal intensity which correlates with the amount of amplified DNA.

Results

Flow Testing

Genomic DNA was spiked into a simulated forensic buffer containing 2% SDS and phase-separated using PCI extraction. The extracted genomic DNA (0.5 mL) was filtered in both the Microcon® DNA Fast Flow and PCR Grade [2x EtO-treated] devices to confirm that processing times and recovery would not be negatively impacted after 2x EtO treatment (flow curves shown in Figure 2). Genomic DNA concentration from 0.5 mL to <50 µL (>10x concentration) was achieved in 12 to 14 minutes. The 2x EtO-treated Microcon® DNA Fast Flow PCR Grade Device performed similarly to the non-EtO-treated devices. Flow curves may be generated in this same manner to test other sample types, sample volumes, and concentrations accordingly.

Human Genomic DNA Challenge and Recovery

As shown in Figures 3A and 3B, 100 ng of PCI-extracted genomic DNA or genomic DNA in TE buffer, pH 8 was applied to each device and centrifuged at 500 x g until the retentate volume was less than 50 µL ("initial," concentration factor >10x). For some samples, the concentrated retentate was diluted with TE buffer and centrifuged for buffer exchange ("wash"). The Microcon® DNA Fast Flow Device performed similarly to the Microcon® DNA Fast Flow PCR Grade Device. However, a decrease in recovery was observed for both devices when using multiple wash steps.

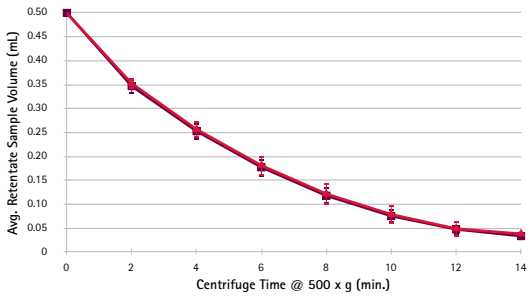
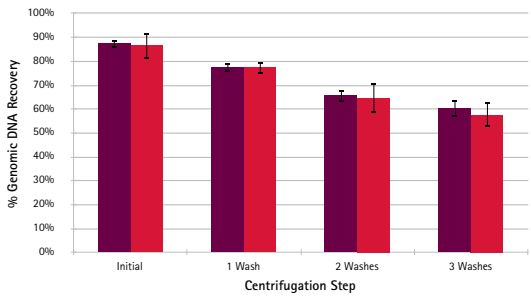


Figure 2. **Equally fast ultrafiltration flow** of Microcon® DNA Fast Flow (**Dark Red**) and Microcon® DNA Fast Flow PCR Grade (**Red**) devices as determined by measuring retentate volume at various time intervals and plotting retentate volume vs. centrifugation time. Each point represents the mean and standard deviation of four replicates.

A



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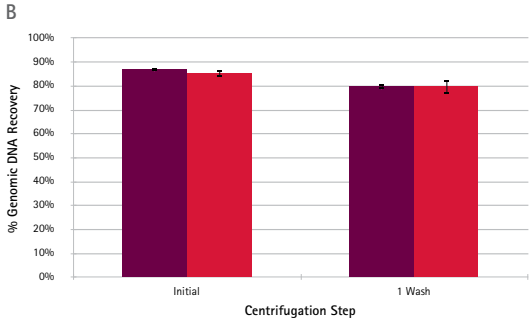

Figure 3. **DNA recovery performance** of Microcon® DNA Fast Flow (Dark Red) and Microcon® DNA Fast Flow PCR Grade (Red) devices as determined by comparison of retentate volume to starting material. Results show percent genomic DNA recovered with respect to centrifugation step. In each case, the starting material was either PCI-extracted DNA (A) or DNA in TE Buffer (B). For PCI samples, three successive wash steps were performed. Each bar represents the mean and standard deviation of four replicates.

Quantitative Polymerase Chain Reaction (qPCR):

For DNA applications, EtO treatment is often used to significantly reduce the level of amplifiable DNA acquired by contamination of devices during manufacturing and handling. To assess the potential for EtO-residual inhibitory effects on PCR, the PCR mix containing 0.02 ng/µL plasmid pLH2 DNA (1 ng) was incubated in the Microcon® DNA Fast Flow devices, amplified and assayed by qPCR for the 1159 bp DNA fragment. Relative PCR inhibition is distinguished by an increase in the samples' Ct values (cycle number required to amplify DNA to reach the critical detection threshold). More available DNA would also reduce values. Most importantly, no relative inhibition of the PCR assay was observed in the 2x EtO-treated Microcon® DNA Fast Flow PCR Grade devices (Figure 4). When compared to the PCR tube control template DNA (test load 1 ng), there was a slight increase in Ct values observed with both Microcon® DNA Fast Flow and Microcon® DNA Fast Flow PCR Grade device samples. This increase was likely due to the small degree of plasmid loss known to occur during Microcon® device processing or tube transfer.

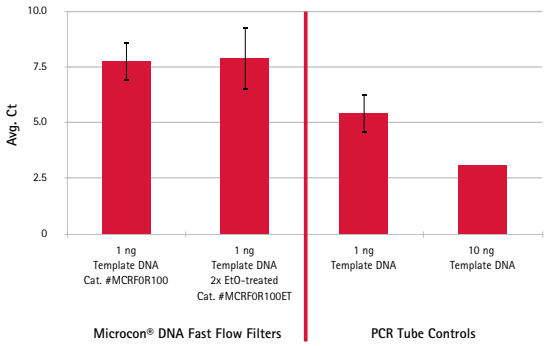

Figure 4. No inhibition of qPCR reactions. 1159 bp DNA qPCR average threshold cycle (Ct) results for Microcon® DNA Fast Flow and Microcon® DNA Fast Flow PCR Grade devices using pLH2 template DNA (1 ng). The qPCR results for the unprocessed PCR Tube Control 1159bp results (1 ng and 10 ng) are also shown. Each bar represents the mean and standard deviation of four replicates.

Discussions

For DNA analyses, many parameters, including yield, integrity, sample purity, and sample volume must be considered when optimizing the preparative workflow for DNA recovery. The standard Microcon® DNA Fast Flow devices have been successfully used for many DNA applications, including amplification techniques such as qPCR. With these assays becoming increasingly more sensitive, concerns arise within the clinical and forensic sectors regarding the potential for false positives or inconsistent findings due to residual contaminating DNA stemming from product manufacturing[2,5]. A dual cycle EtO treatment has been shown to alter the structure of DNA molecules thus preventing replication by DNA polymerases in amplification techniques[5,6,7] while also reducing the loadable DNA profiles[4].

The Microcon® DNA Fast Flow PCR Grade device is manufactured with a dual EtO treatment to address this matter. Our results demonstrate that rapid filtration and overall performance of the Microcon® DNA Fast Flow PCR Grade filter unit is not impacted by this process. The initial 0.5 mL challenge containing 100 ng of genomic DNA was easily concentrated to greater than 10 fold in less than 15 minutes. Equivalent flow was observed with both the standard and dual EtO-treated samples. Smaller sample volumes or different DNA quantities can also be processed, but flow curves should be generated to customize the spin time and concentration factor desired. Importantly, both device formats are also compatible with DNA samples that have been PCI-extracted or are formulated in common nucleic acid storage buffers, such as TE. Successful concentration, wash, and final recoverable yield of the purified genomic DNA were also not affected by the dual EtO treatment. More significantly, when considering the assay requirements of DNA analysis, EtO treatment of the Microcon® devices had no adverse inhibitory effects on performance of the genomic DNA samples in quantitative PCR.

In summary, the Microcon® DNA Fast Flow PCR Grade filter device has been dual EtO treated to significantly reduce the potential risk of contaminating DNA originating from the manufacturing process. The filtration device thus offers a perfect tool for rapid and reliable concentration of DNA samples prior to PCR and other types of analysis.

Ordering Information

Description	Qty/Pk	Catalogue No.
Microcon® DNA Fast Flow Filter	100	MRCF0R100
Microcon® DNA Fast Flow PCR Grade Filter	20	MRCF0R100ET
Microcon® 10K Device	100	MRCPRT010
Microcon® 30K Device	100	MRCF0R030

Product Information and Features

Membrane: Ultracel® low binding regenerated cellulose
Filtration Area, cm^2: 0.32
Device top: Polycarbonate
Membrane support base: Acetal
Filtrate/concentrate tube: Polypropylene
O-ring: Medical-grade silicone rubber
Volume capacity: 0.5 mL
Hold-up volume: <10 µL
Compatible with any centrifuge that can properly accommodate 1.5 mL micro-centrifuge tubes, but variable fixed angle rotors preferred (500 x g and 1,000 x g reverse spin for nucleic acids in Microcon® DNA Fast Flow devices).
10 – 30 kDa Microcon® filter options available (non-Et0)
Applications: Concentration and desalting of nucleic acids, ability to perform multiple wash steps, removal of labeled nucleotides, labeled amino acids, primers, and linkers.

References

1. P. Gill, J. Whittaker, C. Flaxman, N. Brown, and J. Buckleton. 2000. An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. Forensic Sci. Int. 112:17-40.

2. E. Graham. 2008. DNA reviews: low level DNA profiling. Forensic Sci. Med. Pathol. 4:129-131.

3. K. Sullivan, P. Johnson, D. Rowlands, and H. Allen. New developments and challenges in the use of the UK DNA Database: addressing the issue of contaminated consumables. Forensic Sci. Int. 146S:175-176.

4. K. Shaw, I. Sesardić, N. Bristol, C. Ames, K. Dagnall, C. Ellis, F. Whittaker, and B. Daniel. 2008. Comparison of the effects of sterilisation techniques on subsequent DNA profiling. Int. J. Legal Med. 122:29–33.

5. E. Archer, H. Allen, A. Hopwood, and D. Rowlands. 2010. Validation of a dual cycle ethylene oxide treatment technique to remove DNA from consumables used in forensic laboratories. Forensic Sci. Int. Genet. 4:239–243.

6. P. Lawley, and P. Brookes. 1963. Further studies on the alkylation of nucleic acids and their constituent nucleotides. Biochem. J., 89:127-138.

7. M. Altshuler. 2006. PCR Troubleshooting: the Essential Guide. Caister Academic Press, UK.

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