

# Ultrafiltration Devices and Plates Offer Higher-Throughput Options for Cloning and PCR Workflows

# Introduction

It is hard to imagine areas of biological research that have not been touched by molecular cloning. Cloning involves insertion of genetic sequences in mostly bacterial vectors such as plasmids and allows researchers to obtain large number of copies of the inserted sequence. Once cloned, sequences can be readily manipulated and expressed, thereby facilitating functional studies.

Traditional methods for cloning DNA fragments involve the ligation of restriction digest fragments obtained from chromosomes, plasmids, or cosmids into specific sites on a selected cloning vector (see Figure 1). Standard molecular cloning techniques require enzymatic modification and purification steps to prepare DNA sequences for ligation and transformation into bacterial cells. Typically, the first step in the transfer of a DNA sequence for cloning involves digestion of the DNA by restriction enzymes to produce terminal sequence overhangs, also termed cohesive ends, that facilitate ligation into a similarly prepared vector with compatible cohesive ends. Following each digestion step, the released adapter sequences need to be removed so they will not compete with the desired insert during subsequent ligation and significantly increase the number of spurious recombinants. DNA electrophoresis followed by recovery of the desired DNA fragments from the gel is the most frequently used purification method for separating adapters from the desired product.

While restriction digest is still important in the cloning process, aspects of its use have changed. Rather than solely relying on restriction digestion to obtain fragments for cloning, currently the polymerase chain reaction (PCR) often is employed to generate and prepare sequences for cloning. DNA or cDNA resulting from reverse transcription of RNA can serve as templates for PCR amplification. One of the main advantages of PCR is that it provides a straight forward way to introduce restriction sites necessary for directional cloning of the amplified sequence by incorporating them in the amplification primers.

Standard techniques often involve multiple purification steps, each associated with significant product losses, therefore requiring large amounts of starting DNA to guarantee sufficient vector and insert material to complete subsequent ligation and transformation steps.<sup>1</sup> Because of the numerous applications that require production of recombinant DNA molecules, a rapid method is needed for transferring DNA fragments from a PCR product or other source into the desired vector.

These steps can be carried out in a centrifugal ultrafiltration device, such as the Pall Nanosep<sup>®</sup>, for a small number of samples. For higher throughput applications, Pall AcroPrep<sup>™</sup> Advance 96-well filter plates with the same ultrafiltration membrane can be used. As researchers have more and more samples or constructs they want to make, the ability to use filter plates in a high-throughput workflow is an attractive option.

In this paper, we describe the use of these ultrafiltration devices in various steps of the cloning workflow that can provide a more streamlined process and compare them against traditional methods of subcloning PCR-generated fragments. After optimizing the parameters for effective target DNA purification, we synthesized PCR products with terminal adapter sequences containing restriction sites. These adapters were digested, gen¬erating the appropriate cohesive ends for ligation. The resulting DNA products were concentrated and desalted while simultaneously allowing the primers and adapter fragments to pass into the discarded filtrate during a centrifugation step (Figure 1). The ligation reaction of the resulting DNA fragments to digested vector was completed directly within the ultrafiltration device, and then transformed into competent cells. The use of ultrafiltration devices, particularly Acroprep Advance 96-well filter plates, enabled significant savings in time, labor, and starting materials, with numbers of transformed recombinants that roughly equaled those obtained with traditional techniques.

## Figure 1

Directional Subcloning of Restriction Digested PCR Products and DNA Fragments.



#### **Optimized DNA Separation and Recovery Using Ultrafiltration**

Ultrafiltration devices have the ability retain primers, which was evaluated by filtering a solution containing 50 ng of end-labeled 25 bp oligonucleotides in duplicate using different molecular weight cutoff (MWCO) devices.

The effectiveness of ultrafiltration for PCR product purification was examined by observing the recovery of radiolabeled free nucleotides, primers, and PCR products following filtration though a selection of devices containing membranes with different MWCO membranes. PCR reactions were carried out for 30 cycles under standard conditions using primers directed against sequences approximately 200 bp upstream and 200 bp downstream of the plasmid pUC18 polylinker to produce 400 bp DNA fragments. The primers were end-labeled with  $\Upsilon$ -32P-dATP and 1 µL of the radiolabeled nucleotide  $\alpha$ -32P dCTP (3000 Ci/mmol) was added to the PCR reactions to internally label the PCR products at low specific activity. Upon completion of the PCR reactions, samples were pooled to ensure consistency and then split into equal aliquots for desalting and concentration in ultrafiltration devices with a variety of MWCOs. The retained material was recovered in 20 µL TE, separated via gel electrophoresis using a 10% polyacrylamide Tris-borate gel (Bio-Rad, Hercules, CA, USA), and analyzed by autoradiography.

The retention of biological activity during DNA handling is critical. To determine the effect of ultrafiltration on biological activity, we synthesized a 400 bp PCR product containing a centrally located polylinker (described above), purified the product by ultrafiltration or ethanol precipitation, and tested its suitability for digestion. Analysis of the restriction digest fragments of this PCR product allowed us to determine whether the DNA could be recovered without interfering with subsequent enzymatic manipulations.

#### **Rapid Cloning of PCR Fragments**

The utility of ultrafiltration devices in practical cloning applications was assessed by generating a PCR product from the fern *Ceratopteris richardii* corresponding to a 620 bp conserved region of a phytochrome (PHY) photoreceptor genomic DNA sequence.<sup>2</sup> Genomic template DNA from three 14-day-old *C. richardii* gametophyte tissue was isolated by the alkaline lysis method.<sup>4</sup> Forward (5'-AAGGATCCACACGGGAAGTTTTGGCC-3') and reverse (5'-TTAAGCTTGATATTCGGGACTCTGAAAC-3') primers were constructed with sequences corresponding to complementary sequences from a genomic PHY gene. Restriction enzyme adapter sites for *Hind*III and *Bam*HI (underlined in the above sequences) were engineered two base pairs downstream from the 5' ends of each primer, respectively, to generate compatible cohesive ends for cloning into the vector polylinker. The 25 µL PCR reactions were prepared using PCR Master Mix (Qiagen, Valencia, CA, USA) supplemented to 2.5 mM MgCl<sub>2</sub>, 0.2 µM primers, and 1 µL genomic template DNA. The thermal profile of the PCR reactions consisted of an initial denaturation at 90 °C for 1 minute followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 45 °C, and 60 seconds at 72 °C, completed by a post-cycle extension for 3 minutes at 72 °C. A 6 µL aliquot of each reaction was subjected to electrophoresis to confirm the size and purity of the products and to quantitate the fragments for subsequent cloning. The utility of ultrafiltration device-based cloning was compared with standard fragment cloning methods.<sup>3</sup>

#### **Transformation and Recombinant Analysis**

A 3 µL aliquot of ligation product from either the ultrafiltration device or standard method was used to transform competent XL1-Blueu cells (Stratagene, La Jolla, CA, USA). Cells from each transformation were plated onto LB plates with ampicillin to select for transformants and X-gal and IPTG for blue/white colony selection.<sup>5</sup> Transformation efficiencies and white:blue colony ratios were calculated for each preparation, and several white colonies from each were analyzed by PCR for insertion of the phytochrome fragment. White colonies from each preparation were transferred to a fresh LB + ampicillin plate and allowed to grow overnight at 37 °C. A swipe of each colony was picked with a sterile toothpick and resuspended in 7.0 µL water. After lysing the cells for 10 minutes at 98 °C, a PCR mixture containing Qiagen Master Mix, 2.5 mM MgCl<sub>2</sub>, and 2 µM primers was added; the PCR reactions were then cycled according to the same procedure used to amplify the original phytochrome gene sequences. PCR products were subjected to agarose gel electrophoresis to assay the proportion of desired recombinants obtained by the standard and the ultrafiltration device methods.

## Results

#### Separation and Recovery of DNA Fragments Using Ultrafiltration

Ultrafiltration, both centrifugal devices and Acroprep Advance 96-well filter plates, are capable of separating two mixed DNA fragments as long as their sizes are significantly different. When either a 50 bp or 500 bp fragment is purified using a variety of MWCO devices, the 3K, 10K, and 30K devices retain both species of DNA. In contrast, the 100K device is able to retain the larger fragment and allow the smaller fragment to pass into the filtrate (Figure 2A). This observation indicates that a 100K centrifugal ultrafiltration device and Acroprep Advance 96-well filter plates can be used effectively for the removal of primers, polylinkers, and adapters from mixed DNA fragment solutions.

#### Figure 2

Size Separation of DNA Using Ultrafiltration for Primer and Nucleotide Removal



# **Separation of Primers and Adapters**

The ability of centrifugal ultrafiltration devices and Acroprep Advance 96-well filter plates with a variety of different MWCO membranes to remove primers from a solution was determined by filtering a solution containing end-labeled 25 bp oligonucleotides (Figure 2B). The 30k ultrafiltration devices retained close to 90% of the radiolabeled oligonucleotide. Based on the differential separation seen in panels A and B of Figure 2, an experiment was devised to verify which ultrafiltration devices would be most useful for the removal of primers and free nucleotides from PCR reactions. End-labeled primers together with radiolabeled nucleotides were added to a PCR reaction and a 400-bp product was synthesized using standard PCR methods. The resulting solution contained labeled DNA products as well as the primers and unincorporated nucleotides. Consistent with data in panels A and B of Figure 2, the 3K ultrafiltration device only removed a fraction of the free nucleotides while the 10K and 30K devices effectively removed free nucleotides but not the primers (Figure 2C). At the same time, the 300K devices allowed all of the DNA and precursors to pass into the filtrate. Finally, the 100K device was able to differentially separate the PCR products from the mixture of primers and nucleotides in a single pass. In addition to differential separation, the retained material in the 100K device was concentrated, desalted, and ready for downstream enzyme modifications.

It is critical that the act of concentration and PCR product purification using ultrafiltration does not interfere with the downstream modifications required for further digestion and ligation. To determine whether ultrafiltration damages or contaminates DNA samples, experiments were performed to test the ability of the DNA to be cleaved by a variety of restriction enzymes. Pooled, unlabeled PCR fragment samples were purified using either ultrafiltration or ethanol precipitation and then each was split into four samples. One sample served as a control while the others were digested with three different restriction enzymes that were selected for their sensitivity to sub-optimal conditions. The data indicate that all three enzymes digest the ultrafiltration-prepared target DNA to completion, as evidenced by the presence of a 200 bp doublet rather than an undigested 400 bp fragment (Figure 3A). In contrast, PCR samples purified by ethanol precipitation did not digest to completion when using the more demanding Xbal enzyme, suggesting that residual salts or ethanol had inhibited the reaction.

## Figure 3

Digestion Efficiency of DNA Purified by Ultrafiltration or Ethanol Precipitation and in serial digestion of PCR amplified fragments



PCR techniques allow researchers to amplify a DNA sequence from a target template mixture. To facilitate molecular cloning, 5'-terminal sequences containing restriction enzyme recognition sites (adapters) can be included during primer synthesis. This strategy aids subsequent ligation of a digested PCR fragment into an appropriately digested and purified vector. Because of their small size, adapter fragments can be removed by ultrafiltration following restriction digestion while simultaneously concentrating the sample and allowing buffer exchange. The ability of ultrafiltration to facilitate the molecular cloning of a PCR product was demonstrated using a serial restriction digest performed on a phytochrome gene fragment. The fragment was amplified from *Ceratopteris richardii* genomic DNA template using gene-specific primers containing short terminal *Hind*III and *Bam*HI adapters. The removal of free nucleotides was shown for the PCR sample purification [Figure 3B, lanes marked PCR, before (U) and after (F) ultrafiltration]. As shown in Figure 3B, successive *Hind*III and *Bam*HI digestion steps each followed by additional ultrafiltration do not result in a significant loss of sample, (lanes marked *Hind*III and *Bam*HI). Similar results were obtained for serial digestions performed in the *Hind*III to *Bam*HI direction and for simultaneous digestion with both enzymes (data not shown). Serial digestion of the cloning vector was also performed, and the small excised polylinker was efficiently removed from the ligation-ready vector by ultrafiltration in a 100K ultrafiltration device (data not shown).

# **Rapid Preparation of DNA Fragments**

The ultimate test of the effectiveness of an ultrafiltration device method is to prepare vector and insert DNA by ultrafiltration, and then to ligate and transform the products into cells. The recovery of recombinants obtained by this method can then be compared with that observed using DNA purified by standard gel-based methods. Electrophoretic methods are able to completely separate the primer and adapter sequences, but they require subsequent purification of the desired DNA fragment from the gel prior to use. By contrast, ultrafiltration methods have no electrophoresis steps, but trace amounts of primers, adapters, and polylinkers may be retained in the membrane which can potentially interfere with downstream cloning steps. Therefore, it was important to determine whether these trace contaminants would significantly hinder recombination of the desired fragment.

The preparation of vector and PCR products using standard methods involved the separate digestion and purification of both vector and insert sequences. The use of ultrafiltration for DNA sample preparation allowed two variations of the ultrafiltration protocol to be performed. In the first method (UF Method A), the vector and insert DNA molecules were digested and purified separately, and mixed only at the ligation step. In the second method (UF Method B), the vector and insert DNA molecules were and insert DNA molecules were mixed together at the start of the restriction digestion, allowing them to be digested, purified, and ligated in a single 100K ultrafiltration device.

Regardless of which method was used to generate recombinants, the number of recombinants recovered was roughly the same for three independent experiments (Figure 4). The largest difference was in the time and labor involved performing the procedure. Gel electrophoresis purification steps that normally require 2 to 3 hours to perform could be replaced by a simple 5-minute ultrafiltration step. The rapid method using device- or filter plate-based ultrafiltration gave more non-recombinant colonies than the standard method, consistent with the fact that trace polylinker sequences were likely to be present. However, the use of the lacZ marker system effectively alleviates this shortcoming by coloring these colonies blue. In addition, using a third or fourth enzyme that cuts the polylinker, but not the insert, could help remove most of the non-recombinant clones.<sup>5</sup>

#### Figure 4

Cloning DNA Fragments Prepared by Different Procedures: Number of Recombinant Colonies and PCR Screening Results.



Recombinant insert analysis was done by performing colony PCR with the same primer set used to generate the insert. Colonies were considered positive for the insert if the PCR produced a band of the appropriate size. Putative recombinant colonies (white) were screened and, for the standard method, 17 out of 19 colonies (89%) contained the correct insert. When the rapid method transformants were screened, 14 out of 17 colonies (82%) contained the correct insert (Figure 4B). The slightly higher number of spurious recombinants for the rapid method is likely due to the presence of trace adapter sequences. It is apparent from these data that any possible carry-over of enzymes did not interfere with the recovery of recombinants. The fidelity of the rapid subcloning method was comparable to that of traditional methods and therefore is sufficient for routine subcloning procedures.

# Discussion

The availability of selective and differential culture media allows the use of rapid molecular cloning techniques. The ability to purify and digest DNA vectors and inserts using centrifugal and 96-well filter plate ultrafiltration devices not only allows rapid subcloning with smaller initial quantities of DNA, but also helps to circumvent some of the other obstacles frequently encountered during standard gel purification-based methods. Among the most serious of the problems of traditional techniques are carry-over of salts and residual ethanol or other organic solvents that can inhibit downstream enzymatic reactions. Furthermore, the yields for DNA recovery from gels and precipitation are often low, requiring large amounts of starting sample and extensive labor. In this report, we demonstrate that ultrafiltration in both low- and high-throughput workflows is well suited for the high-yield, rapid recovery of dilute DNA samples without the retention of components that may inhibit biological activity. We present a method for cloning PCR products or other DNA fragments with significant advantages of time, labor, and materials over classical methods while maintaining comparable yields of desired recombinants. This report shows that the ultrafiltration method has distinct, quantifiable advantages over other commonly-used subcloning techniques. The speed and efficiency of this method, along with the availability of centrifugal devices and filter plates with different MWCO membranes, suggest this basic method could be adapted to cloning strategies for DNA of different sizes from many sources.

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