Nanosep® Centrifugal Ultrafiltration Devices and PCR: Before and After

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Introduction

The ability to detect specific sequences of DNA has been at the core of the phenomenal success of molecular biology. One of the most powerful and revolutionary procedures used today to analyze DNA sequences is based simply on the unique biochemistry of DNA replication and is called Polymerase Chain Reaction, or PCR.

This powerful technique allows researchers to "amplify" specific DNA sequences from samples that contain only a few individual DNA molecules with sequences complementary to the primers. However, PCR methods are not without problems. The extreme sensitivity of PCR increases the probability that false priming or DNA contamination will lead to the amplification of the wrong molecule.

One way to minimize artifacts that arise from impurities is to work in an extremely controlled environment and to purify the reagents and primers prior to use. But even in a controlled environment, the sample itself can contain components that interfere with PCR or cause false priming. Using Nanosep centrifugal ultrafiltration devices to remove these contaminants prior to PCR can help to ensure correct results.

After the completion of a PCR reaction, the buffer (a mixture of primers and free nucleotides) and the newly synthesized DNA fragments need to be separated so that the synthesized DNA can be used for downstream applications. This separation can be achieved by several means, such as alcohol precipitation, gel filtration, or ultrafiltration. In this study, we evaluated the use of Nanosep centrifugal ultrafiltration devices at several steps in the PCR process: reagent and solution preparation, primer purification/concentration, and PCR product cleanup and recovery.

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Experimental Analysis

Preparation of Reagents and Solutions for PCR

PCR is an extremely sensitive method for the detection and amplification of specific DNA sequences. This sensitivity can result in the amplification of minute amounts of contaminating DNA. This is a particular problem with ancient DNA or forensic human DNA sequences because one contaminating molecule from a technician's skin or hair cell will cause false results.

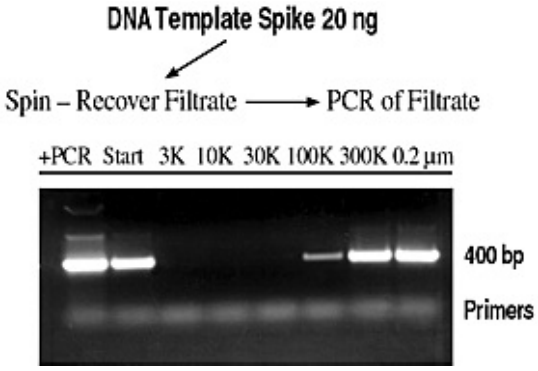
Removal of contaminating DNA from stock reagents or master mixes can be done using ultrafiltration. To ensure that it is free from DNA, the final material synthesis buffer can be spun in a 3K or 10K Nanosep centrifugal device prior to aliquoting into PCR tubes.

Methods

We tested the ability of Nanosep centrifugal devices to remove contaminating DNA by adding a 500 ng of plasmid pUC18 to 1.5 mL TE (10 mM Tris, 1 mM EDTA, pH 8) prior to aliquoting the sample. The sample was divided into seven 200 µL fractions, one fraction was kept as a control (START, 33 ng/uL) and the other fractions were centrifuged in Nanosep centrifugal devices containing a selection of ultrafiltration and microfiltration membranes. Following centrifugation (5,000 x g, 10-15 minutes, 25 °C), a 3 µL sample was taken from the filtrate of each fraction and added to each PCR reaction containing 45 µL PCR mix (Invitrogen) containing 2 µL of a 20 nmole pUC18-complementary primer mix. A PCR reaction of 25 cycles under standard conditions was performed, and 25 µL of each reaction was electrophoresed and

stained with ethidium bromide (Figure 1). A reaction containing 200 ng pUC18 was used as a PCR-reaction positive control.

Figure 1
Removal of DNA Template from PCR Stock Solutions



Results and Conclusions

By utilizing a PCR run of only 25 cycles, an approximation of the amount of template can be made by comparing the relative intensity of the 400 bp DNA bands. As anticipated, the positive control had the highest synthesis. The experimental samples had equivalent levels of synthesis for the unfiltered material (START) as well as samples filtered through the 0.2 µm and 300K devices, indicating that these devices allowed the introduced template to pass completely. In contrast, the 3K, 10K, and 30K devices gave no detectable PCR product, indicating the complete removal of the 3,000 bp template. Filtrate from the 100K device gave a weak PCR product band suggesting that some of the template was able to pass through this device.

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Purification and Concentration of PCR Primers

The use of synthetic oligonucleotides has revolutionized many molecular biology methods. They are critical to the PCR reaction because they act as primers for the synthesis of DNA from sequence-specific start points. Oligonucleotides are synthesized using chemical means. After synthesis, purification steps are required to purify the full-length oligonucleotides from the synthesis reaction mixture. Prior to use in PCR, desalting is required to remove residual by-products from the synthesis, cleavage, and deprotection procedures. Ultrafiltration using centrifugal concentrators is an efficient way to desalt and concentrate oligonucleotides.

Methods

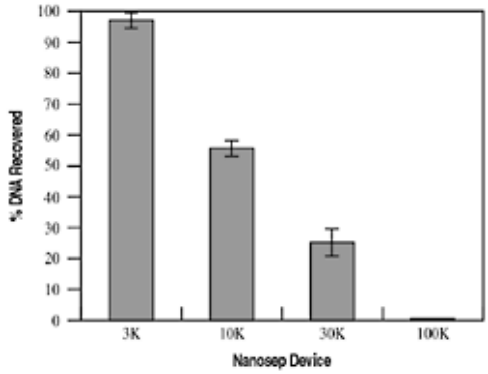
Nanosep® centrifugal devices were evaluated for their ability to retain primers by filtering a 400 µL solution containing 50 ng of end-labeled 25 bp oligonucleotides in duplicate using different molecular weight cutoff (MWCO) devices. These devices were centrifuged for 10 minutes (100K, 30K, 10K) to 30 minutes (3K) at 5,000 x g. The retained material was resuspended in 40 µL TE, diluted to 400 µL, and the recovered, radiolabeled DNA was quantified using a scintillation counter. Duplicate 400 µL samples from the pooled starting material were counted and used to calculate the percent recovery.

Results and Conclusions

The Nanosep 3K centrifugal device retained close to 90% of the radiolabeled oligonucleotide (Figure 2). The choice of a higher MWCO device allowed for a shorter spin time but resulted in decreased yields. If the intention was to pass all of the primers, then the 100K device would have been the best choice.

Figure 2

Retention of Small Oligonucleotides



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Cleanup and Recovery of PCR Products

The final PCR reaction may contain up to a microgram of amplified DNA that can be used for a variety of molecular biology applications. These applications may be more or less sensitive to the remaining components of the PCR reaction mix. Certain restriction enzymes as well as DNA ligase are particularly sensitive to the presence of contaminants in DNA samples. Because a PCR reaction mixture contains a variety of salts, free nucleotides, glycerol, proteins, and primers, most downstream applications will require some sort of PCR cleanup.

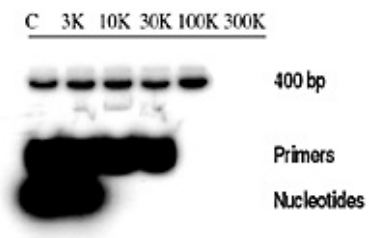
The separation of buffer components, free nucleotides, and primers from the PCR product can be performed in a variety of ways:

1. **Precipitation**, using chemical solubility properties to selectively separate DNA. The primary drawbacks for using this method to purify PCR products are the time loss and incomplete removal of co-precipitating buffer components and contaminants.
2. **Chromatography**, using size exclusion particles or affinity to glass to purify DNA from the PCR mixture components. This technique is costly, generally requires significant handling, and samples must be concentrated after elution from the matrix.
3. **Ultrafiltration** involves the isolation and concentration of PCR products using size exclusion membrane devices. It is rapid, requires very little handling, yields are high, DNA is undamaged, and the concentrated DNA is free of contaminants that may inhibit downstream reactions.

Methods

Two experiments were conducted to demonstrate the use of ultrafiltration for the cleanup of PCR reactions. The first experiment showed the separation of free nucleotides, primers, and PCR products. A small amount of labeled dCTP was added to the PCR reaction mix (Invitrogen) and primers were end-labeled using dATP. The reaction was run for 30 cycles using standard conditions with a small amount of radioactive nucleotides and radioactive primers. Ten reactions were pooled and 100 µL aliquots were diluted to 500 µL for centrifugation in a variety of MWCO devices. The retained material was recovered in 20 µL TE, electrophoresed using a 10% polyacrylimide Tris-borate gel (BioRad), and analyzed by autoradiography (Figure 3).

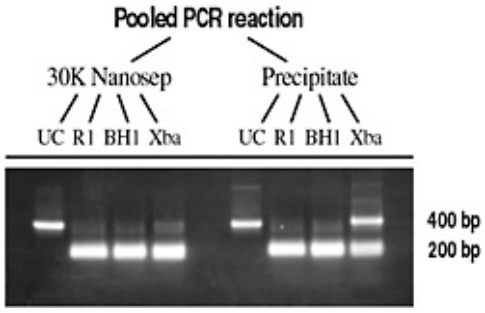
Figure 3
Membrane Selection for PCR Cleanup



The second experiment showed that ultrafiltration not only rapidly recovers PCR products, but purifies them to a higher quality than the standard precipitation protocol. A pooled mixture of PCR products was divided in half. One half was precipitated with 1/10 volume sodium acetate and 2 volumes ethanol, chilled at -20 °C for 2 hours, centrifuged at high speed for 30 minutes, rinsed with 70% ethanol, and air dried prior to resuspension in 40 µL water. The second fraction was diluted to 500 µL, added to a 30K Nanosep® centrifugal device, and centrifuged for 15 minutes. The retained material was resuspended in 40 µL water. Both 40 µL samples were divided into 10 µL fractions. One fraction was used as the uncut (UC) control and the others were diluted into a 20 µL restriction digest reaction according to manufacturer's instructions (EcoRI=RI, BamHI=BH1, Xba1=Xba). These samples were digested for 30 minutes at 37 °C, electrophoresed in a 1.5% agarose gel, and DNA bands were visualized by staining with ethidium bromide

(Figure 4).

Figure 4
Ultrafiltration Desalting Improves Enzyme Activity



Results and Conclusions

Based on the data in Figure 3, the 100K Nanosep centrifugal device demonstrates the best combination of PCR product retention along with complete primer and nucleotide removal. If the desire is to ensure the removal of the buffer and free nucleotides, but not primers, then the 30K or 10K devices will retain the PCR product while removing buffer components. Cleanup using the 30K device may be needed if the PCR product is smaller than 200 bp and if the presence of primers does not inhibit downstream applications.

Downstream applications such as restriction digestion can vary in their sensitivity to residual components of the PCR reaction depending on the enzyme chosen. The EcoRI restriction enzyme tends to be hardy and cuts DNA effectively under suboptimal conditions, while Xba1 restriction enzyme activity is extremely sensitive to the buffer composition. The data (Figure 4) clearly shows that the Xba1 enzyme is unable to digest to completion the 400 bp PCR fragment for a sample that has been precipitated. In contrast, the sample that was rapidly purified with the 30K device was completely digested using Xba1.

This study shows that Nanosep centrifugal devices can be used in PCR applications to assure high quality PCR products. The resulting DNA is present in concentrated form and free of small molecular species that may interfere with further reactions.

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Recommendations

10K Device: Primers
30K Device: DNA Template
100K Device: PCR Cleanup

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