Purification and Handling of DNA Fragments

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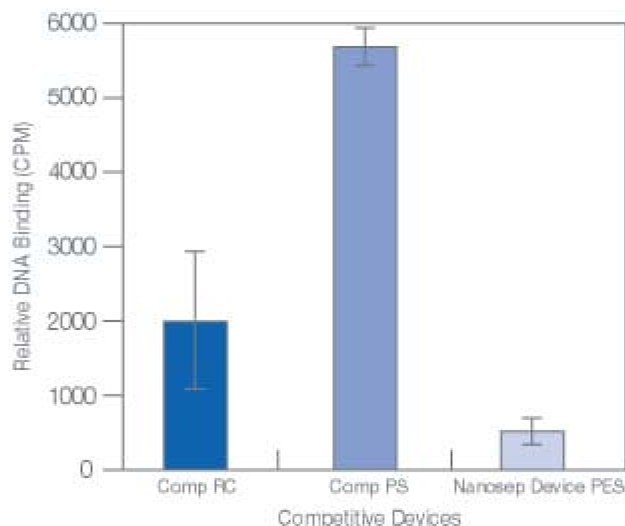
Introduction

From a biochemical standpoint, DNA is a rather simple molecule. But, unlike proteins, biochemical extraction methods will not help to separate one similarly-sized DNA molecule from another. Agarose gel electrophoresis separates DNA molecules by size at a high resolution. The ability to analyze the number and size of the DNA fragments resulting from sequence-specific restriction endonuclease digestion has given researchers a powerful tool for DNA analysis and manipulation. These methods have led to a proliferation of technologies that use DNA fragments as both targets and molecular probes for detection systems and genetic engineering. As described below, using microfiltration and ultrafiltration devices for DNA fragment purification and handling provides the best combination of low cost, easy handling, and high sample quality. Nanosep® centrifugal devices demonstrate superior performance in these applications.

The majority of applications require much more than simply analyzing restriction digest DNA fragments on agarose gels. For these applications, the DNA fragments are cut, ligated, sequenced, transformed, electrophoresed, and labeled. The handling of these sensitive and often very small biomolecules requires specific proactive laboratory procedures to prevent the loss or contamination of valuable samples. In addition, the DNA fragments may require desalting, buffer exchange, or elution from agarose during any of a number of steps. High recoveries of DNA are ideal, but often not achieved by these procedures. The purification and recovery of DNA from either agarose gels or other complex reaction solutions can be done in several ways:

1. **Electroelution**, using an electric charge with either dialysis bags or a specialized electrophoresis device to capture DNA migrating out of a gel fragment. Often this method requires vigilance, and the recovered DNA sample requires an additional concentration/desalting step.
2. **Affinity Chromatography**, using either glass beads, resins, or glass fiber membranes to bind DNA which is eluted after washing. This method is labor intensive, can cause shearing, and leaves glass particles and ethanol in the DNA sample.
3. **Precipitation**, using chemical solubility properties to selectively separate DNA from other alcohol-soluble components. This method can result in the loss of DNA (especially from dilute solutions), is time consuming, and gel/buffer contaminants can be co-precipitated with the DNA. In addition, over-dried pellets may not dissolve completely.
4. **Column Chromatography** relies on size separation in a gel matrix. This technique often requires the addition of carrier tRNA to achieve high yields. Column chromatography requires significant handling and dilutes the original sample.
5. **Microfiltration/Ultrafiltration**. This method allows the rapid purification and concentration of the DNA using size exclusion membrane devices. There is very little handling, yields are high, DNA binding is low (Figure 1), DNA is undamaged, and the concentrated DNA is free of contaminants and ready for downstream reactions.

Figure 1
Nanosep Devices Exhibit Low DNA Binding



From a pooled DNA sample (50 ng/mL) containing a 400 bp ^{32}P-labeled PCR fragment, triplicate 400 µL aliquots were centrifuged at 5,000 x g in a Nanosep 100K (5 minutes) or competitive 100K (15 minutes) device. The retained DNA was removed with 40 µL TE (10 mM EDTA, pH 8) and the upper sample receiver was completely immersed in 5 mL scintillation cocktail and counted for 60 seconds. A counts-per-minute (CPM) value of 5,000 roughly corresponds to 10% of the total radioactive DNA in the sample. Competitor's regenerated cellulose (Comp RC), competitor's polysulfone (Comp PS), Nanosep device with polyethersulfone Omega™ membrane (Nanosep Devices PES).

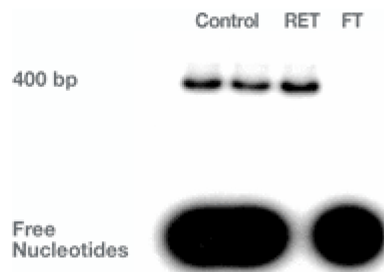
Based on the above requirements, the method of choice to achieve the best combination of cost, handling, and sample quality is the use of microfiltration and ultrafiltration (UF) centrifugal devices to desalt, purify, and recover valuable DNA fragment samples. We present data for three common examples of DNA fragment handling and purification. Nanosep centrifugal UF devices were used to clean up labeling reactions and purify DNA from standard agarose gels as well as enzyme digested gels.

Removal of Free Nucleotides from Labeling Reactions

One of the most common procedures in a molecular biology lab is the addition of radionucleotides to a DNA synthesis reaction to make radioactive DNA from a specific DNA template. These sequence-specific labeled DNA fragments can be used to hybridize to specific DNA or RNA sequences bound to membranes for detection by autoradiography. Recently, a variety of non-radioactive methods for tagging and detecting biological markers have been developed. These methods use enzyme activity tags that can produce color, chemiluminescence, or fluorescence signals.

In all labeling methods, a target molecule is modified using enzymes to attach a molecular tag in the presence of reaction buffers and small molecule substrates. Despite efforts to increase labeling efficiencies, these reactions do not incorporate all of the tag, and often the market molecule is present in excess. Depending on the system, the incorporation ranges from 10-80%, leaving a significant quantity of unbound label in the final reaction mixture. To reduce background noise on blots or to prevent problems with subsequent downstream reactions, purification steps are often required to remove the reaction buffer components and unincorporated label molecules from the labeled DNA. Nanosep centrifugal devices with ultrafiltration membranes are ideal for the rapid removal of reaction components from the labeled product (Figure 2).

Figure 2
Effective Removal of Free Nucleotides



Methods
To demonstrate the removal of free nucleotides, PCR labeling reactions were run with 100 ng pUC18, 20 nmole primers, and PR Supermix (Invitrogen, Carlsbad, CA) supplemented with 10 uCl ^{32}P-dCTP. This reaction consistently produced a 400 bp low-specific-activity PCR product.

On average, 5% of the available label was incorporated into the PCR product. PCR reactions were pooled and aliquoted into 400 µL control samples and 400 µL samples for centrifugal separation. A 30K Nanosep centrifugal device was spun at 5,000 x g for 15 minutes. The retentate was recovered using two 20 µL TE (10 mM Tris, 1 mM EDTA, pH 8) rinses and then diluted to 400 µL. Control and experimental samples were electrophoresed (20 µL) using a 10% Tris Borate polyacrylamide gel according to manufacturer's (Bio-Rad Laboratories, Hercules, CA) instructions. Free nucleotides (FN), retained DNA (RET), flow-through (FT), unfiltered sample (in duplicate control).

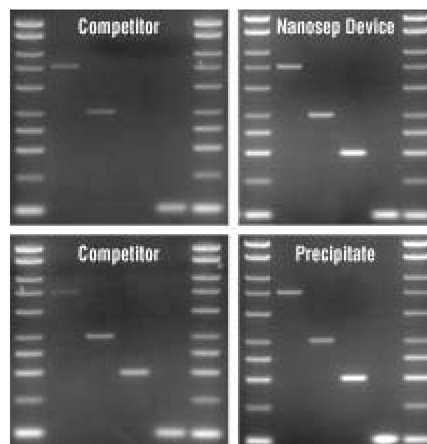
Results and Conclusions
The 30K Nanosep centrifugal device with Omega™ ultrafiltration membrane efficiently removes free nucleotides completely without losing the valuable 400 bp PCR product in the process (Figure 2). The recovery was rapid and high yields of concentrated product were achieved with minimal handling.

Purification of DNA Fragments from Agarose Gels

For most researchers, simply visualizing DNA fragments in agarose gels was not adequate; therefore, numerous methods were developed to purify DNA fragments away from the agarose gel matrix. These methods made it possible to use specific, electrophoretically separated DNA fragments for further analysis and cloning.

DNA can be isolated and purified from a gel slice in a simple two-step procedure using Nanosep and Nanosep MF centrifugal devices. In the first step, a Nanosep device is used to separate DNA from the gel matrix. The agarose gel will be retained by a Nanosep MF containing 0.2 or 0.45 µm Bio-Inert® membrane, and the DNA-containing gel buffer will freely flow through. A second Nanosep device is used to concentrate and wash the DNA. This device is chosen with a MWCO that retains the DNA and allows contaminant to pass through into the filtrate. This method is more rapid and reliable than standard methods relying on electroelution, dialysis, or precipitation (Figure 3).

Figure 3
Recovery of DNA from Agarose



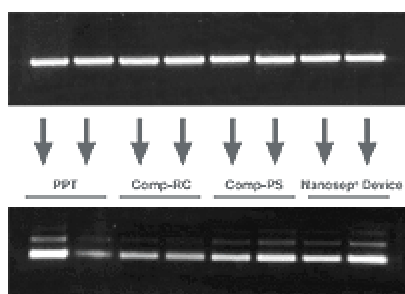
Methods
Four micrograms total DNA from a commercially available 1Kb ladder (Invitrogen) was electrophoresed in a 0.8% agarose preparative gel in Tris-acetate, EDTA (TAE) buffer preparative gel (not shown). Gel slices containing four different molecular weight bands (8.1, 3.1, 1.6, 0.5 Kbp) were excised, frozen, macerated, and centrifuged in a Nanosep device with 0.2 µm Bio-Inert membrane. The DNA-containing filtrate was diluted and divided into four 400 µl fractions. One aliquot was precipitated using 1/10 volume sodium acetate and 2 volumes ethanol, incubated 2 hours at -20 °C, centrifuged at high speed for 30 minutes, dried and resuspended in 20 µL Tris-EDTA (TE). The other aliquots were centrifuged through either a Nanosep 100K MWCO (10 minutes) or competitive 100K MWCO (20 minutes) device, and the retentate was recovered in 20 µL TE (two, 10 µL rinses). These samples were electrophoresed in a 0.8% agarose TAE gel along with marker lanes containing 1 µg of a 1Kb ladder. Equal band intensities approximate 100% recovery.

Results and Conclusions
DNA recovery using Nanosep 100K devices was greater than 90% based on band intensity. The four different molecular weight bands represent independent experiments. Precipitated DNA bands also gave high yields but would contain many gel buffer contaminants not found in ultrafiltered DNA samples. DNA recovery with competitive devices was generally less reliable with recoveries ranging from 0-80%. One competitor (Figure 3, upper-left panel) regularly suffered device seal failures in 10% of the devices tested.

Purification of DNA Fragments from Enzyme-Digested Agarose Gels

Recently, enzymes that digest and liquefy agarose have been used to free DNA from an agarose gel slice. While the DNA is now free in solution, a number of electrophoresis contaminants remain in the solution that will likely inhibit downstream handling and modification procedures. Because agarase-digested agarose is in a liquid state, it is now able to pass through an ultrafiltration membrane as a small molecule. This allows DNA from the original sample to be purified and concentrated in one spin using a Nanosep centrifugal device (Figure 4).

Figure 4
DNA Recovery from Liquefied Agarose



Click graphic to enlarge

Method
The ability of Nanosep centrifugal devices to purify DNA from agarose-digested gel slices was evaluated as follows. Eight aliquots from pooled PCR reactions containing a 400 bp PCR product were electrophoresed in a 1% Low-Melting-Point (LMP) agarose-TAE gel (Figure 4, upper panel). Eight individual bands were excised and treated with agarase according to manufacturer's instructions (Invitrogen). After agarase digestion, duplicate samples were precipitated with sodium acetate and ethanol according to the agarase manufacturer's protocol. The other sets of duplicate samples were centrifuged in either Nanosep or competitive 30K UF centrifugal devices for 15 minutes (Nanosep device) to 30 minutes (competitors' devices). Using care to avoid a small pellet of undissolved agarose, the retentate was recovered in 30 µL TE and electrophoresed in a 0.8% TAE agarose gel. Precipitation (PPT), competitor regenerated cellulose (Comp RC), competitor polysulfone (Comp PS), high-flux polyethersulfone (Nanosep Device PES).

Results and Conclusions
Nanosep 30K MWCO devices gave higher and more reproducible yields of the DNA from the liquefied gel than the other devices or techniques. The low recovery for one of the precipitated samples may have been due to the inability to fully resuspend the dried pellet away from the small amount of undigested agarose that also pelleted during centrifugation.

Note: the Competitive RC is the same device lot as the high DNA binding RC shown in Figure 1. DNA binding may play a role in the lower yields for the Competitive RC.

Summary

These results demonstrate that use of Nanosep and Nanosep MF centrifugal devices is a simple and reliable way to purify nucleic acids from agarose gel slices. These centrifugal devices offer several advantages over other methods: minimal handling is required, the procedure is quick, and recoveries are high. The end product is DNA in a concentrated form that is free of soluble contaminants that may interfere with further reactions.