



## PCR: Before and After

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### Background

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 help prevent artifacts that arise from impurities is to work in an extremely controlled environment and to purify the reagents and primers prior to use. Even in a controlled environment, the sample itself can contain components that interfere with PCR or cause false priming. Using Nanosep® 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.

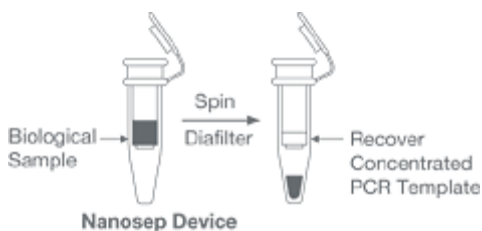
### Preparation of Biological Samples for PCR

#### Description

Biological samples that contain DNA templates come from a variety of sources. Many of these sources contain small molecules that can inhibit the function and accuracy of PCR amplification. For example, heparin and EDTA (commonly used to preserve blood samples) can interfere with PCR reactions. Removal of small molecules while concentrating template DNA can be done by ultrafiltration.

#### Step-by-Step Procedure

1. Dilute the template containing sample to 500  $\mu$ L with water or TE (10 mM Tris/1 mM EDTA, pH 8) and transfer to a suitable Nanosep UF device (Table 2).
2. Centrifuge at 5,000  $\times$  g for 10 to 30 minutes.
3. Optional: To perform discontinuous diafiltration, add a sufficient amount of buffer to bring the sample volume to 500  $\mu$ L and centrifuge again. Usually two cycles of dilution and concentration will remove over 99% of salts and over 90% of small molecular weight contaminants. If a higher level of purity is desired, repeat the dilution and concentration steps for a third time. Multiple diafiltration steps will decrease overall yields; therefore, quality versus yield considerations must be made.
4. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to 20  $\mu$ L TE or water.



## Preparation of Reagents and Solutions for PCR

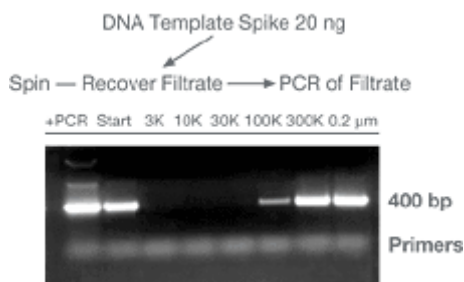
### Description

The extreme sensitivity of PCR can result in amplification of minute amounts of contaminating DNA. This is a particular problem with ancient DNA or forensic human DNA samples because one contaminating molecule from a technician's skin or hair cell can invalidate results.

Removal of contaminating DNA from stock reagents or master mixes can be done using ultrafiltration. To ensure that it is free of DNA, the final material synthesis buffer can be spun in a 3K or 10K Nanosep® UF device prior to aliquoting into PCR tubes (Figure 12). Note: Never bring amplified samples into the same work area that is used to prepare reagents or set up future PCR reactions. Always use aerosol-resistant tips for pipetting.

For full details, see Pall Life Sciences technical report, "[Nanosep Centrifugal Ultrafiltration Devices and PCR: Before and After](#)".

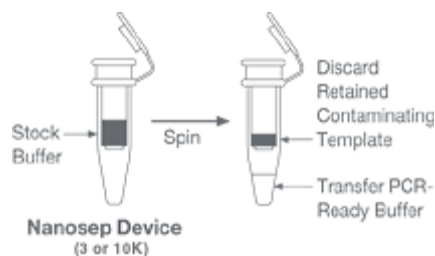
**Figure 12**  
Removal of DNA Template from PCR Stock Solutions



*DNA template sample of 500 ng plasmid pUC18 in TE was divided into seven 200 μL fractions, one fraction was kept as a control (START, 33 ng/μL) and the other fractions were centrifuged in Nanosep ultrafiltration devices. Following centrifugation, 3 μL was added to each PCR reaction containing 45 μL PCR mix (Life Technologies) and 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. No PCR product was detected using filtrate samples from the 3K, 10K, and 30K devices, demonstrating that they effectively removed the template spike. Filtrate from the 100K device gave a weak PCR product band, suggesting that some of the template was able to pass through this device. DNA template passed freely through the 0.2 μm and 300K devices.*

### Step-by-Step Procedure

1. Always use new or "set-aside" chemicals to make stock PCR reagents. Use care to avoid adding any new contaminants during preparation.
2. Prepare reagents away from the area used to process PCR products.
3. Centrifuge the stock reagents or master mixes (without primers, templates, or enzymes) in an appropriate 3K or 10K UF device (Table 2).
4. Transfer filtrate to sterile tubes. Sub-aliquotes are suggested to minimize future contamination of entire stocks.
5. For extra insurance, ultrafiltration can be used to filter the master mix or commercial buffer mix immediately prior to the start of PCR.



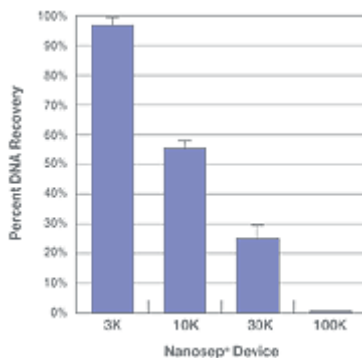
## Purification and Recovery of PCR Primers

### Description

Synthetic oligonucleotides are a critical component of the PCR reaction as they act as primers for the synthesis of DNA from DNA sequence-specific start points. Oligonucleotides are synthesized by chemical means. After synthesis, purification steps are required to purify the full-length oligonucleotides from the synthesis mixture. Prior to PCR, desalting is required to remove residual by-products from the synthesis, cleavage, and deprotection procedures. Ultrafiltration using centrifugal concentrators is an efficient, high-yield way to desalt and concentrate oligonucleotides (Figure 13).

For full details, see Pall Life Sciences technical report, "[Nanosep Centrifugal Ultrafiltration Devices and PCR: Before and After](#)".

**Figure 13**  
Purification and Recovery of 25 bp Oligonucleotides



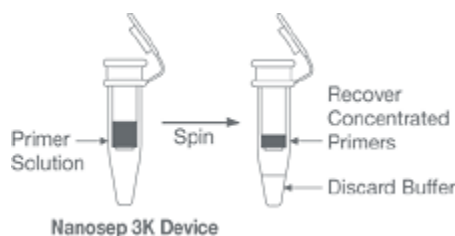
[Click graph to enlarge](#)

*A 400  $\mu$ L solution containing 50 ng end-labeled 25 bp oligonucleotides was filtered, 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  $\times$  g. The retained material was resuspended in 40  $\mu$ L TE, diluted to 400  $\mu$ L, and then recovered. Radiolabeled DNA was quantified using a scintillation counter. The Nanosep 3K device retained close to 90% of the radiolabeled oligonucleotide. 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 be the best choice.*

### Step-by-Step Procedure

1. For oligonucleotides up to 50 bases long, use a Nanosep 3K device. For longer oligonucleotides, the Nanosep 10K device can be used.
2. Place the oligonucleotide solution in the sample reservoir. Dilute the sample to a volume of 500  $\mu$ L with TE (10 mM Tris/1 mM EDTA, pH 8) or water.
3. Optional: To perform discontinuous diafiltration, add a sufficient amount of buffer to bring the sample volume to 500  $\mu$ L and centrifuge again. Usually two cycles of dilution and concentration will remove over 99% of salts and over 90% of small molecular weight contaminants. If a higher level of purity is desired, repeat the dilution and concentration steps for a third time. Multiple diafiltration steps will decrease overall yields; therefore, quality versus yield considerations must be made.

4. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to 20  $\mu\text{L}$  TE or water.



## Cleanup and Recovery of PCR Products

### Description

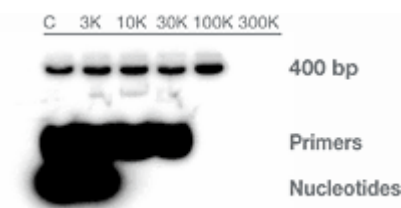
The final PCR reaction may contain up to a microgram of amplified DNA that can be used for a variety of molecular biology applications. These applications may be more or less sensitive to the residual components of the PCR reaction mix. Certain restriction enzymes and particularly DNA ligase are very 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 cleanup of the PCR product.

Purification of the PCR product away from buffer components, free nucleotides, and primers 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 involved and incomplete removal of co-precipitating buffer components and contaminants.
2. **Chromatography**, using size exclusion resin 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 (Figure 14). It is rapid, there is very little handling, yields are high, DNA is undamaged, and the concentrated DNA is free of contaminants that may inhibit downstream reactions (Figure 15).

For full details, see Pall Life Sciences technical report, "[Nanosep Centrifugal Ultrafiltration Devices and PR: Before and After](#)".

**Figure 14**  
Purification and Recovery of PCR Products



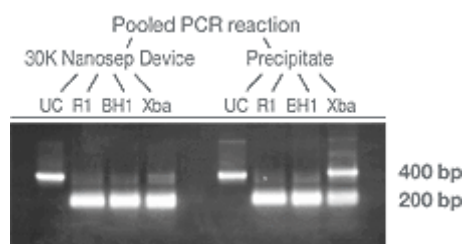
A small amount of  $^{32}\text{P}$ -labeled dCTP was added to the PCR reaction mix (Life Technologies) containing  $^{32}\text{P}$ -end labeled primers. The reaction was run for 30 cycles using standard conditions. Ten reactions were pooled and 100  $\mu\text{L}$  aliquots were diluted to 500  $\mu\text{L}$  and centrifuged in the indicated MWCO devices. The retained material was recovered in 20  $\mu\text{L}$  TE, electrophoresed using a 10% polyacrylimide Tris Borate gel (BioRad) and analyzed by autoradiography. The 100K Nanosep device demonstrated 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 10K device may be used if the PCR product is smaller than 200 bp and the presence of primers does not inhibit

*downstream applications.*

### Step-by-Step Procedure

1. Avoid pipetting the oil or wax into the subsequent steps. Oil can be removed by freezing the sample at  $-70\text{ }^{\circ}\text{C}$  and drawing off the surface liquid, or the sample can be successfully removed by careful pipetting with a narrow bore pipette tip from below the surface of the oil.
2. Dilute the reaction mixture to  $500\text{ }\mu\text{L}$  with water or TE (10 mM Tris/1 mM EDTA, pH 8) and transfer to a Nanosep 30K or 100K UF device (Table 2).
3. Centrifuge at  $5,000\text{ } \times g$  for 10 minutes (greater speeds increase PCR product loss).
4. Optional: To perform discontinuous diafiltration, add a sufficient amount of buffer to bring the sample volume to  $500\text{ }\mu\text{L}$  and centrifuge again. Usually two cycles of dilution and concentration will remove over 99% of salts and over 90% of small molecular weight contaminants. If a higher level of purity is desired, repeat the dilution and concentration steps for a third time. Multiple diafiltration steps will decrease overall yields; therefore, quality versus yield considerations must be made.
5. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to  $20\text{ }\mu\text{L}$  TE or water.

**Figure 15**  
Biological Activity of Purified PCR Products



*A pooled mixture of PCR products was divided in half. One fraction was precipitated with 1/10th volume sodium acetate and two volumes ethanol, chilled at  $-20\text{ }^{\circ}\text{C}$  for 2 hours, centrifuged at high speed for 30 minutes, rinsed with 70% ethanol and air dried prior to resuspension in  $40\text{ }\mu\text{L}$  water. The second fraction was diluted to  $500\text{ }\mu\text{L}$  and centrifuged for 15 minutes in a 30K Nanosep device. The retained material was resuspended in  $40\text{ }\mu\text{L}$  water and divided into  $10\text{ }\mu\text{L}$  aliquots for analysis. One fraction was electrophoresed as the uncut (UC) control and the others were diluted into a  $20\text{ }\mu\text{L}$  restriction digest reaction according to manufacturer's instructions (EcoRI = RI, BamHI = BH1, Xba1 = Xba). These samples were digested for 30 minutes at  $37\text{ }^{\circ}\text{C}$ , electrophoresed in a 1.5% agarose gel, and DNA bands were visualized by staining with ethidium bromide. Our data show clearly that the XbaI enzyme is unable to digest to completion the 400 bp PCR fragment from a sample that has been precipitated. In contrast, the sample that was rapidly purified with the 30K device was completely digested using XbaI, indicating that use of this device removed contaminants that could interfere with digestion.*