Purification of PCR Products in Corning® 96 Well Filterplates

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Introduction

A common requirement in the manipulation of nucleic acids to perform sequencing, cloning or hybridization assays is polymerase chain reaction (PCR) amplification of a DNA region of interest. The result of this reaction is the amplified desired sequence along with a mixture of nucleotides, primer oligonucleotides, and buffer components. This sequence of interest is usually purified prior to subsequent manipulation. Many methods are employed in this effort including gel electrophoresis, silica based affinity matrices, precipitation, and ion exchange or size exclusion chromatography columns (1,2,3). For low throughput applications, these matrices are applied to single spin column devices. However, with an increasing number of samples a multiwell format such as the 96 well microplate is required to process more samples in the same time without increasing the cost. A new 96 well filter bottom microplate from Corning Incorporated can be utilized effectively to aid in this task.

The purpose of this article is to portray the utility of Corning's new filterplate by describing one of many sample preparation applications suited to this product. Conducting purification of DNA from a PCR reaction using a gel filtration matrix based approach will highlight features of the Corning® 96 well filter bottom microplate. The method used here is an adaptation of that described by Hood et al. (1). Commercially-available silica-based affinity products as well as gel filtration matrix-based products have been included in this comparative analysis.

Materials and Methods

Enzymatic amplification

As a model system, a 500-nucleotide sequence of bacteriophage Lambda DNA was used to analyze the performance of the purification technique. Enzymatic amplification was performed using the GeneAmp® PCR Reagent Kit (PE Applied Biosystems, Foster City, CA). Primer sequences are shown in Figure 1. The reaction mix consists of 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.05 U/µL AmpliTaq® Polymerase, 0.2 µM of each primer, 200 µM of each nucleotide, and 0.01 ng/µL of full length bacteriophage Lambda DNA as a template for amplification. 75 µL of this mix was added to all wells of a Corning® 96 well Thermowell® microplate.

PCR reactions were carried out using a Perkin Elmer GeneAmp® System 9600 or 9700 Thermal Cycler. The initial starting temperature was held at 94°C for 5 min, followed by 30 cycles of amplification programmed as follows: denature

Nucleotide — Region for Primer 1

7131		5'	GATGAGTTCG	TGTCCGTACA	ACTGGCGTAA	TCATGGCCCT
7171	TCGGGGCCAT	TGTTTCTCTG	TGGAGGAGTC	CATGACGAAA	GATGAACTGA	TTGCCCGTCT
7231	CCGCTCGCTG	GGTGAACAAC	TGAACCGTGA	TGTCAGCCTG	ACGGGGACGA	AAGAAGAACT
7291	GGCGCTCCGT	GTGGCAGAGC	TGAAAGAGGA	GCTTGATGAC	ACGGATGAAA	CTGCCGGTCA
7351	GGACACCCCT	CTCAGCCGGG	AAAATGTGCT	GACCGGACAT	GAAAATGAGG	TGGGATCAGC
7411	GCAGCCGGAT	ACCGTGATTC	TGGATACGTC	TGAACTGGTC	ACGGTCGTGG	CACTGGTGAA
7471	GCTGCATACT	GATGCACTTC	ACGCCACGCG	GGATGAACCT	GTGGCATTTG	TGCTGCCGGG
7531	AACGGCGTTT	CGTGTCTCTG	CCGGTGTGGC	AGCCGAAATG	ACAGAGCGCG	GCCTGGCCAG
7591	AATGCAATAA	CGGGAGGCGC	TGTGGCTGAT	TTCGATAACC	3'	

Region for Primer 2

Figure 1. Target sequence of bacteriophage Lambda and primers for amplification reactions.

at 94°C for 30 sec, anneal at 55°C for 30 s, extend at 72°C for 30 sec; after cycle 30, samples were held at 72°C for 5 min. When samples were not processed immediately after the run, they were held at 4°C until removed from the cycler. An aliquot of the unpurified amplification product was examined by agarose gel electrophoresis prior to further manipulation. One should note that the PCR protocol was designed to yield a distinct primer dimer band upon agarose gel electrophoresis for illustrative purposes.

Purification of amplified DNA sequence

Commercial purification products were used as recommended by the manufacturer. To compare the microcentrifuge-tube-based products, 50 µL of PCR reaction was used in all protocols. The eluant volume was adjusted to 50 µL with elution buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The multi-column spin-plate was prepared using the Corning® 96 well styrene bodied filter plate with a supported 0.2 µm hydrophilic PVDF membrane disk at the bottom of each well (Cat. No. 3504). The Plate was placed on the top of a 1 mL 96 well polypropylene assay block to collect wash liquid. Sephacryl® S-400 HR (Amersham Pharmacia Biotech, Inc. Piscataway, NJ) was diluted 1:1 in 10 mM Tris, 1 mM EDTA, pH 8.0. Using a multi-channel pipette, 230 µL of this diluted gel filtration media was transferred into all wells of the 96 well filter plate. The assembly was then centrifuged at 600 x g for 2 min in a Sigma 4K15C centrifuge equipped with a microplate rotor. This was repeated until approximately 700 µL of the diluted filtration matrix had been added and packed into a mini-column of about two-thirds the height of the well. The columns were then washed three times with 100 µL of elution buffer using the same centrifuge settings as described above.

The prepared plate was then placed on a Corning® 96 well polypropylene V-bottom collection plate. 40 µL of the PCR reaction was loaded slowly with a multichannel pipette. For best results, care was taken so as to not disturb the top of the gel bed. The assembly was placed in the centrifuge and spun at 800 x g for 2 min. Depending on the recovery and purity desired 10 to 20 µL of additional elution buffer was added to each well, and the filter plate was spun again at 800 x g for 2 min. The resulting eluate could now be used for sequencing or hybridization assays.

Agarose gel electrophoresis

Purity and recovery of the samples was evaluated by electrophoresis on a 1.2% agarose gel. 2.0 µL of the purified samples and an equivalent amount of unpurified amplification product were electrophoresed to separate the product band from the primer-dimer band. The gel was stained with SYBR® Green 1 fluorescent dye (Molecular Probes, Eugene, OR) to visualize the DNA bands, and then analyzed using the FluorImager® with the ImageQuant® software (Molecular Dynamics, Sunnyvale, CA)

DNA Sequencing

Sequence data on purified PCR fragments was generated with an ABI PRISM® system using a BigDye™ sequencing Kit (PE Applied Biosystems Foster City, CA). The reactions were performed as recommended by the manufacturer. Primers were of the same sequence as that used in the amplification reaction. Three gel filtration purified samples were randomly chosen from wells of the 96 well collection plate. 2 µL of purified sample plus 20 ng of primer were used for each sequencing reaction. Each sequencing reaction was dried, then resuspended in 6 µL of loading dye. 1.5 µL per sample was loaded in each lane for sequencing.

Results and Discussion

Today, the use of centrifugation-driven purification devices is a routine occurrence in many laboratories. Depending on the approach taken the resulting sample quality can differ significantly. DNA purity is commonly assessed by gel electrophoresis. This technique was chosen for the work described here. The differences between some of the many commercially available microcentrifuge-based devices are readily apparent upon examination of the gel image in Figure 2. The starting material for this comparison was the result of a non-optimal PCR reaction. Typically, a well-optimized reaction will not give this large excess of primer dimer; its use here is to illustrate the removal of these contaminating sequences from the product. There is a large difference in the result depending on the type of purification matrix chosen. The matrix Sephacryl®-400 HR, used to purify samples in lanes 4-6, was chosen to be loaded into the 96 well filterplate. This size exclusion media gave good results compared to the silica-based devices (lanes 13-18) and was the obvious choice among the gel filtration based devices if purity of the DNA is required.

A typical result of the simultaneous purification of 96 samples is shown in Figure 3. In the separation portrayed here, a recovery of 80% was achieved while reducing the contaminating primer-dimer up to five-fold. The gel that was analyzed to generate the chart in Figure 3 is shown in Figure 4. The analysis of all 96 wells on a single gel has been performed repeatedly (n=5), resulting in a range of variations in the intensity of all 96-500 base pair bands from 14 to 30%. Optical density measurements of the purified DNA at 260 nm have shown an average of 25% variation in concentration of the purified sample for an entire plate.

The mini-gel filtration device described here is not a novel purification technique. In applications where size separation is required, this 96 well spin-column device can greatly increase the throughput as well as reduce the overall cost. The cost for the purification protocol described here, including all materials, is about one quarter of the cost of a popular commercial purification kit. Using this protocol, two plates can be prepared in about 30 min in a two-bucket centrifuge rotor. The protocol routinely achieves 50 to 70% recovery of amplified DNA without significant contamination from nucleotides or small fragments and primer dimer. Using size-exclusion-based separation, recovery and purity of a sample are determined by several factors: column height, sample volume and concentration, molecular size, driving force, and duration of centrifugation (1). The typical amount of DNA purified in this work was in the range of 200 ng to 2.0 µg in a volume of 40 µL. It has been shown here that the purification achieved with this technique produces product of sufficient purity for further manipulation. Little or no background interference was observed in the sequencing reaction of the purified product (Figure 4). The sequence produced from the gel filtration-purified PCR reactions was of equal quality to that obtained from a commercially available silica-based 96 well purification device (Data not shown).

The filter bottom plate described above can be considered a universal multicolumn device. There are many other chromatography matrices that would work equally well in the 96 well format. Any of the gel filtration media in the market today can be utilized in the Corning® 96 well filterplate to achieve a wide variety of separations. With the use of affinity gels, such as those functionalized with protein A or G, one could purify monoclonal antibodies from ascities in a high throughput format. This would aid in the screening

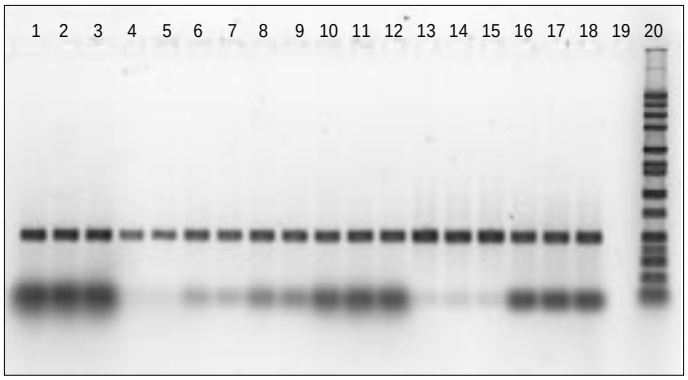

Figure 2. Purity of DNA using different commercially available microcentrifuge tube devices. Lanes 1-3 are unpurified PCR samples, lanes 4-12 are triplicates of 3 different gel filtration based units with different size exclusion properties. Lanes 13-18, (n=3) show the purification results using two different commercial products with a silica based affinity matrix. Lane 1 is Hi-Lo™ (DNA Marker (Bionexus Inc.).

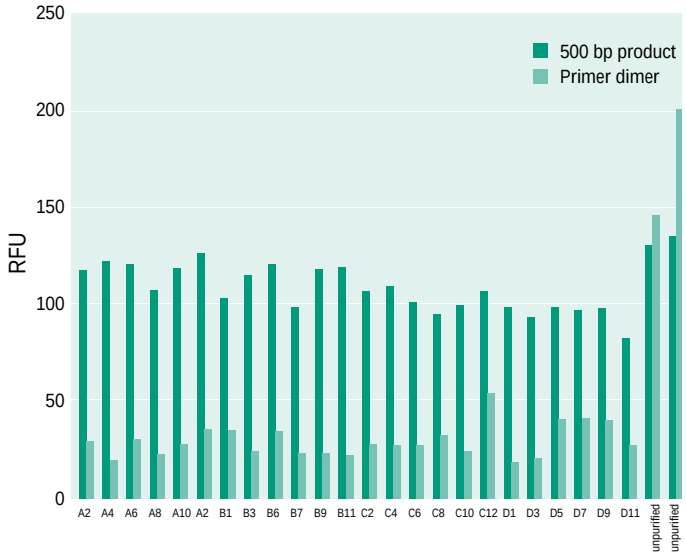

Figure 3. Fluorescence intensity analysis of the image in Figure 4. X-axis labels are well positions from a 96 well plate. Sample recovery based on fluorescence intensity of the band of interest is 80%. The variation in recovery for these 24 representative wells out of 96 samples is 11.2%.

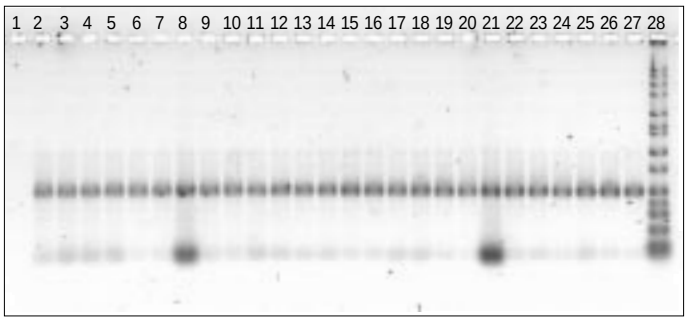

Figure 4. Amplification product purified in the 96 well filterplate with gel filtration media. Lanes 8,21 are the unpurified reaction products. Lane 28 is Hi-Lo™ (DNA Marker (Bionexus Inc.). The remaining lanes show the results of 24 samples from a 96 well purification run representing the entire plate.

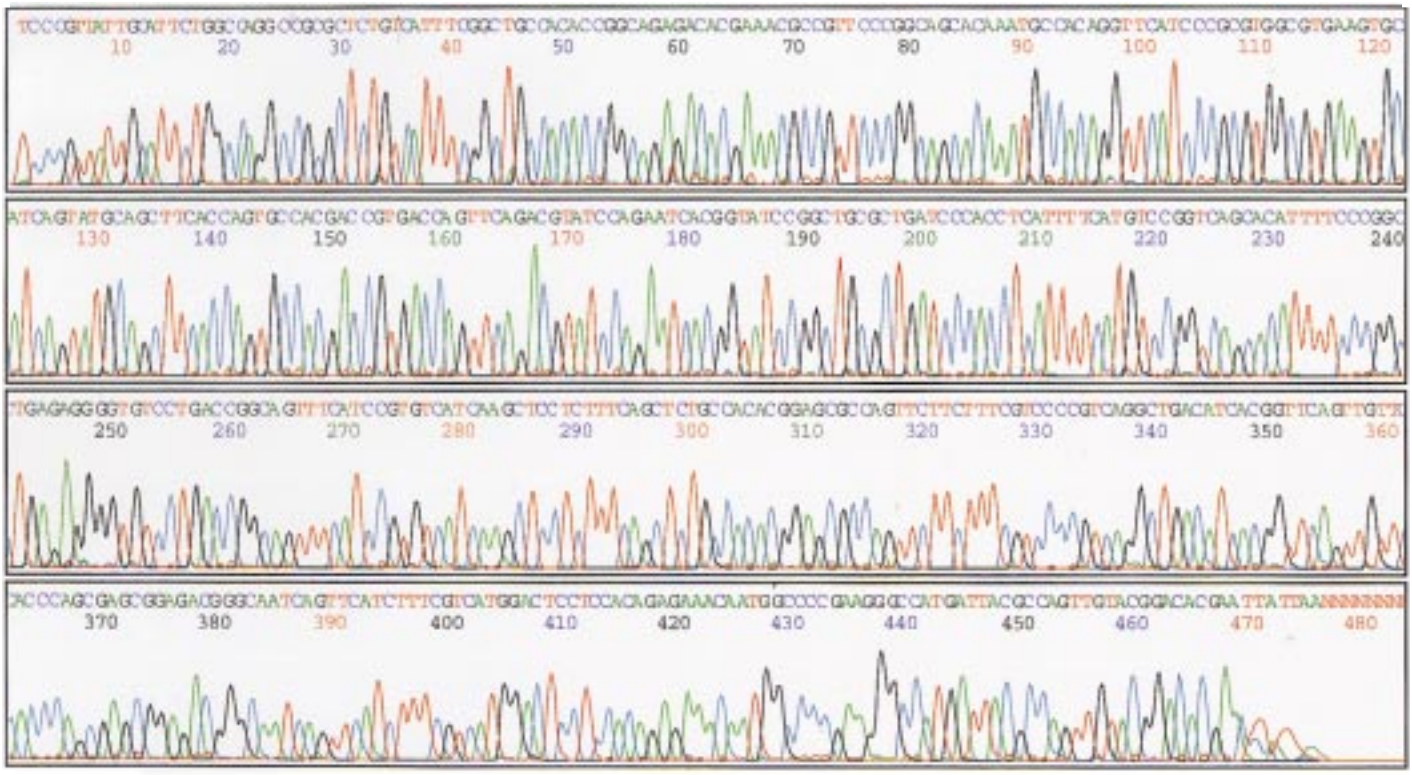

Figure 5. Sequence electropherogram of a 500 bp region of the Lambda phage. Sample template was purified using the Corning® filterplate loaded with a gel filtration media.

of multiple cell lines or growth conditions. This platform can also be used with bead- or silica particle-based systems to purify both proteins and nucleic acids from a wide variety of sources. Additional filterplate products from Corning Incorporated are being evaluated at this time. Several different membrane types will greatly expand the applications potential of this product.

For further information, please contact Cutomer Service, Corning Incorporated, Science Products Division, Advanced Life Science Products (USA), Tel: 1-800-492-1110, Fax 978-635-2476.

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