Laboratory

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Application Note

AcroPrep™ Advance 96-Well Long Tip Filter Plate for Nucleic Acid Binding for High-Throughput Purification of Plasmid DNA

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1. Introduction

The demand for high purity plasmid DNA continues to increase in molecular biology research. Advances in cloning techniques have greatly increased the number of samples, necessitating high-throughput small-scale plasmid preparation.

Various applications such as drug delivery, preclinical studies, and production of DNA vaccines require different amounts of high quality plasmid DNA. Typically, selection and optimization of suitable plasmid DNA for these applications start with small scale cultures of 1-1.5 mL volume. Plasmid DNA purified from these bacterial cultures provides material for clone verification. Further characterization is often accomplished by techniques such as polymerase chain reaction (PCR), restriction enzyme digestion combined with gel analysis, and Sanger sequencing.

The alkaline lysis method of plasmid DNA isolation is commonly combined with further purification over silica membranes. In this method, *E. coli* cells harboring plasmids are lysed under alkaline conditions in the presence of a detergent. After neutralization of the lysate, the plasmid DNA is separated from precipitated bacterial genomic DNA and bacterial proteins. The plasmid DNA remains in solution after the precipitate is separated via centrifugation or filtration.

Here we describe the use of AcroPrep Advance 96-well Long Tip Filter Plate for Nucleic Acid Binding (Pall NAB plate) for small scale plasmid DNA purification. These plates contain a unique quartz-based media suitable for nucleic acid isolation and purification and a new long tip geometry that minimizes hanging-drops and crosstalk. Combined, these improvements translate into higher throughput, increased sensitivity and signal-to-noise ratios, and reduced processing times.

The plasmid DNA purification protocol described in this application note relies on standard reagents that can readily be obtained and prepared in-house by the researcher. Clarification of the lysate is accomplished via filtration with the Pall AcroPrep Advance filter plates for lysate clearance or via centrifugation. The 96-well Pall NAB plate offers researchers an attractive platform for small-scale plasmid DNA purification by providing increased throughput and consistency without sacrificing the quality. The purified plasmid DNA is suitable for downstream applications such as PCR, restriction enzyme digestion, and Sanger sequencing.

Hanging-drop and crosstalk volumes for Pall NAB plates were evaluated in a typical plasmid DNA purification process. The data illustrate that the new long tip geometry found in Pall NAB plates significantly minimizes the hanging-drop formation and crosstalk when compared to its predecessor with short tip geometry.

2. Materials & Methods

2.1 Plasmid DNA Purification Protocol for Pall NAB plates

Table 1

Buffers and their compositions for pDNA purification via Pall NAB Plates

Buffer	Composition
Resuspension Buffer	50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/mL RNase A
Lysis Buffer	200 mM NaOH, 1% SDS
Neutralization Buffer	3.0 M Potassium Acetate, pH 5.5
Binding Buffer	6 M Guanidine-HCl
Wash Buffer	10 mM Tris-HCl, pH 7.5, 80% Ethanol
Elution Buffer	10 mM Tris-HCl, pH 8.0, 1 mM EDTA

One liter of *E. coli* DH5α carrying high-copy number plasmid pGEM◆-luc (Promega) was grown overnight at 37 °C in LB broth supplemented with 100 µg/mL ampicillin. Aliquots of the culture solution (1.5 mL/well) were transferred to two deep well plates with 2 mL well volume. The cells were pelleted at 5,000 x g for 10 min, and the supernatant was decanted.
Purifications were carried out using the standard buffer solutions presented in Table 1. Resuspension buffer (150 µL/well) was added to the plate, and the cell pellets were resuspended. Then, lysis buffer was added (150 µL/well), and the plate was shaken using a plate shaker for 2 min. Next, neutralization buffer was added (150 µL/well), and the plate was shaken for 2 min. Lysate clarification was carried out via vacuum filtration at 33.9 kPa (10 in. Hg) using the Pall AcroPrep Advance filter plate for lysate clearance (PN: 8175) or via centrifugation (1,000 x g for 5 min).

Lysate cleared by centrifugation or filtration was transferred to the Pall NAB plate, and binding buffer (450 µL/well) was added and mixed by pipetting up and down. Next, the plasmid DNA was bound to the membrane by vacuum filtration at 50.8 kPa (15 in. Hg) on a vacuum manifold (Pall, PN: 5017). After that, wash buffer (600 µL/well) was added, and vacuum was applied to remove the washing solution. This washing process was repeated. Then, the outlet of the filter plate was blotted on an absorbent towel to dry. For the elution of the bound pDNA, elution buffer was added to the wells (70 µL/well), and the plate was incubated at room temperature for one minute. Then, the elution was performed via either vacuum at 50.8 kPa (15 in. Hg for 1 min) or via centrifugation (1,000 x g for 5 min). The elution volume in the receiver plate was determined as the weight difference between the pre-weighed empty and the filled collection plate. The average elution volume per well was then calculated by dividing this weight difference by the number of filled wells.

2.2 Plasmid DNA Purification with Commercially Available Kits

Two commercially available plasmid DNA purification kits, CK1 and CK2, were utilized as recommended by the manufacturer.

2.3 Downstream Applications for Purified Plasmid DNA

The purity (A_{260}/A_{280}) of the eluted plasmid DNA was measured using a NanoDrop◆ 8000 spectrophotometer (Thermo Fisher Scientific).

The purified plasmid DNA was used for the following downstream applications:

1) PCR amplification using standard T7 and SP6 primers. The PCR product was visualized on a 1% agarose gel stained with ethidium bromide.
2) Restriction digestion with enzymes *Sph*I and *Eco*RI. Undigested and digested plasmid DNA were visualized on a 1% agarose gel stained with ethidium bromide.
3) Sanger sequencing. Sequence of the luciferase insert was verified using standard T7 and SP6 primers. Average read-length of high quality sequence was determined.

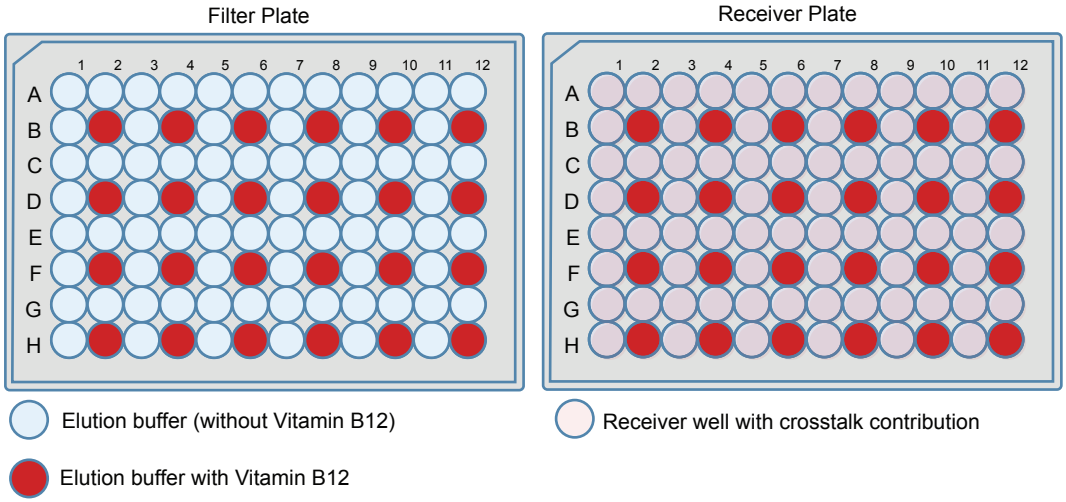
2.4 Hanging-drop and Crosstalk Volume for Plasmid DNA Purification

Hanging-drop and crosstalk volume determination took place following the DNA elution step of a regular plasmid isolation procedure as described in section 2.1. To determine the influence of the tip geometry, these experiments were carried out with long tip Pall NAB plates and Pall's previous generation AcroPrep Advance 96-well filter plates for DNA purification (PN 8132), which were equipped with short tips. Hanging-drop volume determination following plasmid DNA elution via vacuum filtration was facilitated by using an elution buffer that was colored crimson through the addition of 0.5% vitamin B12 (elution buffer/VitB12).

Upon completion of vacuum filtration, after all the wells were emptied, the volume of the hanging-drops that did not release under the influence of the vacuum was measured. The outlet of the plate was blotted on a pre-weighed dry blotting paper to absorb the hanging-drops. The wetted blotting paper was weighed, and the hanging-drop volume was calculated by dividing the weight differential by the number of filled wells.

To determine the crosstalk volume, elution buffer and elution buffer/VitB12 were loaded into the plasmid DNA binding plate according to the arrangement depicted in Figure 1. The solutions were eluted by vacuum filtration and collected into a pre-weighed receiver plate. The total elution volume in the receiver plate was determined by measuring the weight difference of the pre-weighed empty collection plate and the plate filled with elution buffer. The elution volume per well was calculated by dividing this weight by the number of filled wells in the plate. Then, a calibration curve was obtained measuring the OD_{500} via the calibration standards prepared using elution buffer and elution buffer/VitB12. The cross-talk volume per receiver well was quantified via the equation: $V_1 = (C_2/C_1)V_2$, where $C_1 \equiv$ original VitB12 concentration in the buffer, $V_1 \equiv$ crosstalk volume (µL), $C_2 \equiv$ VitB12 concentration of the neighboring receiving well solution, and $V_2 \equiv$ average volume of the solution (µL) in a receiver plate well.

Figure 1



The arrangement of elution buffer (without VitB12) and elution buffer/VitB12 in a 96-well plate. In filter plate (left), wells are filled with elution buffer alone (white wells), or with elution buffer spiked with Vitamin B12 (red wells). Crosstalk volume in receiver plate (right) is determined by measuring Vitamin B12 concentration in adjacent wells (pink wells)

3. Results and Discussion

3.1 Plasmid DNA Purification

Plasmid DNA yield generally depends on bacterial strain, plasmid copy number and size, growing conditions, and culture medium. Typically, a total plasmid DNA yield of up to 15 µg/well can be expected from a culture of *E.coli* strain DH5α with a high copy number plasmid.

Plasmid DNA yield was assessed by A_{260} absorbance. The results are summarized in Table 2. Using standard reagents, Pall NAB plates generated an average plasmid DNA yield of 6.4 µg/well for the centrifugation-based approach and 7.1 µg/well for the filtration-based approach. While the costlier commercial kits CK1 and CK2 yielded higher amounts of plasmid DNA, downstream applications such as polymerase chain reaction, PCR, and Sanger sequencing generally require only sub-microgram quantities of plasmid DNA. Performance of plasmid DNA in those applications therefore provides a more meaningful measure.

Purity of the plasmid DNA was assessed by A_{260}/A_{280} ratio. DNA with an A_{260}/A_{280} ratio of 1.8 to 2.0 is generally regarded as ''pure.'' A lowered ratio indicates copurification of "contaminants'' such as proteins whose aromatic amino acids contribute to absorbance at 280 nm. As shown in Table 2, plasmid DNA isolated with the Pall NAB plates, both via vacuum filtration and centrifugation, had average A_{260}/A_{280} ratios of 1.87, indicating that the Pall NAB plates yield high quality plasmid DNA. The commercially available plasmid DNA purification kits yielded plasmid DNA of similar quality with only marginally higher A_{260}/A_{280} ratios.

Table 2

Plate/Kit	Lysate Clarification	Average Yield (µg/well)	A_{260}/A_{280}
Pall NAB Plates	Centrifugation	6.4	1.87
	Filtration	7.1	1.87
Commercial Kit 1 (CK1)	Filtration	10.4	1.91
Commercial Kit 2 (CK2)	Filtration	10.9	1.92

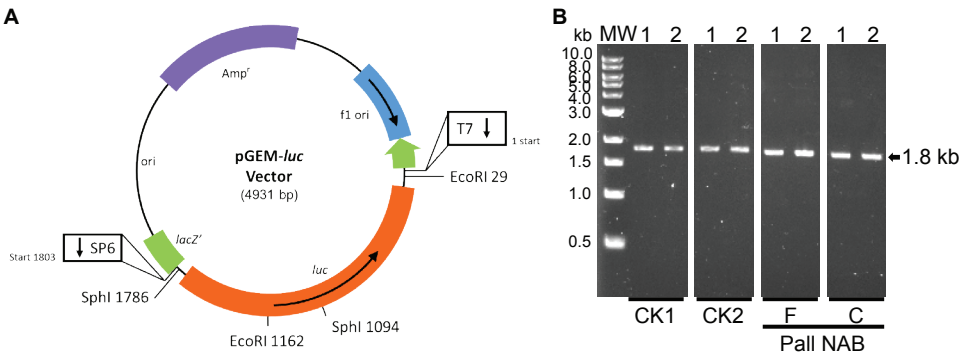
The yield and purity (A_{260}/A_{280}) for high-copy number plasmids. E. coli DH5α cells transformed with pGEM-luc vector were grown overnight in LB medium containing 100 µg/mL of ampicillin. The pGEM-luc vector plasmids were purified using the Pall NAB plates and two commercially available plasmid DNA purification kits. Yield and purity (A_{260}/A_{280}) of the plasmid DNA were determined with a NanoDrop UV/Vis spectrophotometer.

3.2 Quality and Suitability of Purified Plasmid DNA for Downstream Applications

3.2.1 PCR Amplification and Agarose Gel Electrophoresis

Suitability of the purified plasmid DNA for PCR analysis was demonstrated by using it as the template for amplification with standard T7 and SP6 primers. As indicated in Figure 2A, this approach should generate a 1.8 kb PCR amplicon. Quality, purity, and structural integrity of the plasmid DNA were evaluated by electrophoresis of PCR products on a 1% agarose gel stained with ethidium bromide, and the resulting electropherograms are shown in Figure 2B. The PCR products obtained from plasmid samples purified with the Pall NAB plates and two commercially available kits (CK1 and CK2) demonstrate the presence of the expected 1.8 kb fragment as a single clear band of similar intensity without other contaminating products. The consistent appearance of the tight DNA bands and the absence of low molecular weight smear demonstrate the excellent quality of the plasmid DNA obtained with the Pall NAB plates.

Figure 2



Agarose gel analysis of PCR products of the high-copy number plasmids. Purified plasmid DNA (pGEM-luc) was subjected to PCR amplification using standard T7 and SP6 primers. A) pGEM-luc vector circle map showing the annealing locations of T7 and SP6 primers and restriction site locations for restriction enzymes EcoRI and SphI (adapted from Promega technical bulletin TB104). B) Agarose gel electrophe-rogram showing the 1.8 kb PCR products of plasmid DNA samples purified using commercial kits 1 and 2 (CK1 and CK2), and Pall NAB plates via filtration (F) and centrifugation (C). Lanes 1 and 2 indicate two replicate sample runs.

3.2.2 Restriction Enzyme Digestion

In molecular biology, restriction enzymes play a vital role in both construction of plasmid DNA molecules and screening of the resulting constructs by restriction mapping. These applications require plasmid DNA of high quality to allow restriction enzymes to cut with high fidelity. Under sub-optimal conditions, restriction enzymes, as for instance *Eco*RI, are known to exhibit star activity, where the enzymes cut non-canonical sequences.

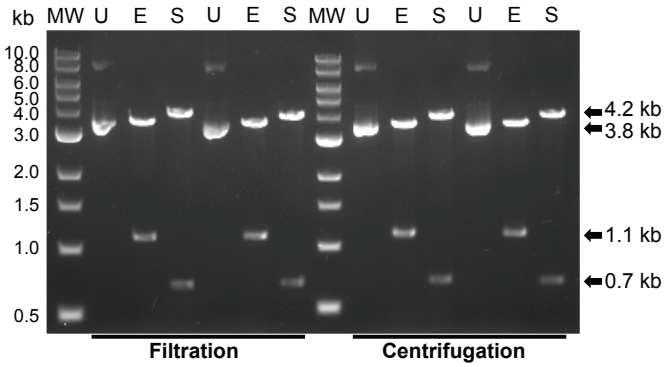
High quality and integrity of the purified plasmid DNA were demonstrated through restriction digests with enzymes *Eco*RI and SphI followed by agarose gel electrophoresis of the resulting products. The expected *Eco*RI and *Sph*I digestion fragment sizes are tabulated in Figure 3A. The resulting agarose gel electropherogram is shown in Figure 3B.

In Figure 3B the lanes marked with U represent undigested plasmid DNA and show a predominant band of approximately 3 kb reflecting the supercoiled conformation. An additional band with a molecular weight >8 kb represents a multimeric form of the plasmid. No nicked digestion resistant forms of the plasmid were detected.

Restriction digestion fully resolved the supercoiled form and its concatemers to linear fragments. Only distinct bands of the expected sizes were obtained for all the samples treated with *Eco*RI or *Sph*I enzyme (Lanes E and S, respectively, in Figure 3B), indicating the absence of DNase activity in the purified plasmid DNA samples. Similar results were obtained in digests of plasmid DNA purified with commercially available kits CK1 and CK2 (data not shown).

Figure 3

Restriction Enzyme	Restriction Fragment	
	Number	Size (bp)
Eco RI	1	3,798
	2	1,133
Sph I	1	4,239
	2	692



Restriction enzyme digestion of high-copy number plasmids. Plasmid pGEM-luc isolated from E. coli DH5α using the Pall NAB plates was digested with enzyme EcoRI or SphI for 1 hour at 37 °C. Digested samples and undigested control samples were resolved on a 1% agarose gel and stained with ethidium bromide. A) Expected plasmid DNA fragment sizes for EcoRI and SphI digestions. B) Gel electropherogram with undigested plasmid and EcoRI and SphI digestion products of plasmid isolated with Pall NAB plates. Method by which precipitate was removed from lysate following neutralization step is indicated by labels Filtration and Centrifugation. Lanes marked MW indicate dsDNA molecular weight marker; lanes marked U indicate undigested samples; lanes marked E and S indicate EcoRI and SphI digested samples, respectively.

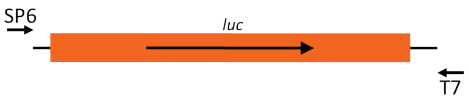
3.2.3 Sanger DNA Sequencing

Sanger sequencing is often used for final verification of plasmid integrity. In order to demonstrate the quality of the purified DNA for this application, the insert of the purified plasmid DNA was sequenced using standard primers directed against T7 and SP6 promoters that flank the insert (Figure 4A).

The average QV20+ read lengths (total number of bases in the entire trace that have a base calling accuracy > 99%) are tabulated in Figure 4B. The read length obtained by Sanger sequencing is governed by a combination of sequencing chemistry, instrumentation, and plasmid DNA quality. Extra-long sequencing protocols allow read lengths of approximately 900 bases. Average read lengths of the sequences obtained from plasmid DNA purified using the Pall NAB plates and commercially available kits all were greater than 975 bases, indicating the excellent quality of the plasmid DNA. Figure 5C shows a representative electropherogram obtained for the plasmid DNA purified via Pall NAB plate.

Figure 4

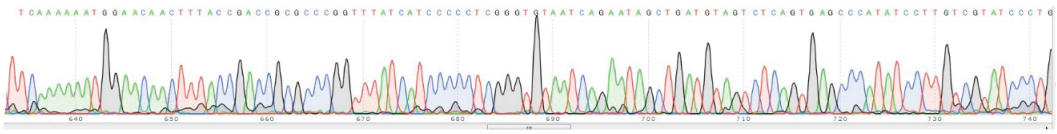
A



B

Plate/Kit	Lysate Clarification	QV20+ Read Length	
		Average	SD
Pall NAB Plate	Centrifugation	976.2	22.6
	Filtration	994.5	19.4
Commercial Kit 1	Filtration	987.5	18.3
Commercial Kit 2	Filtration	987.5	24.5

C



DNA sequencing of the purified high-copy number plasmids. A) The insert flanked by standard T7 and SP6 promoters (Figure 2A) was sequenced using the Sanger technique. B) Average QV20+ read lengths obtained for the plasmid DNA purified with Pall NAB plates and commercial kits. Three samples from each condition were analyzed. C) Resulted sequencing electropherogram for the plasmid DNA purified from Pall NAB plate.

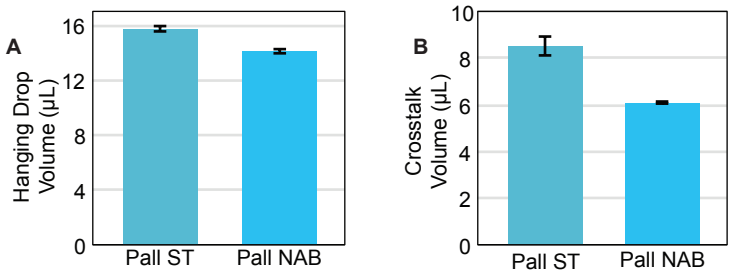
3.3 Hanging-drops and Crosstalk

In multi-well plate context, minimization of hanging-drop formation is critical for maintaining sample integrity and reducing the potential for crosstalk between adjacent wells.

Crosstalk occurs when the content of one well of the filter plate contaminates samples in adjacent wells of the receiver plate. Utilization of 96-well filter plates that minimize crosstalk is vital, especially when dealing with analytes for which the signal-to-noise ratio is very close to that of the blank, or where small amounts of contaminants can generate false positive results as for instance PCR applications. Therefore, design attributes which minimize hanging-drops and crosstalk can dramatically improve performance consistency, reproducibility, and data quality.

Hanging-drop and crosstalk volumes for the Pall NAB plates with long tip geometry were quantified and benchmarked against Pall's previous generation plasmid DNA purification plates (Pall ST) with short tip geometry. The results, summarized in Figure 5, show a direct correlation between hanging-drop and crosstalk volume. The hanging-drop volume was found to be 10% lower for the Pall NAB plates compared to Pall's previous generation plasmid DNA purification plates. Correspondingly, the crosstalk volume was determined to be ~30% lower for the Pall NAB plates.

Figure 5



Hanging-drop and crosstalk volumes for the Pall NAB plates and Pall's previous generation plasmid DNA purification plates (Pall ST). A) In hanging-drop experiments, three plates of each type were tested, and the averages were calculated. B) In crosstalk experiments, the wells were filled with elution buffer (24 wells) and elution buffer/VitB12 (72 wells) as shown in Figure 1. After 1 min incubation, the solutions were evacuated, and the receiver plates were analyzed for crosstalk. Three plates of each type were tested, and the averages were calculated (216 data points = 72 wells for elution buffer (without VitB12) x 3 plates).

4. Conclusion

Pall understands that researchers want high quality plasmid DNA when preparing precious samples. This led Pall to create the Pall NAB plate with a quartz membrane, which provides fast flow rates, efficient purification of target DNA, and removal of contaminants without clogging.

Pall NAB plates allowed purification of plasmid DNA with good yield and high quality using generic laboratory reagents prepared in-house. High quality of the purified plasmid DNA was demonstrated by its excellent performance in PCR amplification (Figure 2), restriction enzyme digestion (Figure 3), and Sanger sequencing (Figures 4). DNA fragments of expected size were obtained following the PCR amplification and restriction enzyme digestion, and high quality sequencing results with long read lengths were obtained in Sanger sequencing, all confirming that the purified plasmid DNA was excellently suited for these downstream applications. The results are comparable with those obtained with plasmid DNA purified using commercially available kits. This indicates that Pall NAB plates are able to generate plasmid DNA of the same high quality as can be obtained with commercially available kits at greatly reduced cost.

Plasmid DNA purification using the Pall NAB plates is effective via centrifugation or vacuum filtration. The filtration approach is user friendly and it eliminates any possibility of sample loss or contamination.

The advanced fluid directors on the outlet of the Pall NAB plates minimize hanging-drop formation, which in turn diminishes well-to-well crosstalk. The data obtained for hanging-drops and crosstalk (Figure 5) clearly illustrate that the Pall NAB plates reduce hanging-drop and crosstalk contribution by about 10% and 30%, respectively, compared to the Pall's previous generation plasmid DNA purification plates.

5. Summary

- The nucleic acid binding quartz membrane in the AcroPrep Advance 96-well long tip filter plate for nucleic acid binding plate is optimized for high quality plasmid DNA purification.
- AcroPrep Advance 96-well long tip filter plate for nucleic acid binding plate allow purification of plasmid DNA using cost effective generic lab reagents, while maintaining downstream results that are comparable to those obtained with costlier commercially available kits.
- Redesigned outlet tips reduce well-to-well crosstalk by minimizing the formation of hanging drops.

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