Plasmid DNA Downstream Process

For gene therapy and plasmid-based DNA vaccine development

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Introduction

Widespread application of plasmid DNA (pDNA) in vaccines and gene therapy is driving increased demand. As a response, plasmid manufacturing must become more efficient with improved productivity. Today, plasmids represent an innovative key technology used in a variety of next-generation applications, from viral vector production to mRNA *in vitro* transcription.

The manufacturing schemes for pDNA were first developed in the mid-1980s and have since relied on well-established traditional production processes, typically fermentation using a microbial source, usually *E. coli.*

There are several challenges facing pDNA manufacturing due to its large size, high viscosity, shear sensitivity and similarities between pDNA and impurities. Furthermore, the purification of pDNA is difficult; the starting material, which is typically clarified lysate from alkaline lysis of bacterial cells after neutralization, has a complex composition with fewer than 3% of the content being pDNA while the remaining 97% represent impurities.

This process development guide provides you with guidance for your plasmid DNA downstream process development, including clarification, TFF, chromatography and sterile filtration unit operations.

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Cell Harvest, Lysis, Neutralization & Clarification of Plasmid DNA

1. Recommendations

Plasmid DNA (pDNA) is typically produced via fermentation using a microbial source. Following *E. coli* fermentation, the primary downstream purification begins with harvesting of the cells, lysis, and clarification. During cell harvest, cells are concentrated, and the fermentation broth is removed via centrifugation or microfiltration tangential flow filtration (MF-TFF).

For MF-TFF, open-channel, flat-sheet TFF devices such as Prostak™ cassettes with Durapore® 0.1 or 0.2 µm microfiltration membranes or Pellicon® cassettes with Durapore® V screen or Biomax® 1,000 kD V screen ultrafiltration (UF) membranes are recommended (Table 1).

The harvested *E. coli* cells are then disrupted to release the plasmid DNA. Lysis is most often performed via an alkaline method. Alkaline lysis with 0.1–0.5 N NaOH with 0.1–0.2% SDS or Triton® X-100 is commonly used. Lysis time and mixing should be optimized.

Precipitation/flocculation is the first step to separate the supercoiled pDNA by selectively precipitating and removing impurities (high molecular weight RNA and genomic DNA, proteins and endotoxins) typically by use of 0.7–3 M potassium acetate with or without $CaCl_2$ (1.0–1.5%), pH range 5.0–7.5.

Lysate can be clarified using depth filtration, such as Clarisolve® filter or Millistak+® HC and Millistak+® HC Pro filters, to achieve high filtration capacity and yield. These filters are available in a wide range of formats with sizes from 0.014 m^2 to 1.1 m^2. Preclarification/pretreatment significantly affects the capacity of the depth filter and process development should therefore be carefully considered for optimization of the step. Yield from the clarification step is generally >90%. Table 2 summarizes the recommended filters for clarification.

Table 1. Recommended modules for harvest step.

Option	Harvest	Bioburden
Option 1	Prostak™ 0.1 or 0.2 or 0.45 µm filter	Millipore Express® SHC 0.5/0.2 µm filter
Option 2	Pellicon® cassette with Biomax® or Ultracel® 1,000 kD membrane, V screen	

Table 2. Recommended filters for the clarification step.

Option	Primary	Secondary (if needed)	Bioburden
Option 1	Clarisolve® 60 HX filter	Milligard® PES 1.2/0.45 µm filter	Millipore Express® SHC 0.5/0.2 µm filter
Option 2	Millistak+® HC D0HC filter		
Option 3	Millistak+® CE20 filter	Millistak+® CE50 or Polysep™ II 1.0/0.5 µm filter	

Note – Filter selection and capacity depend on whether feed is pretreated/untreated. Pre-treatments will have a significant impact on performance.

2. Overview

When harvesting pDNA, using MF-TFF and normal flow filtration (NFF) attributes, parameters, and considerations outlined in Table 3 are important.

Table 3. Overview of MF-TFF and NFF step.

Attributes	Parameters	Key Considerations
Filtration capacity	Filter selection (chemistry and pore sizing)	Viscosity of pDNA solution
Filtration flux	Filtration endpoint	Shear sensitivity of pDNA
pDNA yield	Driving force	High pH of lysis – near denaturization point of pDNA
Impurity reduction (gDNA, protein, and RNA)	Feed treatments	High solids content from fermenter
Bioburden protection and reduction	Mixing formulation (pH, conductivity, buffer components)	

2.1. Cell harvest

2.1.1. Attributes

Bacterial cells containing the plasmid of interest are typically harvested by either centrifugation or tangential flow filtration (TFF). Centrifugation is often more cost-effective for the harvest step when smaller batch volumes (<10 L) or larger batch volumes (>1,000 L) need to be processed.

2.1.2. Parameters

Bacterial cells containing the plasmid of interest are typically harvested based on OD600nm. Harvest OD600 depends on the type of media used in fermentation and the type of fermentation. OD-based harvest parameters are outlined in Table 4.

Table 4. Harvest OD values for different types of fermentation media.

Fermentation media	Harvest OD600
LB media	3–5
Super broth media	Up to 8
Super broth media with glycerol	25–35

(Reference: based on Input from Industry)

High cell density fermentation techniques for culturing *E. coli* have been developed to improve productivity and obtain high cell density.[2]

The goal of fermentation is to maximize cell density of dry cell weight at approximately 40–60 g/L and pDNA titers of approximately 1 g/L. It was possible to reach 2.2 g/L with use of optimized vectors and optimization of the fermentation process.

TFF devices used in a harvest step include MF membranes such as Durapore® (PVDF) 0.1 µm or 0.22 µm or 0.45 µm V screen (suspended screen) membranes and open grade UF such as Biomax® (PES) or Ultracel® (Regenerated Cellulose) 1,000 kDa V screen membranes. When using membrane cut-offs such as these, it is important to utilize a two-pump (permeate-controlled) TFF system.[3] The TFF harvest step typically involves a 2–5X volumetric concentration followed by a 3–5 volume diafiltration for washing out spent media components and extracellular impurities prior to further downstream purification. TFF harvest is typically operated at low transmembrane pressure (TMP; 3–5 psi) and ΔP (<7 psi) with a control on the permeate flux (Table 5).

Table 5. Operating parameters for MF-TFF.

Parameters	Value
Device	Durapore® 0.1 µm or Durapore® 0.22 µm or Durapore® 0.45 µm or Biomax® 1,000 kDa, V screen or Ultracel® 1,000 kDa, V screen membranes
Volumetric loading	10–60 L/m^2
Feed flow	7–9 L/min/m^2
TMP	<0.5 bar
Average flux	20–30 LMH
Volumetric concentration factor	2 to 5
Diafiltration volume	3 to 5

(Reference: Internal data)

E. coli cells could be harvested into a pellet by batch centrifugation using 4,500–6,000 g for ~15–20 min (at room temperature or ~4 ºC). Other types of centrifuges such as continuous-feed, intermittent-solids-discharge, disc-stack, batch-discharge or solid-bowl could also be used on the harvest step.

2.2. Cell lysis

2.2.1. Attributes

The methods used for cell disruption can be divided into two main categories – chemical (alkali, detergents, enzymes, osmotic shock) and physio mechanical (heat, shear, agitation, ultra-sonification, and freeze-thawing) lysis.

Alkaline lysis (NaOH at pH ~12) accompanied by detergents such as sodium dodecyl sulfate (SDS) and Triton® X-100 is the most common approach. The detergent solubilizes the cell walls and the alkaline environment denatures genomic DNA. It is important to optimize the lysis incubation time as it directly impacts the quality and quantity of plasmid DNA. Longer incubation time could lead to irreversible denaturation of plasmid DNA and shear degradation of genomic DNA. It is critical to have efficient but not too aggressive mixing employed on the alkaline lysis step to ensure there are no pH extremes causing irreversible denaturing of the plasmids or degrading it due to excessive shear.

A completely different method for cell lysis involves the use of newly developed autolytic *E. coli* strains. The pDNA is recovered by autolytic extraction under slightly acidic, low-salt buffer conditions and treatment with a low concentration of nonionic detergent. Genomic DNA remains associated with the insoluble cell debris and is removed by solid-liquid separation using a thermal flocculation followed by coarse filtration.[1]

2.2.2. Parameters

During the alkaline lysis method, cells are treated at specific, narrow range of pH (typically around pH 12) at which the genomic DNA will be irreversibly denatured, while the pDNA double chain remains intact (pH range of 12.0 to 12.5). The optimum pH value varies depending on the type of plasmid and host strain. A deviation of more than 0.1 pH unit from the optimum value may affect the yield and it is therefore critical to maintain a tight control of the pH range during alkaline lysis; at a pH >12.5, pDNA becomes irreversibly denatured and if the pH is too low, genomic DNA won't be completely denatured and could complicate further downstream purification process.

The incubation time for a standard alkaline lysis is fairly short and the step is usually completed typically within 5 minutes. The degree of lysis could be controlled by measuring viscosity/residence time in a vessel.

In a laboratory setting, mixing is often performed gently by hand, which is not feasible at larger scales.

For achieving complete but gentle mixing of large lysis volumes, batch mixing in a mechanically agitated vessel (specialized vessel design with utilizing baffles, low power number impellers, feed lines) and/or continuous flow-through devices/in-line static mixers have been used, taking into consideration viscous non-Newtonian properties of the lysate. Mobius® single-use mixers can be very effective for batch lysis.

2.3. Precipitation/flocculation

2.3.1. Attributes

Precipitation/flocculation is the first step in removing host cell contaminants in a pDNA manufacturing process. Neutralization can be done using a high concentration of sodium or potassium acetate with or without surfactant, RNAse, or $CaCl_2$. This step causes precipitation of detergent solubilized proteins including high molecular weight genomic DNA. Smaller, covalently closed circular pDNA renatures into double stranded molecules and remains in a soluble state. RNAse can be added into the neutralization buffer for degradation of high molecular weight RNA impurities (RNA could be present at least 20X amount of pDNA). Some chaotropic salts, such as lithium chloride, ammonium acetate, and calcium chloride have the additional advantage of precipitating high molecular weight RNA together with the proteins. Polyethylene glycol (PEG) and polyethylenimine (PEI) can also be used for precipitation of genomic DNA.

2.3.2. Parameters

Rapid neutralization occurs with high-salt buffer (such as sodium or potassium acetate at concentration of 0.7 M–3.0 M and pH ~5–7.5, with/without 1.0–1.5% $CaCl_2$) in the presence of a detergent (1% SDS).

A low-cut off PEG precipitation (at 4% w/v) can also be used for precipitation of genomic DNA with up to 20% (w/v) of the precipitate formed during the step. Homogenous mixing during neutralization and precipitation is critical to maintain pDNA quality.

Based on our internal data, impurities such as high molecular weight RNA and genomic DNA, proteins and endotoxins can be selectively precipitated using high salt buffer, PEG and PEI. Proper optimization is recommended.

To separate the precipitated solids, typical clarification methods such as settling with decanting, depth filtration and centrifugation are used. Product loss has been observed occasionally with filtration, and therefore filters with low adsorption are preferred.

2.4. Clarification

2.4.1. Attributes

Clarification unit operations for pDNA processes should enable removal of solid content from the feed stream. Feed streams can either be untreated, pretreated or preclarified. Post chemical lysis and neutralization with sodium or potassium acetate leads to development of large floccules/precipitates.

Pretreatment has a major impact on the clarification filter capacity and must be selected carefully along with a consideration of the scalability of the process. Pretreatment options include use of gravity settling and separation, PEG, PEI, bag filters stainless steel screen filters, paper filters, and centrifugation.

2.4.2. Parameters

To achieve the desired attributes, clarification operations should ensure proper filter selection to handle the solids load of the lysate. Depth filters are ideal, as capacity can be high and adsorptive interactions are masked by the high salt concentration of lysate feed allowing high yield. Feed flux and filtration endpoints can be optimized to ensure minimal

filter area is used and high yield of pDNA is achieved. Additionally, product recovery operation such as blow down and buffer flushing should be considered.

3. Technical Data

The data presented in this section is derived from our internal database.

3.1. Harvest

Biomax® or Ultracel® 1,000 kDa V screen membranes or Durapore® V screen MF TFF membranes are used for harvest at low TMP and permeate control. Normalized water permeability (NWP) recovery post use is >90%. The load challenge reported for Biomax® 1,000 kDa membrane ranges from 10–60 L/m^2 with an optimum permeate flux around 25–30 LMH.

Centrifugation is one of the preferred methods for harvesting at lab scale; at large scale, centrifugation process can be cumbersome and provide low yield. Disk stack centrifuges operating at high speed with intermittent ejection gave supercoiled plasmid yields as low as 40% because of shear damage during discharge.[4]

3.2. Lysis and neutralization

Cell lysis is typically carried out at pH 12–12.5 with 0.2% SDS, followed by neutralization using potassium acetate (0.7–3 M). Typically, neutralization is carried out at approximately 5.0. but has been reported at pH 6.0 and pH 7.5. Use of $CaCl_2$ is common for RNA precipitation during neutralization.

Floccules generated during the neutralization step after undisturbed incubation commonly float on top of the liquid.

Preclarification methods reported in our internal database show pretreatment by use of a range of approaches were used approximately 75% of the time; in 25% of the studies, no treatment or prefiltration was used (Figure 1).

Preclarification method

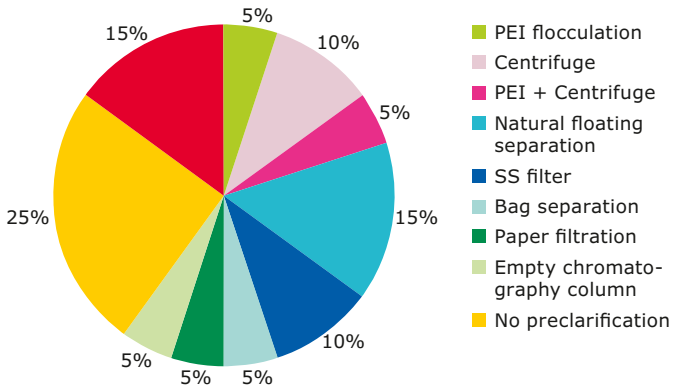

Figure 1. Various reported pretreatment/prefiltration conditions.

3.3. Clarification

A review of internal data for clarification filtration of post lysis and neutralization feeds showed that filtration capacity varies significantly based on whether the feed is pretreated or untreated.

Feed quality impacts the NFF operation. Our internal database shows two kinds of feed, either pretreated (feed turbidity 20 to <500 NTU) or untreated feed (feed turbidity >1,000 NTU).

The pretreatment condition reported in the majority of studies in our database was gravity separation of floccules and solution; solutions were carefully filtered without disturbing floccules/sediments and a product loss of approximately 20% was reported in floccules.

Another pretreatment method includes use of stainless-steel filter, bag filter, empty column, paper filter, centrifuge, PEI flocculation and centrifugation, and use of Polygard® CR 1 µm/Polygard® CR 50 µm filters. Capacity of the Polygard® CR filters were in range of 0.55–8 L/inch.

Filters commonly used for pretreated or untreated feed are listed in Table 6. Average capacity of the filters is shown in Figure 2.

Table 6. Recommended filters, conditions and capacity expected capacity ranges.

			Pretreated		Untreated	
Filter	**Media**	**Pore rating**	**Operating flux (LMH)**	**Avg. capacity range (L/m²)**	**Operating flux (LMH)**	**Avg. capacity range (L/m²)**
Clarisolve® 60 HX filter	Polypropylene	7.5–60 µm	100–150	150–300	100–150	50–300
Clarisolve® 40 MS filter	Polypropylene + cellulosic + inorganic filter aid	0.6–40 µm	100–150	190–460	100–150	50–250
Millistak+® HC D0HC filter	Diatomaceous earth and cellulose	0.6–8.0 µm	90–150	115–200	90–150	25–100
Millistak+® HC C0HC filter	Diatomaceous earth and cellulose	0.2–2.0 µm	100–150	85–300	100–150	30–100
Millistak+® CE20 filter	Cellulose	5.0–10.5 µm	60–200	100–400	50–100	50–100
Millistak+® CE50 filter	Cellulose	0.6–1.0 µm	60–200	100–285		
Millistak+® HC Pro-DOSP filter	Polyacrylic + Silica	0.6–8.0 µm	100–150	100–275	100–150	150–200

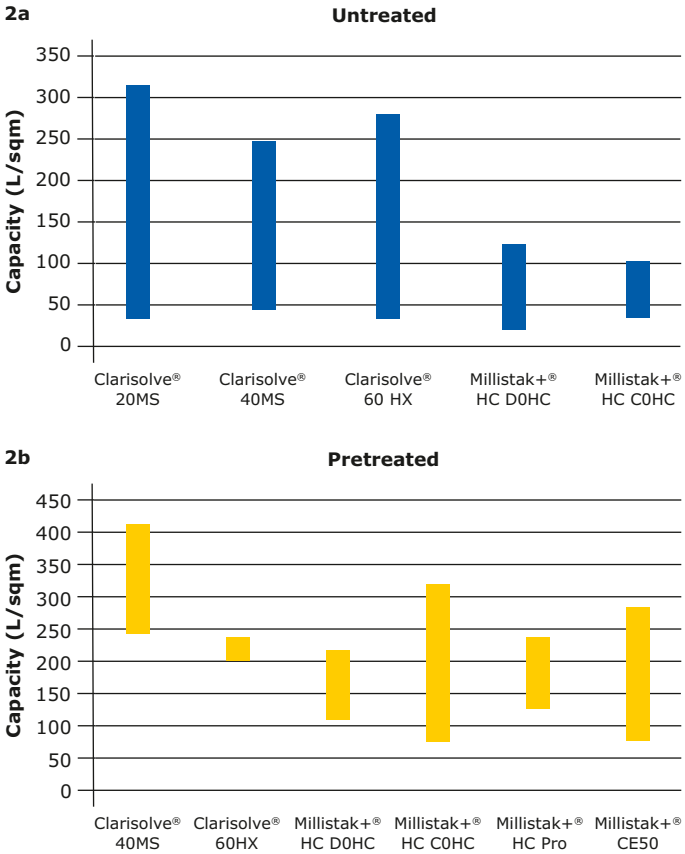

Figure 2a/b. Average capacity range of Clarisolve®, Millistak+® HC, and Millistak+® HC Pro filters for pretreated and untreated feed.

Milligard® PES 1.2/0.45 µm filter can be used as a secondary filter for the Clarisolve® filter. Reported capacity for the Milligard® PES 1.2/0.45 µm filter after Clarisolve® filter is >150 L/m^2. A secondary filter such as Millistak+® HC XOHC and Millistak+® HC Pro XOSP filters, can also be evaluated if required but recovery needs to be monitored.

Millistak+® CE 50 filter is generally reported to be used as primary or secondary filter based on feed conditions.

A combination of Millistak+® CE20 or CE30 or CE40 filter as primary filter with Millistak+® CE50 filter as secondary filter can also be evaluated. The reported capacity for Millistak+® CE20 filter is >300 L/m^2; Millistak+® CE30 filter is >150 L/m^2, Millistak+® CE40 filter is >100 L/m^2; whereas for Millistak+® CE50 filter reported capacity ranged from 80–320 L/m^2.

Recovery of >90% is reported with Clarisolve® and Millistak+® filters. Clarification recovery for Millistak+® filters can be increased using a chase with salt containing buffer.

It is observed that the clarification unit operation is run at low flux considering viscosity of feed. Typical operation flow was in range of 60–150 LMH.

Use of Millipore Express® SHC filter has been reported as a bioburden reduction filter after clarification with average capacity range of 400–650 L/m^2 based on feed quality.

4. References

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Tangential Flow Filtration (UF/DF) of Plasmid DNA

Recommendations

Pellicon® 2 cassette with Biomax® 100 kDa C-screen/ V-screen can be used for concentration and diafiltration with high loading and yield. The V-screen configuration is recommended for high concentration or high viscosity feed streams.

Table 1. Recommended filter options for UF/DF step.

Options	UF/DF Membrane
Option 1	Pellicon® 2 cassette with Biomax® 100 kDa C-screen
Option 2 (high concentration/viscosity)	Pellicon® 2 cassette with Biomax® 100 kDa V-screen

Overview

Attributes

Precipitated plasmid is separated, concentrated, washed and then re-suspended in the appropriate buffer. This is typically accomplished using tangential flow filtration (TFF) as this technique is easily scalable, highly selective and cost-effective.

Because the starting concentration of plasmids is generally much lower than that of a typical antibody or recombinant protein feed stream, use of TFF prior to chromatography also functions as a concentration step to further improve downstream purification.

This membrane-based separation and concentration step needs to be optimized to achieve high performance without compromising the plasmid integrity. TFF relies on the size difference between pDNA and contaminants present in the lysate such as linear DNA, RNA and endotoxins. Therefore, the TFF membrane must have an appropriate molecular weight cut-off (MWCO) to retain this pDNA and allow sieving of contaminants and the initial buffer. In addition to these retention and purification capabilities, TFF should be able to manage the increased viscosity throughout the process step and have a high capacity to enable an acceptable footprint at scale.

Parameters

The performance of a TFF step depends on the feed conditions, MWCO, feed and filtrate/permeate flux and system pressure. The desired plasmid purity, formulation, and concentration specification without product damage can be achieved through optimization of these hydraulic parameters.

Challenges

Due to their structure, plasmids can sometimes pass through pores that are smaller than their apparent molecular weight. This sieving can be more predominant with flux increase. The sieving coefficient also increases at higher ionic strength due to reduction in the effective plasmid size observed in these conditions[1].

Additionally, the DNA can be shear-sensitive and tends to increase with plasmid size[2]. The result can be degradation and reduction of the overall yield.

Technical Data

The selected molecular weight cutoff (MWCO) depends on the pDNA structure and can range from 30 kDa to 300 kDa. The standard rule of thumb is to use a membrane cutoff that is 3–5 X tighter in pore diameter than the diameter of the product of interest; for common plasmid sizes of 5–20 kbp, 100 kD is often selected.

Loss of the pDNA in the permeate can potentially be addressed by polarizing the membrane (using full recirculation mode with permeate diverted into the feed tank) prior to starting the TFF run with the

permeate line directed to exhaust. This will create a stable polarization layer that will improve the retention.

Additionally, base buffer salt concentration, concentration of pDNA, presence of RNA, transmembrane pressures (TMP) and delta P should be optimized for effective retention of the product. Higher salt concentration has been shown to reduce the plasmid radius[1]. In these conditions, the plasmid structure seems to be more tightly twisted, exhibiting a condensed effective size.

In terms of parameters, a lower TMP is favored. Use of a two-pump, permeate controlled system is preferred for 100 kDa and larger MWCO[3]. Depending on the specific configuration of the membrane used, the step is typically operated at TMP ≤10 psi for a permeate flux of ~20–50 LMH. The plasmid is usually completely retained at low filtrate flux and sieving can be observed at higher fluxes[4].

The feed flux chosen for the concentration and diafiltration typically ranges between 4 and 6 LMM to reduce shear stress that can ultimately lead to DNA degradation. High loading in the range of 70 to 140 L/m² can be achieved if these pressure and flux parameters are well optimized with the correct membrane.

As viscosity also increases, particularly at concentrations approaching and exceeding 10 mg/mL, tight screens are not recommended. Coarse (C-screen) and open channel or V-screen TFF device configurations should be applied for medium (5–10 X) to higher concentration (30–50 X) activities; TFF process optimization is required, however.

Table 2. Operating parameters for MF-TFF.

Parameters	Value
Device	Pellicon® 2 with Biomax® 100 kDa C-screen
Volumetric loading	70–140 L/m²
Feed flux	4–6 LMM
Permeate average flux	20–50 LMH
TMP	≤10 psi
Volumetric concentration factor (VCF)	3–50 (V-screen for high concentration)
Diafiltration volume (DF)	3–10

References

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Chromatographic Purification of Plasmid DNA

1. Challenges

Manufacturing schemes for plasmid DNA (pDNA) were first developed in the two decades since the mid 1980s and have relied on well established traditional production processes and products.[1]

The purification of pDNA is difficult; the starting material, which is typically clarified lysate from alkaline lysis of bacterial cells after neutralisation, has a complex composition with no more than 3% of the content being pDNA while the remaining 97% represent impurities. Most of the critical impurities are negatively charged (RNA, genomic DNA, endotoxins), similar in size (open circular pDNA, genomic DNA, high molecular weight RNA), and in hydrophobicity (endotoxins).[2] Final bulk pDNA must meet quality specifications set by regulatory agencies and should be free from host cell proteins, genomic DNA, RNA, and endotoxins, and more than 90% of the pDNA should be the supercoiled isoform.[3]

Most existing large-scale purification processes are based on chromatographic methods, which offer comparatively high resolution, and leverage a range of different chromatographic modalities, either singly or combined. Plasmid molecules present a challenge for conventional chromatographic media, however. These media were originally designed for protein purification; plasmid molecules are much bigger than proteins and as such cannot enter the pores, causing low binding capacity and slow mass transfer.[4,5] Additional challenges presented by chromatographic purifications are low recovery, high pressure drop/long processing times due to the viscosity of plasmid solutions, resolution of isoforms, and potential fouling.

The most commonly used techiques for plasmid purification are anion exchange chromatography (AEC) and hydrophobic interaction chromatography (HIC). Both techniques have been implemented for capture or intermediate purification/polishing and are often combined.[1,4] Size exclusion chromatography (SEC) is sometimes included as part of the downstream scheme; it is typically chosen as the last step due to its disadvantages of low throughput and slow kinetics.[1,3] HIC is able to separate the native supercoiled pDNA from pDNA isoforms, from more hydrophobic nucleic acid impurities (RNA, genomic DNA, denatured pDNA), and from endotoxins.[3] AEC achieves the removal of proteins, low molecular weight RNA (resolution of high molecular weight RNA is limited), and endotoxins. The efficiency of AEC is, however, highly dependent on sample composition according to its pretreatment and origin; a sufficiently high salt concentration in the load should be applied in order to maximize pDNA capture.[2,6]

Widespread application of pDNA in vaccines and gene therapy is driving increased demand and as a response, plasmid manufacturing must become more efficient with improved productivity. Intensification of chromatographic steps can help address this demand and has led to an exploration of the use of convective media (monoliths, membranes, fibre based technologies).

2. Recommendations

2.1. Salt supplemented lysate as feed for anion exchange purification

The standard feed used as starting material for purification runs was original *E. coli* lysate[7], clarified by centrifugation and subsequent depth filtration, and directly supplemented with NaCl (120–250 mM, depending on resin or membrane type) to eliminate RNA interference, at pH 5.0, 74–82 mS/cm, containing pDNA size 5.7 kbp. This feed was used in two ways, either concentrated by TFF (pDNA titer of 0.2–0.3 mg/mL) with resins in order to reduce the loading time or non-concentrated (pDNA titer of about 0.05 mg/mL) with a membrane adsorber.

The optimal salt concentration for supplementation was pre-determined prior to the purification runs for each resin/membrane adsorber using a batch assay in microtiter plate format, measuring plasmid binding capacity at increasing sodium chloride concentrations. The principle is demonstrated with the examples of Fractogel® EMD DEAE (M) and Fractogel® EMD DMAE (M) resins in Figure 1.

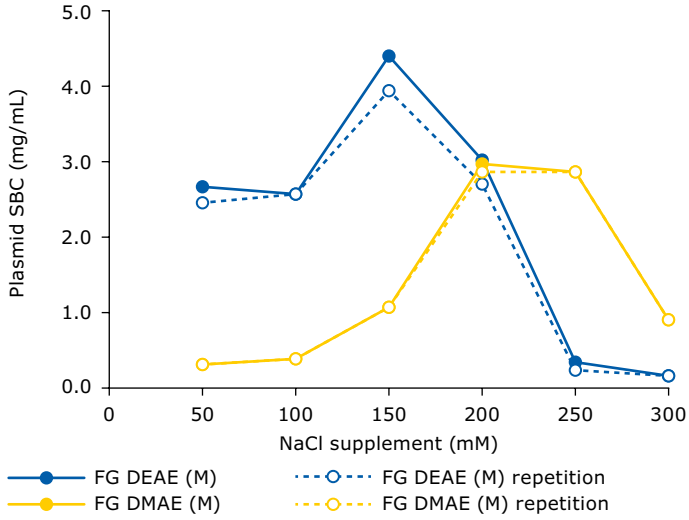


Figure 1. Batch assay for determination of optimal NaCl concentration for lysate supplementation. Static binding capacities (SBC) were measured in 96-well filter plates (1 mL per well). Plasmid feed was original clarified lysate (pH 5.0, 67 mS/cm) supplemented with increasing NaCl concentrations. FG = Fractogel® EMD resin.

2.2. Performance overview of anion exchange products

Table 1. Performance overview of anion exchange resins and membrane adsorber for purification of plasmid DNA. FG = Fractogel® EMD resin.

Recommended Process step	Resin/Membrane Absorber	Dynamic Binding Capacity (mg/mL)	Residence Time, 10 cm BH (min)	CV/ min	RNA Removal	Yield ccc-form	Purity (A260 based)
High-throughput capture	Natrix® Q	~10	0.1–0.03	10–33	>95%	≥80%	>80% pDNA
	Eshmuno® Q	~2.5	3–0.3	0.3–3.3	>95%	~75%	>95% pDNA
Intermediate Purification/ Polishing	FG DEAE (M)	~2.5	4–2	0.25–0.5	>95%	≥80%	>95% pDNA
	FG DMAE (M)	~3	4–2	0.25–0.5	>95%	≥95%	>95% pDNA

2.3. High-throughput capture using anion exchange chromatography

2.3.1. Natrix® Q chromatography membrane

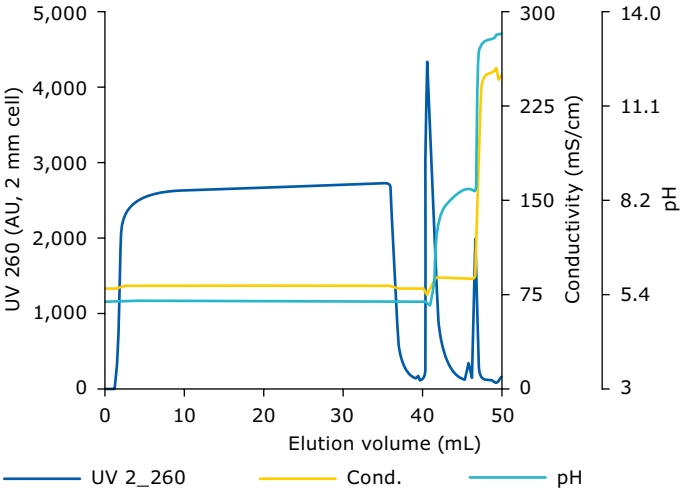
Plasmid with ≥80% pure plasmid DNA and about 10% residual RNA (A260 based) can be obtained with a yield of about 80% in a total run time of about 30 minutes, using Natrix® Q chromatography membrane with a binding capacity of approximately 10 mg/mL for capture from NaCl supplemented clarified lysate at very high flow rate. Greater than 95% of the initial large RNA excess can be removed.

Experimental conditions are summarized in Table 2. Figure 2 illustrates the results of the capture run.

Table 2. Experimental conditions for using Natrix® Q chromatography membrane for capture of plasmid DNA.

Parameters	Value
Chromatography membrane	Natrix® Q chromatography membrane
Priming	50 MV buffer A: 1 M K-acetate + 160 mM NaCl, pH 5.0, 77–78 mS/cm
Equilibration	5 MV buffer A
Load	Clarified lysate (1 M K-acetate buffer matrix, pH 5.0, 67 mS/cm) directly supplemented with NaCl (ca. 175 mM) to 80 mS/cm, variable volume Fraction collection: 5 mL size
Wash	20 MV buffer A Fraction collection: 1 mL size
Elute	30 MV buffer B: 100 mM Tris pH 9 plus 1 M NaCl Fraction collection: 5 mL size
CIP	20 MV 1 M NaOH + 2 M NaCl
Re-equilibration	10 MV 50 mM Tris pH 8 plus 2 M NaCl 40 MV buffer A
Flow rate	For elution 5 MV/min = 0.2 min residence time (RT) For all other steps 10 MV/min = 0.1 min RT

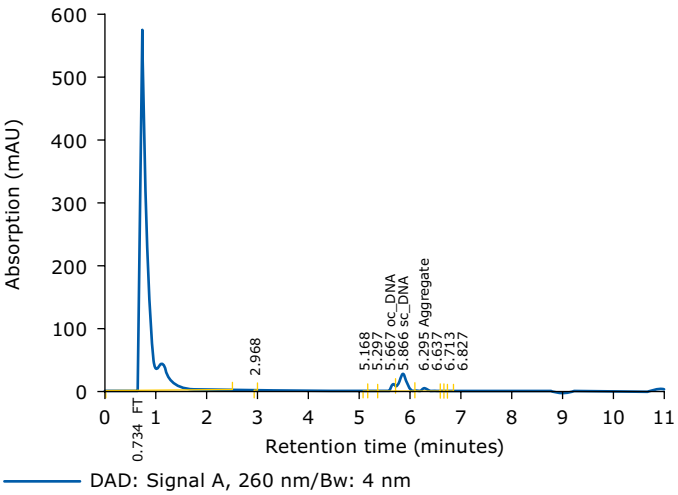
A



B

Fraction	ccc-form amount (µg)	ccc-form rel. amount (%)	oc-form rel. amount (%)
load	1,497	100	100
breakthrough/ wash	23	2	37
eluate pool	1,216	81	68

C



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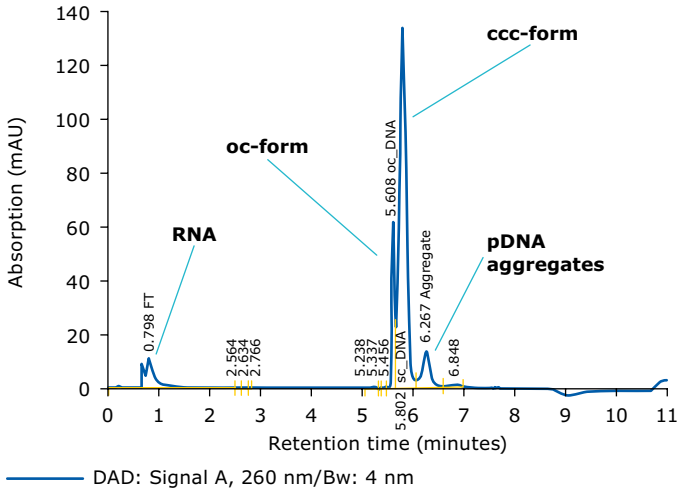


Figure 2. Plasmid capture from clarified lysate supplemented with NaCl using Natrix® Q chromatography membrane. **A:** AKTA™ chromatogram. **B:** Mass balance. **C:** Analytical chromatogram of clarified lysate using AEX HPLC with a TSKgel DNANPR column. **D:** Analytical HPLC chromatogram of the elution pool. "ccc-form" is the covalently closed circular isoform of a plasmid. "oc-form" is the open circular isoform of a plasmid.

Non-concentrated clarified lysate (pDNA titer ca. 0.045 mg/mL, 81% ccc-form, 19% oc-form) was loaded to a Natrix® Recon Mini device (0.2 mL membrane volume, MV) at 9 mg ccc-form of pDNA/ mL MV. Ninety-eight percent of the host cell RNA was maintained in the flow-through. Plasmid purity in the eluate was calculated from the A260 area of the respective individual peak and the A260 total peak area as determined by analytical AEX HPLC. Distribution of plasmid isoforms as well as low level of residual RNA is visible.

Total loading time with the membrane adsorber was about 18 minutes for the load volume of about 35 mL using a residence time of 0.1 minute. For capturing the same amount of pDNA (in form of about five-fold concentrated lysate with a titer of 0.21 mg/mL) with a chromatography resin like Fractogel® EMD DEAE (M) resin, which allows only moderate flow rates, loading time using the concentrated lysate would be 94 minutes. Binding capacity of the Fractogel® resin under these loading conditions is approximately 2.5 mg/mL, which would require 0.64 mL of packed resin, and applying a residence time of 8 minutes for the load step.

The feasibilty of loading non-concentrated feeds as large volumes within a short period of time is enabled by fast flow properties of chromatography membranes, which makes an additional concentration step unnecessary.

2.3.2. Eshmuno® Q chromatography resin

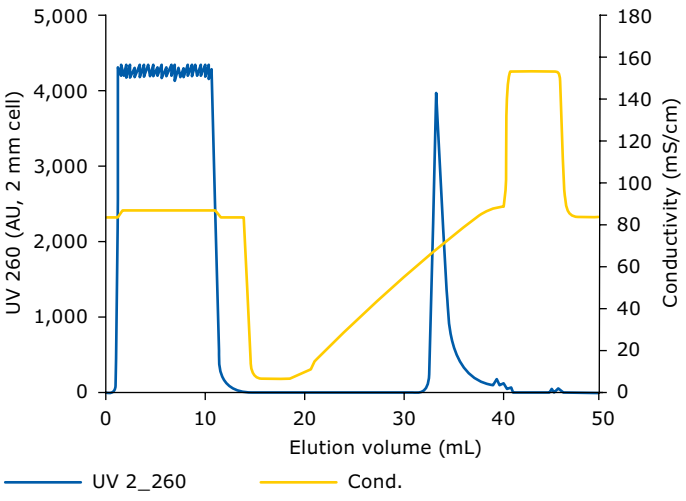
Plasmid preparations with >95% pure plasmid DNA and <5% residual RNA (A260 based) can be obtained with a yield of approximately 75%, using Eshmuno® Q resin with a binding capacity of about 2.5 mg/mL for capture from NaCl-supplemented clarified lysate at a high flow rate. Greater than 95% of the initial large RNA excess can be removed.

Experimental conditions are summarized in Table 3. Figure 3 illustrates the results of the capture run.

Table 3. Experimental conditions for using Eshmuno® Q resin for capture of plasmid DNA.

Parameters	Value
Chromatography resin	Eshmuno® Q resin, 1 mL MiniChrom column 0.8×2.0 cm ID/L
Equilibration	5 CV buffer A1: 1 M K-acetate + X mM NaCl, pH 5.0, 82 mS/cm
Load	Clarified lysate (1 M K-acetate buffer matrix, pH 5.0, 67 mS/cm) directly supplemented with NaCl (ca. 250 mM) to 84 mS/cm, variable volume Fraction collection: 1.5 mL size
Wash 1	3 CV buffer A1 Fraction collection: 1.5 mL size
Wash 2	3 CV buffer A2: 100 mM Na-acetate, pH 5.0 Fraction collection: 1.5 mL size
Elute, part 1	Linear gradient 0–100% A2/B in 20 CV Fraction collection: 1.5 mL size
Elute, part 2	Step gradient 3 CV buffer B: 100 mM Na-acetate + 1 M NaCl, pH 5.0 Fraction collection: 1.5 mL size
Strip	3 CV strip buffer: 2 M NaCl in 50 mM Tris pH 8.0
Flow rate	For load 0.33 mL/min = 3 min residence time (RT) For all other steps 0.25 mL/min = 4 min RT

A



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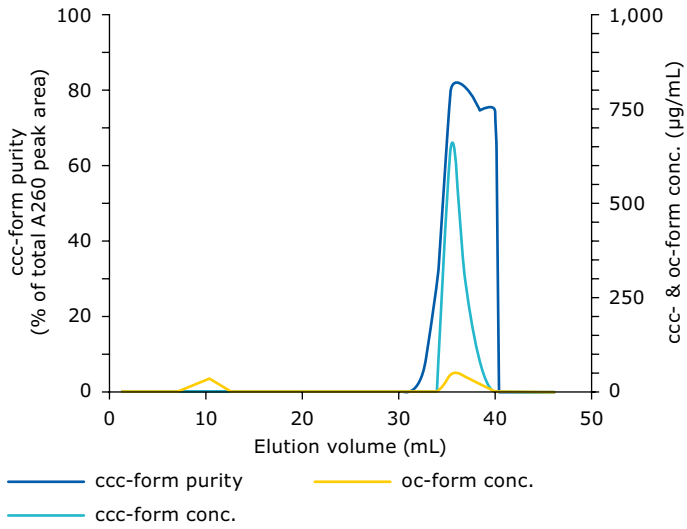


C

Fraction	ccc-form amount (µg)	Total pDNA (µg)	ccc-form (%) of total pDNA
load	2,107	2,677	79
breakthrough	5	132	N/A
eluate pool	1,529	1,699	90

pDNA-form	Recovery (%)	Elution yield (%)
total pDNA	68	63
ccc-form	73	73

D

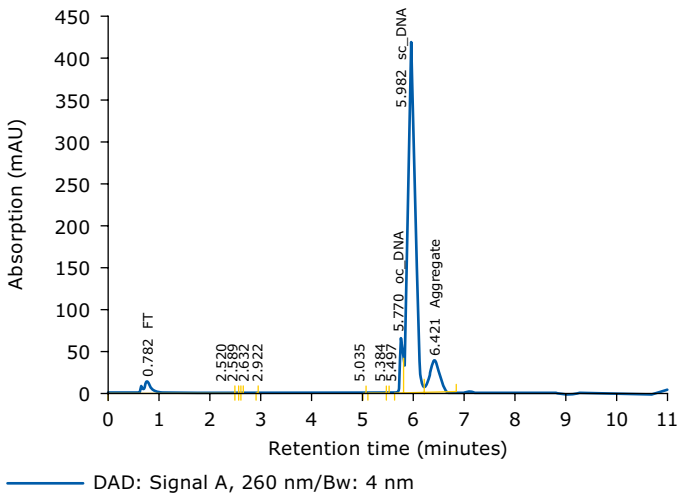


Figure 3. Plasmid purification from clarified lysate supplemented with NaCl using Eshmuno® Q resin. **A:** AKTA™ chromatogram. **B:** Isoform distribution and purity of ccc-form. **C:** Mass balance. **D:** Analytical chromatogram of the pDNA elution peak center fraction using AEX HPLC with a TSKgel DNA-NPR column.

Concentrated clarified lysate (pDNA titer ca. 0.21 mg/mL, 79% ccc-form, 21% oc-form) was loaded to a 1 mL MiniChrom column packed with Eshmuno® Q resin at 2.7 mg of pDNA/mL CV. Plasmid purity in the eluate was calculated from the A260 areas as determined by analytical AEX HPLC. Distribution of plasmid isoforms as well as low level of residual RNA is displayed.

2.4. Intermediate purification and polishing using anion exchange chromatography

2.4.1. Fractogel® EMD DEAE (M) and Fractogel® EMD DMAE (M) resins

Fractogel® EMD DEAE (M) and Fractogel® EMD DMAE (M) resins are well suited for intermediate purification or polishing of plasmid DNA due to their moderate binding capacity and flow as well as good resolution due to a medium bead size (d50: 48–60 µm), clearing residual impurities like RNA and endotoxin efficiently.

Plasmid with ≥95% pure plasmid DNA and <5% residual RNA (A260 based) can be obtained with pDNA yields of >80% (for the DEAE resin) and >95% (for the DMAE resin) with binding capacities of 2.5 mg/mL and 3 mg/mL, respectively, for purification from NaCl supplemented clarified lysate. Greater than 95% of the initial large RNA excess can be removed.

Experimental conditions are summarized in Table 4. Figure 4 illustrates the results of the purification run with the example of Fractogel® EMD DEAE (M) resin.

Table 4. Experimental conditions for using Fractogel® EMD DEAE (M) and Fractogel® EMD DMAE (M) resins for intermediate purification and polishing of plasmid DNA.

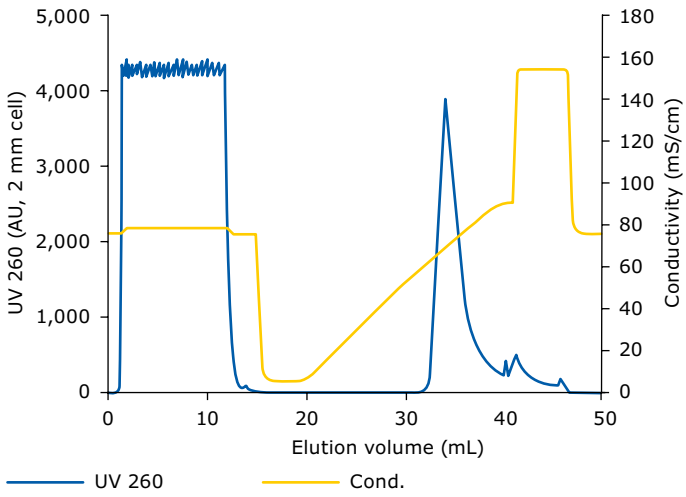
a) Fractogel® EMD DEAE (M) resin

Parameters	Value
Chromatography resin	Fractogel® EMD DEAE (M) resin, 1 mL Scout column 0.8×1.9 cm ID/L
Equilibration	5 CV buffer A1: 1 M K-acetate + X mM NaCl, pH 5.0, 74 mS/cm
Load	Clarified lysate (1 M K-acetate buffer matrix, pH 5.0, 67 mS/cm) directly supplemented with NaCl (ca. 120 mM) to 76 mS/cm, variable volume Fraction collection: 1.5 mL size
Wash 1	3 CV buffer A1 Fraction collection: 1.5 mL size
Wash 2	3 CV buffer A2: 100 mM Na-acetate, pH 5.0 Fraction collection: 1.5 mL size
Elute, part 1	Linear gradient 0–100% A2/B in 20 CV Fraction collection: 1.5 mL size
Elute, part 2	Step gradient 3 CV buffer B: 100 mM Na-acetate + 1 M NaCl, pH 5.0 Fraction collection: 1.5 mL size
Strip	3 CV strip buffer: 2 M NaCl in 50 mM Tris pH 8.0
Flow rate	For load 0.125 mL/min = 8 min residence time (RT) For all other steps 0.25 mL/min = 4 min RT

b) Fractogel® EMD DMAE (M) resin

Parameters	Value
Chromatography resin	Fractogel® EMD DMAE (M) resin, 1 mL Scout column 0.8×1.9 cm ID/L
Equilibration	5 CV buffer A1: 1 M K-acetate + X mM NaCl, pH 5.0, 82 mS/cm
Load	Clarified lysate (1 M K-acetate buffer matrix, pH 5.0, 67 mS/cm) directly supplemented with NaCl (ca. 250 mM) to 84 mS/cm, variable volume Fraction collection: 1.5 mL size
Wash 1	3 CV buffer A1 Fraction collection: 1.5 mL size
Wash 2	3 CV buffer A2: 100 mM Na-acetate, pH 5.0 Fraction collection: 1.5 mL size
Elute, part 1	Linear gradient 0–100% A2/B in 20 CV Fraction collection: 1.5 mL size
Elute, part 2	Step gradient 3 CV buffer B: 100 mM Na-acetate + 1 M NaCl, pH 5.0 Fraction collection: 1.5 mL size
Strip	3 CV strip buffer: 2 M NaCl in 50 mM Tris pH 8.0
Flow rate	For load 0.125 mL/min = 8 min residence time (RT) For all other steps 0.25 mL/min = 4 min RT

A



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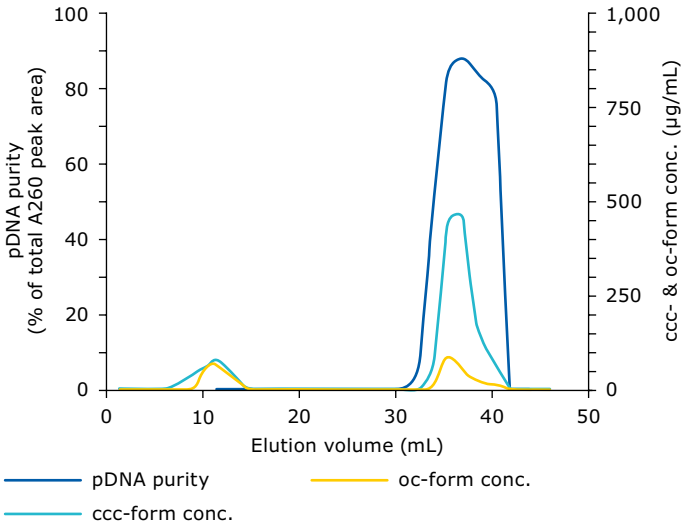


C

Fraction	ccc-form amount (µg)	Total pDNA (µg)	ccc-form (%) of total pDNA
load	2,588	3,318	78
breakthrough	373	609	N/A
eluate pool	1,840	2,117	87

pDNA-form	Recovery (%)	Elution yield (%)
total pDNA	82	64
ccc-form	86	71

D

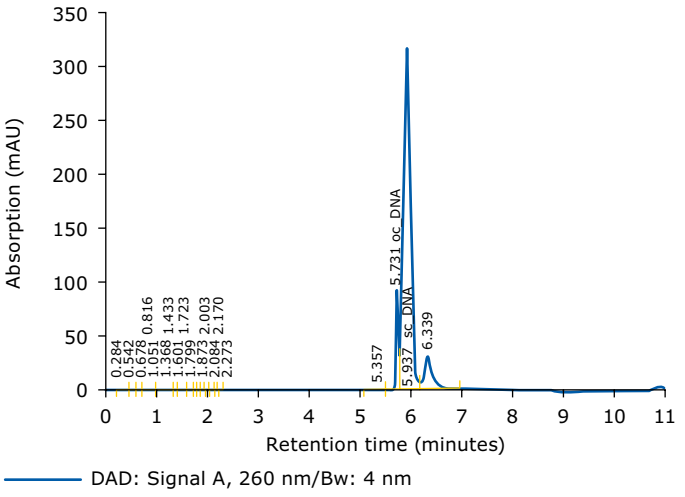


Figure 4. Plasmid purification from clarified lysate supplemented with NaCl using Fractogel® EMD DEAE (M) resin. **A:** AKTA™ chromatogram. **B:** Isoform distribution and purity of ccc-form. **C:** Mass balance. **D:** Analytical chromatogram of the pDNA elution peak center fraction using AEX HPLC with a TSKgel DNA-NPR column.

Concentrated clarified lysate (pDNA titer ca. 0.21 mg/mL, 78% ccc-form, 22% oc-form) was loaded to 1 mL MiniChrom columns packed with Fractogel® EMD DEAE (M) and Fractogel® EMD DMAE (M) resins at 3.3 mg of pDNA/mL CV and 2.9 mg of pDNA/mL CV, respectively. Plasmid purity in the eluates was calculated from the A260 areas as determined by analytical AEX HPLC. Distribution of plasmid isoforms as well as the low level of residual RNA is shown with the example of Fractogel® EMD DEAE (M) resin.

2.5. Compatibility of anion exchange chromatography with HIC conditions

Hydrophobic interaction chromatography (HIC) is one of the most commonly used chromatographic techniques for purification of pDNA, in addition to AEC. Prior to purification with HIC, plasmid solutions are adjusted with a high concentration of ammonium sulfate to achieve binding on the resin.[4] Two arrangements of HIC and AEX (AEX -> HIC or HIC -> AEX) are used in large-scale production processes for pDNA.[1]

Figure 5 lists the binding capacities of selected AEX resins for pDNA in ammonium sulfate- and sodium chloride-containing solutions. pDNA binding to these AEX resins tolerated the presence of elevated concentration of ammonium sulfate, and as such, offers the potential for direct processing of the eluate pool from HIC capture.

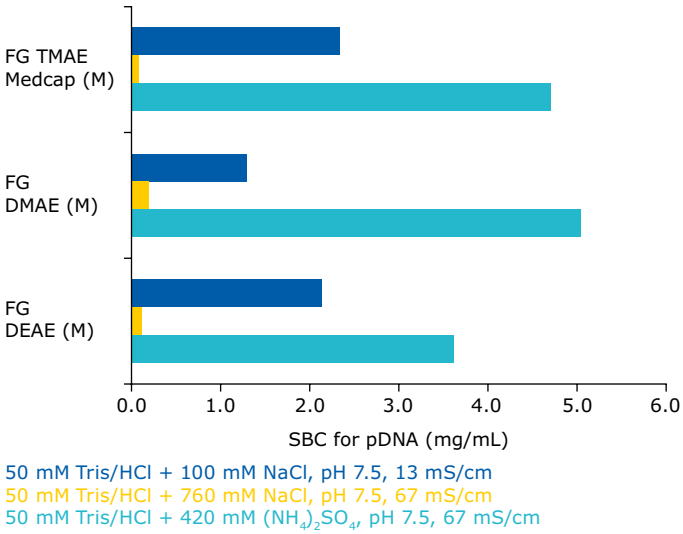


Figure 5. Static binding capacities of tentacle AEX resins with purified pDNA (pEGFP-N1, 4.7 kbp, prepared in-house) in ammonium sulfate and sodium chloride-containing solutions. Measured in batch assay mode using 96-well filter plates (1 mL per well).

2.6. Low performance of anion exchange chromatography using original, non-treated lysate

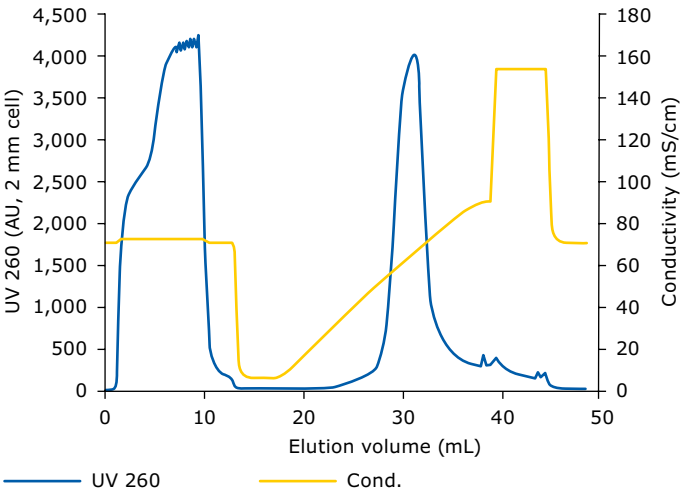
Plasmid of low purity with about 20% pure plasmid DNA still containing 80% RNA (A260 based) can be obtained with a low yield of 60%, using Fractogel® EMD DEAE (M) resin with a binding capacity of approximately 1.5 mg/mL for purification from concentrated clarified original, non-treated lysate, without NaCl supplementation.

Experimental conditions are summarized in Table 5. Figure 6 illustrates the results of the purification run.

Table 5. Experimental conditions for using Fractogel® EMD DEAE (M) resin for capture of plasmid DNA.

Parameters	Value
Chromatography resin	Fractogel® EMD DEAE (M) resin, 1 mL Scout column 0.8×1.9 cm ID/L
Equilibration	5 CV buffer A1: 1 M K-acetate, pH 5.0, 67 mS/cm
Load	Clarified original lysate (1 M K-acetate buffer matrix, pH 5.0, 67 mS/cm), variable volume Fraction collection: 1.5 mL size
Wash 1	3 CV buffer A1 Fraction collection: 1.5 mL size
Wash 2	3 CV buffer A2: 100 mM Na-acetate, pH 5.0 Fraction collection: 1.5 mL size
Elute, part 1	Linear gradient 0–100% A2/B in 20 CV Fraction collection: 1.5 mL size
Elute, part 2	Step gradient 3 CV buffer B: 100 mM Na-acetate + 1 M NaCl, pH 5.0 Fraction collection: 1.5 mL size
Strip	3 CV strip buffer: 2 M NaCl in 50 mM Tris pH 8.0
Flow rate	For load 0.125 mL/min = 8 min residence time (RT) For all other steps 0.25 mL/min = 4 min RT

A



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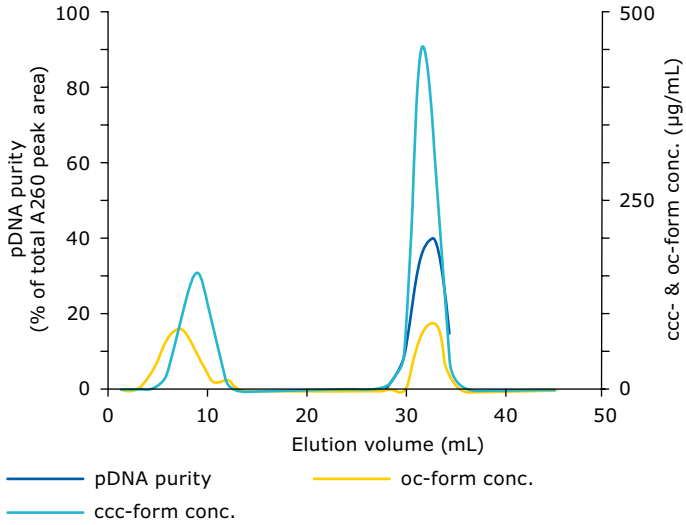

C

Fraction	ccc-form (µg)	Total pDNA (µg)
load	2,190	2,901
breakthrough	577	960
eluate pool	1,257	1,515

pDNA-form	Recovery (%)	Elution yield (%)
total pDNA	85	52
ccc-form	84	57

D

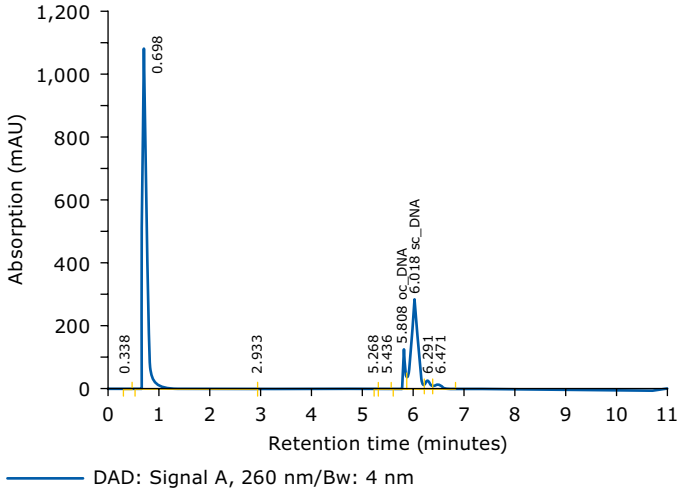

Figure 6. Plasmid purification from clarified original, non-treated lysate using Fractogel® EMD DEAE (M) resin. **A:** AKTA™ chromatogram. **B:** Isoform distribution and purity of ccc-form. **C:** Mass balance. **D:** Analytical chromatogram of the pDNA elution peak center fraction using AEX HPLC with a TSKgel DNA-NPR column.

Concentrated clarified lysate (pDNA titer ca. 0.30 mg/mL, 75% ccc-form, 25% oc-form) was loaded to a 1 mL Scout column packed with Fractogel® EMD DEAE (M) resin at 2.9 mg of pDNA/mL CV. Plasmid purity in the eluate was calculated from the A260 areas as determined by analytical AEX HPLC. Distribution of plasmid isoforms as well as high level of residual RNA is shown.

Figure 7 lists the binding capacities of AEX resins for pDNA, also using clarified original, non-treated lysate. Of the existing resin portfolio tested, only Fractogel® EMD DEAE (M) resin exhibited sufficient binding in the direct capture of pDNA. For all other resins pDNA capture was impacted by RNA binding.

Only when RNA interference is eliminated, the potential of anion exchange resins for pDNA purification unfolds (compare to Figure 8).

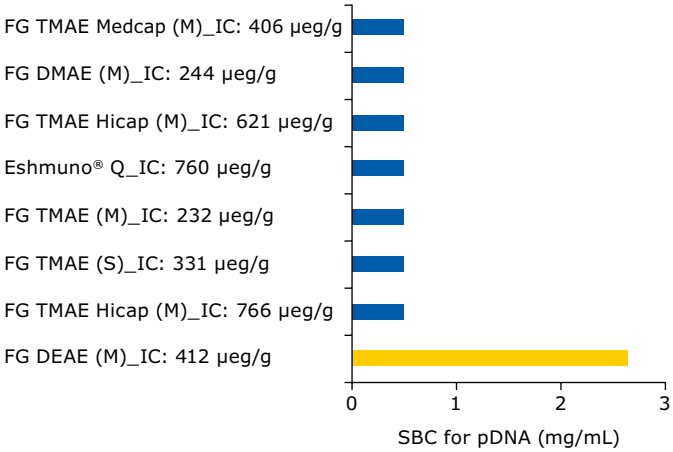

Figure 7. Static binding capacities of tentacle AEX resins with original clarified lysate (pH 5.0, 67 mS/cm) without any RNA treatment/removal. Measured in batch assay mode using 96-well filter plates (1 mL per well). IC = ionic capacity. FG = Fractogel® EMD resin.

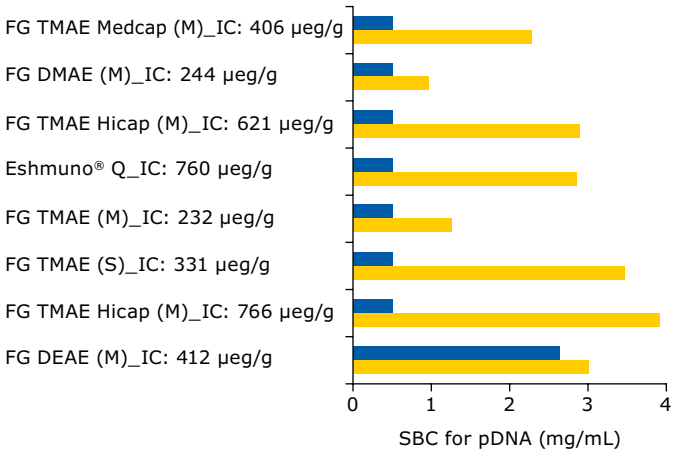

Figure 8. Static binding capacities of tentacle AEX resins with original clarified lysate (pH 5.0, 67 mS/cm) with RNase treatment. Measured in batch assay mode using 96-well filter plates (1 mL per well)

The data presented in this section confirm the significant interference of host cell RNA with the performance of anion exchange chromatography for plasmid DNA purification. This interference can be especially challenging when host cell RNA is present in large excess such as in *E. coli* lysates, particularly if such lysates are used as starting material (feed) without application of adequate countermeasures.

3. References

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Sterilizing Grade Filtration Unit Operations for Plasmid DNA Processes

Recommendations

Millipore Express® SHC can be used to achieve high filtration capacity, flux and yield for Plasmid DNA (pDNA) filtration in a variety of formats including pre-sterile capsules with sizes ranging from 0.014 m^2 to 3.0 m^2. Capacity and yield of the unit operation can vary significantly, especially with larger plasmids (~10 kbp and greater), and as such, process development should be carefully considered for optimization of the step.

Overview

Attributes

Sterilizing Grade Filtration unit operations for pDNA processes should include:

- A membrane with the ability to remove bioburden from the feed stream
- A device and filtration system that can prevent the introduction of bioburden
- A membrane that can reduce particulates, provides high capacity and high flux, and allows pDNA to flow through

Parameters

A Sterilizing Grade Filtration unit operation for pDNA processing can be optimized by changing the:

- Membrane used for sterile filtration
- Filtration device and system used
- Driving force (flowrate or pressure)
- Formulation of the pDNA solution
- Purity of the pDNA solution
- Conformation of the pDNA (supercoiled, linear, open-circular, etc.)
- Endpoint of the filtration

Key Considerations

The large size of pDNA can present a challenge for sterile filtration unit operations, as the product can be retained by the filters, leading to yield loss and low filtration capacity. Additionally, large pDNA molecules can be shear sensitive and use of a sterile filtration step has the potential to cause shear-induced denaturization of the product. Viscosity must also be considered as flow rates for sterile filtration steps can be low due to viscous material. Finally, a sterile filter must be proven to retain bacteria, which can be problematic for pDNA vaccines containing adjuvants.

Attributes	Parameters	Issues
Sterility assurance	Membrane pore size	Large size of pDNA
Particulate reduction	Membrane chemistry	Shear sensitivity of pDNA
Filtration capacity and flux	Driving force	Viscosity of pDNA solution
pDNA yield	Formulation	Bacterial retention for adjuvanted pDNA solutions
	Filtration endpoint	

Table 1. Key considerations for sterile filtration of pDNA solutions.

Technical Data

Process parameters should be optimized to achieve highest sterilizing grade filtration performance. While some plasmids present unique filtration challenges, for many smaller plasmids of less than 10 kbp, development of a robust unit operation could be as simple as confirming filter sizing using Vmax™ or Pmax™ methodology.[1]

A review of internal data for sterilizing grade filtration of pDNA feeds showed that filtration capacity, flux and yield can vary significantly, depending on the size of the plasmid, with larger plasmids presenting the greatest filtration challenge. Other researchers have also shown that filtration performance declines as plasmid size increases. The most significant filtration challenge occurs with pDNA of 20 kbp and larger – although 10–20 kbp pDNA often also cause filtration issues.[2,3] Table 2 summarizes the review of internal data and published studies.

Plasmid DNA Size (kbp)	Expected Sterilizing Grade Filtration Yield (%)	Expected Sterilizing Grade Filtration Capacity (L/m²)
<10	>90	>50
10–20	>80	Variable
>20	<80	<20

Table 2. Expected performance for sterilizing grade filtration of purified pDNA based on internal studies and literature search.

While the size of pDNA impacts sterilizing grade filtration performance, internal data and published studies both show that buffer composition can alter the plasmid conformation and subsequent radius of gyration. Specifically, salt concentrations have been shown to directly impact both the radius of gyration and diffusion coefficient of pDNA (Table 3).[4,5,6]

NaCl Concentration[a] (mM)	R_s[b] (nM)	D[c] (m^2/s)
10	6.9	4.0×10^{-12}
40	5.8	5.2×10^{-12}
100–300	4.5	5.5×10^{-12}

Table 3. Plasmid DNA properties.

[a] In TE buffer.

[b] From Hammermann et al. (1998) for 2.69 kbp plasmid.

[c] From Nguyen and Elimelech (2007) for 3.0 kbp plasmid with values adjusted to account for TE species in buffer solution (refer to text for details).

Changing the salt concentration has empirically demonstrated a greater than 2× increase in sterilizing grade filtration capacity and yield in internal studies and published studies.[3]

Using membranes for ultrafiltration, a study demonstrated a significant change in the sieving of pDNA with a change in salt concentration, providing further evidence that salt concentration heavily influences membrane filtration of pDNA.[7]

In addition to impact of pDNA size, studies have shown that supercoiled plasmid gives better filtration performance than open-circular; the purity of supercoiled pDNA can thus significantly impact unit operation outcomes of a sterilizing grade filtration step. One study cited an increase of approximately 10× in filtration capacity going from 90% to 95% supercoiled content.[2]

The filtration endpoint has been found to be significant in internal studies. Under constant pressure, plasmid concentration in the filtrate decreases at high flux decay, while constant flowrate operation has shown yield decline when pressure drop increases above a threshold. While both findings suggest that plasmid yield correlates with membrane fouling, detailed studies are needed to investigate the mechanism of action.

Both PVDF and PES membranes have shown success in filtering pDNA solutions. PES is preferred as it tends to have both higher capacity and flux versus PVDF and can be less damaging to larger plasmids.[3] Internal studies have shown higher yield for PES filters, although more detailed studies are needed to confirm this finding.

Data from internal and published studies suggest that altering the pDNA concentration can affect yield and capacity. Some published data have shown increased mass throughput with increased pDNA concentration.[2] Internal data suggest, however, this may not always be true; increased concentration may cause some self-association of pDNA molecules depending on the background buffer and purity, resulting in lower filtration capacity and yield. While concentration of pDNA is certainly a critical operating parameter, specific approaches for optimizing performance via dilution or concentration need to be better defined.

A review of sterilizing grade filtration operation conditions showed that feed flux or pressure has little to no impact on filtration capacity or yield (Table 4). It is possible, however, that high driving force could compromise plasmid integrity from shear, especially for larger plasmids.[3]

Optimization Parameter	Yield	Capacity	Product Integrity
Salt concentration	X	X	
Supercoiled pDNA content (purity)	X	X	
Filtration endpoint	X		
Membrane type – PVDF or PES	X – PES		X – PES
pDNA concentration	X	X	
Feed flux or pressure			X

Table 4. Critical parameters for optimizing plasmid DNA sterilizing grade filtration unit operations.

After a thorough review of published and internal data, critical parameters have been defined and can be applied to process development activities. Critical quality attributes of yield, capacity, and product integrity can be optimized through various parameters.

- Yield can be optimized by increasing salt concentration, increasing pDNA purity, defining the filtration endpoint to avoid extreme fouling, screening membranes, and exploring various pDNA concentrations.
- Capacity can be optimized through increasing salt concentration, increasing pDNA purity, or testing different pDNA concentrations.
- Product integrity through sterilizing grade filtration can be impacted by membrane type and feed flux or pressure.

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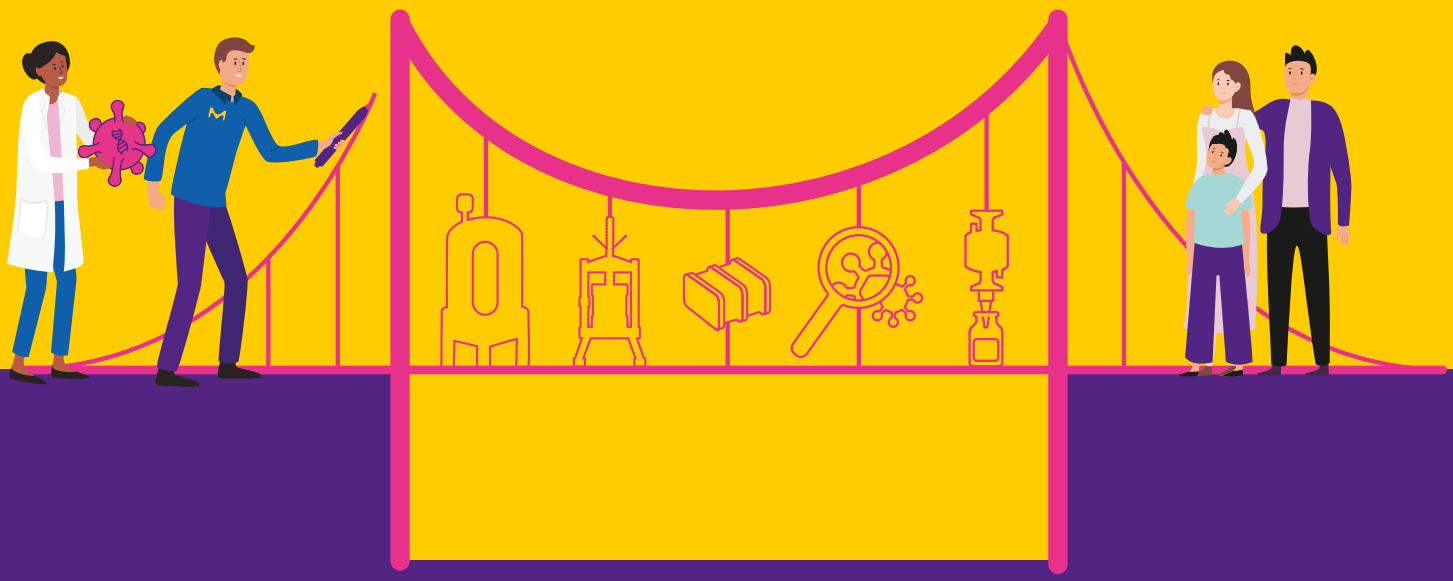

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