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Application Note

Scalable Purification of Plasmid DNA: Strategies and Considerations for Vaccine and Gene Therapy Manufacturing

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Plasmid DNA (pDNA) is an important component of viral vector therapies. These circular double helix DNA molecules can be used as the therapeutic transgene, to code for the viral capsid or as the vaccine itself. DNA vaccines have been approved for use in animals and this approach is being leveraged to combat the SARS-CoV-2 virus. In addition, pDNA is used as the starting material for mRNA vaccines which have also emerged in response to the pandemic and have other applications such as oncology. Furthermore, Plasmid DNA constructs are used for mammalian cell transfection to produce viral vectors used in gene therapy.

pDNA manufacturing presents several challenges. Production suffers from low productivity of microbial fermentation and the purification process is complicated by the fact that plasmids are quite large and possess a highly negative charge. The bacterial lysate contains contaminants with properties similar to pDNA leading to low resolution separation and can be highly viscous. A low flow rate is needed for chromatography and it can be difficult to achieve the desired concentrations at the final tangential flow filtration (TFF) step.

Additionally, pDNA is sensitive to shear stress which can lead to changes in its topological form. Plasmid isoforms include supercoiled (fully intact and wound around itself), open circular (one strand is broken and the molecule relaxes) and linear (both strands are broken with free ends). Supercoiled plasmids are recognized as the most therapeutically relevant and regulatory agencies set expectations for the supercoiled percentage in final drug substance for DNA vaccines.

This white paper describes an end-to-end platform consisting of single-use technologies and validation and testing services for pDNA manufacturing (**Figure 1**). Each of these steps is explored below, along with strategies to optimize and streamline the purification workflow.

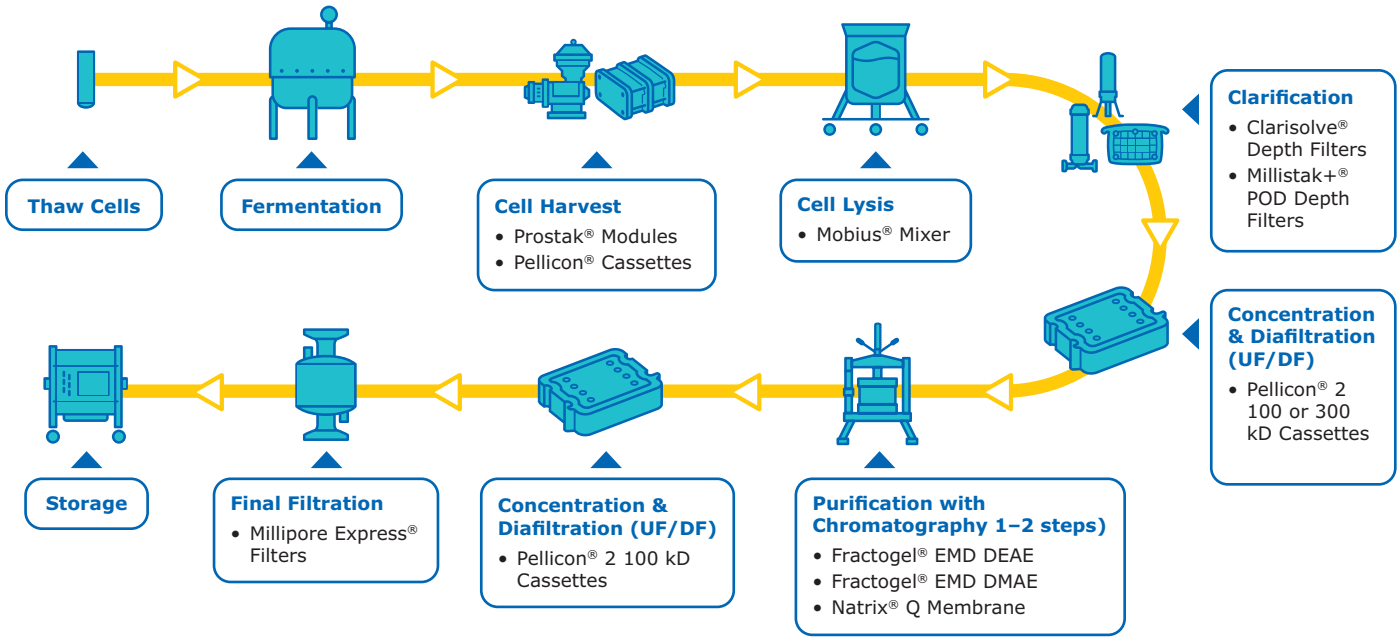


Figure 1. Integrated workflow with capabilities for pDNA purification from harvest to final fill.

Cell Harvest, Lysis and Centrifugation

The harvest step of pDNA purification requires multiple unit operations as plasmids are produced intracellularly in either a batch or fed-batch process. Centrifugation or microfiltration TFF (MF-TFF) is used to remove spent fermentation broth from the *E. coli* cells; at the lower volumes common for pDNA production, MF-TFF can be a better fit due to lower capital costs, process flexibility and easier scalability (**Table 1**). As shown in the table, when defining a MF-TFF step, the high solids load favors selection of an open channel device.

Centrifugation

- Traditional approach is cost effective at large scale (>500 L) or very small scale with swinging bucket (<5 L)
- Special attention is needed due to high shear generation in large scale centrifugation

Microfiltration Tangential Flow Filtration (MF-TFF)

- High solids count favor open-channel formats
- Flat-sheet TFF devices work well in this application
  - Linear scalability
  - Wide range of formats and installation options

MF-TFF Typical Operating Parameters

Parameters	Value
Device	Prostak™ Modules or Pellicon® 2
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 volumes	3 to 5

Table 1. Comparison of centrifugation and MF-TFF and operating parameters for bacterial cell harvest.

Once harvested, *E. coli* cells are lysed with an alkaline condition to release the plasmids. This method can be challenging as the lysis pH is close to the denaturization pH of pDNA. Alkali addition rate and mixing are critical, further high viscosity of the feed stream can affect the mixing process. Both the alkaline condition and shear from the mixing may damage supercoiled pDNA. The next step is either clarification for removal of cell debris or initial purification through pretreatment. The lysate is a very challenging feed with high solids load and a complex two phase separation; a froth phase on the top contains cell debris and gDNA and a lysate phase contains the pDNA, RNA and host cell protein on the bottom and it impacts initial purification through pretreatment. Various methods have been explored to condition the feed for improved clarification but no one method has emerged as optimal.

The clarification can be performed by centrifugation or normal flow filtration, as described in cell harvest, centrifugation is not preferred. While centrifugation can handle a high percentage of solids, secondary clarification may be required, and the high shear rate can damage the supercoiled pDNA structure. In contrast, normal flow filtration uses size exclusion and adsorption mechanisms and is a robust clarification method with many installation options and simple scaling and process development. Another important consideration is that the positively charged filtration aid may interact with the plasmid.

For pDNA clarification, media that have lower adsorptive properties are recommended to mitigate the risk of product binding. The filter types that have been proven to be optimal for pDNA clarification are highlighted in **Figure 2**. These depth filters have demonstrated excellent performance over a wide range of conditions, making them a viable platform option. **Figure 3** shows the relatively high capacities for both pre-conditioned and non-conditioned lysate feeds with average capacity above 150 L/m^2 for many filters. Additionally, these depth filters show high yield and good protection of polishing membranes placed after the clarification step.

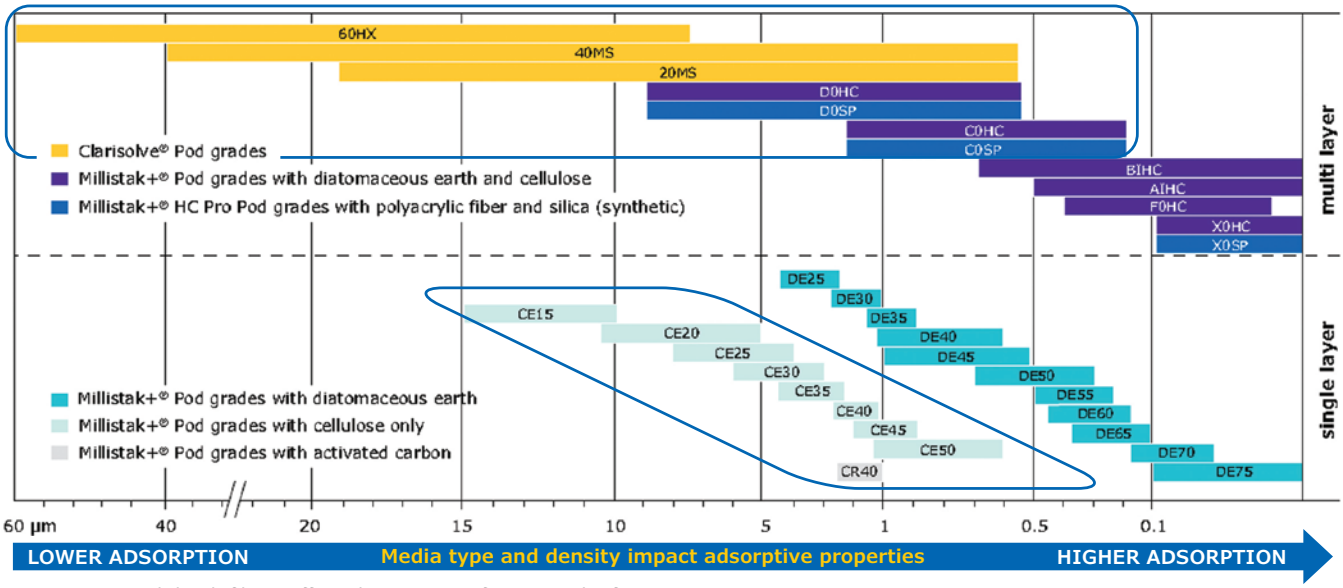
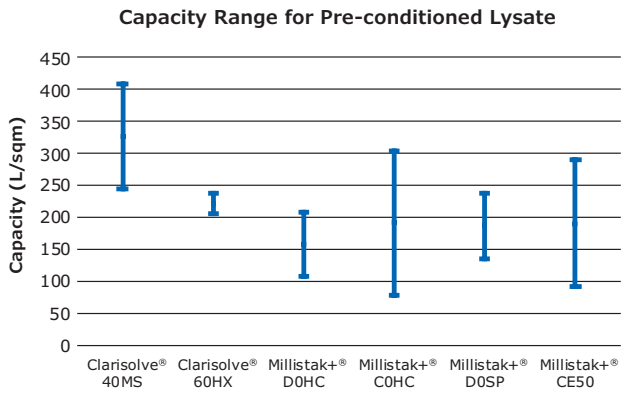
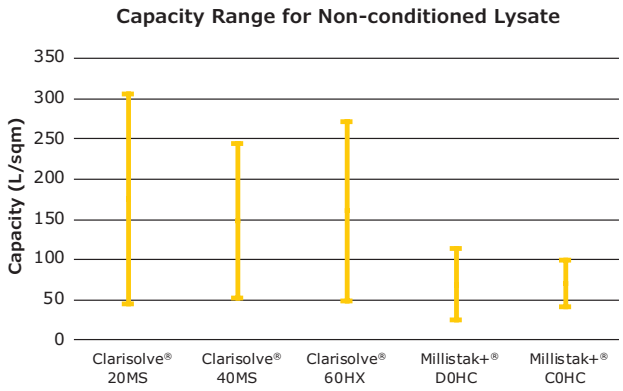


Figure 2. Several depth filters offer robust options for pDNA clarification.





High yields at >90%

- Millistak+® D0HC or C0HC: >80%
- Millistak+® CE: >90%
- Clarisolve®: >90%

- Salt chase recommended
- Bioburden reduction membrane capacity post depth filtration varies – >500 L/m² typical

Figure 3. Depth filter capacity ranges for pre- and non-conditioned lysate.

Table 2 summarizes two case studies in which different depth filters were used for clarification of a $CaCl_2$ precipitated lysate and a non-treated lysate. In the first study, Clarisolve® 60HX was selected and loading was sufficient to allow an installation for a 500 L batch of two process scale pod racks of 14 x 0.55 m² filters. For the non-treated lysate, Millistak+® D0HC, a tighter filter in comparison to the Clarisolve® 60HX, was optimal and only a single process scale pod rack was needed to clarify 500 L of lysate.

Tangential Flow Filtration

Purification of pDNA is a complex operation requiring a combination of unit operations including TFF, chromatography, and sterile filtration.

TFF for pDNA is implemented in either one or two steps and can be accomplished using 30, 100, or 300 kD molecular weight cut off membranes depending on the plasmid size. TFF can be placed between the clarification and chromatography steps, where residual impurities can be washed through diafiltration and pDNA can be concentrated to reduce load volume for chromatography steps. TFF is consistently used as a formulation step at the end of the process to diafilter into the formulation buffer and concentrate to formulation concentration.

When considering process development for TFF, it is important to account for filter fouling, especially for challenging or high impurity feeds. It is common to see higher molecular weight cutoff membranes foul faster than lower molecular weight cutoffs and the fouling depends on permeate flow rate. Two approaches can be considered to control the permeate flow rate:

- A two-pump control where a feed and permeate pump fully control flow rates
- Transmembrane pressure (TMP) control where a single feed pump is used and a retentate valve controls transmembrane pressure which drives permeate flow

With either mode of operation, a critical permeate flux exists that causes filter fouling, as particles are not swept off the membrane fast enough to enable stable operation and are instead sent into the pore structure. An example of critical flux levels for TMP control operation is shown in **Figure 4**. With TMP control, the TMP causing critical flux can be quite low for 100 kD and higher molecular weight cutoffs, making the TMP window of operation for manufacturing very tight. As such, two-pump control is preferred to create greater consistency for process scale up.

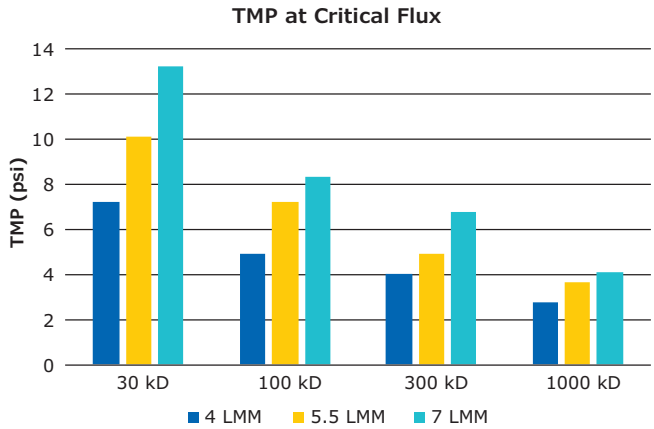


Figure 4. Transmembrane pressure at critical flux.

	Description	Filter	Flux (LMH)	Volumetric Throughput at 15 psi dP (L/m²)	Installation for 500 L with 1.5x safety factor
Case Study #1	• Clarisolve® 60HX • Alkaline lysis + $CaCl_2$ precipitation • gDNA and RNA being removed	Clarisolve® 60HX	200	160	14 x 0.55m²
Case Study #2	• Millistak+® D0HC • Alkaline lysis without pretreatment • gDNA removed	Millistak+® D0HC	100	150	8 x 1.1m²

Table 2. Clarification of precipitated and non-treated lysate using different depth filters

Typical TFF operating parameters are shown in **Table 3**. With TFF, it is important to remain mindful of shear and high viscosity, especially for high concentration formulations. Open feed channel screens such as a D-Screen or V-Screen are preferred for this application. Additionally, it is important to realize that operating parameters can change retention properties. A 100 kD molecular weight membrane can separate plasmid isoforms based solely on filtrate flux, as the sieving coefficient for each isoform is dependent on filtrate flux (**Figure 5**). While this study demonstrated the feasibility of TFF for separating plasmid isoforms, it reinforces the fact that processes must be optimized and reproducible for robust concentration and diafiltration of pDNA.

Parameters	Value
Feed Flux (LMM)	4 or less
Permeate Flux (LMH) – for two pump	20–50
TMP (psi) – for TFF control	<10
Loading (L/m^2)	70–140
Concentration Factor	5x–50x
Volumetric concentration factor	2 to 5
Diafiltration volumes	3 to 5

Table 3. Typical TFF operating parameters.

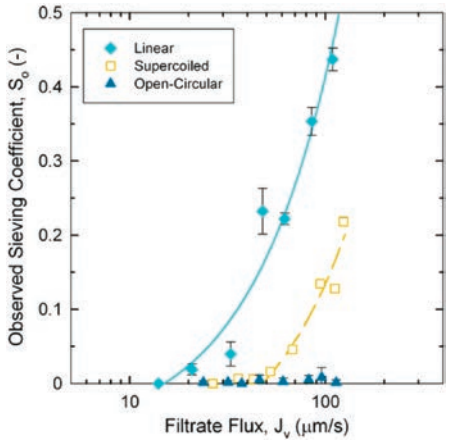


Figure 5. Operating parameters can change retention properties.

Chromatography

Anion exchange chromatography (AEX) and hydrophobic interaction chromatography (HIC) are commonly used for pDNA purification. AEX is highly effective at binding negatively charged nucleic acids and is widely used for pDNA purification; many impurities are also negatively charged and co-binding and separation can be challenging. Despite this shortcoming, AEX has demonstrated robust clearance of proteins, RNA, gDNA and endotoxin. Plasmid isoforms, on the other hand, are very challenging to separate with ion exchange. In this case, HIC can be used and is well-placed following AEX due to the high salt eluate pool.

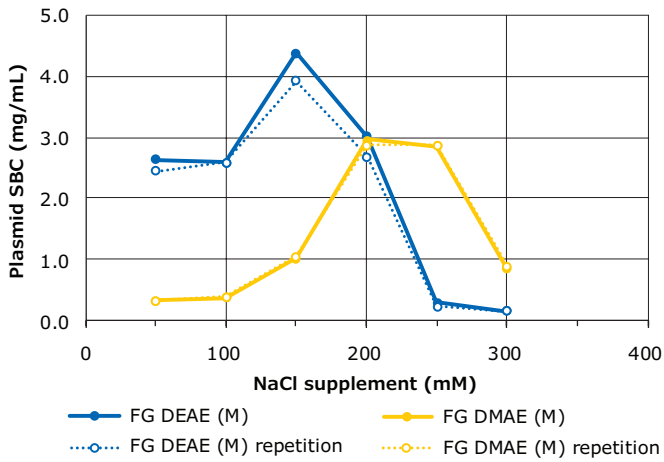
Chromatographic purification can be accomplished using either resins and membranes. While resins offer flexible installations and good selectivity, they suffer from low binding capacity, as plasmids are too large to diffuse into the resin bead thus limiting access to binding sites. Additionally, high pressure drops due to elevated viscosity of feed streams can lead to flow limitations and long processing times. In contrast, membranes have large pores and do not depend on diffusion for binding site access; binding capacity and flow rates can therefore be much higher.

Fractogel® EMD DEAE and EMD DMAE weak anion exchange resins are a versatile solution offering unique selectivity and best in class capacity and are ideal for intermediate purification. Among the benefits of these AEX resin are:

- Unique binding selectivity
- Best-in-class binding capacity of 2–4 mg/mL due to the unique tentacle chemistry which extends the binding area beyond the bead surface
- High yield: 80% to 95% of ccc-form (covalently closed circular)
- Removal of residual endotoxin
- Good resolution due to moderate bead size (d50: 48–60 µm)
- Moderate flow: 4–8 minutes residence time

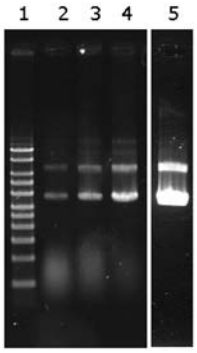
While RNA reduction methods such as $CaCl_2$ precipitation, RNAse treatment and TFF have been explored, the best approach to prevent RNA binding and reduce interference with pDNA binding on Fractogel® EMD DEAE is direct salt supplementation. In this method, clarified lysate is adjusted with NaCl to mitigate RNA co-binding, which gives high binding capacity for pDNA. A static binding capacity versus NaCl supplement curve shows a clear optimal salt concentration where RNA (but not plasmid) binding is suppressed (**Figure 6**). This mechanism is confirmed by the accompanying gel where Fractogel® EMD DEAE eluate purity is highest with the optimal salt supplementation for high pDNA binding.

A.



B.

Purity of Fractogel® EMD DEAE capture eluates from lysates with varying NaCl supplement



1	Marker
2	0 mM NaCl
3	100 mM NaCl
4	120 mM NaCl
5	150 mM NaCl

Figure 6. Binding of pDNA from lysate (A) and purity of capture eluates from lysates with varying NaCl supplement (B).

Given the advantage of this salt supplementation method, a preferred approach to pDNA purification is to load NaCl-supplemented clarified lysate directly on the AEX to remove RNA, endotoxin, gDNA and host cell protein. This AEX direct capture results in >95% RNA removal, >95% pDNA purity and >90% pDNA yield (**Figure 7**). An important note and significant advantage of this approach is that direct capture eliminates complex development of a pre-treatment and TFF step prior to chromatography.

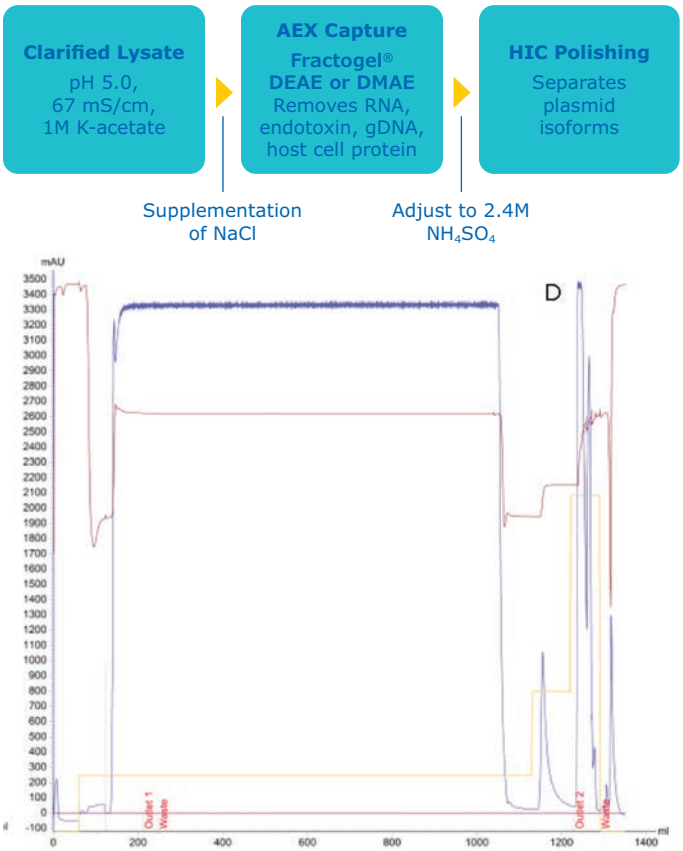


Figure 7. Salt supplementation for AEX direct capture eliminates need for pre-treatment and TFF.

An alternative approach to the direct capture with AEX is to use Fractogel® weak anion exchangers as a polishing step after HIC capture. Though not preferred due to potential for precipitate formation when adjusting the lysate to HIC binding conditions, this method can be effective. Minimal to no feed adjustment between HIC and AEX is required, as these anion exchangers have been shown to maintain high binding of pDNA even with high ammonium suflate concentrations (**Figure 8**).

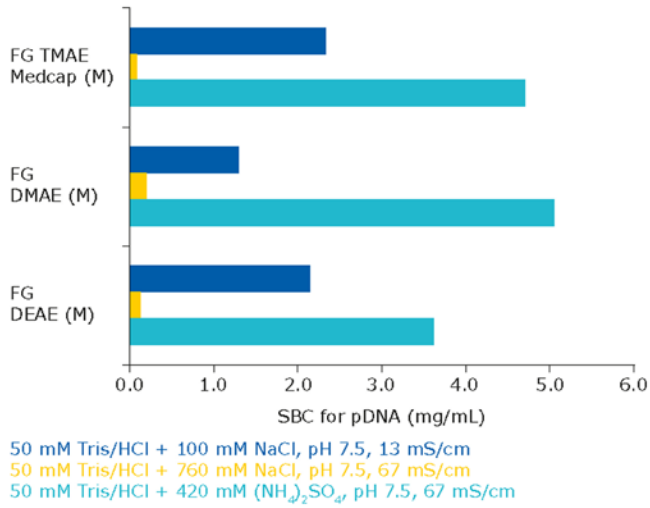


Figure 8. pDNA binding to Fractogel® AEX resins tolerates presence of elevated concentration of $(NH_4)_2SO_4$.

Natrix® Q chromatography membrane is an AEX single-use membrane providing superior productivity and is ideally suited for the capture of small and mid-sized plasmids (<20 kbp). It is a composite membrane with high mechanical strength and hydraulic permeability. The nominal pore size of 0.4 µm permits high flow rates. Re-use in a rapid cycling operation allows for cost reductions while caustic stability enables efficient cleaning. The high capacity of Natrix® Q for pDNA has been demonstrated at 5–10 mg/mL with >95% RNA removal and short cycle times (~35 minutes).

Using the same salt supplementation method as described above, Natrix® Q gave very high binding capacities for a small (5.7 kbp) and medium (13 kbp) sized plasmids, at 10 and 4 mg/mL respectively (**Table 4**). In addition to high binding capacity, Natrix® Q provided good purity with high yield. For a large plasmid at 20 kbp, however, performance declines as the plasmid size approaches the size of the membrane pores. Decreased membrane permeability was observed, resulting in lower capacity and process performance.

Feed used:

- Original *E. coli* lysates from alkaline lysis, clarified by centrifugation/depth filtration, supplemented with optimal NaCl to eliminate RNA interference, pH 5.0, finally 0.22 µm filtered
- Plasmids ranging from small to large size
- Varying initial pDNA purity ranging from 0.7%–4%

Plasmid Size	Plasmid Titer (µg/mL)	Initial pDNA Purity (A260 based)	Initial Lysate Conductivity (mS/cm)	Optimal NaCl Supplement (mM)	Final Lysate Conductivity (mS/cm)	Residence Time (min)	Operating Capacity (mg/mL MV)	Yield (%)	pDNA Eluate Purity (A260 based)
5.7 kb	45	4.0%	69	160	82	0.1	~10	≥80	≥80% pDNA
13 kb	33	2.4%	72	130	82	0.1	~4	≥77	≥90% pDNA
20 kb	25	0.7%	79	100	86	0.2	~1	≥65	≥62% pDNA

Table 4. pDNA purification using Natrix® Q chromatography membrane.

Table 5 summarizes the use of Natrix® Q for high throughput capture with direct salt supplementation to enable high binding capacity of pDNA. Alternatively, Fractogel® EMD DEAE or EMD DMAE can be used for polishing purification following HIC with good capacity, high selectivity, and high yield.

Feed used:

- Original *E. coli* lysate, clarified by centrifugation and subsequent depth filtration, directly supplemented with NaCl (120–250 mM, depending on resin or membrane type) to eliminate RNA interference, pH 5.0, 74–82 mS/cm
- pDNA size 5.7 kbp, pDNA titer 45 µg/mL

Recommended Process Step	Resin / Membrane Adsorber	Dynamic Capacity (mg/mL)	Cycle Time (min)	Residence Time w(min)	RNA Removal	Yield ccc-Form	Purity (A260 based)
High-throughput capture	Natrix® Q	~10	~0.5 h	0.1–0.03	>95%	≥80%	>80% pDNA
Polishing Purification	Fractogel® EMD DEAE (M)	~2.5	~3 h	4	>95%	≥80%	>95% pDNA
	Fractogel® EMD DMAE (M)	~3	~3 h	4	>95%	≥95%	>95% pDNA

Table 5. Summary of the performance of Natrix® Q and Fractogel® EMD DEAE or EMD DMAE for purification of pDNA.

Sterile Filtration

Once purified and formulated, pDNA undergoes sterile filtration for sterility assurance and patient safety. While this step may appear to be a relatively simple operation, filtration of pDNA can be challenging due to the large size of plasmids, high viscosity of the solution and bacterial retention for adjuvanted formulations.

Many process parameters must be considered to optimize sterilizing grade filtration unit operations with the most significant being salt concentration, plasmid purity and plasmid concentration (**Table 6**). Filtration endpoint, membrane type and driving force can also affect quality attributes of the unit operation. In addition, the effective size of the pDNA can be altered by changing the ionic concentration of the background buffer solution.

Another major consideration for pDNA sterile filtration is plasmid size; smaller plasmids (<10 kbp) are rarely limited by yield or filtration capacity for sterilizing grade filtration steps while larger plasmids often encounter yield and capacity issues, especially those at or above 20 kbp. In these cases, careful process development should be performed.

Millipore Express® SHC (PES) is preferred choice, with optimized process parameters can deliver the best performance for sterile filtration. Depending on size average capacity of 20 L/m^2 to 200 L/m^2 is reported based on internal data base.

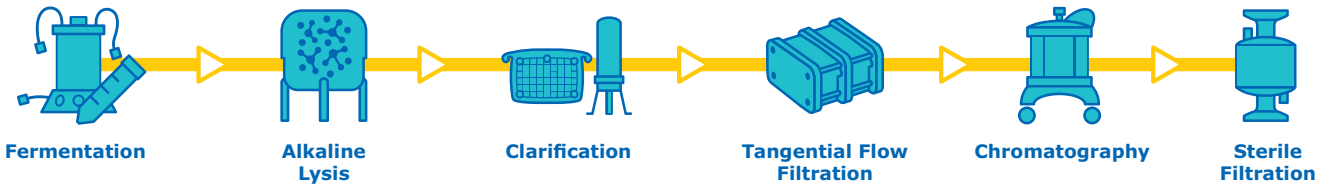
Sterile filtration is also suggested at intermediate steps like after clarification, UFDF and chromatography for Bioburden control. Millipore Express® SHC filter has been reported as a bioburden reduction filter after clarification with average capacity range of 400–650 L/m^2 based on feed quality.

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 6. Impact of optimization parameters on yield, capacity and product integrity.

An Optimized Platform for pDNA Purification

pDNA has become an important therapeutic modality in the form of DNA vaccines and as the basis for RNA vaccines and gene therapy vectors. These biologicals can be made in a relatively short time span and do not need to be stored at low temperatures as DNA is a very stable molecule. Purification of pDNA for use as vaccine doses, however, present several challenges. Fortunately, a comprehensive set of technologies are available to optimize the entire workflow, from lysis to sterilizing grade filtration, and enable robust and scalable manufacturing of pDNA (**Table 7**).



Lysis	Mobius® Mix	Extract DNA, denature gDNA, potentially precipitate or degrade RNA
Clarification	Clarisolve® 60HX or Millistak+® POD Depth Filters	Remove cell debris and precipitated impurities (gDNA, RNA)
TFF	Pellicon® 30 kD, 100 kD, 300 kD	Concentrate and remove small impurities
Chromatography	Natrix® Q or Fractogel® EMD DEAE or EMD DMAE	Remove RNA, gDNA, HCP, and endotoxin
Sterile Filtration	Millipore Express® SHC	Sterility assurance

Table 7. Integrated solutions for pDNA purification.

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