Millipore®

Preparation, Separation, Filtration & Monitoring Products

Tech Note

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).

The Life Science business of Merck operates as MilliporeSigma in the U.S. and Canada.

MERCK

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.

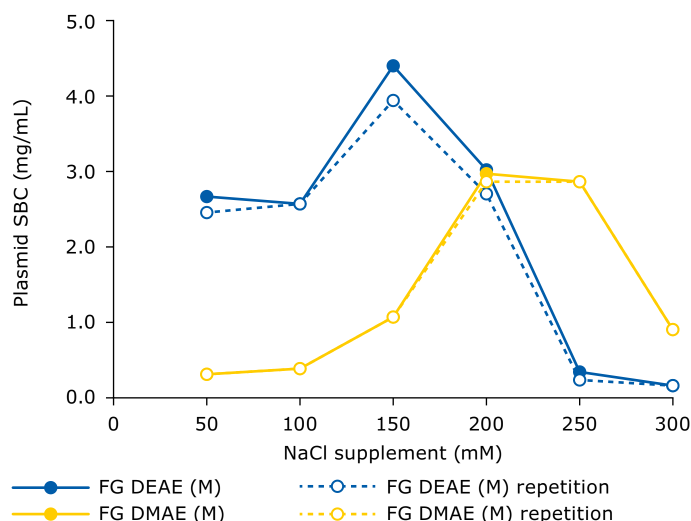


Figure 1. Batch assay for determination of optimal NaCl concentration for lysate supplementation. Static binding capacities (SBC) were measured in 96-well filter plates (1 mL per well). Plasmid feed was original clarified lysate (pH 5.0, 67 mS/cm) supplemented with increasing NaCl concentrations. FG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

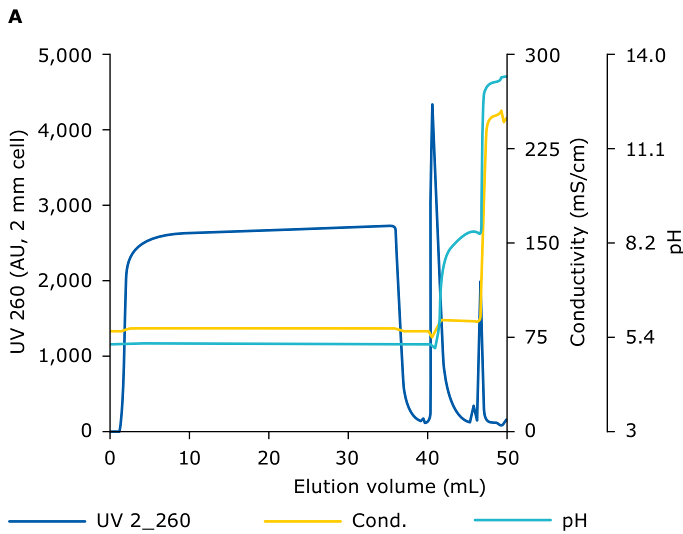
Plasmid with ≥80% pure plasmid DNA and about 10% residual RNA (A260 based) can be obtained with a yield of about 80% in a total run time of about 30 minutes, using Natrix® Q chromatography membrane with a binding capacity of approximately 10 mg/mL for capture from NaCl supplemented clarified lysate at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

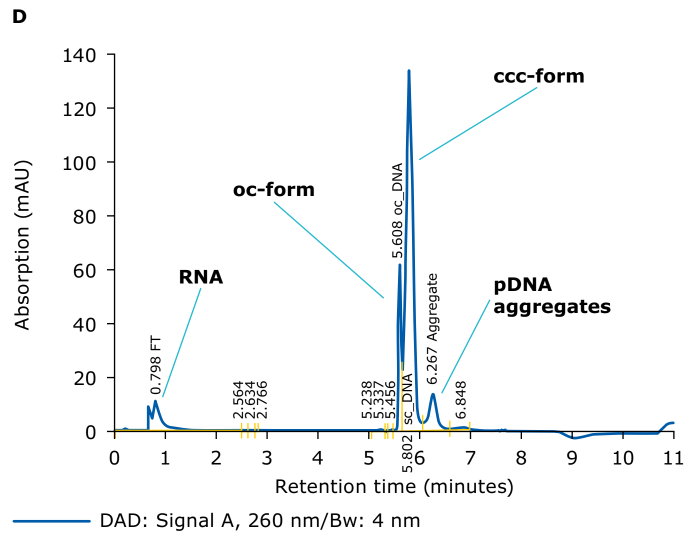
Table 2. Experimental conditions for using Natrix® Q chromatography membrane for capture of plasmid DNA.

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



D



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

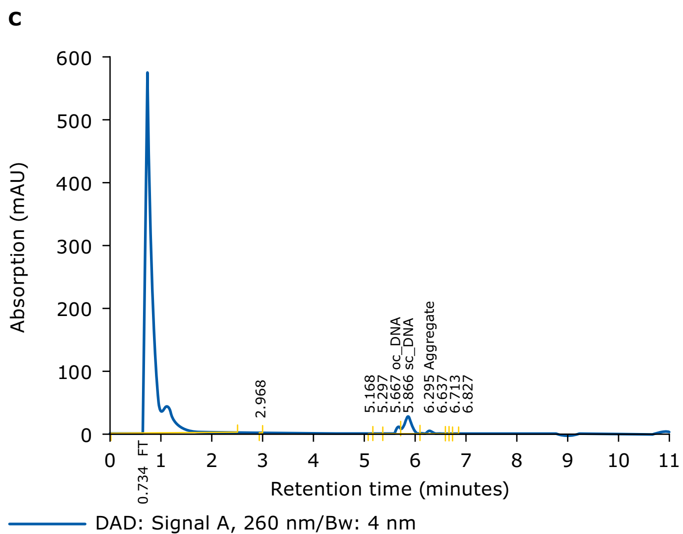


Figure 2. Plasmid capture from clarified lysate supplemented with NaCl using Natrix® Q chromatography membrane. **A:** AKTA™ chromatogram. **B:** Mass balance. **C:** Analytical chromatogram of clarified lysate using AEX HPLC with a TSKgel DNANPR column. **D:** Analytical HPLC chromatogram of the elution 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

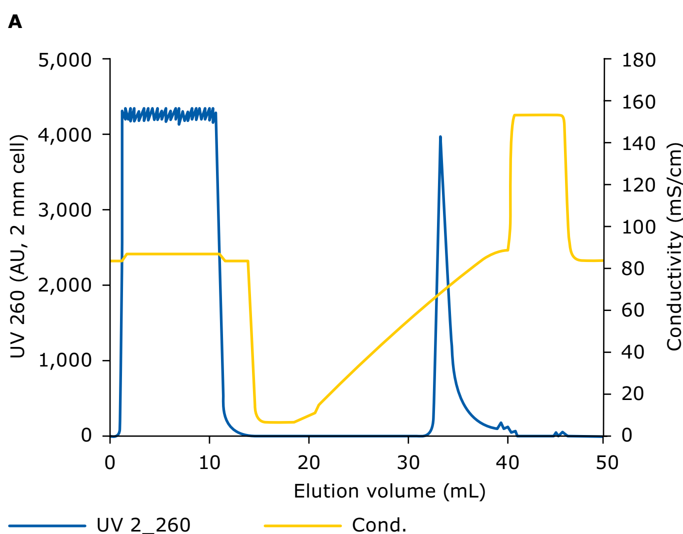
Plasmid preparations with >95% pure plasmid DNA and <5% residual RNA (A260 based) can be obtained with a yield of approximately 75%, using Eshmuno® Q resin with a binding capacity of about 2.5 mg/mL for capture from NaCl-supplemented clarified lysate at a high flow rate. Greater than 95% of the initial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

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

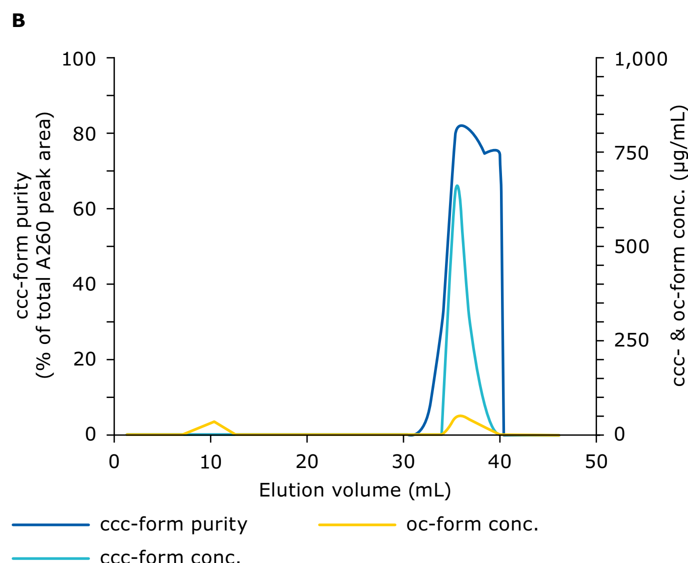
Table 3. Experimental conditions for using Eshmuno® Q resin for capture of plasmid DNA.

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



B



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

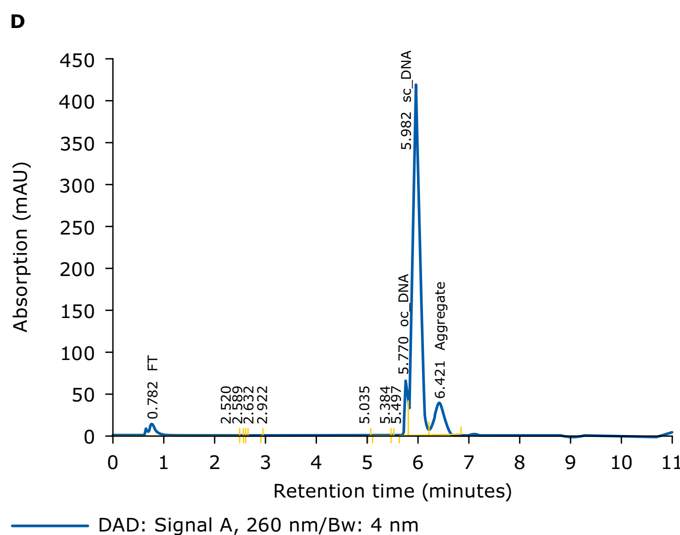


Figure 3. Plasmid purification from clarified lysate supplemented with NaCl using Eshmuno® Q resin. **A:** AKTA™ chromatogram. **B:** Isoform distribution and purity of ccc-form. **C:** Mass balance. **D:** Analytical chromatogram of the pDNA elution peak center fraction using AEX HPLC with a TSKgel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

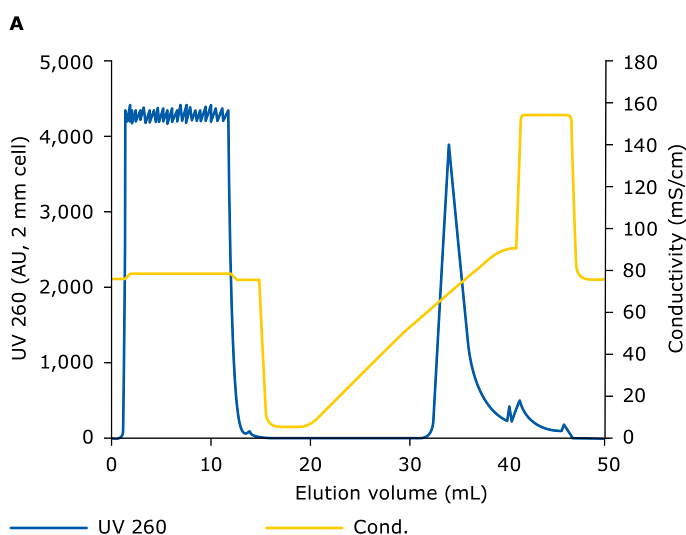
Table 4. Experimental conditions for using Fractogel® EMD DEAE (M) and Fractogel® EMD DMAE (M) resins for intermediate purification and polishing of plasmid DNA.

a) Fractogel® EMD DEAE (M) resin

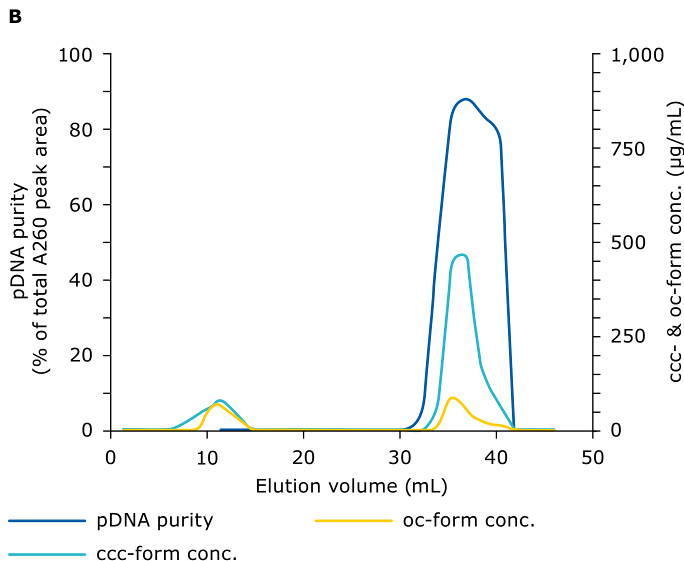
Parameters	Value
Chromatography resin	Fractogel® EMD DEAE (M) resin, 1 mL Scout column 0.8×1.9 cm ID/L
Equilibration	5 CV buffer A1: 1 M K-acetate + X mM NaCl, pH 5.0, 74 mS/cm
Load	Clarified lysate (1 M K-acetate buffer matrix, pH 5.0, 67 mS/cm) directly supplemented with NaCl (ca. 120 mM) to 76 mS/cm, 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



B



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

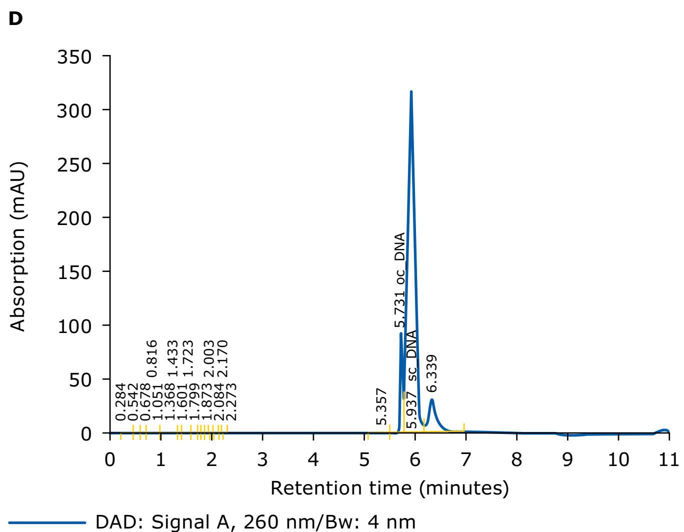


Figure 4. Plasmid purification from clarified lysate supplemented with NaCl using Fractogel® EMD DEAE (M) resin. **A:** AKTA™ chromatogram. **B:** Isoform distribution and purity of ccc-form. **C:** Mass balance. **D:** Analytical chromatogram of the pDNA elution peak center fraction using AEX HPLC with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

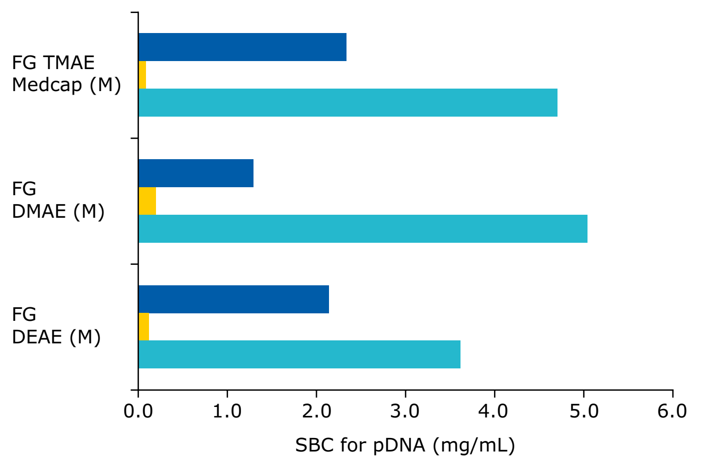


Figure 5. Static binding capacities of tentacle AEX resins with purified pDNA (pEGFP-N1, 4.7 kbp, prepared in-house) in ammonium sulfate and sodium chloride-containing solutions. Measured in batch assay mode using 96-well filter plates (1 mL per well).

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

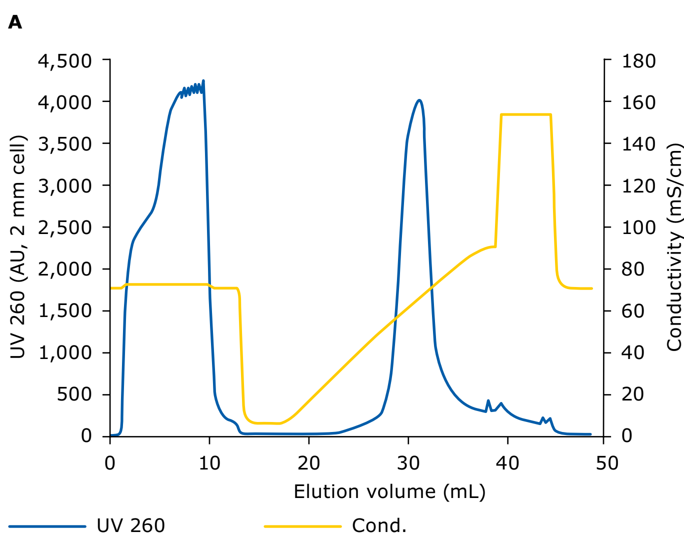
Plasmid of low purity with about 20% pure plasmid DNA still containing 80% RNA (A260 based) can be obtained with a low yield of 60%, using Fractogel® EMD DEAE (M) resin with a binding capacity of approximately 1.5 mg/mL for purification from concentrated clarified original, non-treated lysate, without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

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

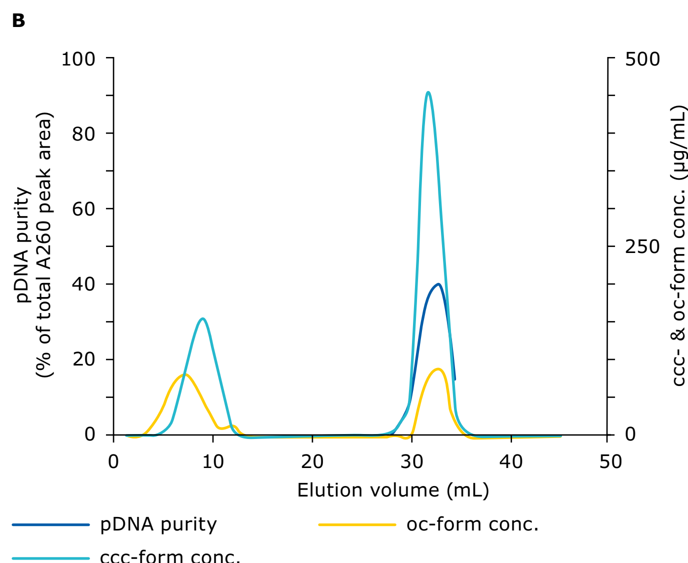
Table 5. Experimental conditions for using Fractogel® EMD DEAE (M) resin for capture of plasmid DNA.

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



B



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

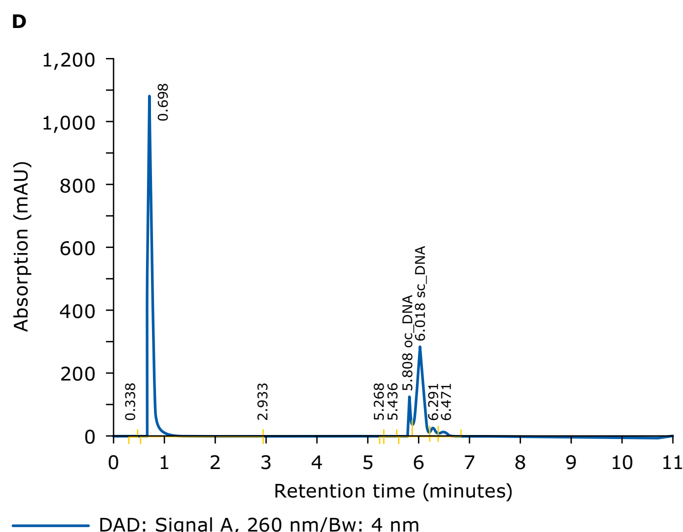


Figure 6. Plasmid purification from clarified original, non-treated lysate using Fractogel® EMD DEAE (M) resin. **A:** AKTA™ chromatogram. **B:** Isoform distribution and purity of ccc-form. **C:** Mass balance. **D:** Analytical chromatogram of the pDNA elution peak center fraction using AEX HPLC with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

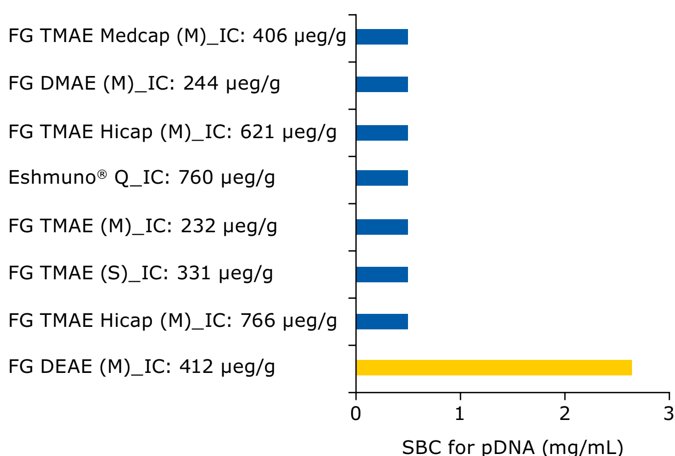


Figure 7. Static binding capacities of tentacle AEX resins with original clarified lysate (pH 5.0, 67 mS/cm) without any RNA treatment/removal. Measured in batch assay mode using 96-well filter plates (1 mL per well). IC = ionic capacity. FG = Fractogel® EMD resin.

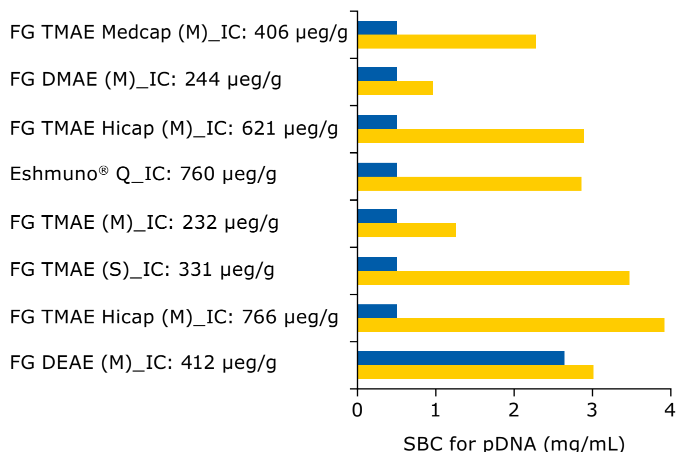


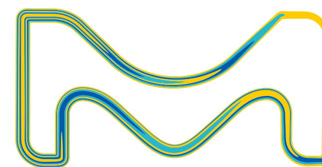
Figure 8. Static binding capacities of tentacle AEX resins with original clarified lysate (pH 5.0, 67 mS/cm) with RNase treatment. Measured in batch assay mode using 96-well filter plates (1 mL per well)

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

1. Xenopoulos, A. and Pattnaik, P. (2014) Production and purification of plasmid DNA vaccines: is there scope for further innovation? Expert Rev. Vaccines **13**, 1537–1551
2. Stadler, J., Lemmens, R. and Nyhammar, T. (2004) Plasmid DNA purification. J. Gene Med. **6**, S54–S66
3. Diogo, M.M., Queiroz, J.A. and Prazeres, D.M.F. (2005) Chromatography of plasmid DNA. J. Chromatogr. A **1069**, 3–22
4. Urthaler, J., Buchinger, W. and Necina, R. (2005) Improved downstream process for the production of plasmid DNA for gene therapy. Acta Biochim. Pol. **52**(3), 703–11
5. Tarmann, C. and Jungbauer, A. (2008) Adsorption of plasmid DNA on anion exchange chromatography media. J. Sep. Sci. **31**, 2605–2618
6. Eon-Duval, A. and Burke, G. (2004) Purification of pharmaceutical-grade plasmid DNA by anion-exchange chromatography in an RNase-free process. J. Chomatogr. B **804**, 327–335
7. Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**, 1513–1523

Merck KGaA
Frankfurter Strasse 250
64293 Darmstadt
Germany



For additional information, please visit **MerckMillipore.com**
To place an order or receive technical assistance, please visit **MerckMillipore.com/contactPS**

Merck, Millipore, the Vibrant M, Natrix, Eshmuno and Fractogel are trademarks of Merck KGaA, Darmstadt, Germany or its affiliates. All other trademarks are the property of their respective owners. Detailed information on trademarks is available via publicly accessible resources.
© 2020 Merck KGaA, Darmstadt, Germany and/or its affiliates. All Rights Reserved.

MK_AN6573EN Ver. 0.1
08/2020