

mRNA purification using anion exchange chromatography at ambient temperature

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Introduction and Objectives

Due to the negative charge exhibited by nucleotide products, such as mRNA, anion exchange chromatography (AEX) in bind/elute mode is an effective purification step. Typically, short mRNA of less than 500 nucleotides (nt) can be purified by traditional anion exchange chromatography using a linear salt gradient.¹ However, the charge characteristics of larger mRNA products over 500 nucleotides can make elution recovery challenging. In addition to the standard strong multi-point binding of these large products, RNA molecules can form highly complex quaternary structures with other RNA transcripts, further complicating purification. The strong interaction of these molecules with the stationary phase can be weakened by operating under denaturing or partially denaturing conditions, which can be achieved by elevating temperature to approximately 65 °C or by adding chaotropic reagents.² However, implementing elevated temperature solutions in a Good Manufacturing Practice (GMP) environment is challenging. Consequently, there is a significant industry need for a method that allows for both high binding capacity and high yield in this purification step while utilizing standard elution chromatographic methods.

Polishing steps to eliminate mRNA-related impurities, such as dsRNA, aggregates, and DNA/RNA hybrids, are required for meeting product quality specifications. This application note presents case studies that demonstrate preparative purification of mRNA molecules exceeding 500 nt. Furthermore, it displays a novel, scalable method designed to achieve high yields, making it suitable for large-scale GMP manufacturing of mRNA therapeutics.

Preparative mRNA purification – Salt gradient vs. dual gradient

Based on the results of initial batch assay (data not shown), the combinations of high salt, high pH and chaotropic salt were identified as the effective parameters for improving mRNA elution from an AEX ligand. Furthermore, to identify the optimal elution methods, two elution techniques of salt gradient and dual gradient were evaluated using the strong anion exchange membrane absorber **Natrix[®] Q Micro** 0.2 mL with quaternary amine as ligand. An 858 nt mRNA encoding erythropoietin (EPO) protein containing chemically modified 5-methoxyuridine (5-moU) and a chemically unmodified 1,929 nt mRNA encoding firefly luciferase (FLuc) protein were utilized as the feed material for this experiment. Experimental procedures are summarized in Table 1. Figure 1 illustrates the results of purification run.

Table 1.

Chromatographic procedure for bind/elute **Natrix[®] Q** device operation.

Step	Description	MV
Salt gradient	Membrane: Natrix[®] Q Micro 0.2 mL	-
Equilibration 1	Buffer A1: 50 mM Tris, 150 mM NaCl, pH 8.0	50
Load	<i>In vitro</i> transcription (IVT) mRNA (EPO-5moU: 858 nt, FLuc: 1,929 nt) in Buffer A1 10 µg mRNA/0.2 mL Natrix[®] Q	2.5
Wash	Buffer A1	25
Elution 1	Buffer B1: 50 mM Tris, 2 M NaCl, pH 8.0, from 0 to 100% B1	250
Dual gradient	Flow path primed with buffer (A2 and B2)	
Equilibration 2	Buffer A2: 50 mM Tris, 0.5 M Arginine, pH 9.0	50
Elution 2	Buffer B2: 50 mM Tris, 0.5 M Arginine, 2 M NaCl, pH 11.0, from 0 to 100% B2	250
Elution 2 hold	Buffer B2: 50 mM Tris, 0.5 M Arginine, 2 M NaCl, pH 11.0	50
CIP	1 M NaOH, 2 M NaCl	50
Re-equilibration	Buffer A1	50

In terms of behaviour of mRNA EPO, the partial mRNA EPO eluted broadly during NaCl gradient elution at neutral pH while the rest of the mRNA is still bound to Natrix® Q (Figure 1A). In contrast, bound mRNA EPO was completely eluted by increasing NaCl concentration and pH in the presence of 0.5 M arginine as a chaotropic salt at approximately 100 mL volume (Figure 1B).

Regarding longer mRNA FLuc, most of mRNA FLuc showed low elution efficiency by NaCl gradient due to the strong hydrogen binding as expected (Figure 1A). Subsequently, bound mRNA FLuc was completely eluted by the dual gradient as well as mRNA EPO (Figure 1B). mRNA with different nucleotides in length showed similar behavior during chromatographic operation. In addition to major peak of ssRNA, three other peaks were observed on later phase of dual gradient elution. It is postulated that these are mRNA-related impurities generated in the process of *in vitro* transcription. As a result of this evaluation, dual gradient method at ambient temperature enables the effective elution, high selectivity and impurities removal for RNA transcripts of greater than 800 nt in length.

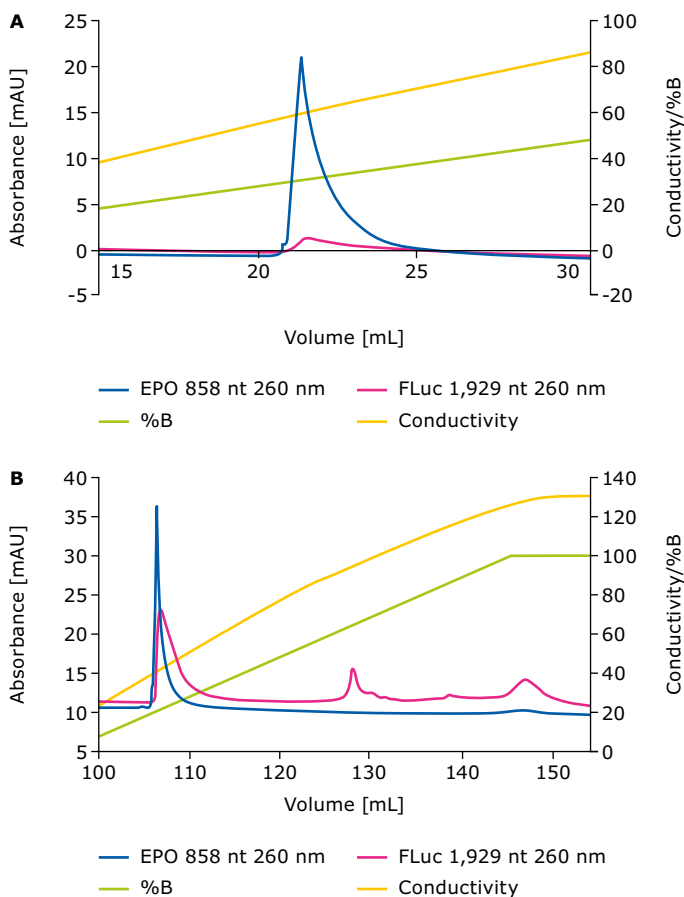


Figure 1. Comparison of mRNA EPO (blue) and mRNA FLuc (pink) chromatograms using Natrix® Q Micro 0.2 mL. **A.** Salt gradient elution at neutral pH in the absence of chaotropic salt. **B.** Dual gradient with increasing pH and salt concentration in the presence of chaotropic salt.

Easy to translate to step elution with high yield

In manufacturing, chromatography operation step elution method is typically preferred for pilot and commercial manufacturing scales. To mimic a manufacturing setup, step elution was applied and the mRNA yield was determined in the fractions. Experimental procedures are summarized in Tables 2 and 4. Figures 2 and 3 and Tables 3 and 5 illustrate the results of step elution.

For EPO, total yield was 92.6% and 95.0% for Natrix® Q and Eshmuno® Q, respectively (Table 3). For FLuc, total yield was 77.2% and 75.3% for Natrix® Q and Eshmuno® Q, respectively (Table 5). In this case, slight variations in yield were observed depending on the length of mRNA. In particularly, mRNA FLuc had a high proportion of impurities, resulting in a low yield compared to ssRNA alone of EPO. Even with this assumption, this dual step elution method was still able to maintain high yields.

Table 2.

Chromatographic procedure for bind/elute operation.

Step	Description	MV/CV
Equilibration	Buffer A1: 50 mM Tris, 150 mM NaCl, pH 8.0	20/5
Load	IVT mRNA (EPO: 858 nt) in Buffer A1 10 µg mRNA/0.2 mL Natrix® Q 10 µg mRNA/1 mL Eshmuno® Q	2.5/1
Wash 1	Buffer A1	10/5
Wash 2	Buffer A2: 50 mM Tris, 0.5 M Arginine, pH 9.0	15/5
Elution 1	40% buffer B1: 50 mM Tris, 0.5 M Arginine, 2 M NaCl, pH 11.0	30/7.5
Elution 2	100% buffer B1	30/7.5
CIP	1 M NaOH, 2 M NaCl for Natrix® Q 0.5 M NaOH for Eshmuno® Q	30/5
Re-equilibration	Buffer A1	50/15
Flowrate	For all steps: 2 mL/min = residence time 6 sec for Natrix® Q 0.335 mL/min = residence time 3 min for Eshmuno® Q	-

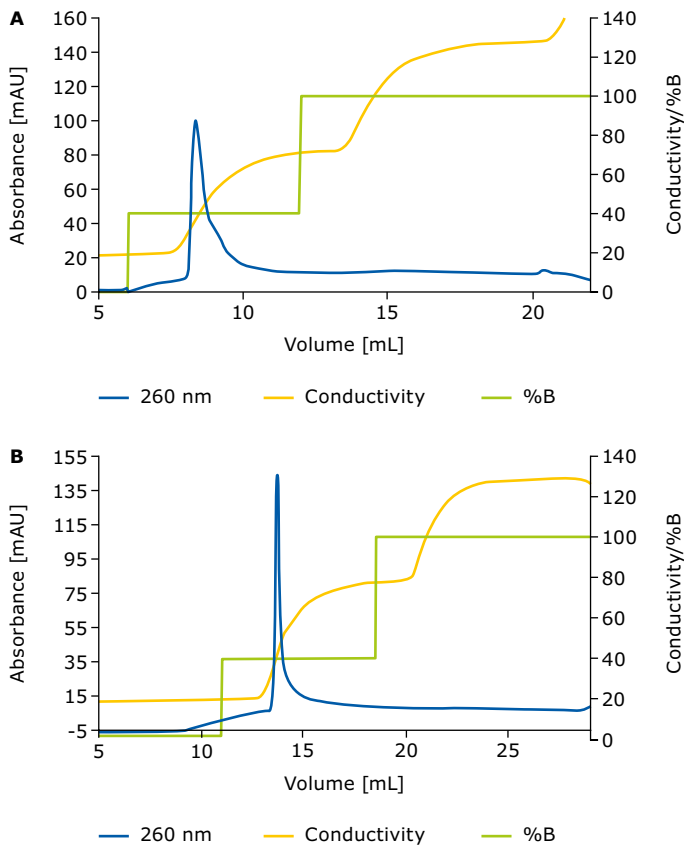


Figure 2. Chromatogram of step elution using Matrix® Q (**A**) and Eshmuno® Q (**B**) for mRNA EPO.

Table 3. Mass balance of step elution for mRNA EPO.

AEX	Fraction	Load [µg]	Eluate [µg]	Yield Pool [%]
Matrix® Q	40%B	10.8	10.0	92.6
Eshmuno® Q	40%B	12.0	11.4	95.0

Table 4. Chromatographic procedure for step elution operation.

Step	Description	MV/CV
Equilibration	Buffer A1: 50 mM Tris, 150 mM NaCl, pH 8.0	20/5
Load	IVT mRNA (FLuc: 1,929 nt) in Buffer A1 10 µg mRNA/0.2 mL Matrix® Q 10 µg mRNA/1 mL Eshmuno® Q	2.5/1
Wash 1	Buffer A1	10/5
Wash 2	Buffer A2: 50 mM Tris, 0.5 M Arginine, pH 9.0	15/5
Elution 1	40%B with Buffer B1: 50 mM Tris, 0.5 M Arginine, 2 M NaCl, pH 11.0	30/7.5
Elution 2	70%B with Buffer B1	30/7.5
Elution 3	90%B with Buffer B1	30/7.5
Elution 4	100%B with Buffer B1	30/7.5
CIP	1 M NaOH, 2 M NaCl for Matrix® Q 0.5 M NaOH for Eshmuno® Q	30/5
Re-equilibration	Buffer A1	50/15
Flowrate	For all steps: 2 mL/min = residence time 6 sec for Matrix® Q 0.335 mL/min = residence time 3 min for Eshmuno® Q	-

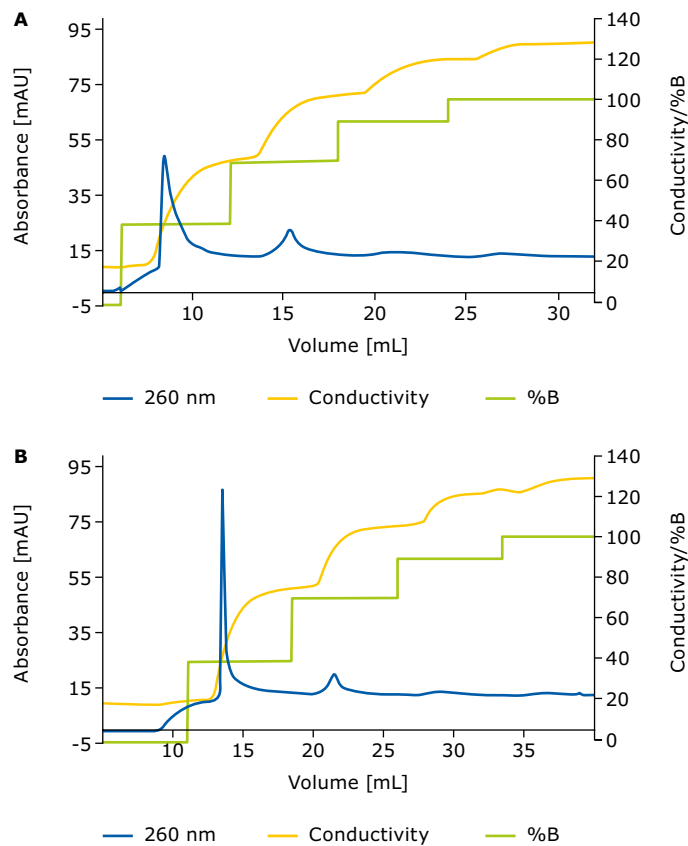


Figure 3. Chromatogram of step elution using Matrix® Q (**A**) and Eshmuno® Q (**B**) for mRNA FLuc.

Table 5.

Mass balance of step elution for mRNA FLuc.

AEX	Fraction	Load [µg]	Eluate [µg]	Yield Pool [%]	Total Yield [%]
Natrix® Q	40%B	11.5	5.52	48.0	77.2*
	70%B		2.04	17.7	
	90%B		0.84	7.3	
	100%B		0.60	4.2	
Eshmun® Q	40%B	11.95	4.3	36.0	75.3*
	70%B		2.3	19.2	
	90%B		1.8	15.1	
	100%B		0.6	5.02	

* Total yield is likely to be improved further approximately 10% higher by using appropriate buffer blank.

In order to determine impurities removal, the levels of dsRNA of load and eluate were also quantified by double-strand RNA ELISA kit (J2 based, Exalpha Biologicals, 10613002). As shown in Table 6, anion exchange chromatography demonstrated superior dsRNA removal with more than 97% compared to the load.

Table 6.

Mass balance of dsRNA.

mRNA	Fraction	Load [%]	Eluate [%]	dsRNA removal [%]
EPO	40%B	<1.0	ND	100
FLuc	40%B	4.6	0.2	97.9
	70%B		ND	-
	90%B		ND	-
	100%B		ND	-

ND: not detected

Salt gradient elution at fixed high pH

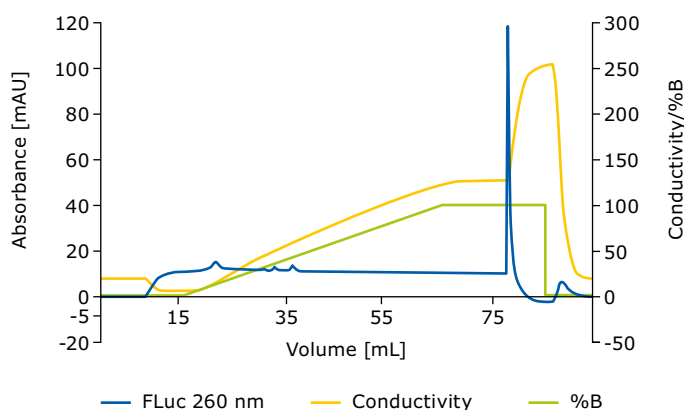
The potential of salt gradient elution method at fixed high pH was evaluated. In this trial, pH 10.5 was selected to avoid the alkaline hydrolysis of mRNA, which is degraded rapidly at the alkaline pH >11.0.³ Experimental procedures are summarized in Table 7. Figure 4 illustrates the results of chromatographic run.

In Figure 4, Natrix® Q maintains the peak separation performance whilst most of the mRNA bound does not elute from Natrix® Q by salt gradient, therefore resulting in a low yield compared to dual gradient elution. This data indicates that pH and salt concentration would work jointly to improve the mRNA elution from a strong anion exchanger.

Table 7.

Chromatographic procedure of salt gradient at pH 10.5 for mRNA FLuc.

Step	Description	MV
Equilibration	Buffer A1: 50 mM Tris, 150 mM NaCl, pH 8.0	50
Load	IVT mRNA (FLuc: 1,929 nt) in Buffer A1 10 µg mRNA/0.2 mL Natrix® Q	2.5
Wash	Buffer A2: 50 mM Tris, 0.5 M Arginine, pH 10.5	25
Elution	Buffer B1: 50 mM Tris, 0.5 M Arginine, 2 M NaCl, pH 10.5, from 0 to 100% B1	250
Elution hold	Buffer B1: 50 mM Tris, 0.5 M Arginine, 2 M NaCl, pH 10.5	50
CIP	1 M NaOH, 2 M NaCl	50
Re-equilibration	Buffer A1	50
Flowrate	For all step: 2 mL/min = residence time 6 sec	-

**Figure 4.**

Chromatogram of salt gradient elution at pH 10.5 for mRNA FLuc.

Conclusion

The critical elution parameters to improve mRNA elution recovery from a strong anion exchanger were tested. Our findings demonstrate that strong anion exchange membranes and resins with quaternary amine are effective for preparative mRNA purification at ambient temperature. We identified that optimal pH, salt concentration, and chaotropic salt concentration work synergistically to improve mRNA elution performance. Furthermore, the dual gradient elution method, which involves increasing both pH and salt concentration in the presence of chaotropic salt, resulted in effective mRNA elution with high yields and efficient separation of dsRNA from ssRNA. Our results indicate that satisfactory recoveries and purities can be achieved without the need for elevated temperatures during elution. This novel scalable method enables a wide range of larger mRNA purification during capturing and polishing step, and would be potentially applicable for the GMP manufacturing of mRNA-based therapeutics and vaccines.

References

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