

Application Note

## Plasmid Isoform Separation and Quantification by Anion-Exchange Chromatography (AEX)

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Hua Yang, Stephan M. Koza, Weibin Chen

Waters Corporation



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## Abstract

The purity and quality of plasmids play a critical role in the production of gene therapies and vaccines. Among all the isoforms of a plasmid, the supercoiled plasmid is the active form, while the open circular and linear forms are considered impurities. Therefore, it is important to monitor the levels of these undesired plasmid isoforms and quantify the percentage of supercoiled plasmid. This application note demonstrates that an ACQUITY UPLC H-Class Bio System and a Waters Protein-Pak Hi Res Q strong anion-exchange Column can be used to separate and quantify plasmid isoforms.

### Benefits

- Optimized Waters Protein-Pak Hi Res Q Column separation of plasmid DNA isoforms
- Quantification of individual plasmid isoforms in the isoform mixture

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## Introduction

Plasmids are small circular double-stranded DNAs and have been increasingly used in the manufacturing of gene therapies and vaccines in recent years. The production of plasmid DNA mostly results in the supercoiled form, which is the desired conformation due to its excellent stability and favorable antigenicity. However, other forms of plasmid, such as open circular and linear forms, are usually present due to conformational changes that occur during bioprocessing. The open circular form results when the plasmid is nicked on one strand, while the linear form is produced when the plasmid is nicked on both strands at or near the same site. These isoforms, along with others, are considered plasmid impurities, because they may affect the efficacy or the safety of the products.<sup>1,2</sup> For this reason, FDA guidelines recommend that the purity of the supercoiled form of the plasmid be greater than 80%.<sup>2,3</sup> To ensure high purity and quality, the industry expectation for the supercoiled plasmid purity level is usually greater than 90%.<sup>2,3</sup> Consequently, reliable measurement on the relative abundance of plasmid isoforms becomes critical to ensure batch process, formulation, and storage consistency.

For decades, the use of agarose gel electrophoresis has been considered as routine analytical method for separating plasmid isoforms.<sup>4,5</sup> However, this technique has drawbacks such as difficulty in quantification, low throughput, and manual-handling steps. Capillary electrophoresis (CE) has also been used for plasmid isoform

analysis,<sup>4</sup> but it struggles to analyze samples that are in the complex matrix such as cell lysate. Anion-exchange chromatography (AEX) separates analytes based on their differences in the amount and localization of negative surface charges. It is well accepted that AEX is a robust, reproducible, easily automated analytical technique, requiring small amounts of samples. Since plasmid DNAs are negatively charged due to the phosphate groups on the backbone, AEX is a natural choice for plasmid-related separation and analysis.<sup>4,5</sup>

In this application note, we show that various levels of the three major isoforms of the plasmid DNA, namely, supercoiled, open circular, and linear form, can be separated and quantified on a Waters Protein-Pak Hi Res Q Column fitted to an ACQUITY UPLC H-Class Bio System.

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## Experimental

### Sample Description

ΦX174 RFI (supercoiled form, N3021L), ΦX174 RFII (open circular form, N3022L) and XhoI restriction enzyme (R0146S) were purchased from New England BioLabs. The linear form of the ΦX174 plasmid was obtained by digesting ΦX174 RFI with XhoI. XhoI was later removed from the digestion mixture using a Monarch PCR & DNA cleanup kit (T1030S) from New England BioLabs.

### LC Conditions

System:	ACQUITY UPLC H-Class Bio
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell, wavelength: 260 nm
Vials:	Polypropylene 12 x 32 mm Screw Neck, with Cap and Pre-slit PTFE/Silicone Septum, 300 µL volume, 100/pk (p/n: 186002639)
Column(s):	Protein-Pak Hi Res Q, 5 µm, 4.6 x 100 mm (p/n: 186004931)
Column temp.:	30 °C

Sample temp.:	10 °C
Injection volume:	0.6–10 µL
Flow rate:	0.4 mL/min
Mobile phase A:	100 mM Tris-HCl
Mobile phase B:	100 mM Tris base
Mobile phase C:	3 M Tetramethylammonium chloride (TMAC)
Mobile phase D:	Water
Buffer conc. to deliver:	20 mM

#### Gradient Table (AutoBlend Plus Method)

Time (min)	Flow (mL/min)	pH	Salt (mM)	Salt curve
0	0.4	7.4	0	
1	0.4	7.4	0	11
2	0.4	7.4	1690	6
4	0.4	7.4	1690	6
14	0.4	7.4	1750	6
16	0.4	7.4	2400	6
16.1	0.4	7.4	0	11
32	0	7.4	0	11

In the above gradient table, the buffer is 20 mM Tris pH 7.4. The initial salt concentration is set to 0 mM to ensure all the analytes are strongly bound onto the column. Then the salt concentration is increased rapidly

to 1690 mM and equilibrated for 2 min before the separation gradient starts. The salt concentration increases linearly to 1750 mM in 10 min for the isoform separation. Then it is ramped up to 2400 mM in 2 min to strip off any remaining bound molecules. Finally, an equilibration step to the initial condition takes place, preparing for the next injection.

An equivalent gradient table for a generic quaternary LC system is shown below:

Time (min)	%A	%B	%C	%D
0	17.8	2.2	0.0	80.0
1	17.8	2.2	0.0	80.0
2	17.8	2.2	56.3	23.7
4	17.8	2.2	56.3	23.7
14	17.8	2.2	58.3	21.7
16	17.8	2.2	80.0	0.0
16.1	17.8	2.2	0.0	80.0
32	17.8	2.2	0.0	80.0

## Data Management

Chromatography software:

Empower 3 FR 4

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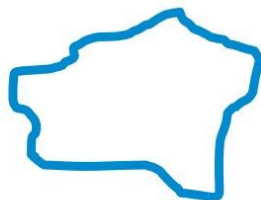
## Results and Discussion

As shown in Figure 1, plasmid isoforms display differences in shape and conformation. AEX exploits interaction between the positively charged stationary phase and the negatively charged phosphate groups on the backbone of the plasmid DNA. Although the overall molecular weight and charges are similar for

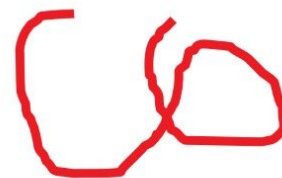
different isoforms, their local charge densities are different due to their conformations. As a result, these isoforms can be separated on an AEX column.



Supercoiled (SC)



Open Circular (OC)



Linear (L)

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*Figure 1. An illustration of major isoforms of plasmids.*

Figure 2 shows a mixture of three isoforms of  $\Phi$ X174 plasmid (~5.4 kbps) separated on a Waters Protein-Pak Hi Res Q strong anion exchange Column. The isoforms are identified by matching the retention time with that of individually injected isoforms. Since supercoiled DNA has higher charge density than the more relaxed open circular and linear form, it has stronger interaction with the stationary phase, resulting in later elution than the other two forms.

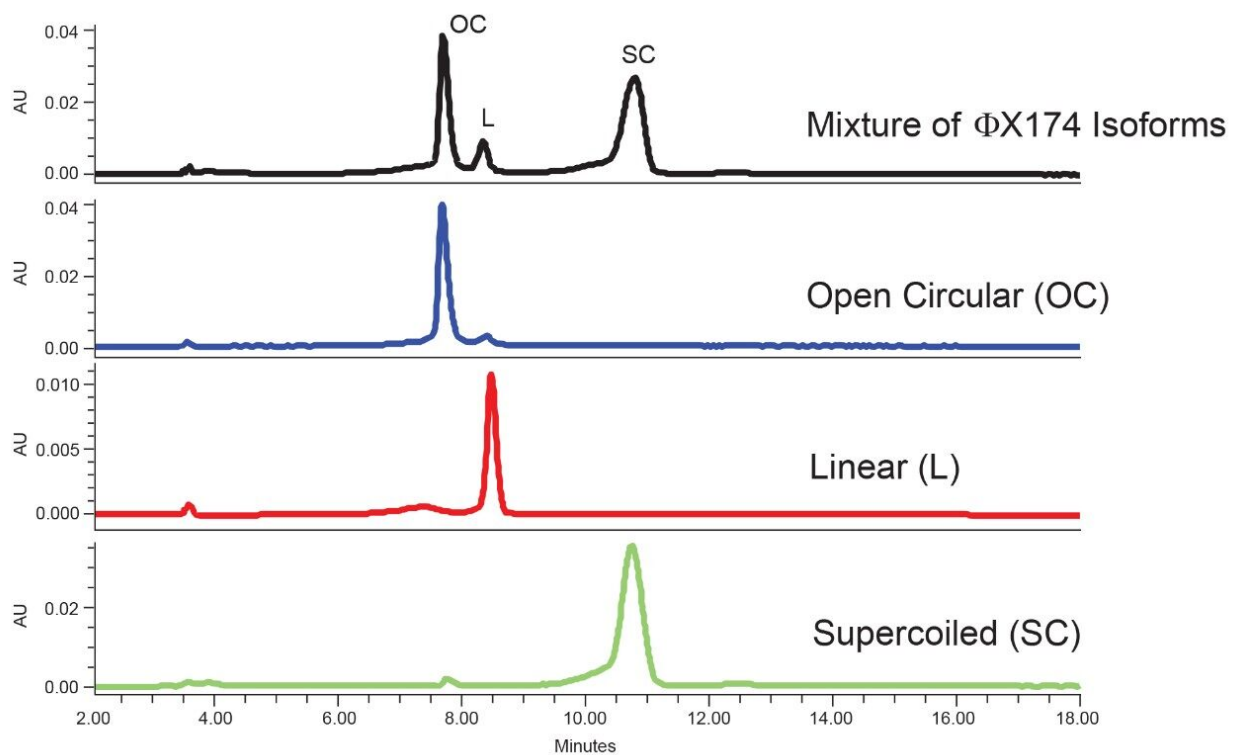


Figure 2.  $\Phi$ X174 plasmid isoform separation on a Waters Protein-Pak Hi Res Q Column. 20 mM Tris pH 7.4, 1.69–1.75 M tetramethylammonium chloride in 10 min. Flow rate: 0.4 mL/min.

The optimization of the AEX separation was carried out by changing the pH and the column temperature (Figure 3). When the pH of the mobile phases is increased, the resolution between the open circular form and the linear form decreases slightly, as does the resolution between the linear form and the supercoiled form. Therefore, lower pH value (pH 7.4) was chosen. When the column temperature is increased, the resolution between the open circular form and the linear form decreases, while the resolution between the linear form and the supercoiled form increases. Since the linear form and the supercoiled form are well separated at all the temperatures tested, the lower column temperature (30 °C) was chosen to ensure good resolution between the open circular form and the linear form.

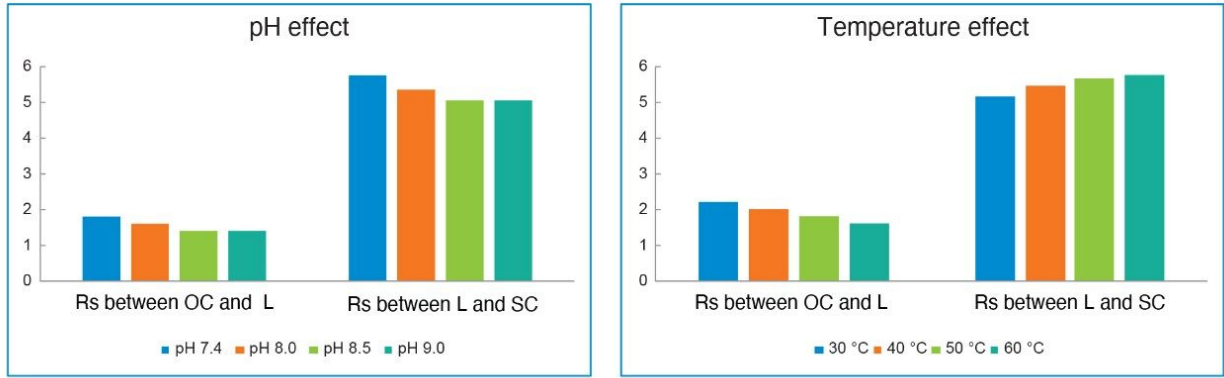
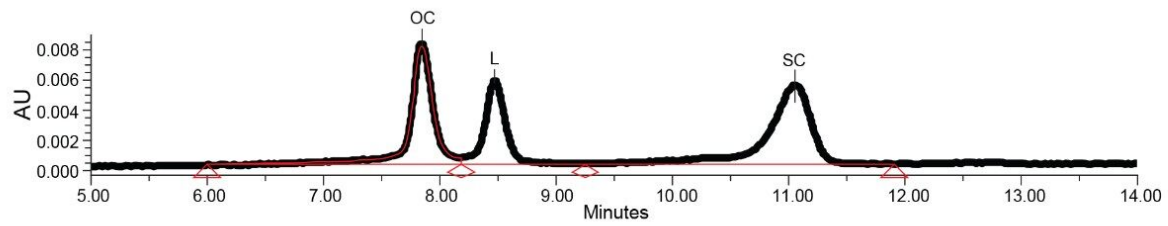


Figure 3. Effect of pH and temperature on the  $\Phi X174$  plasmid isoform separation.

Isoform mixtures were diluted serially and separated on a Waters Protein-Pak Hi Res Q Column (Figure 4A) to evaluate the quantitation linearity of the method. The total mass load on column ranges from 59 ng to 1875 ng. No difference in selectivity is observed upon varying mass load. Calibration curves were constructed by plotting the peak area of the individual isoform against the total amount of the isoform mixture (Figure 4A, insert). Excellent linearity is obtained for all three isoforms. Figure 4B shows the percentage of the peak area of each isoform. Consistent results are achieved down to approximately 117 ng total mass load, and no specific loss of any of the isoforms is observed. Overall, the results show that relative quantification of the plasmid isoforms can be achieved using a Waters Protein-Pak Hi Res Q Column.



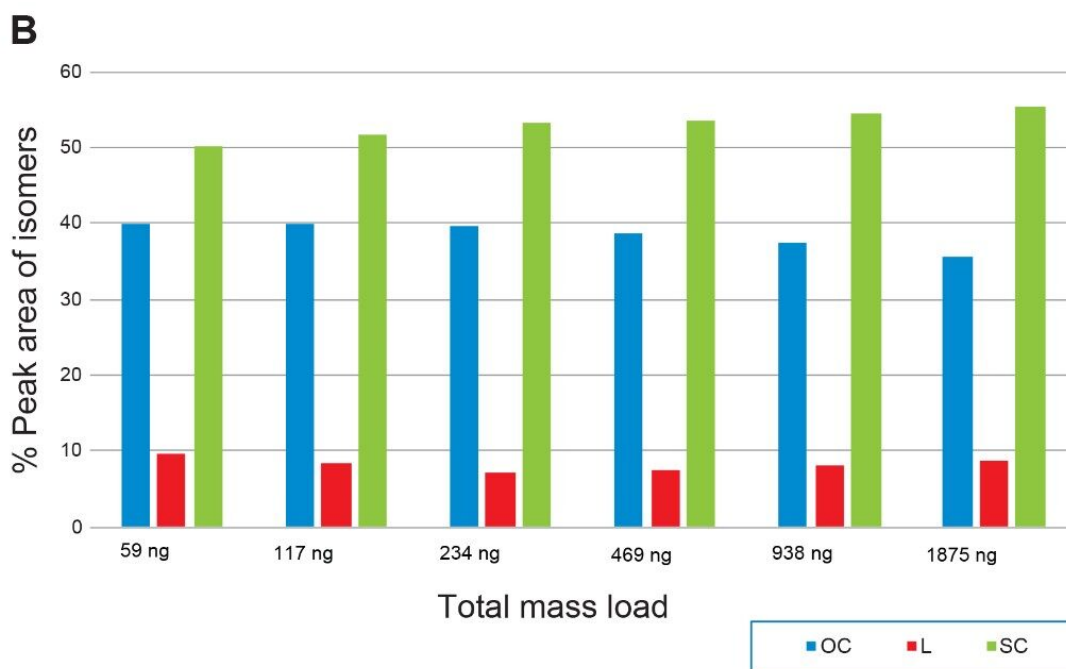
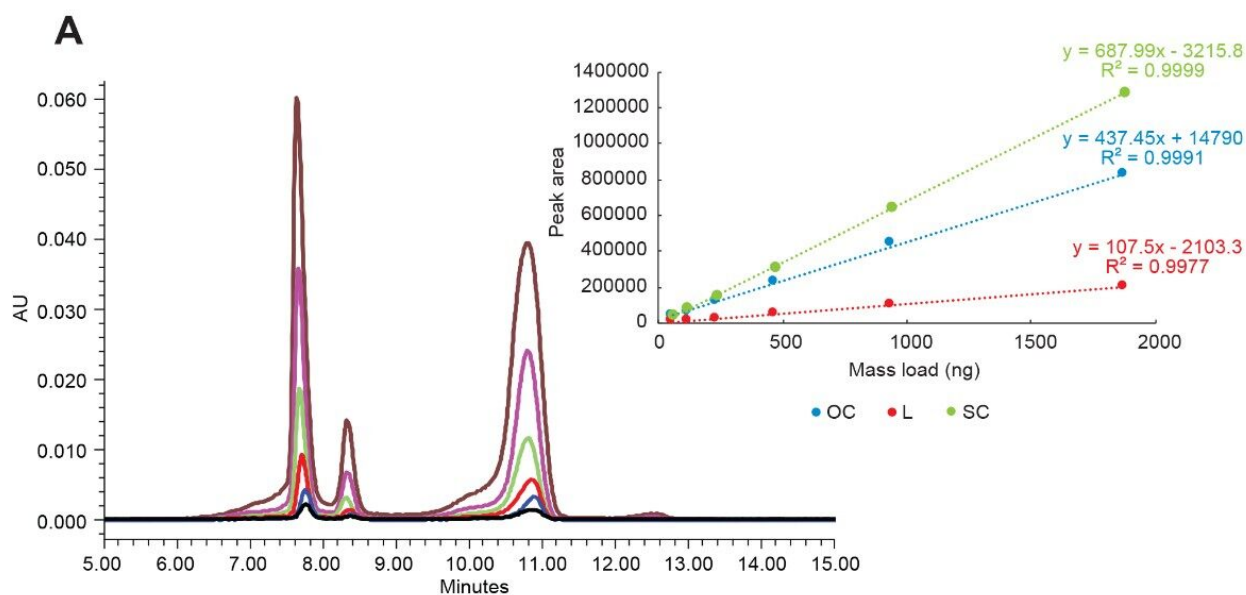


Figure 4A. Various amount of  $\Phi$ X174 plasmid isoform mixtures were loaded and separated on the Protein-Pak Hi Res Q Column. Total mass load ranged from 59 ng to 1875 ng. Insert: Peak area of the individual isoform is plotted against the total amount of the isoform mixture. Effective linearity is obtained for all three isoforms. A peak is observed after the supercoiled plasmid, and it's presumably to be the dimer.

Figure 4B. Measured relative abundances of plasmid isoforms in a mixture as a function of total mass load on column.

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## Conclusion

Anion-exchange chromatography has advantages of consuming small amounts of sample and being able to yield robust and reproducible results using a standard LC instrumentation. While this technique has been widely used for plasmid purification/preparation,<sup>1,2</sup> it has great potentials, as an analytical technique, in separating and quantifying plasmid isoforms, and provides a simple means for plasmid purity testing. Indeed, while not specifically investigated in this work, the substantial binding strength of plasmid DNA to the quaternary ammonium ligands of the column used may allow for plasmid analysis in low purity lysate without an additional sample clean-up step or the use of DNA specific dyes. Here, we report an analytical method which separates supercoiled, open circular, and linear form of a 5.4 kbp plasmid, using a Waters Protein-Pak Hi Res Q strong anion exchange Column. The relative quantification of the three primary plasmid forms are consistent for total sample loads ranging from approximately 117 ng to 1875 ng.

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## References

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