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Note d'application

Slalom-Aided Anion Exchange Chromatography for Enhanced Analysis of Plasmid DNA Topological Impurities

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Abstract

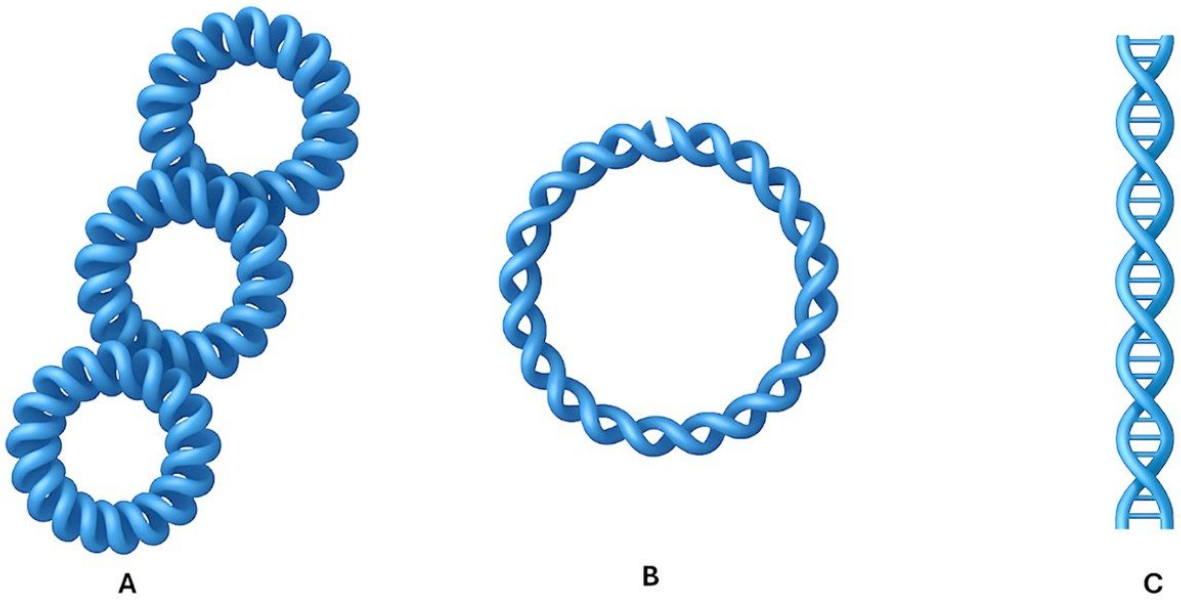

Figure 1. Structural representations of plasmid DNA isoforms: (A) supercoiled, (B) open circular, and (C) linear.

Accurate characterization of nucleic acid therapeutics is essential to the development and quality control of gene therapy products and biologics based on recombinant expression. Plasmids, viral genomes, and linear DNA fragments often exist in multiple structural forms (topological conformations), including supercoiled, open circular, and linear configurations, which differ in biological activity and regulatory relevance. While anion exchange chromatography (AEX) is widely used for nucleic acid purification, it often lacks the ability to consistently resolve structural isomers across a broad size range. Recent evidence has revealed flow-dependent retention behavior for large nucleic acids that cannot be explained by charge-based electrostatic interactions alone.

In this application note, the observation of slalom chromatography effects during anion exchange separations with a Protein-Pak™ Hi Res Q Column is described. Through manipulating these effects, it is possible to optimize both a conventional ion exchange (IEX) retention mechanism plus a slalom-based hydrodynamic mechanism

that enhances selectivity for large, conformationally unique DNA species with differing elasticities. Using a single salt gradient method, the structural isoforms of pBR322 [4.4 kbp], ΦX174 virion DNA [5.4 kbp], and a Cas9 plasmid [7.0 kbp] have been successfully resolved. In addition to the conventional salt gradient applied in AEX, these results demonstrate that the flow rate can be independently tuned to fully resolve the three topological isomers, irrespective of their size, and that the alteration of selectivity in the example separations is not necessarily due to changes in the gradient steepness of the IEX mechanism. Ultimately, these AEX plus slalom chromatography insights will enable chromatographers to deliver increasingly robust nucleic acid analysis tuned as appropriately as possible for a specific separation selectivity.

Benefits

- Baseline resolution of DNA isoforms achieves complete separation of supercoiled, open circular, and linear forms of pBR322, ΦX174 RFI/RFII, and Cas9 plasmid
- Dual mode retention mechanism demonstrates separation driven by both anion exchange and flow-dependent slalom chromatography mechanisms
- Single method offers broad applicability using a single gradient method on the Protein-Pak Hi Res Q Column to analyze diverse nucleic acid structures with flow rate optimization to achieve desired selectivity through slalom effects

Introduction

The rapid growth of cell and gene therapies, particularly plasmid-based therapeutics, viral vector formulations, and genome-editing platforms, has driven an increasing demand for analytical methods that can robustly separate and characterize complex nucleic acid mixtures. Within these mixtures, plasmids and other related drug substances commonly exist as multiple topological isoforms, specifically supercoiled (SC), open circular (OC), and linear (L) forms. Among these, the SC form is typically the desired conformation due to its superior transfection efficiency, while OC and L forms are considered process-related impurities. These structural variants significantly impact therapeutic efficacy, transfection efficiency, cell culture yield in recombinant expression, and overall product and drug substance quality, thereby making their accurate identification, separation, and quantitation critically important.[1]

AEX has become a preferred method for separating nucleic acids due to its high resolution, scalability, and

compatibility with aqueous-based biological systems.[2] Conventional AEX methods have been developed to resolve structurally similar isoforms, especially larger nucleic acids greater than 5 kbp in length.[3–6] In particular, the separation of the open circular plasmid DNA and the linear double-stranded DNA (dsDNA) isoform is not systematically observed on an AEX column when changing the size of those topological isomers. Independently, slalom chromatography has recently been reintroduced as a method for separating large nucleic acids based on their rigidity and elongation. Its separation mechanism is driven by shear forces under high flow conditions and the weak entropic elasticity of double-stranded DNA. Plasmid DNA, which are constrained by their geometry, will not extend to the same degree as linear DNA when subjected to mild shearing forces. As a result, the higher the flow rate (and pressure across the column), the higher the selectivity between the plasmid DNA and the linear DNA.[7–9] However, slalom chromatography will generally struggle to separate OC and SC isomers as they share similar persistence lengths.

In the present study, for the first time, the synergy between traditional AEX and slalom retention mechanisms using a Protein-Pak Hi Res Q Column has been systematically investigated. More specifically, two experimental variables have been purposely manipulated to optimize the separation of 3 unique plasmids. The salt gradients (for AEX selectivity) and shear force-inducing flow rates were adjusted to optimize the slalom-based selectivity of the separations. The dual-mode AEX–slalom separation was evaluated and optimized for three biologically relevant DNA constructs: ΦX174 RF I/II (5.4 kbp), pBR322 (4.4 kbp), and a 7.0 kbp Cas9-encoding plasmid. Using defined mobile phase conditions consisting of Tris buffer with 5% urea and a precisely controlled sodium chloride (NaCl) gradient, flow-rate-dependent resolution of OC, L, and SC topological forms was observed. The resulting data elucidates key operational parameters that can be leveraged to significantly enhance resolution, method reproducibility, and scalability, ultimately achieving a powerful analytical approach tailored for emerging gene therapy applications.

Experimental

Three nucleic acid samples were used to evaluate the performance of anion exchange separations and the influence of slalom effects for resolving plasmid topological isoforms:

- pBR322 (New England Biolabs), a circular plasmid DNA (4361 bp), was combined with its linearized form to obtain a mixture of OC, L, and SC species
- ΦX174 RF I / RF II (New England Biolabs) was prepared as an equimolar mixture of the supercoiled (RF I) and

open circular (RF II) forms of the ΦX174 replicative form DNA (5386 bp)

- pCMV-Cas9 Plasmid (Sigma-Aldrich, CAS9P-1EA), an expression vector containing a CMV promoter and kanamycin resistance marker, was evaluated in its OC, L, and SC forms (7037 bp)

Analyte Composition				
Component	**Description**	**Topologies**	**Length**	**Product Identifiers**
pBR322 combined with Linearized	Plasmid DNA from E. coli [Double-stranded]	OC, L, SC	4361 bp	pBR322 [N3033L]
ΦX174 RF I / RF II	Equimolar mixture of RF I (supercoiled) and RF II (open circular) [Double-stranded]	OC, L, SC	5386 bp	PhiX174 RF I DNA [N3021L] PhiX174 RF II DNA [N3022L]
pCMV-Cas9	Expression plasmid with CMV promoter, KanR [Double-stranded]	OC, L, SC	7037 bp	pCMV-Cas9 [CAS9P-1EA]

LC Conditions

Note on Flow Cell Selection: A 5 mm titanium flow cell (ACQUITY™ PDA, Waters™ p/n: 205000613 < https://www.waters.com/nextgen/global/shop/service-parts--kits/205000613-flowcell-acquity-pda-5-mm-titanium.html>) was used to minimize metal–analyte interactions during analysis. Prior experiences with SEC-UV demonstrated that titanium's chemical inertness reduces the adsorption of nucleic acids and phosphate species, supporting consistent peak shape, signal stability, and reproducible performance, critical for accurately profiling diverse DNA constructs, including plasmids and viral genomes.[10]

LC system:	ACQUITY™ Premier System with: – Binary Solvent Manager (BSM, p/n: 186018000) – Flow-Through Needle Sample Manager (SM-FTN, p/n: 186018002) – Column Manager-A (CM-A, p/n: 186018003)
Detector:	ACQUITY™ Premier eLambda PDA (p/n: 186018007) with 5 mm Titanium Flow Cell (p/n: 205000613)
Columns:	Protein-Pak Hi Res Q Column, 5 µm, 4.6 mm x 100 mm (p/n: 186004931)
Mobile phase A:	20 mM Tris, 5% (w/v) urea, pH 9.0, 0.1 µm filtered
Mobile phase B:	20 mM Tris, 5% (w/v) urea, 1.0 M NaCl, pH 9.0, 0.1 µm filtered
Vials:	TruView™ pH Control LCMS Certified Clear Glass Vials, 12 x 32 mm, Screw Neck (Waters, p/n: 186005663CV)
Column temperature:	35 °C
Sample temperature:	5 ± 3 °C
Flow rates:	0.1, 0.3, 0.5, and 0.8 mL/min
Seal wash:	10% HPLC-grade Methanol / 90% 18.2 MΩ*cm resistivity (Milli-Q®) water (v/v)
Samples & injection volume:	pBR322 w/ Linearized - 10 µL

	ΦX174 RF I / RF II - 1 µL pCMV-Cas9 – 10 µL
Gradient:	Refer to the tables below
System control & data acquisition:	Empower™ 3.9.x (tested with 3.9.0)
Detection wavelength:	260 nm
Sampling rate:	20 points/sec

Gradient Table - 0.1 mL/min

Gradient Table - 0.1 mL/min			
Time (min)	**Flow (mL/min)**	**%A**	**%B**
Initial	0.1	40	60
75.0	0.1	5	95
75.5	0.1	0	100
100.0	0.1	0	100
100.5	0.1	10	90
125.0	0.1	40	60
160.0	0.1	40	60

Gradient Table - 0.3 mL/min

Gradient Table - 0.3 mL/min			
Time (min)	**Flow (mL/min)**	**%A**	**%B**
Initial	0.3	40	60
25.00	0.3	5	95
25.17	0.3	0	100
33.33	0.3	0	100
33.50	0.3	10	90
41.67	0.3	40	60
53.33	0.3	40	60

Gradient Table - 0.5 mL/min

Gradient Table - 0.5 mL/min			
Time (min)	**Flow (mL/min)**	**%A**	**%B**
Initial	0.5	40	60
15.0	0.5	5	95
15.1	0.5	0	100
20.0	0.5	0	100
20.1	0.5	10	90
25.0	0.5	40	60
32.0	0.5	40	60

Gradient Table - 0.8 mL/min

Gradient Table - 0.8 mL/min			
Time (min)	**Flow (mL/min)**	**%A**	**%B**
Initial	0.8	40	60
9.38	0.8	5	95
9.44	0.8	0	100
12.50	0.8	0	100
12.56	0.8	10	90
15.63	0.8	40	60
20.00	0.8	40	60

Disclaimer

The Protein-Pak Hi Res Q Column has a recommended maximum pressure of 2175 psi. At 0.8 mL/min, the column pressure exceeded this limit (2800–3100 psi). While acceptable separation was achieved, routine use under these conditions may reduce column lifespan. This column is not specifically designed to maximize slalom effects.

Results and Discussion

To evaluate the performance of AEX chromatography where slalom effects might be at play, three structurally

distinct DNA samples with different sizes were selected: pBR322 (4361 bp), an equimolar mixture of ΦX174 RF I and RF II (5386 bp), and pCMV-Cas9 (7037 bp). Each analyte contained OC, L, and SC forms. All separations were performed using a Protein-Pak Hi Res Q Column, employing 20 mM Tris (pH 9.0), 5% (w/v) urea, and a 7.5 mL linear NaCl gradient (0.6–1.0 M). Flow rate was varied to assess the impact of shear-dependent retention on resolution.

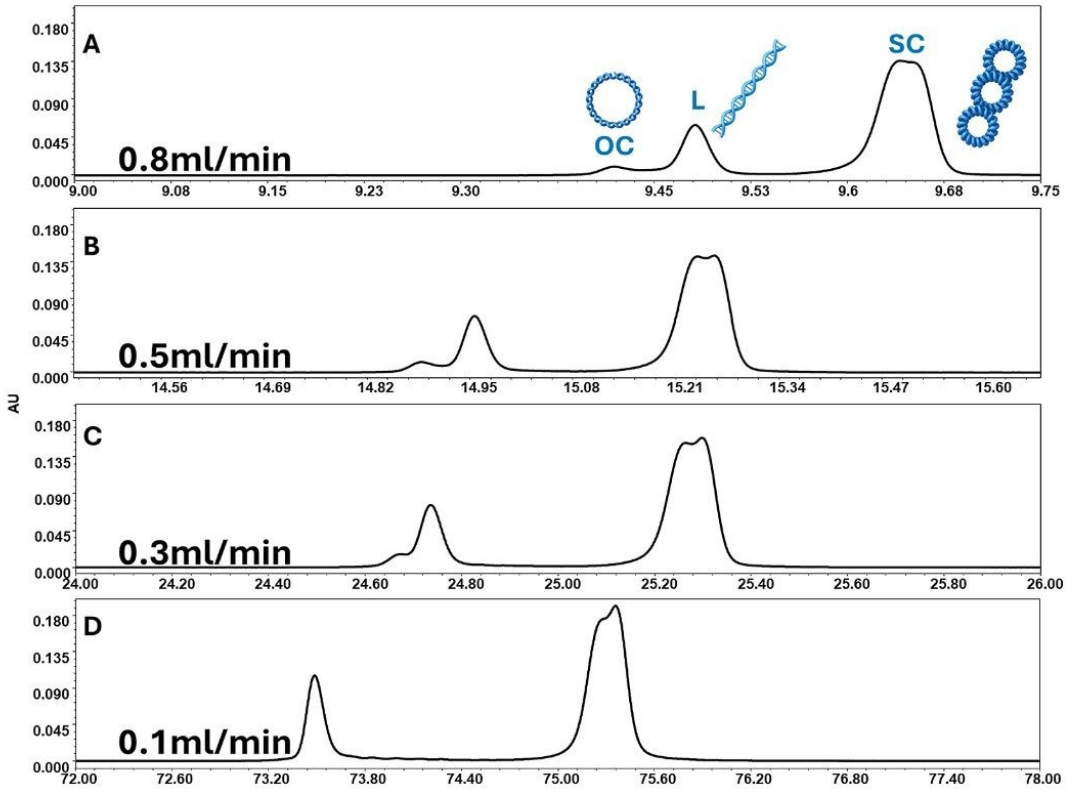

Figure 2. Volume-normalized (the separation window is fixed at 0.6 mL) stacked chromatograms show flow rate-dependent separation of plasmid pBR322 isoforms [4361 bp] (supercoiled [SC], open circular [OC], and linear [L]).

This illustrates the separation of pBR322 isoforms. At 0.1 mL/min, OC and L forms co-elute (D). As the flow rate increased to 0.3 and 0.5 mL/min, resolution between all three isoforms improved (B,C). At 0.8 mL/min, SC, L, and OC peaks were baseline-resolved (A).

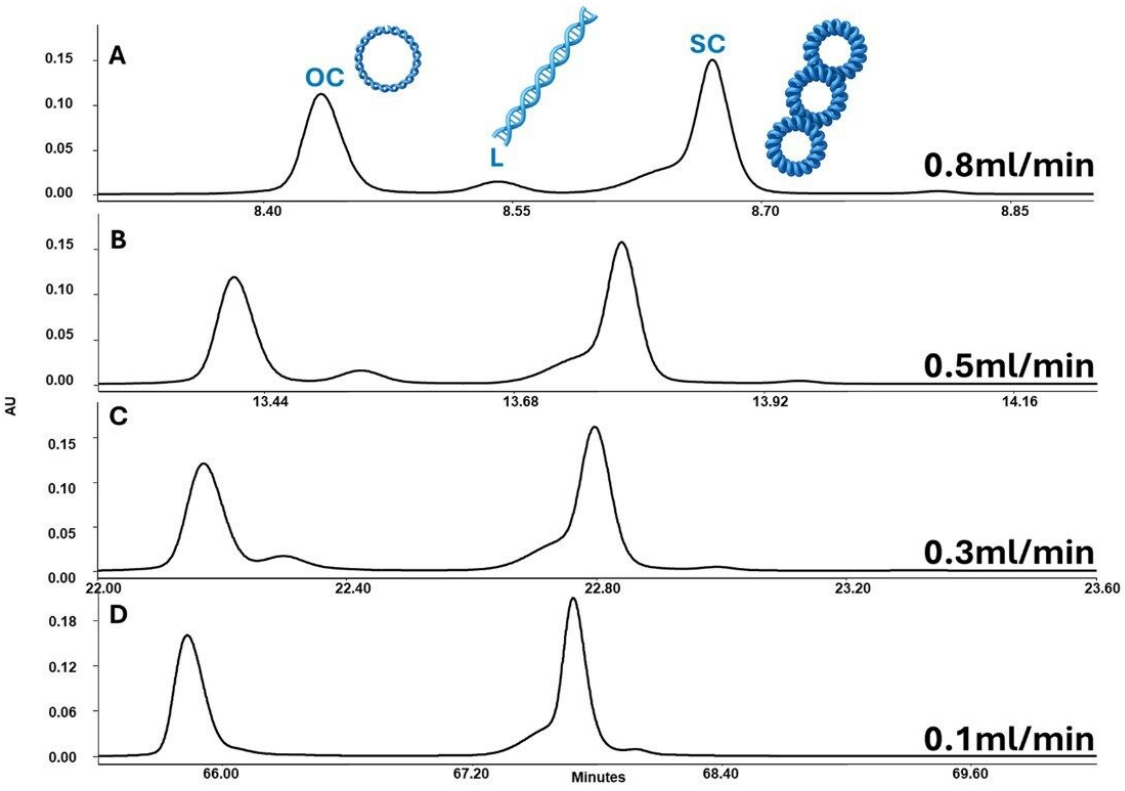

Figure 3. Volume-normalized (the separation window is fixed at 0.48 mL) stacked chromatograms show flow rate-dependent (A-D) separation of ΦX174 RF I (supercoiled, SC), RF II (open circular, OC) [5386 bp], and linear (L) DNA forms using AEX–slalom chromatography.

This presents results for the ΦX174 RF I / RF II mixture, where SC and OC forms are present without a significant linear component. As with pBR322, resolution improved with increasing flow rate. The optimal separation was achieved at 0.8 mL/min (A), yielding well-resolved and symmetric SC and OC peaks. These results demonstrate the platform's ability to resolve topological isomers of small circular DNA with minimal optimization and confirm the generalizability of the flow rate–dependent slalom mechanism across different analytes.

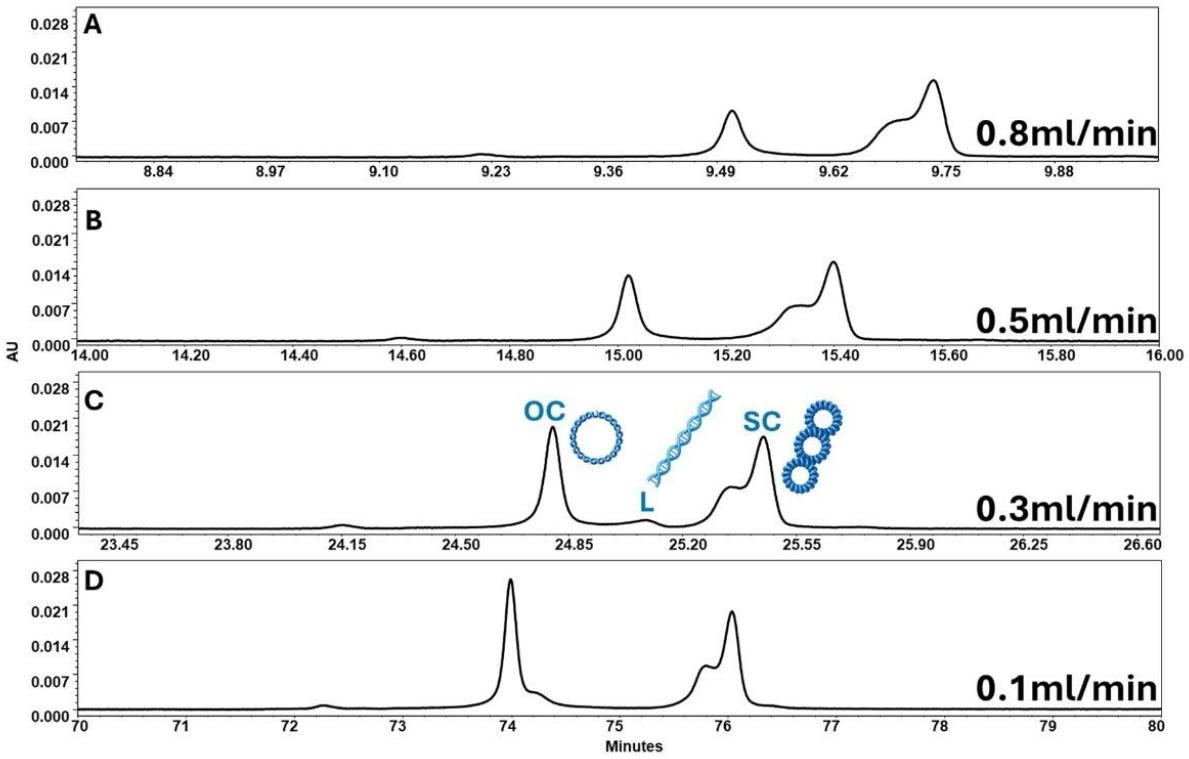

Figure 4. Volume-normalized (the separation window is fixed at 1 mL) stacked chromatograms show flow rate-dependent separation of Cas9 plasmid [7037 bp] isoforms using AEX–slalom chromatography. Baseline resolution of open circular (OC), linear (L), and supercoiled (SC) isoforms was achieved at 0.3 mL/min (C). At 0.5 and 0.8 mL/min, the linear form co-eluted with the SC peak and was not distinguishable (A,B). At 0.1 mL/min, L co-eluted with OC (D).

The three separation examples above demonstrate that combining a conventional salt gradient with an appropriate flow rate enables systematic resolution of the three DNA isoforms, regardless of their size. This is facilitated by the fact that the gradient retention volume of the linear isoform increases more steeply with flow rate compared to the open-circular and supercoiled isoforms. These results demonstrate that intermediate flow rates maximize topological resolution for large plasmids by balancing slalom-induced selectivity and AEX-induced selectivity.

Conclusion

This application note demonstrates the utility of Protein-Pak Hi Res Q Column and strong anion-exchange chromatography for resolving the three plasmid DNA isoforms, supercoiled (SC), open circular (OC), and linear (L), across a wide range of DNA sizes, including pBR322 (4.361 kbp), ΦX174 RF I/II (5.386 kbp), and the pCMV-Cas9 (7.037 kbp) plasmid. Using a mobile phase system containing 5% urea and a NaCl gradient, the method achieved excellent resolution of all topological forms by tuning the flow rate applied throughout the gradient method. Notably, this strategy offers a streamlined alternative to traditional method development approaches that require manual changes in mobile phase additives or compositions. Indeed, flow rate was found to influence resolution significantly due to the difference in entropic elasticity of DNA plasmid (open-circular, supercoiled) and linear isoforms. For example, optimum flow rates were found to be equal to 0.8, 0.5, and 0.3 mL/min for the separation of pBR322, ΦX174 RFI RFII Mixture, and pCMV-Cas9 plasmids, respectively. Higher flow rates are needed to increase the retention of shorter linear DNA isoforms relative to the open circular plasmid. These results highlight the method's adaptability and precision for analyzing plasmid topology, providing analysts with enhanced control critical for assessing identity, integrity, and purity in plasmid-based therapeutics and gene therapy workflows.

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