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应用纪要

A New Alternative to Gel Electrophoresis: Higher Resolution and Faster Analysis of Large Nucleic Acids by Rigorously Designed GTxResolve™ 250 Å Slalom Columns

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Abstract

This application note discusses the benefits of slalom chromatography (SC) for the analysis of large nucleic acids. Slalom chromatography is a size-based separation technique that offers high-resolution discrimination of nucleic acid species exceeding 3 kilobase pair (kbp) in length by leveraging differences in their molecular stretching and relaxation dynamics. These new columns employ high-purity, hydrophilic 2.5 µm particles packed in MaxPeak™ Premier Column hardware to minimize unwanted non-specific interactions during separations. The particles are tailored specifically to facilitate the separation of nucleic acid sample components through flow retardation due to the semi-rigid nature of large double-stranded DNA (dsDNA) under flow rate-related shear force conditions. The molecules

exhibit entropic extension under the chromatographic shear forces (~10k psi), and differences in relaxation time from stretched to random coil conformations facilitate the separation of nucleic acid components varying in structural elasticity and rigidity. These columns exhibit excellent inertness, high resolution, recovery, and reproducibility at both batch-to-batch and column-to-column levels using standard mobile phases. Different than a size exclusion column, this slalom chromatography column is manufactured for reliable, repeat use under ultra performance liquid chromatography (UPLC™) (8 to 12k psi) pressure conditions. As such, slalom chromatography provides an intriguing new alternative to agarose gel electrophoresis or capillary gel electrophoresis to analyze a restriction map of DNA involving large dsDNA species in <6 minutes with enhanced resolution. The ability of slalom chromatography to analyze large nucleic acids such as plasmids, DNA digests or restriction maps, messenger RNA (mRNA), and double-stranded RNA (dsRNA) impurities should enable quicker development of efficacious and more globally accessible cell and gene therapy (CGT) medicines.

Benefits

- Fast, high-resolution separations of mega-size nucleic acids in <6 minutes
- Excellent reproducibility across multiple columns and packing material batches
- Excellent analyte recovery with minimal or no nonspecific adsorption
- High-throughput and analytical precision with beyond gel-like separation capabilities
- Robust chromatography with the ability to run more than 400 injections

Introduction

The increasing complexity of genetic medicines requires continuous advances in analytical techniques. Unlike traditional drugs involving small molecules (<1000 Da), genetic medicines use DNA and RNA to achieve tailored medical interventions. For example, the instructions provided by mRNA can be used to modify the cellular gene expression either by correcting a disease-causing genetic condition through protein replacement or by introducing a novel protein into the cell.[1] Despite the promise and performance of mRNA drugs, the analytical strategies to maintain quality control are not fully developed.[2] The mRNA synthesis involves the identification of a gene sequence, DNA synthesis, restriction digests, and purification of the gene product to clone into a suitable plasmid for subsequent

amplification and generation of an appropriate template for *in vitro* transcription (IVT). These activities involve several analytical steps, such as agarose gel or capillary gel electrophoresis. Although these are traditional methods for evaluating DNA quality, resolving large nucleic acids can require up to and beyond 1 hour long runs. In addition, it can become difficult to optimize a method and appropriately select the best gel media.

Slalom chromatography is an analytical technique that provides separation selectivity to analyze large nucleic acids using pressure versus electrophoretic driven effects. This chromatographic approach provides an attractive alternative to the time-consuming use of conventional agarose gel electrophoresis.[3–4] The elution pattern of slalom chromatography follows a pattern of small to larger size, which is opposite to what is observed by size exclusion chromatography (SEC). The largest nucleic acid components in a slalom separation will experience flow retardation. Specifically, a large dsDNA species will undergo entropic extension, a non-equilibrium state, under the shear stress or shear force exerted by the mobile phase. Conversely, the entropic elasticity of linear dsDNA compels the polymer to revert to the equilibrium configuration, referred to as entropic elasticity stress. Ratio of external shear force/stress to entropic elasticity stress is related to Weissenberg number, which is a product of relaxation time (τR) and fluid shear rate ($\dot{\gamma}$) . When Wi>10, the DNA polymer stays in non-equilibrium extended configuration, and at Wi<10, the DNA remains in equilibrium random configuration.[5]

$$\frac{\textit{Shear force}}{\textit{Entropic elasticity force}} \approx \tau R \dot{\gamma} = Wi$$

As such, the dsDNA will exhibit a so-called persistence length of ~500 Å or greater and act as a semi-rigid polymer. Its long relaxation time to transition from a stretched form to a random coil conformation creates a delay in elution. Resolution and selectivity are maximized when the average extension length of the double stranded analyte of interest is about half of the particle size. The mobile phase shear stress depends on the flow rate, solvent viscosity, and temperature. With the advent of ultra-high pressure liquid chromatography (UHPLC), low-adsorption systems, column hardware, highly pure hydrophilic packing materials, and specialty-packed column beds, it has become possible to explore

and implement slalom chromatography with simple, fully aqueous buffered mobile phases.[6]

In this application note, the reproducible high-resolution separation of large DNA species is documented using GTxResolve 250 Å Slalom Columns. A packing material with a 250 Å average pore diameter is applied in this new technology. Although the large nucleic acids of a slalom LC sample do not access the intraparticle pore volume of this packing material, there is a chance to apply SEC mode separations to other smaller analytes if they have hydrodynamic radii ranging from 20 to 400 Å. This detail aside, slalom chromatography with the new column is straightforward to apply. Large DNA molecules (>3 kbp) can be resolved with reproducibly higher recovery across multiple columns and batch materials. This type of separation capability provides the opportunity to generate a restriction map of large DNA, such as bacteriophage or plasmid DNA in under 6 minutes, thereby making it a well-suited high-throughput analysis method.

Experimental

LC Conditions

1X TAE buffer:	40 mM Tris-acetate, 1 mM EDTA pH 8.3 made from 10x TAE (Thermo Fisher Scientific p/n: 15558042)
Column storage:	10% acetonitrile, 90% aqueous 25 mM sodium phosphate pH 7.0 + 100 mM potassium chloride
Seal and weak wash:	20% methanol: 80% water
Sample:	Lyophilized dsDNA 23K Ladder (p/n: 186011286). Resuspend in 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0)

Spermidine:	1M solution was prepared by dissolving spermidine (Sigma-Aldrich #124-20-9) in 18.2 M Ω*cm water
LC system:	ACQUITY™ UPLC I-Class Bio System (or equivalent) consisting of QSM H-Class (Quaternary Solvent Manager with 50 µL mixer), TUV Detector - 1000 psi MAX Pressure 70 bars Max Pressure 500 nL, Sample Manager FTN (Ver. 2023 03 02), 15 µL needle size (p/n: 700012820) Assy, Needle, FTN-15 µL, HPS, TXT w/Gd&st), 100 µL sample syringe size, Injection valve flow through (p/n: 700011791), External Pre-Heater, CH-30A, with Active Preheater (APH) MP35N, 0.004” ID, 18.5’’ (p/n: 430005558) and post column tubing to TUV: 0.004’’ ID 22.5’’ LG MP35N Welded Tube (p/n: 430002856), Assy, Pre-HRT to Column, Reusable MP35N (p/n: 700011809)
Column:	Waters™ GTxResolve 250 Å Slalom Column, MaxPeak Premier, 2.5 µm, 4.6 x 300 mm and dsDNA 23K Ladder (p/n: 176006046)
Mobile phase A:	1X TAE buffer, pH 8.3
Column equilibration:	Column conditioning is done by ramping the flow rate by 0.1 mL increments to reach the flow rate of 1.4 mL/min over 20-min and

	continued for another 40-min.
Vials:	QuanRecovery™ with MaxPeak HPS 12 x 32 mm Screw Neck Vial, 300 µL, 100/pk (p/n: 186009186 (vial) p/n: 186005827 (caps))
Column temperature:	40☒°C
Sample temperature:	5☒±☒3☒°C
Sample amount:	1 µL
Gradient:	Isocratic
Sampling rate:	40 *HZ*

Results and Discussion

The GTxResolve 250 Å Slalom Column has been designed to quickly separate large DNA and RNA based on their stretched and non-equilibrium hydrodynamic size under shear flow conditions in a UHPLC system (6-7). The column uses a holistic level of inertness from the particle to the frit while maintaining batch-to-batch and column-to-column performance. The GTxResolve Slalom Column hardware also includes a hydrophilic high performance surface (HPS) on its frits and column tube. In addition, the Slalom Column contains particles having an average nominal 2.5 µm particle diameter and nominal pore size of 250 Å as well as a proprietary hydroxy terminated polyethylene oxide (PEO) silane bonding phase. The large analytes of interest will be fully excluded from the pores, while the smaller impurities which can diffuse into the pores will be eluted later than the target analytes by a mixed mode size exclusion/slalom chromatography separation. There are potential advantages to having this extra separation selectivity available for method development. Future work will demonstrate a pertinent example.

The slalom separation maximum is always achieved when the average extended lengths of linear dsDNAs are equal to a critical length, which is approximately half the particle diameter ($L_{ext} = 0.5*d_p$).[5–6] Most advantageously, the relaxation times of linear dsDNAs achieved under shear flow conditions are two orders of magnitude shorter than those expected in the absence of flow. This enables the detection of the longest linear dsDNAs up to 25 kbp without irremediable loss in column performance. Figure 1 illustrates a comparison of the analyte movements under a low flow rate 0.3 mL/min (back pressure of 2,000 psi) where SEC conditions predominate vs. high flow rate @1.4 mL/min (back pressure of 9880 psi). Note how the shear force elongates the nucleic acid.

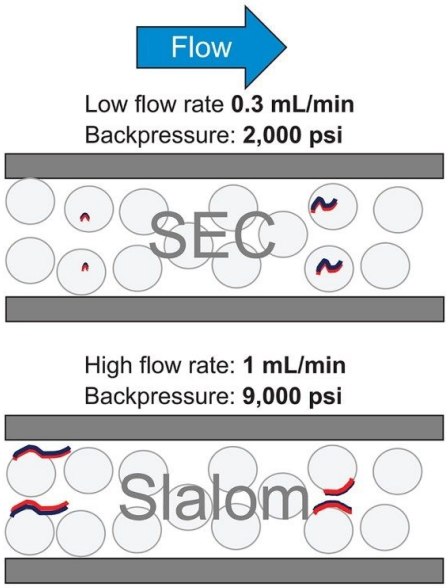

Figure 1. Slalom chromatography conditions achieved by column back pressure induced shear forces. The top panel shows the separation and early elution of larger molecules at low flow rate during SEC, whereas the bottom panel depicts the retardation of large molecules due to the back pressure induced shear forces of slalom chromatography.

Improved Resolution of Larger dsDNA Species by Slalom Chromatography

To test the applicability of slalom chromatography to large nucleic acids, the separations of a 23K

dsDNA ladder comprised of 2, 2.3, 4.3, 6.5, 9.4 and 23 kbp dsDNA species using 1X TAE buffer were investigated. Sample components were monitored by their absorbance at 260 nm. The elution profile of the dsDNA components observed on GTxResolve 250 Å Slalom Column (4.6 x 300 mm) was converted into a simulated slalom chromatography separation model based on the resolution of component species. This format of the slalom chromatogram was then compared to actual results obtained with an agarose gel (Figure 2, Table 1). While the dsDNA species with <4 kbp are well resolved on an agarose gel, the resolution of larger species (>4kbp) was superior on a GTxResolve 250 Å Slalom Column, wherein a 4.5X improvement in resolution between 9.4 and 23 kbp species was observed. This strongly supports the utility of slalom chromatography for analysis of larger dsDNA >3 kbp in size.

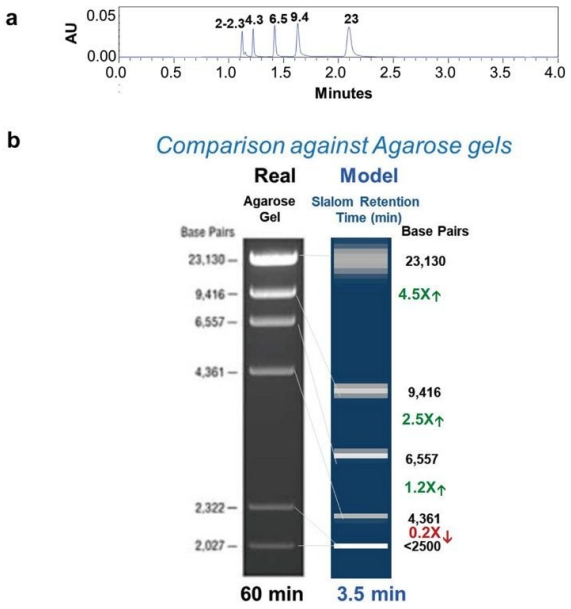

Figure 2. a) Slalom chromatography-based separation of dsDNA species in the 23K ladder. b) Comparison of agarose gel electrophoresis based separation against the corresponding model generated from the observed resolution for various dsDNA species. Resolution differences are marked in green and red.

dsDNA (kbp)	Retention time (min)	USP resolution (HH)	Resolution difference vs agarose gel
2–2.3	1.111		
4.3	1.216	3.85	0.2X
6.5	1.416	6.79	1.2X
9.4	1.624	5.5	2.5X
23	2.105	7.13	4.5X

Table 1. Slalom chromatography-based separation of dsDNA components of 23K ladder.

Column-to-Column and Batch-to-Batch Reproducibility of GTxResolve 250 Å Slalom Columns

Robust analytical separations require consistent performance across multiple columns and batches. To assess the column-to-column and batch-to-batch reproducibility of slalom separations, 23 kbp dsDNA ladder components were evaluated using five different Waters GTxResolve 250 Å Slalom Columns packed from a single batch of packing material as well as the columns packed with three unique batches of packing material. Retention times (RT), column pressure and USP resolution values (HH) were evaluated across columns for the 4.3, 6.5, and 9.4 kbp species. These Waters slalom columns displayed exceptional reproducibility as demonstrated by very low percent relative standard deviations (%RSD) in retention times and resolution metrics (Table 2).

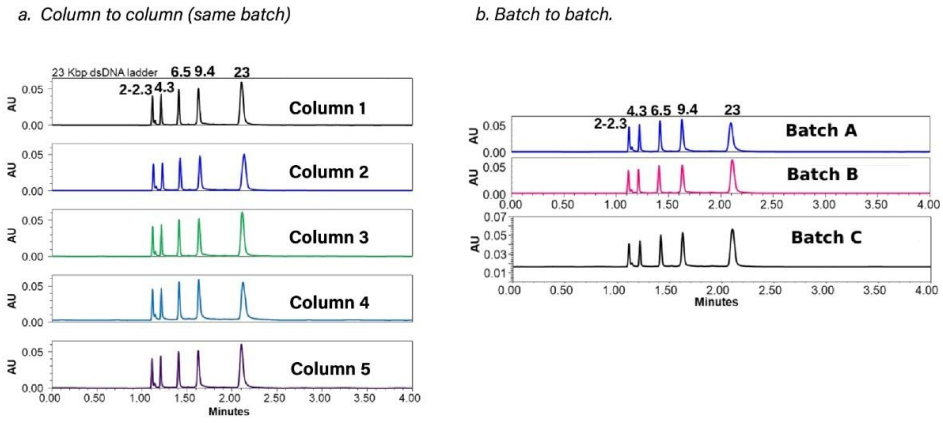

Figure 3. Column-to-column and batch-to-batch reproducible behavior of GTxResolve 250 Å Slalom Columns. The components of 23K dsDNA Ladder were evaluated for chromatography peak quality metrics and the reproducibility of their elution times.

		5 columns same batch		3 batches	
Ds DNA (Kbp)	Attribute	Column-to-column		Batch-to-batch	
		Average	% RSD	Average	% RSD
4.3	Retention time	1.21 min	**0.55**	1.22	**0.39**
6.5	Retention time	1.41 min	**0.52**	1.41	**0.39**
	USP R_s (HH)	7.25	**4.77**	7.37	**5.04**
9.4	Retention time	1.63 min	**0.45**	1.63	**0.16**
	USP R_s (HH)	5.95	**4.01**	5.88	**5.95**
NA	Back pressure	10099.7 psi	**1.0**	10475	**4.32**

Table 2. Retention time and resolution metrics across multiple columns and batches.

High Recovery of dsDNA Components Across Multiple Columns and Batches

Recovery of sample components from a chromatographic column is critical to any analysis. Therefore, the total recovery of optical signal at A260 nm in the column elution was analyzed and compared to that of no column/union injection. These investigations revealed that 91-95% of the optical signal observed for no column/union injection was recovered from each of the investigated GTxResolve 250 Å Slalom Columns indicating minimal on-column losses due to non-specific adsorption. High recovery of sample components supports the utility of the slalom column for impurity analysis such as those in plasmid linearization studies or DNA impurity identification IVT reactions.

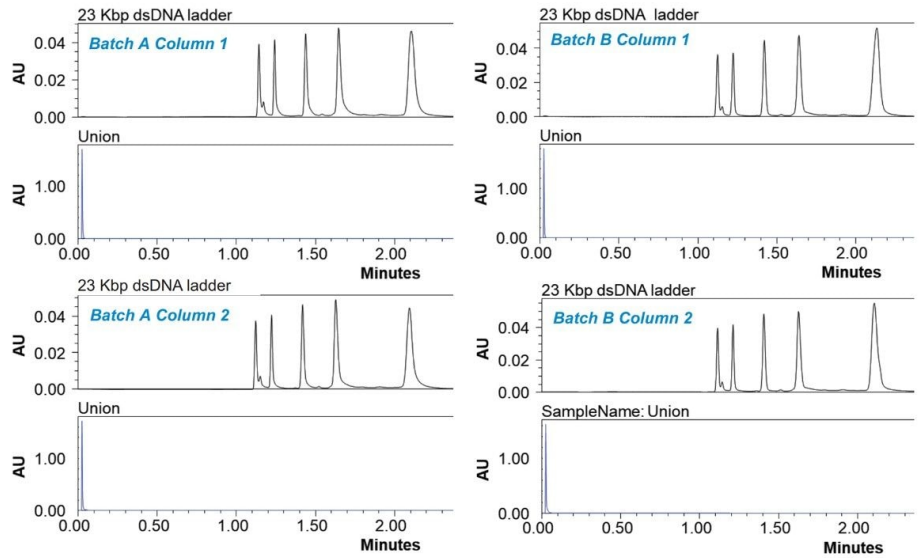

Figure 4. Determining the recovery of dsDNA components across multiple batches and columns.

Robust Lifetime

Since slalom chromatography operates at high backpressure, it was of interest to test the effects of making hundreds of injections on a Waters GTxResolve 250 Å Slalom Column. A study was performed to monitor 400 consecutive injections of the 23 kbp dsDNA ladder. In order to reduce performance variability, a 10 µL injection of 1M spermidine was incorporated in every 10^{th} injection, with conditions involving gradual lowering of the flow rate (1.4 mL/min to 0.5 mL/min) followed by conditioning for 20 minutes with flow rates ramping again from 0.5mL/min to 1.4 mL/min. This was found to be an effective procedure for extra trace amounts of nucleic acid components that could accumulate over the course of repeat injections. It is recommended that new slalom chromatography users also consider this routine cleaning procedure along with the use of a system suitability standard like the 23kbp DNA ladder.

Figure 5 shows the overlay of the chromatographic profiles of all 400 injections of 23 kbp ladder from 3 different vials. All dsDNA peaks aligned well, and there was no broadening or emergence of spurious peaks over the course of lifetime study. Key metrics such as elution time, total peak area, and the overall

peak profile remained unchanged from the first injection through the 400th. Negligible variations in peak intensities were attributed to the small differences in dsDNA component amounts in the three different vials— not to the variations in chromatographic performance. Overall, the variability in performance was minimal, with the RSD values for resolution on example peak pairs 4.3 & 6.5 kbp and 6.5 & 9.4 kbp found to be < 1% over 400 injections. Similarly, the change in resolution was <1.5% between the first and 400th injections. Further, the column did not exhibit any significant change in back pressure after 400 injections as the difference between the first and 400th injections was -0.8% (Table 3).

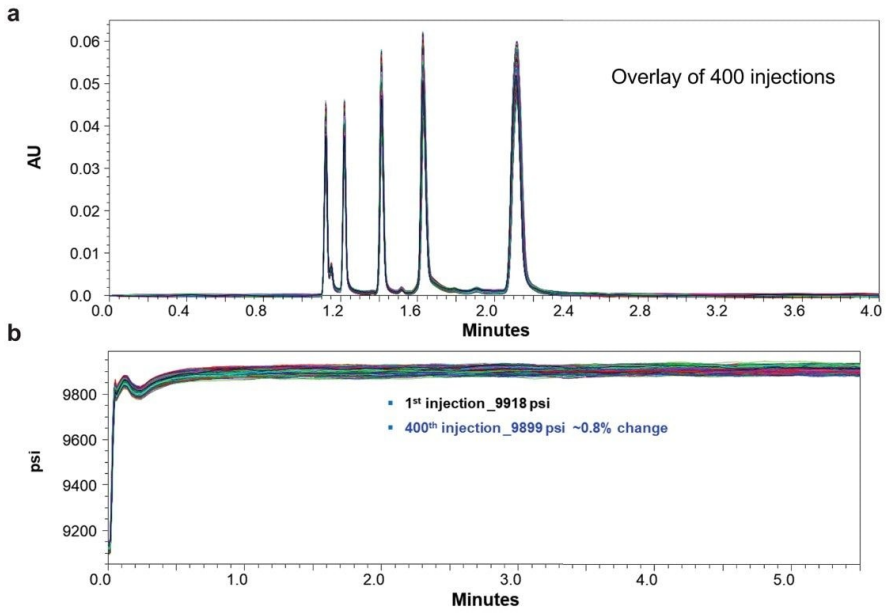

Figure 5. Robust lifetime of GTxResolve 250 Å Slalom Columns a) Overlay of 23K DNA ladder traces from 400 injections. b) Column back pressure traces. Minimal change between 1st and 400th injection.

USP HH resolution peak pair	400 inj		% RSD
4.3 / 6.5 Kbp	6.20		0.38
6.5 / 9.4 Kbp	5.46		0.35
Peak pair	**1st**	**400th**	**% change**
4.3 / 6.5 Kbp	6.09	6.18	1.47
6.5 / 9.4 Kbp	5.38	5.46	1.49
Backpressure	9918 psi	9899 psi	-0.8%

Table 3. Robust column in use lifetime as seen with a 400 injection test.

Conclusion

This application note demonstrates the utility of Waters GTxResolve 250 Å Slalom Columns for high-resolution, reproducible analyses of large nucleic acids relevant to CGT workflows and more. Using a 23 kbp ladder with component dsDNA lengths varying from 2-2.3 kbp to 23kbp, it was confirmed that these GTxResolve Slalom Columns facilitate separations with high resolution, minimal secondary interactions, and remarkable reproducibility across multiple columns and batches. Specific benefits resulting from the design of the GTxResolve 250 Å Slalom Columns include:

- A 10-fold reduction in analysis time of (<6 minutes) compared to 60 minutes or more required for agarose gel or capillary gel electrophoresis
- Improved resolution for nucleic acids larger than 3 kbp compared to agarose gels
- Accurate quantification of sample components with high precision and the capability to collect sample components for downstream analysis
- Excellent robustness with low variability in the analytical data even after 400 injections

Waters GTxResolve 250 Å Slalom Columns make slalom chromatography easy to implement. More importantly, they can be used to expedite important nucleic acid analyses, such as generating restriction maps of recombinant plasmids or measuring nucleic acid purity during the development and manufacturing of new drug substances.

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