Going big, small, and low: semi-prep columns with small particles for polynucleotide applications

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Abstract

Purpose: Demonstrate high resolution separation of polynucleotide molecules on a 21.2x150mm column packed with a 4µm supermacroporous, divinylbenzene particles.

Methods: Ion-Pairing Reverse Phase (RP) separation methods using water/acetonitrile mobile phases with TEAA

Results: High resolution separation of DNA compounds across a wide molecular weight range using reduced flow rates on a conventional (U)HPLC system.

Introduction

Purification and recovery of sample components for functional use and/or further evaluation by orthogonal analytical methods is a common technique in pharma and biopharma industries. Analytical columns provide high resolution separations of components but are limited by the ability to load sufficient sample quantities for recovery and downstream processing of target analytes. Large diameter (e.g., ≥10mm) semi-prep and prep chromatography provides higher levels of sample loading but can suffer from poorer resolution compared to analytical methods due to the use of larger particles to minimize backpressure. Here we compare the separation of DNA on a 2.1x50mm and 21.2x150mm columns packed with DNAPac RP 4µm media. The small particles provide high resolution separations while the supermacroporous structure of the particles enables separation across a wide range of molecular weights[1]. The use of low flow rates enables use of a conventional (U)HPLC system and the elution of samples at higher concentrations relative to high flow rate methods.

Materials and methods

Sample preparation

All samples were used as-received. Any required dilution of samples was done using 18.2 MΩ deionized water.

Test method(s)

Reverse Phase liquid chromatography separations of dsDNA samples were compared for a 2.1x50mm or 2.1x100mm Thermo Scientific™ DNAPac™ RP analytical column (PNs 088924 and 088923, respectively) and a 21.2x150mm DNAPac RP Semi-Prep column (PN 080922-1521232). For all separations Mobile Phase A was 100 mM TEAA and mobile phase B was 75% Mobile Phase A + 25% Acetonitrile unless denoted otherwise. All separations were performed at ambient temperature and UV detection at 260nm. A conventional (U)HPLC chromatography system with a binary pump, autosampler and detector without column compartment was used for all separations with differences in analytical and semi-prep setup denoted in Table 1.

Table 1. Components for analytical and semi-prep setup

Part	Analytical setup	Semi- prep setup
Pump static mixer	150 µL	350 µL
AS sample loop	25 µL	1000 µL
Detector cell	Semi-Micro Bio Flow Cell	Analytical Flow cell
Viper tubing	0.1x350mm MP35N	0.5mm ID MP35N

Data Analysis

Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System (CDS) was used for data acquisition and analysis

Results

Sample separation range on analytical and semi-Prep Column

The 2.1x100mm analytical column and 21.2x150mm semi-prep column were found to have the same separation range for different BP lengths of dsDNA as shown in Figure 1. The analytical column achieved near baseline resolution in ~14 minutes for each analyte. The gradient was scaled volumetrically for the semi-prep column and evaluated used a long gradient at 4 mL/min and a short gradient at 8 mL/min with each semi-prep separation showing baseline resolution of analyte. A larger semi-prep flow cell was used at 8 mL/min to minimize total system pressure to 155 bar also resulting in lower signal strength.

Figure 1. Comparison of dsDNA Separation ranging from 35 – 10,000 BP on 2.1x100mm and 21.2x150mm columns. Sample: 0.03 mg/mL for each analyte.

Format: 2.1x50mm (Top)
Gradient:

Time (min)	%A	%B
0.0	70	30
15.0	30	70
15.1	0	100
18.1	0	100
18.2	70	30
24.0	70	30

Flow rate: 0.20 mL/min
Inj. volume: 1 µL

Format: 21.2x150mm (Middle)
Gradient:

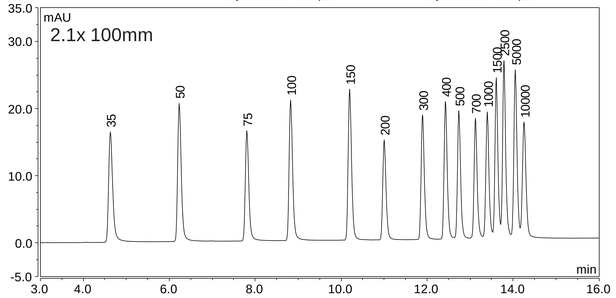
Time (min)	%A	%B
0.0	70	30
13.12	70	30
125.62	30	70
126.37	0	100
148.33	0	100
149.62	70	30
178.12	70	30

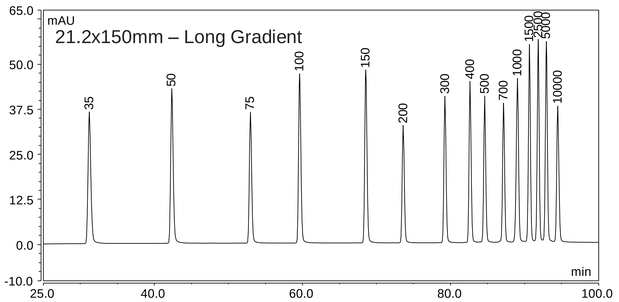
Flow rate: 4.0 mL/min
Inj. volume: 153 µL

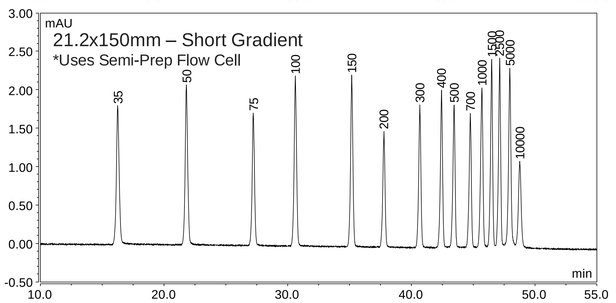
Format: 21.2x150mm (Bottom)
Gradient:

Time (min)	%A	%B
0.0	70	30
6.56	70	30
62.81	30	70
63.19	0	100
74.16	0	100
74.81	70	30
89.00	70	30

Flow rate: 8.0 mL/min
Inj. volume: 153 µL



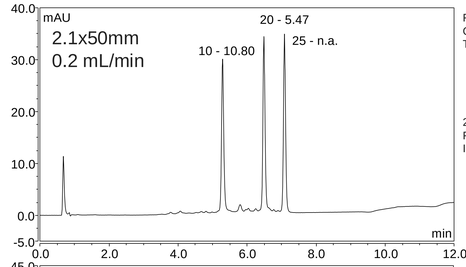




High resolution analysis of short (10-15 BP) dsDNA

Purification and sample recovery is the primary application for semi-prep and prep columns. Samples commonly have multiple associate variants and/or impurities from their manufacture process. High resolution separations are thus required to ensure collected sample is of sufficient purity for downstream processing and evaluation. Figure 2 shows the scaling of an analytical method to a semi-prep method with the volumetric gradient kept constant and scaled for column bed volume. Despite using a reduced linear flow rate (0.08 mL/min for 2.1mm ID → 8.15 mL/min for 21.2mm ID), the 21.2x150mm column shows higher resolution for each peak compared to the 2.1x50mm column, which may be partially attributable to column length. The bottom chromatogram shows an enlarged view of the 21.2x150mm separation. From this data, the 21.2x150mm column provides high resolution separations with the ability to separate minor variants for recovery of highly pure sample.

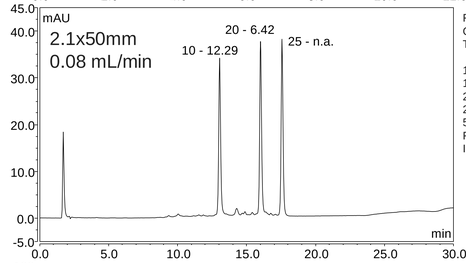
Figure 2. Comparison of dsDNA Separation 10, 20, and 25 BP on 2.1x50mm and 21.2x150mm columns with scaled volumetric gradients. Sample: 0.10 mg/mL for each analyte. Peak label is shown as BP length – resolution (EP).



Format: 2.1x50mm
Gradient:

Time (min)	%A	%B
0.0	80	20
7.5	40	60
7.6	10	90
9.6	10	90
9.7	80	20
20.0	80	20

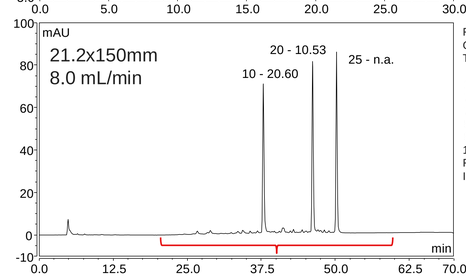
Flow rate: 0.2 mL/min
Inj. volume: 1 µL



Format: 2.1x50mm
Gradient:

Time (min)	%A	%B
0.0	80	20
18.75	40	60
19.00	10	90
26.50	10	90
26.75	80	20
55.00	80	20

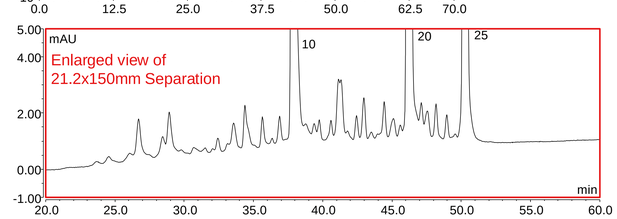
Flow rate: 0.08 mL/min
Inj. volume: 1 µL



Format: 21.2x150mm
Gradient:

Time (min)	%A	%B
0.0	80	20
14.83	80	20
71.08	40	60
71.20	10	90
81.20	10	90
81.30	80	20
105.00	80	20

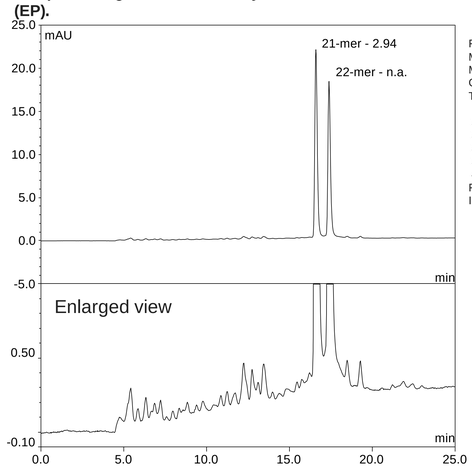
Flow rate: 8.0 mL/min
Inj. volume: 306 µL



High resolution analysis of 21-mer and 22-mer ssDNA

The resolution of the 21.2x150mm column is highlighted in figure 3 showing the separation of two ssDNA samples differing in length by a single nucleotide. Baseline separation is easily achieved for the peaks with a resolution of 2.94. The enlarged view shows the detection of additional minor variants for each of the analytes. The resolving power of the column enables removal of these variants especially for shorter oligonucleotides with baseline resolution being achieved.

Figure 3. 21-mer and 22-mer ssDNA separation on a 21.2x150mm column. Sample: 20 mg/mL for each analyte. Peak label is shown as BP length – resolution (EP).



Format: 21.2x150mm
Mobile Phase A: 100 mM TEAA
Mobile Phase B: Acetonitrile
Gradient:

Time (min)	%A	%B
0.0	94	6
30.0	90	10
30.1	75	25
35.0	75	25
35.1	94	6
48.0	94	6

Flow rate: 8.0 mL/min
Inj. volume: 50 µL

For each of the examples given, the gradients can be further refined to shorten the analysis time and/or focus on separation and recovery of an analyte with a specific length or molecular weight. Optimized gradients with column coupling to a fraction collector can be used for subsequent down stream evaluation by an orthogonal chromatography method and/or using a functionality assay.

Conclusions

- A 21.2x150mm DNAPac RP column provides equivalent separation range to standard analytical DNAPac RP formats enabling sample analysis over a wide molecular weight range
- High resolution variant/impurity separation is observed for ssDNA and dsDNA with single nucleotide difference baseline resolution for ~20-mer analytes
- High resolution separation is achieved a reduced linear flow rates relative to the analytical column enabling separation on a standard analytical (U)HPLC at ambient temperature.

References

Ma, K. et al., (June 2023). *A versatile reversed phase platform for short, intermediate, and long nucleic acid analysis*, HPLC 2023, Duesseldorf, Germany.

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