DNAPac RP Column

A versatile reversed phase platform for short, intermediate, and long nucleic acid analysis

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Abstract

Purpose: To demonstrate efficient Reverse Phase separation of nucleic acids varying in length from 10 to 10,000 nucleotides using a 4.0 µm polymeric, supermacroporous (SMP) resin with divinylbenzene chemistry.

Methods: A Thermo Scientific™ Vanquish™ Horizon UHPLC system is used for analyzing nucleic acids samples.

Results: Excellent separation of nucleic acids samples from 10 to 10,000 nucleotides using a Thermo Scientific™ DNAPac™ RP column compared to conventional C18 silica columns.

Introduction

Several important classes of nucleic acid-derived therapeutics have recently emerged. The lengths and structures of these therapeutics varies depending on their mechanism of action. Small interfering RNA (siRNA) is a class of double-stranded RNA molecules, 20–25 nucleotides in length, able to block gene expression by degrading target mRNA via the RNA interference (RNAi) pathway. The CRISPR/Cas9 genome editing system utilizes the Cas9 endonuclease to cleave DNA as directed by a guide ribonucleic acid (gRNA, ~100 nucleotides) that recognizes the target nucleic acid sequence. Recently two highly effective COVID-19 vaccines based on the mRNA sequence encoding a modified version of the SARS-CoV-2 spike protein (4,000 nucleotides) were developed and approved by the FDA.

The most common approach of manufacturing synthetic siRNA or gRNA is through solid phase synthesis based on phosphoramidite chemistry followed by chromatographic purification. While mRNA is manufactured via an enzymatic process, the final products are generated in conjunction with other potential impurities such as truncated mRNA. The impurity profiles of siRNA, gRNA, and mRNA require thorough characterization of these synthetic products.

In this study, we demonstrate efficient reverse phase and ion-pairing reverse phase separation of nucleic acids varying in length from 10 to 10,000 nucleotides using a 4.0 µm polymeric, supermacroporous (SMP) resin with divinylbenzene chemistry The SMP particle possesses a wide range of pores (50 Å- 2000 Å) to provide accessible surface area for separating nucleotides across a wide molecular weight range. In addition to that, the polymeric media is highly stable to extremes of pH (0-14) and temperature (up to 100 ºC). The chromatographic profiles of conventional C18 silica columns are compared to the profile from the SMP column. The results demonstrate the versatility of a chromatographic material possessing a wide range of pores sizes to enable analysis of nucleotide-based therapeutics across a wide molecular weight range.

Materials and methods

Sample

8-Combo DNA:

GACTGACTGACT - 12

GACTGACTGACTGACT - 16

GACTGACTGACTGACTGACT - 20

GACTGACTGACTGACTGACTGACT - 24

GACTGACTGACTGACTGACTGACTGACT -28

GACTGACTGACTGACTGACTGACTGACTGACT -32

GACTGACTGACTGACTGACTGACTGACTGACTGACT -36

GACTGACTGACTGACTGACTGACTGACTGACTGACTGACT -40

ΦX174-BsuRI digest (72, 1181 194, 234, 271, 281, 310, 603, 872, 1078 and 1353 bp).

98-100 mer DNA:

GATTGTAGGTTCTCTAACGCTGATTGTAGGTTCTCTAACGCTGATTGTAGGTTCTCTAA CGCTGATTGTAGGTTCTCTAACGCTGATTGTAGGTTCTC – 98

GATTGTAGGTTCTCTAACGCTGATTGTAGGTTCTCTAACGCTGATTGTAGGTTCTCTAA CGCTGATTGTAGGTTCTCTAACGCTGATTGTAGGTTCT – 99

GATTGTAGGTTCTCTAACGCTGATTGTAGGTTCTCTAACGCTGATTGTAGGTTCTCTAA CGCTGATTGTAGGTTCTCTAACGCTGATTGTAGGTTCTA – 100

FastRuler low range DNA ladder (50, 200, 400, 850 and 1.5k bp).

FastRuler middle range DNA ladder (100, 400, 850, 2k and 5k bp).

Columns

DNAPac RP column, 4 µm, 2.1 × 50 mm (P/N 088924)

Conventional C18 column, 2.5 µm, 300 Å, 2.1 × 50 mm

Conventional C18 column, 1.7 µm, 300 Å, 2.1 × 50 mm

Data analysis

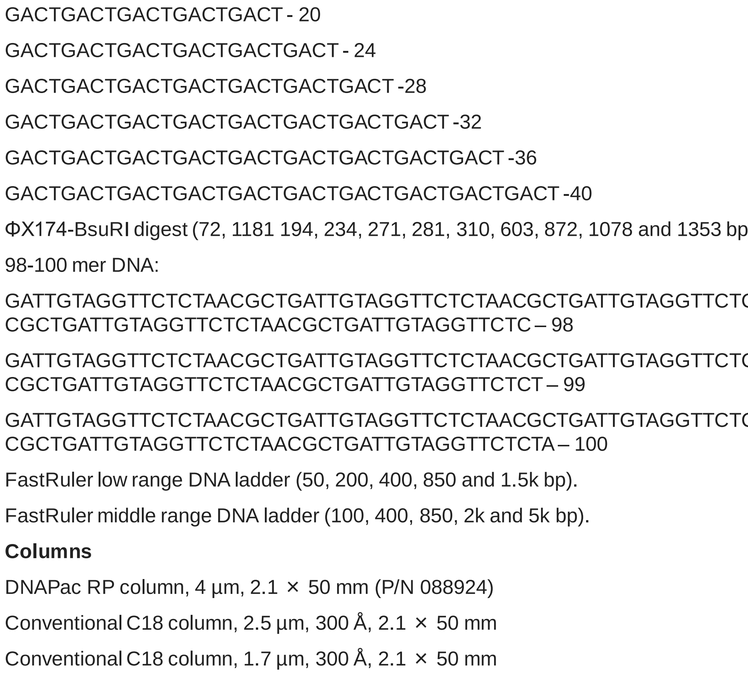
The Thermo Scientific™ Chromeleon™ 7.2.10 Chromatography Data System (CDS) was used for data acquisition and analysis.

Results

DNAPac RP Column Particle Pore Size Distribution

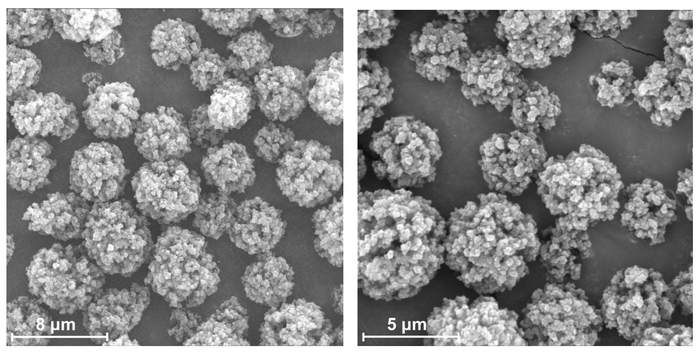
In Figure 1, the Brunauer-Emmett-Teller (BET) analysis using N_2 shows the DNAPac RP particles possess a wide range of pores (50 Å - 2000 Å) to provide accessible surface area for separating nucleotides across a wide molecular weight range. SEM images are shown in Figure 2 to provide visual evidence of the broad range of pore distribution.

Figure 1 BET analysis of DNAPac RP column particles



SEM images are shown in Figure 2 to provide visual evidence of the broad range of pore distribution.

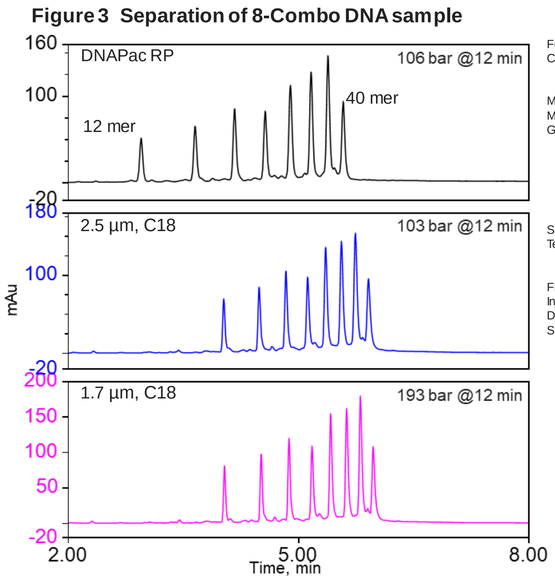
Figure 2 SEM image of DNAPac RP column particles



Short nucleic acid sample testing (12 – 40 mer)

In Figure 3, a mixture of 12, 16, 20, 24, 28, 32, 36 and 40 mer DNAs with mixed base composition (5 µM) are separated using a linear gradient. Triethylammonium acetate (TEAA) is used as an ion pairing reagent. For DNAPac RP column, all eight components can be baseline separated within the 9 min gradient. The smaller pores of the DNAPac RP column enable the sharp peaks and high resolution of short nucleic acid samples. The resolution between each peak and overall peak spread using DNAPac RP column is superior compared to conventional C18 silica columns.

Figure 3 Separation of 8-Combo DNA sample



Format: 2.1 X 50 mm
Column: Top: DNAPac RP, 4 µm, 50 - 2000 Å; Middle: C18, 2.5 µm, 300 Å; Bottom: C18, 1.7 µm, 300 Å
Mobile phase A: 0.1M TEAA
Mobile phase B: ACN
Gradient:

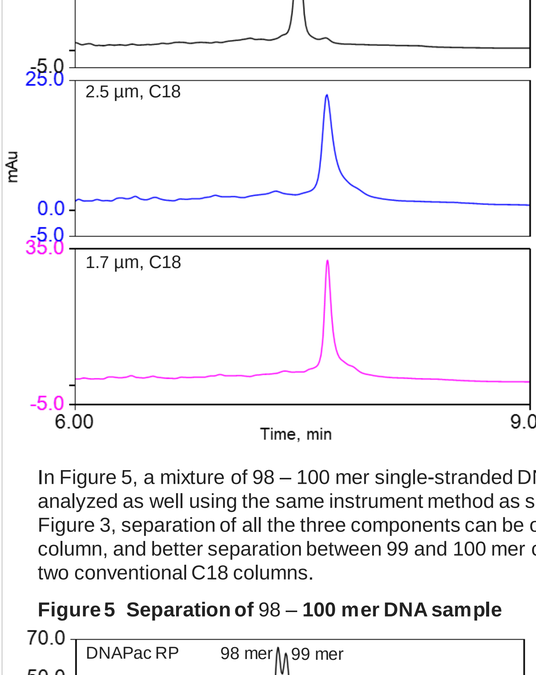
Time (min)	%A	%B
-5.0	97	3
0.0	97	3
9.0	87	13
9.1	75	25
13.0	75	25

System: Vanquish H
Temperature: : 60 ºC; Preheater 60 ºC; Post column cooler 45 ºC
Flow rate: 0.4 mL/min
Inj. volume: 2 µL
Detection: UV (260 nm)
Sample: 8 Combo DNA (5 µM)

Intermediate nucleic acid sample testing (98 – 100 mer)

In Figure 4, an intermediate length 100 mer single-stranded DNA sample (20 µg/mL) is analyzed using the same instrument method as shown in Figure 3. A sharp and well resolved 100 mer DNA peak can be obtained using the DNAPac RP column due to the existence of the wide range of pores (50 Å – 2000 Å). The 100 mer peak using conventional 2.5 µm C18 silica column is much wider. For 1.7 µm C18 column, the variant to the right of the 100 mer peak is not well resolved as using DNAPac RP column.

Figure 4 Separation of 100 mer DNA sample



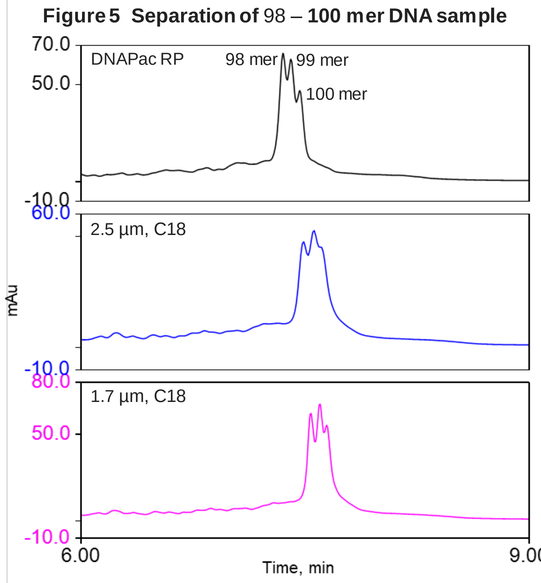
Format: 2.1 X 50 mm
Column: Top: DNAPac RP, 4 µm, 50 - 2000 Å; Middle: C18, 2.5 µm, 300 Å; Bottom: C18, 1.7 µm, 300 Å
Mobile phase A: 0.1M TEAA
Mobile phase B: ACN
Gradient:

Time (min)	%A	%B
-5.0	97	3
0.0	97	3
9.0	87	13
9.1	75	25
13.0	75	25

System: Vanquish H
Temperature: : 60 ºC; Preheater 60 ºC; Post column cooler 45 ºC
Flow rate: 0.4 mL/min
Inj. volume: 5 µL
Detection: UV (260 nm)
Sample: 100 mer DNA (20 µg/mL)

In Figure 5, a mixture of 98 – 100 mer single-stranded DNA sample (20 µg/mL each) is analyzed as well using the same instrument method as shown in Figure 3. As shown in Figure 3, separation of all the three components can be obtained using DNAPac RP column, and better separation between 99 and 100 mer can be achieved compared to the two conventional C18 columns.

Figure 5 Separation of 98 – 100 mer DNA sample



Format: 2.1 X 50 mm
Column: Top: DNAPac RP, 4 µm, 50 - 2000 Å; Middle: C18, 2.5 µm, 300 Å; Bottom: C18, 1.7 µm, 300 Å
Mobile phase A: 0.1M TEAA
Mobile phase B: ACN
Gradient:

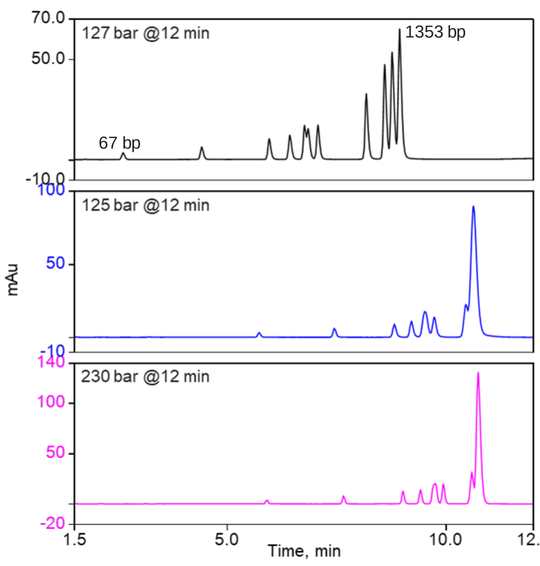
Time (min)	%A	%B
-5.0	97	3
0.0	97	3
9.0	87	13
9.1	75	25
13.0	75	25

System: Vanquish H
Temperature: : 60 ºC; Preheater 60 ºC; Post column cooler 45 ºC
Flow rate: 0.4 mL/min
Inj. volume: 5 µL
Detection: UV (260 nm)
Sample: 98:99:100 mer 1:1:1 (20 ug/mL)

Long nucleic acid sample testing (up to 5000 bp)

Figure 6 depicts the resolution of double-stranded DNA fragments generated from plasmids digested by the restriction endonuclease BsuRI (HaeIII). Fragments ranging from 67 bp to 1353 bp are well separated on the DNAPac RP column in 12 minutes. Separation of 603 – 1353 bp cannot be achieved for conventional C18 silica columns due to lack of large pores

Figure 6 Separation of ΦX174-BsuRI digest dsDNA sample



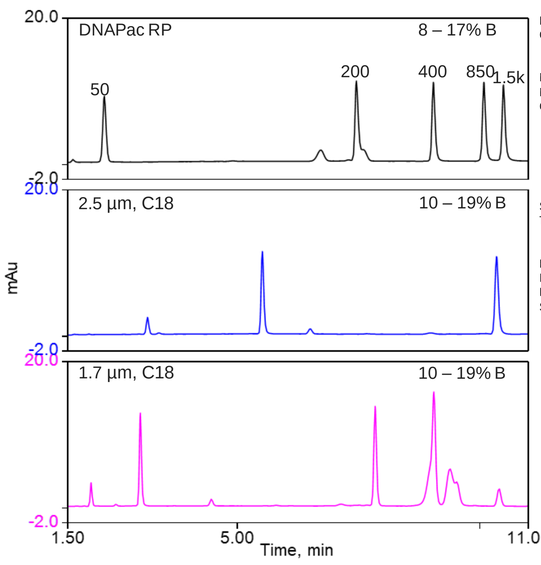
Format: 2.1 × 50 mm
Column: Top: DNAPac RP, 4 µm, 50 - 2000 Å; Middle: C18, 2.5 µm, 300 Å; Bottom: C18, 1.7 µm, 300 Å
Mobile phase A: 0.1M TEAA
Mobile phase B: ACN
Gradient:

Time (min)	%A	%B
-8.0	91	9
0.0	91	9
12.0	82	18
12.1	75	25
16.0	75	25

System: Vanquish H
Temperature: : 50 ºC; Preheater 50 ºC
Flow rate: 0.4 mL/min
Inj. volume: 5 µL
Detection: UV (260 nm)
Sample: dsDNA (100 µg/mL)

Separation of FastRuler low range DNA ladder 50 – 1500 bp (Figure 7) and FastRuler middle range DNA ladder 100 – 5000 bp (Figure 8) further shows the advantage of DNAPac RP column compared to conventional C18 silica columns.

Figure 7 Separation of FastRuler low range DNA ladder (50, 200, 400, 850 and 1.5k bp) sample

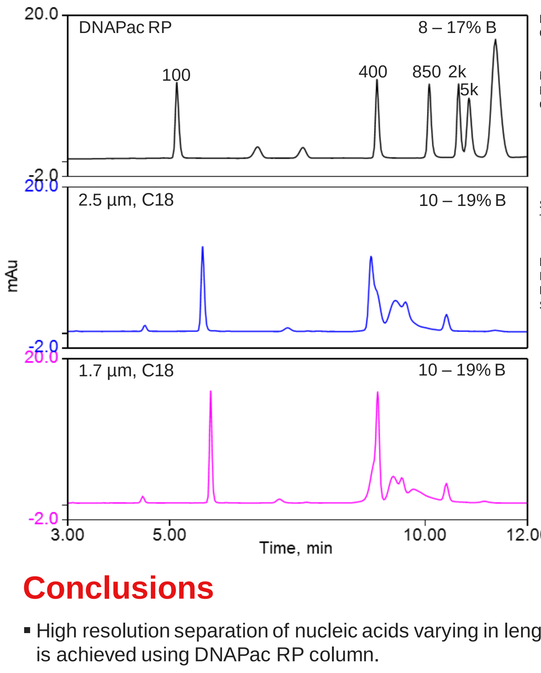


Format: 2.1 × 50 mm
Column: Top: DNAPac RP, 4 µm, 50 - 2000 Å; Middle: C18, 2.5 µm, 300 Å; Bottom: C18, 1.7 µm, 300 Å
Mobile phase A: 0.1M TEAA
Mobile phase B: ACN
Gradient:

Time (min)	%A	%B
-8.0	92	8
0.0	92	8
12.0	83	17
12.1	75	25
15.0	75	25

System: Vanquish H (SpaceGhost)
Temperature: : 55 ºC; Preheater 55 ºC; Post column cooler 45 ºC
Flow rate: 0.4 mL/min
Inj. volume: 5 µL
Detection: UV (260 nm)
Sample: FastRuler LR DNA Ladder

Figure 8 Separation of FastRuler middle range DNA ladder (100, 400, 850, 2k and 5k bp) sample



Format: 2.1 × 50 mm
Column: Top: DNAPac RP, 4 µm, 50 - 2000 Å; Middle: C18, 2.5 µm, 300 Å; Bottom: C18, 1.7 µm, 300 Å
Mobile phase A: 0.1M TEAA
Mobile phase B: ACN
Gradient:

Time (min)	%A	%B
-8.0	92	8
0.0	92	8
12.0	83	17
12.1	75	25
15.0	75	25

System: Vanquish H (SpaceGhost)
Temperature: : 55 ºC; Preheater 55 ºC; Post column cooler 45 ºC
Flow rate: 0.4 mL/min
Inj. volume: 5 µL
Detection: UV (260 nm)
Sample: FastRuler MR DNA Ladder

Conclusions

- High resolution separation of nucleic acids varying in length from 10 to 10,000 nucleotides is achieved using DNAPac RP column.

References

1. Vanhinsbergh, C.J.; Criscuolo, A.; Sutton, J.N.; Murphy, K.; Williamson, A.J.K.; Cook, K.; Dickman, M.J., Characterization and Sequence Mapping of Large RNA and mRNA Therapeutics Using Mass Spectrometry. *Analytical Chemistry* **2022**, 94, 7339–7349.
2. Wei, B.; Wang, J.; Cadang, L.; Goyon, A.; Chen, B.; Yang, F.; Zhang, K. Development of an ion pairing reversed-phase liquid chromatography-mass spectrometry method for characterization of clustered regularly interspaced short palindromic repeats guide ribonucleic acid. *Journal of Chromatography A* 1665 (**2022)** 462839
3. Packer, M.; Gyawali, D.; Yerabolu, R.; Schariter, J.; White, P. A novel mechanism for the loss of mRNA activity in lipid nanoparticle delivery systems. *Nature Communications* **2021** 12:6777

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