Application Note 21533

Separation of Large Double-Stranded DNA (dsDNA) Fragments Using a Wide-Pore Reversed-Phase Chromatography Column

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Key Words
Ion-pair reversed-phase chromatography, double-stranded DNA (dsDNA), DNAPac RP, HPLC

Goal
To demonstrate the separation of dsDNA fragments and a PCR product using a wide-pore, polymer-based DNAPac RP column.

Introduction

Restriction nucleases are enzymes that generate dsDNA fragments of various sizes, and help researchers construct plasmids for expression of modified or novel proteins. In addition, DNAs that contain sequences for alternative gene editing systems (such as CRISPR-Cas9) may also generate plasmids.[1] Restriction enzymes may also be used to identify single nucleotide polymorphisms (SNPs) where the base change alters the specific restriction nuclease site.[2] For all these applications, restriction nuclease DNA fragments are typically purified prior to subsequent use. Agarose and polyacrylamide gels are most often used for separating and recovering dsDNA fragments. Gel electrophoresis is followed by manual excision of the target dsDNA size from the gel and extraction of the DNA from the excised gel. The method is laborious and time consuming and produces relatively low yields. It also requires handling of toxic materials such as acrylamide or ethidium bromide. HPLC provides reliably higher yields, allows direct DNA quantitation, and can be automated using fraction collection. Here we describe the use of a new reversed-phase column for the separation and recovery of dsDNA molecules.

The Thermo Scientific™ DNAPac™ RP column is based on a 4 µm, wide-pore polymer resin, which is stable at high pH and high temperatures. The wide-pore resin (1000–2000 Å) separates large dsDNA molecules as well as single-stranded oligonucleotides. This application note demonstrates the separation of DNA fragments generated from restriction enzymes and DNA ladder components, with automated purification and collection of the PCR product using ion-paired reversed-phase chromatography on the DNAPac RP column.

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Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (Fisher Scientific™ P/N A955-4)
- Triethylammonium acetate (TEAA) 2.0 M (Applied Biosystems P/N 400613)

Samples

- pBR322-BsuRI digest (Thermo Scientific P/N SM0252)
- ΦX174-BsuRI digest (Thermo Scientific P/N SM0271)
- Thermo Scientific™ FastRuler™ Low Range DNA Ladder, ready-to-use (P/N SM1103)
- Thermo Scientific FastRuler Middle Range DNA Ladder, ready-to-use (P/N SM1113)
- Thermo Scientific FastRuler High Range DNA Ladder, ready-to-use (P/N SM1123)
- WPRE PCR product (Generous gift from Sanjay Vasu)

Sample Handling and Electrophoresis Equipment

Vial and closures: 300 µL PP 8-425 Screw Thread Vial (P/N C4013-11), Pre-Assembled Black PP 8-425 Cap with Pre-Slit PTFE/Silicone Septa (P/N C4013-77A)

Agarose Gels: Thermo Scientific™ Invitrogen™ 1.2% E-Gel (P/N G501801) with Invitrogen E-Gel Base

Power supply: FisherBiotech™ FB200Q Electrophoresis Power Supply or equivalent

Visualization: UV/Vis transilluminator

Sample Preparation

pBR322-BsuRI and ΦX174-BsuRI digests were diluted five-fold with DI water to make 100 µg/mL solutions.

FastRuler DNA Ladders and the PCR product were used as delivered.

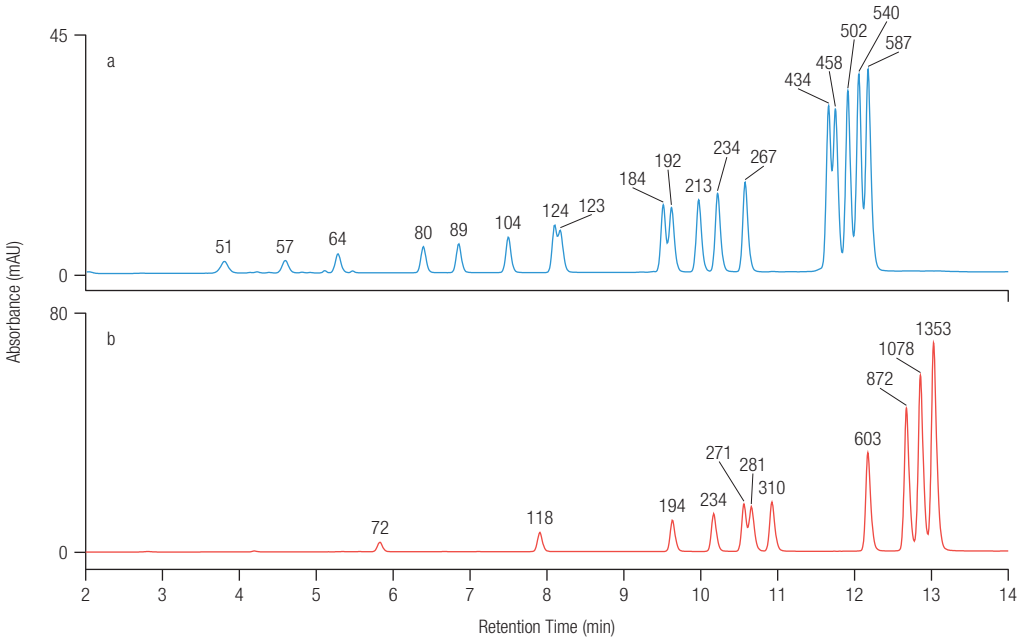
Separation Conditions	
Instrumentation	1. Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of:
	System Base (P/N VF-S01-A)
	Quaternary Pump Flex (P/N VF-P20-A)
	Split-Loop Sampler FT (P/N VF-A10-A)
	Column Compartment H (P/N VH-C10-A)
	Active Pre-heater (P/N 6732.0110)
	Diode Array Detector HL (P/N VH-D10-A)
	Thermo Scientific™ LightPipe™ Flow Cell, Standard, 10 mm (P/N 6083.0100)
	2. Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRSLC system equipped with:
	SR-3000 Solvent Rack (without degasser) (P/N 5035.9200)
	LPG-3400RS Biocompatible Quaternary Rapid Separation Pump with degasser (P/N 5040.0036)
	WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection (P/N 5825.0020)
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000) equipped with 11 µL pre-column heater (P/N 6723.0252)
	VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010) equipped with a micro flow cell (PN 6074.0300)
Columns	DNAPac RP, 2.1 × 100 mm (P/N 088923) DNAPac RP, 2.1 × 50 mm (P/N 088924)
Mobile Phase A	0.1 M TEAA, pH 7.0
Mobile Phase B	0.1 M TEAA in water/acetonitrile (75:25 v/v)
Gradient	As specified in Figures
Flow Rate	As specified in Figures
Column Temperature	As specified in Figures
UV Detector Wavelength	260 nm

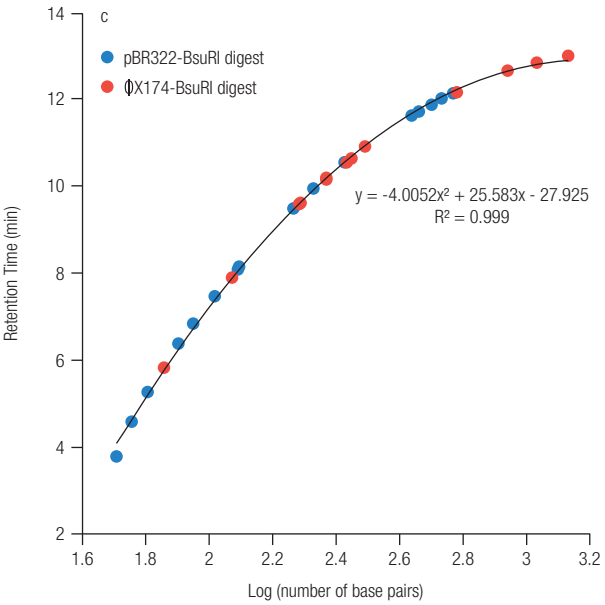
Data Processing

The Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System was used for data analysis.

Results and Discussion

Ion-pair reversed-phase chromatography on the DNAPac RP column was used to separate restriction digest components (Figure 1). At 55 °C, all of the dsDNA digestion fragments from pBR322 plasmid (Figure 1a) and ΦX174 viral DNA (Figure 1b) with BsuRI enzyme were separated. The DNAPac RP column was able to separate fragments differing by as little as a single base pair (bp) up to 100 bp. In addition, the retention of the fragments correlated to the length of the DNA. Retention versus log (number of base pairs) of both pBR322 and ΦX174 digests was plotted in Figure 1c. The data fit well to a second-order polynomial equation, indicating good correlation between the retention time and the length of the fragments.





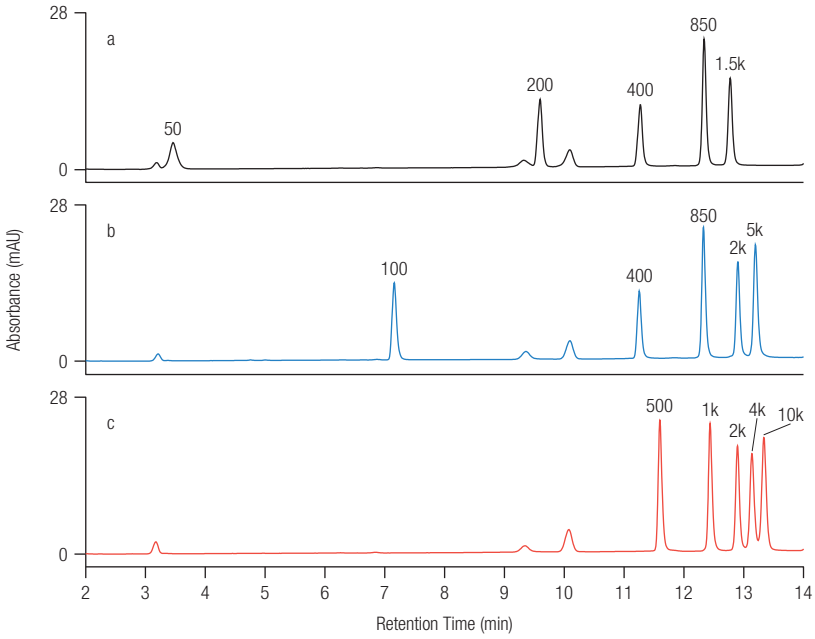
Column:	**DNAPac RP**, 4 µm
Format:	2.1 × 100 mm
Mobile Phase A:	0.1 M TEAA, pH 7.0
Mobile Phase B:	0.1 M TEAA, pH 7.0 in water/acetonitrile (75:25 v/v)
Gradient:	

Time (min)	%A	%B
-14.0	64	36
0.0	64	36
12.0	31	69
12.1	5	95
15.0	5	95

Flow Rate:	0.40 mL/min
Inj. Volume:	5 µL
Temp.:	55 °C
Detection:	UV (260 nm)
Sample:	a. pBR322-BsuRI digest (100 µg/mL) b. ΦX174-BsuRI digest (100 µg/mL)
Peak Label:	Base pairs

Figure 1. Separation of restriction enzyme digests.

To further investigate the DNAPac RP column resolution of large dsDNA fragments, DNA ladders with components up to 10,000 base pairs were examined (Figure 2). All of the DNA ladder components were resolved in less than 14 minutes with the resolution of components differing by approximately 10% for fragments up to around 10,000 bp. The DNA ladder retention time can be compared to the retention time of a DNA fragment to estimate its size.



Column:	**DNAPac RP**, 4 µm
Format:	2.1 × 100 mm
Mobile Phase A:	0.1 M TEAA, pH 7.0
Mobile Phase B:	0.1 M TEAA, pH 7.0 in water/acetonitrile (75:25 v/v)

Gradient:

Time (min)	%A	%B
-14.0	65	35
0.0	65	35
12.0	30	70
12.1	10	90
15.0	10	90

Flow Rate:	0.40 mL/min
Inj. Volume:	10 µL
Temp.:	55 °C
Detection:	UV (260 nm)
Sample:	DNA ladders a. Low range b. Middle range c. High range
Peak label:	Base pairs

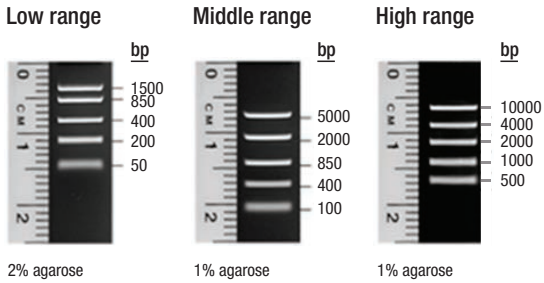


Figure 2. Separation of FastRuler DNA ladders.

Separation of ΦX174-BsuRI digest, which contains components from 72 to 1353 bp, on a DNAPac RP column was compared to competitive columns of similar format (all equivalent 2.1 mm columns, except for brand C, which has a 2.0 mm ID). The same mobile phase and linear velocity were used. The larger pore size DNAPac RP column gave significantly better separation of the larger dsDNA fragments. Differences in separation can be attributed to larger pore size; however, selectivity, particle size, and particle chemistry may have also contributed to the difference in the separation of dsDNA fragments (Figure 3).

The ΦX174-BsuRI digest was used to study the temperature effect on the dsDNA retention and resolution (Figure 4). Resolution and the retention of these dsDNA fragments increased with temperature up to 50 °C without changing the peak intensity pattern. However, at 60 °C, the retention pattern changed and the resolution of the peaks appeared to be higher. Since the intensities of several peaks change at 60 °C, partial denaturation of some dsDNA fragments is likely induced (hyperchromic shift). Two peaks also eluted earlier at 60 °C than at 50 °C, which is consistent with exposure of ssDNA sections within the fragment. At 70 °C, denaturation is obvious as the number of peaks increased from 11 to 18, and many peaks eluted much earlier than at lower temperatures, suggesting full denaturation of some dsDNA into single stranded DNA (ssDNA) forms. At even higher temperatures (80–90 °C), more peaks were observed and all eluted much earlier, indicating full denaturation of all of the dsDNA fragments.

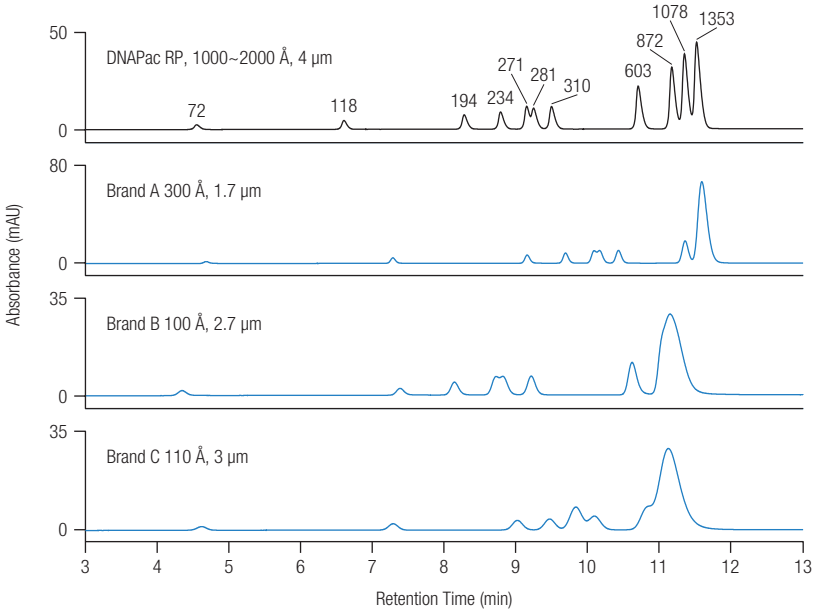
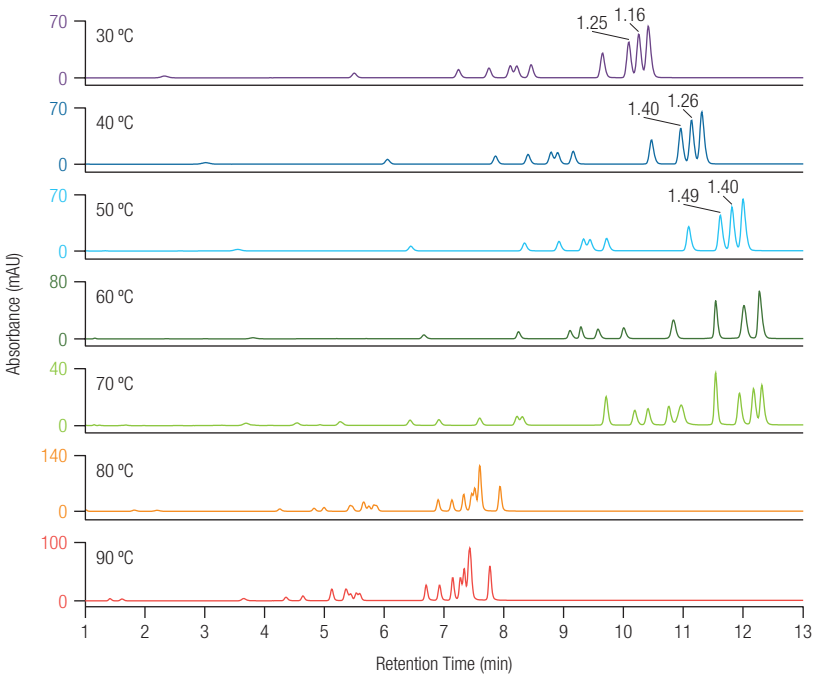


Figure 3. Separation of ΦX174-BsuRI digest on columns with different pore sizes.



Column:	**DNAPac RP**, 4 µm
Format:	2.1 × 100 mm
Mobile Phase A:	0.1 M TEAA, pH 7.0
Mobile Phase B:	0.1 M TEAA, pH 7.0 in water/acetonitrile (75:25 v/v)

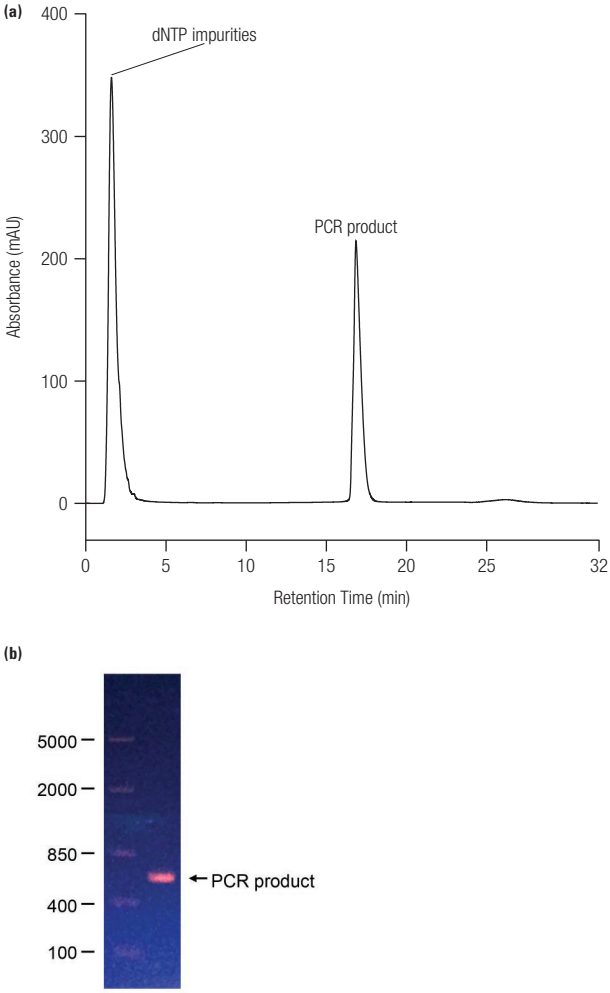
Gradient:

Time (min)	%A	%B
-14.0	60	40
0.0	60	40
12.0	30	70
12.1	5	95
15.0	5	95

Flow Rate:	0.40 mL/min
Inj. Volume:	5 µL
Detection:	UV (260 nm)
Sample:	**ΦX**174-BsuRI digest (100 µg/mL)
Peak label:	Resolution (ep)

Figure 4. Separation of ΦX174-BsuRI digest at different temperatures.

PCR is routinely used in molecular biology labs for amplification of DNA and sequencing. PCR products are typically purified from the dNTPs and other impurities in the reaction prior to use. Using an UltiMate 3000 system with an UltiMate WPS-3000TBFC (Well Plate Autosampler configured for Fraction Collection), a PCR product was separated from its impurities and automatically collected into a well plate (Figure 5).[4,5] The collected sample was then analyzed with a 1.2% agarose E-gel to confirm its purity (Figure 5b). This example demonstrates automated HPLC with fraction collection of dsDNA using the DNAPac RP column, showing facile purification from PCR-related components in 20 minutes.



Column:	**DNAPac RP**, 4 µm		
Format:	2.1 × 100 mm		
Mobile Phase A:	0.1 M TEAA, pH 7.0		
Mobile Phase B:	0.1 M TEAA in water/acetonitrile (75:25 v/v)		
Gradient:	Time (min)	%A	%B
	0.0	56	44
	20.0	32	68
	20.1	10	90
	22.0	10	90
	22.1	56	44
	32.0	56	44
Flow Rate:	0.25		
Inj. Volume:	50 µL		
Temp.:	50 °C		
Detection:	UV (260 nm)		
Sample:	PCR product		

Figure 5. Purification of a PCR product.

Conclusion

- The combination of the DNAPac RP column and Vanquish Flex UHPLC system delivers rapid, high-resolution dsDNA fragment separation up to 10,000 bp.
- Elution of the dsDNA fragments is essentially proportional to fragment length (in bp).
- The UltiMate 3000 system equipped with the WPS-3000 TBFC Well Plate Autosampler configured for Fraction Collection facilitates purification and recovery of dsDNA fragments.

Reference

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Useful Links

AppsLab Library

The eWorkflow and the Chromeleon Backup (cmbx) file can be downloaded at AppsLab Library:
www.thermofisher.com/appslab

For Research Use Only. Not for use in diagnostic procedures

www.thermofisher.com/BioLC

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