High-Resolution Purification of Sheared Lambda Phage DNA Using a Gilson HPLC System

Application Note CL0513

Keywords

High-Performance Liquid Chromatography (HPLC), DNA shearing, DNA separation, methylation, lambda phage, gradient pattern, electrophoresis, next-generation sequencing (NGS)

Introduction

Data in this application note were provided by M. Kawakatsu and Y. Hayashi, Applied Technical Department, M&S Instruments, Inc., Japan.

As the size-selective separation of nucleic acids is a necessary step in many molecular biological analyses performed in clinical, forensic, and other types of laboratories, the development of optimal DNA separation methods is important. Liquid chromatographic separation techniques involve simple automated sample purification steps and provide high resolution of DNA molecules, thereby enabling efficient DNA separation. Furthermore, fragments can be identified and quantified directly when using liquid chromatography methods.

The separation and characterization of modified DNA is particularly relevant, as general interest in next-generation sequencing (NGS) is expanding.[1] This application note describes the successful separation of methylated lambda phage DNA fragments on a Gilson HPLC system using a Sepax PolyRP-1000 column. Prior to HPLC analysis, a gradient method was developed to provide sufficient resolution of the DNA fragments (25-2000 bp). These results demonstrate the feasibility of separating DNA fragments, including those containing methylated modifications, by HPLC. This highly effective separation method can thus be applied in a wide variety of analyses, including the preparation steps of next-generation sequencing workflows.

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Materials & Methods

Gradient Method Development – Materials and Conditions

DNA fragments ranging from 25 to 2000 base pairs (bp) in length were used for the development of a suitable gradient for separating DNA molecules:

HPLC System & Conditions

- Column: Sepax PolyRP-1000, 5 µm, 1000 Å, 4.6 x 250 mm
- Column Temperature: 50°C
- Mobile Phase:
  - A: 0.1M TEAA (pH 7.2)
  - B: 0.1M TEAA (pH7.2) in Acetonitrile
  - Flow Rate: 1 mL/min (unless otherwise specified)
  - Gradient: Varied, as indicated below
- Gilson Binary Pump System: 305 (5SC) & 306 (5SC), 811D Mixer – 700 µL, 805 Manometric Module
- Injection Valve: Rheodyne Model 7125i , 20 µL loop
- UV Detection:
  - 254 nm
  - 155 Dual Wavelength UV/VIS Detector with standard flow cell (5 mm path length)

Gradients Tested

1. %B: 35-70%, 1 to 15 min; flow rate 0.7 mL/min (Figure 2)
2. %B: 40-80%, 1 to 22 min (Figures 3, 4)
3. %B: 50-75%, 1 to 22 min (Figure 4)
4. %B: 50-61%, 1 to 10 min; 61-70%, 10 to 25 min (Figures 4, 5, 6)
5. %B: 50%, 0 to 0.8 min; 50-61%, 0.8 to 10.1 min; 61%, 10.1 to 10.2 min; 61-70%, 10.2 to 25.1 min; 70%, 25.1 to 26.1 min; 70-50%, 26.1-26.3 min; 50%, 26.3 to 56.3 min (final optimized gradient)

DNA Standards

1. 100 bp ladder (Bioneer) (Figure 1)
   Volume: 25 µL
   Concentration: 135 ng/µL
2. 25/100 bp ladder (Bioneer)
   Volume: 25 µL
   Concentration: 150 ng/µL
3. 1 Kbp ladder (Bioneer)
   Volume: 25 µL
   Concentration: 130 ng/µL
4. M3 marker - old (Elchrom)
5. 100 bp ladder - old (Gibco-BRL)

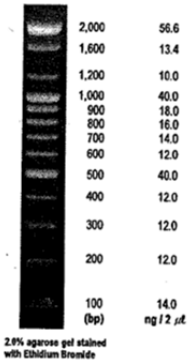

Figure 1. Example DNA ladder (100 bp) showing expected fragment sizes.

Gradient Method Development - Workflow

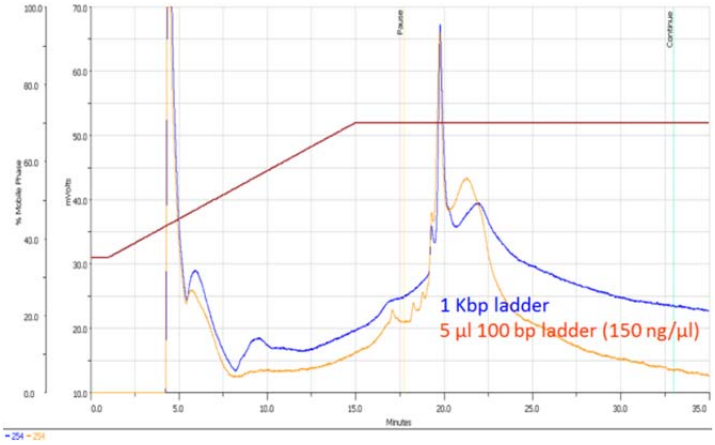

Figure 2. Steep gradient in Method #1 did not resolve the peaks in the 100 bp (orange) and 1 Kb (blue) DNA standards. Gradient 1: 35-70%, 1 to 15 min, flow rate 1 mL/min.

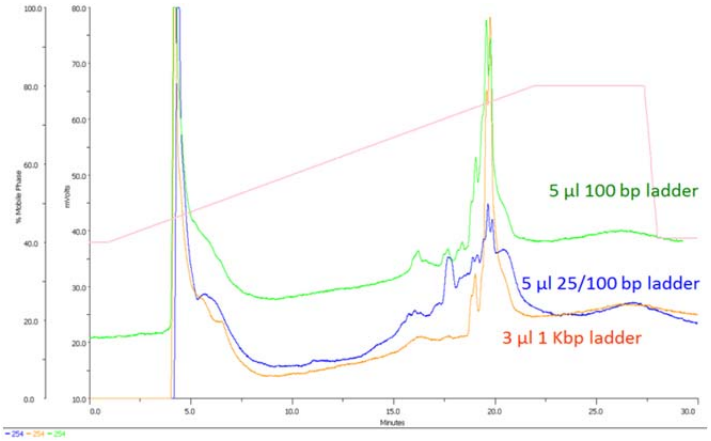

Figure 3. Shallower gradient in Method #2 did not provide adequate separation of the peaks in the 100 bp (green), 25/100 bp (blue) and 1 Kb (orange) ladders. Gradient 2: 40-80%, 1 to 22 min, flow rate 1 mL/min.

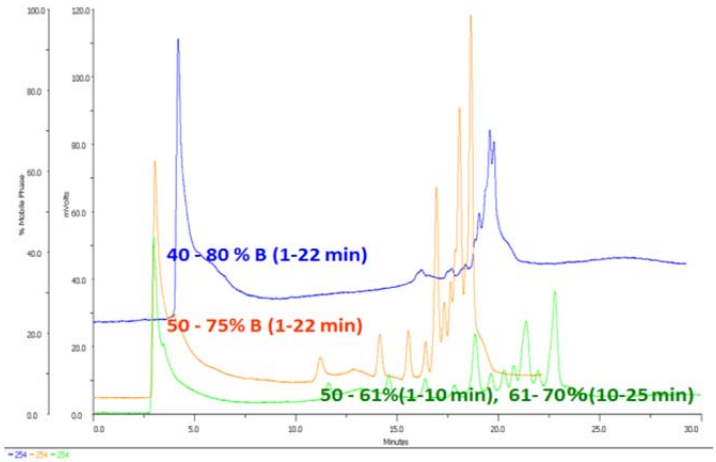

Figure 4. Optimal separation of the peaks in the 100 bp standard was achieved with bi-phasic gradients in Method #4. Gradient 2 (blue): 40-80%, 1 to 22 min; Gradient 3 (red): 50-75%, 1 to 22 min; and Gradient 4 (green) 50-61%, 1 to 10 min & 61-70%, 10 to 25 min.

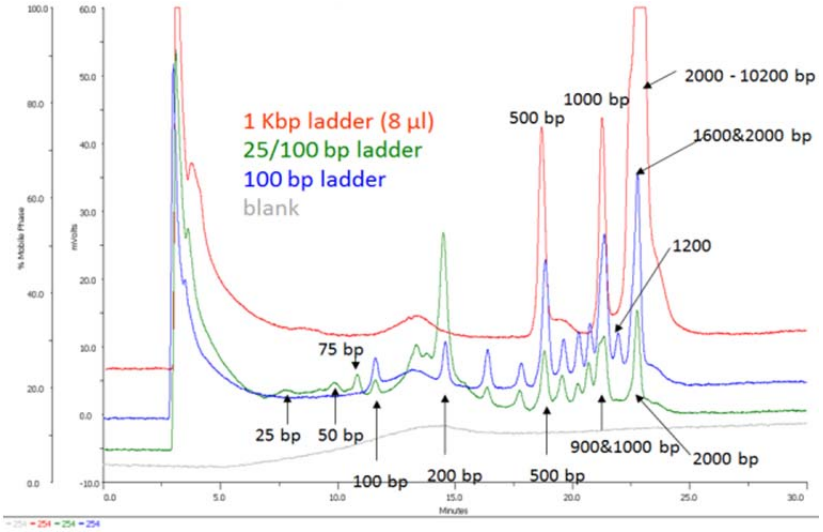

Figure 5. DNA fragment sizes were verified for three DNA ladders separated with Gradient 4.

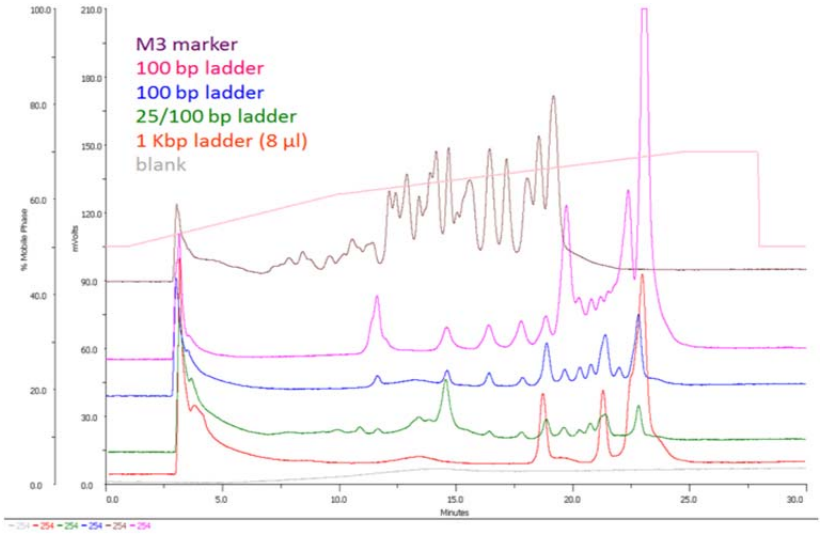

Figure 6. All DNA standards were chromatographed under the optimal gradient conditions. (Refer to Figure 5 for fragment sizes and corresponding peaks.) The optimal gradient method is versatile and can be used across a variety of DNA standards.

Methods - DNA Shearing and Analysis

DNA Shearing

- DNA Sample:
  - Lambda phage DNA, methylated (Sigma-Aldrich)
  - 7 µg in 300 µL TE Buffer (pH 8.0)
- Shearing Conditions:
  - Covaris S2 Focused-ultrasonicator (Covaris, Inc.)
  - Duty Cycle – 20%; Intensity – 8; Cycle/Burst – 200
  - Treatment times: 30 s, 60 s, 120 s, 600 s, 1800 s

HPLC System and Conditions

- Column: Sepax PolyRP-1000, 5 µm, 1000 Å, 4.6 x 250 mm
- Column Temperature: 50°C
- Mobile Phase:
  - A: 0.1M TEAA (pH 7.0)
  - B: 0.1M TEAA (pH7.0) + 25% Acetonitrile
  - Gradient 5: 0-0.8 min (50%), 0.8-10.1 min (50-61%), 10.1-10.2 min (61%), 10.2-25.1 min (61-70%), 25.1-26.1 min (70%), 26.1-26.3 min (70-50%), 26.3-56.3 min (50%)
- Gilson Binary Pump System: 305 (5SC), 306 (5SC), 811D Mixer – 700 µL, 805 Manometric Module
- Injection Valve: Rheodyne Model 7725i, 500 µL loop
- UV Detection:
  - 254 nm
  - 155 Dual Wavelength UV/VIS Detector with standard flow cell (5 mm path length)
- Fraction Concentration Method: Ethanol precipitation

Electrophoresis and Imaging

- 1.5% agarose gel using the AgaroPower kit (Bioneer)
- E-Box used for gel imaging (Vilber-Lourmat)

Results

Results produced by the chromatographic separation of sheared DNA samples using optimized gradient conditions were consistent with the standard electrophoresis separation method, as indicated below in Figures 7, 8, and 9.

Reproducibility of the Covaris S2 DNA shearing method was first confirmed by standard gel electrophoresis, wherein two independent sets of sheared DNA samples were separated and compared (Figure 7).

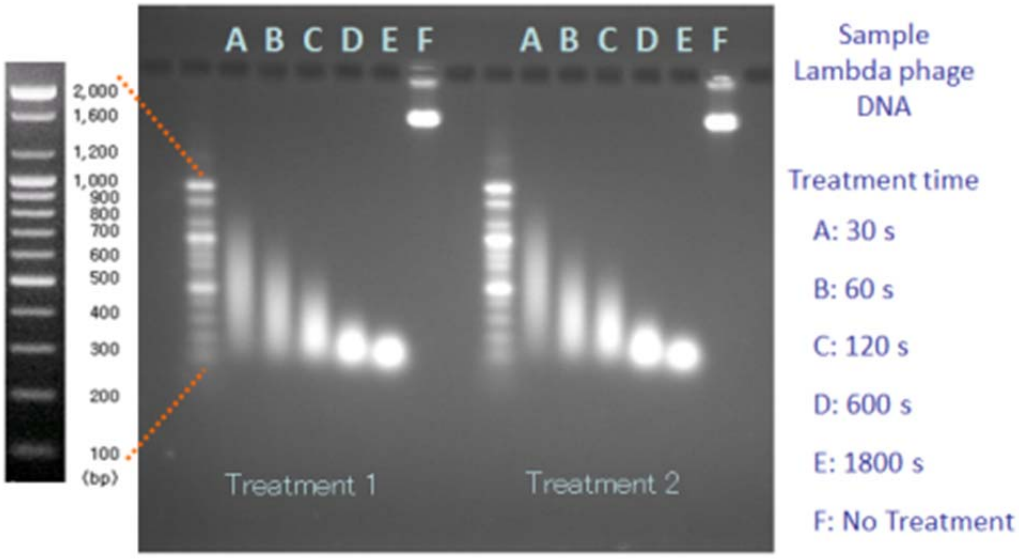

Figure 7. Sheared DNA samples separated by agarose gel electrophoresis. The two replicates showed comparable fragment lengths, indicating adequate reproducibility.

When the Gilson HPLC system combined with a Sepax PolyRP-1000 column was employed as an alternative separation method, the separation of sheared DNA fragments was similar to that detected by standard gel electrophoresis. Using the gradient conditions described (p. 5), adequate fragment separation was achieved. Moreover, when HPLC fractions containing the different fragment sizes (Figure 8) were run on an agarose gel and visualized, apparent fragment resolution was considerably higher (Figure 9) than fragments separated without HPLC.

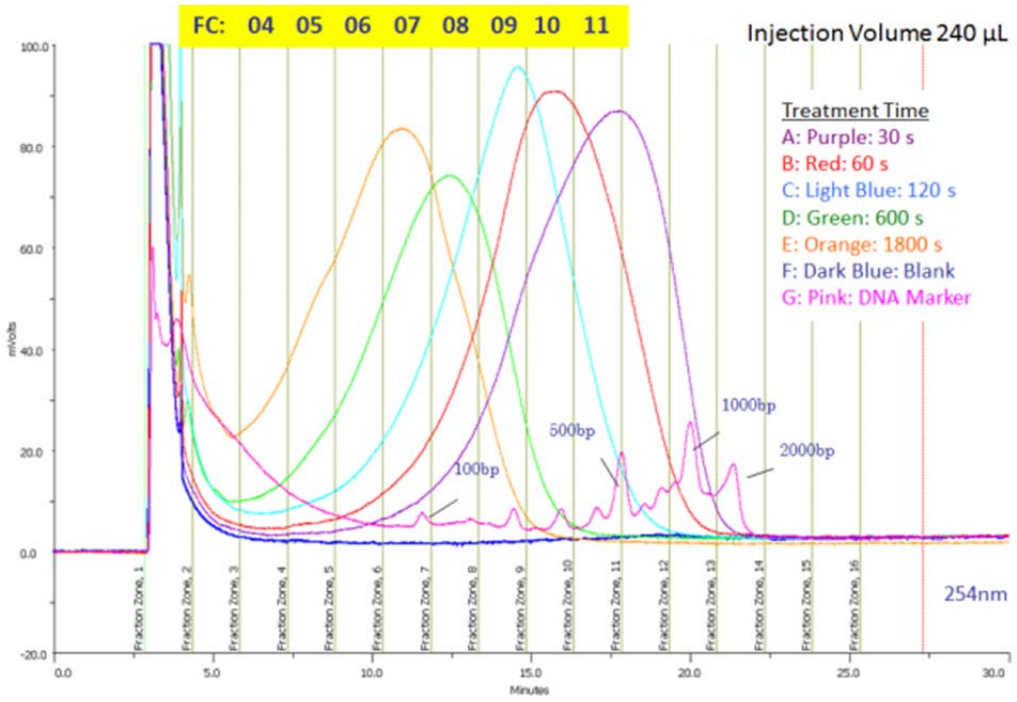

Figure 8. Chromatogram of sheared methylated lambda phage DNA samples. Fractions of each sample, as indicated on the chromatogram (FC 4-11), were collected, ethanol precipitated, and electrophoresed (Figure 9).

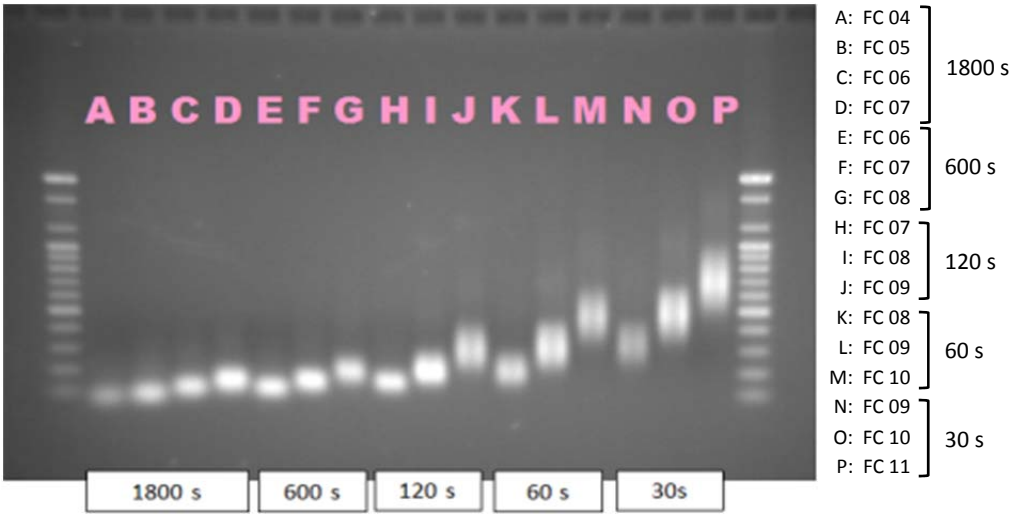

Figure 9. Fractions of chromatography-separated DNA samples sheared for various amounts of time were run on a 1.5% agarose gel for visualization.

Summary

High-resolution separation of sheared methylated DNA fragments ranging from approximately 25 bp to 2000 bp was readily achieved using HPLC with a Gilson binary pump system equipped with a dual wavelength detector. After testing several gradient methods, the optimal conditions were defined for an LC system and a Sepax PolyRP-1000 column. Thus, as an accurate and efficient DNA separation technique, HPLC represents a feasible, if not better, alternative to traditional gel electrophoresis methods for DNA sizing. Moreover, the apparent success of this approach to separating methylated DNA samples implies its suitability as a method to follow the DNA shearing step in next-generation sequencing efforts.

References

1. Shendure, J., Hanlee J. Next-generation DNA sequencing. Nat. Biotechnol. (2008) 26: 1135-1145.