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Note d'application

Separation and Size Assessment of dsDNA Fragments by Anion-Exchange Chromatography (AEX)

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Abstract

Reliably separating dsDNA species and assessing their sizes are important analytical tasks in the fields of cell and gene therapy and vaccines. One application example is that scientists need to confirm the presence and identity of plasmid DNA used as a therapeutic or as the vector to produce a therapeutic transgene. Separating and sizing of dsDNA fragments obtained from restriction enzyme-digested plasmid DNA is recommended as one of the tests for this particular purpose. In this application note, we show that the Waters Protein-Pak Hi Res Q Column can separate a dsDNA ladder ranging from 0.1–10 kbp. The size of the dsDNA fragments obtained from the restriction enzyme digestion of a plasmid can be assessed by comparing the retention time of the fragment with that of the dsDNA ladder.

Benefits

- Optimized Waters Protein-Pak Hi Res Q Column separation of 1 kb Plus DNA Ladder
- Size estimation of dsDNA using the AEX method on Waters Protein-Pak Hi Res Q Column

Introduction

Human gene therapeutic products manipulate gene expression or alter the biological properties of the living cells.[1] These products can be either naked nucleic acids such as plasmid DNA, or the modified microorganisms such as Adeno-associated virus (AAV) that carry the therapeutic genes. In the Investigational New Drug Application (IND), sponsors need to demonstrate the uniqueness of the gene therapy products. Regulatory agencies recommend that the genetic sequence be presented in a schematic diagram that includes a map of relevant regulatory elements such as promotors and restriction enzyme sites. During the manufacture process, the presence and the identity of the therapeutic plasmid DNA or the plasmid DNA used to produce the transgene needs to be tested to demonstrate proper control of the starting material, the intermediates, and the final drug product. One of the recommended tests is the restriction enzyme digestion, where the plasmid DNA is digested into different length of fragments by specific restriction enzymes.[1] The identity of the plasmid DNA can then be evaluated by separating and assessing the size of the fragments which has been traditionally done by agarose or polyacrylamide gel electrophoresis. However, this technique is low throughput and requires many manual-handling steps.

Anion-exchange chromatography (AEX) separates molecules based on their differences in the numbers as well as the localization of negative surface charges that the analytes carry. This analytical technique has many advantages such as being robust, reproducible, yielding quantitative information, being easy to automate, and requiring small amounts of sample. Since the dsDNA fragments are negatively charged due to the phosphate groups on the backbone, we investigated AEX for the separation of dsDNA fragments.

In this application note, we show that a dsDNA ladder ranging from 0.1 kilobase pairs (kbp) to 10 kilobase pairs (kbp) can be separated on a Waters Protein-Pak Hi Res Q Strong Anion-Exchange Column fitted into an ACQUITY UPLC H-Class Bio System. In addition, the ladder separation can be used to estimate the size of dsDNA fragments obtained from restriction enzyme-digested plasmid.

Experimental

Sample Description

1 kb Plus DNA Ladder (N3200L) and pBR322 plasmid-BstNI digest (N3031L) were purchased from New England Biolabs.

LC Conditions

LC system:	ACQUITY UPLC H-Class Bio System
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell, wavelength: 260 nm
Vials:	Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and Pre-slit PTFE/Silicone Septum, 300 µL Volume, 100/pk (p/n: 186002639)
Column(s):	Protein-Pak Hi Res Q column, 5 µm, 4.6 x 100 mm (p/n: 186004931)
Column temp.:	30 ˚C
Sample temp.:	10 ˚C
Injection volume:	0.3 mL
Flow rate:	0.4 mL/min

Mobile phase A:	100 mM Tris-HCl
Mobile phase B:	100 mM Tris base
Mobile phase C:	3 M Tetramethylammonium chloride (TMAC)
Mobile phase D:	Water
Buffer conc. to deliver:	20 mM

Gradient Table (AutoBlend Plus Method, Henderson-Hasselbalch derived)

Time (min)	Flow (mL/min)	pH	Salt (mM)	Salt Curve
0	0.4	7.4	0	–
1	0.4	7.4	0	11
2	0.4	7.4	1620	6
4	0.4	7.4	1620	6
24	0.4	7.4	1780	6
26	0.4	7.4	2400	6
26.1	0.4	7.4	0	11
42	0	7.4	0	11

In the above gradient table, the buffer is 20 mM Tris pH 7.4. The initial salt concentration is set to 0 mM to ensure all the analytes are strongly bound onto the column. The salt concentration is then increased rapidly to 1620 mM and equilibrated for 2 min before the separation gradient starts. The salt concentration increases linearly to 1780 mM in 20 min for the 1 kb DNA Ladder separation. It is then ramped up to 2400 mM in 2 min to strip off any remaining bound molecules. Finally, an equilibration step to the initial condition takes place, preparing for the next injection.

An equivalent gradient table for a generic quaternary LC system is shown below:

Time (min)	%A	%B	%C	%D
0	17.8	2.2	0.0	80.0
1	17.8	2.2	0.0	80.0
2	17.8	2.2	54	26
4	17.8	2.2	54	26
24	17.8	2.2	59.3	20.7
26	17.8	2.2	80.0	0.0
26.1	17.8	2.2	0.0	80.0
42	17.8	2.2	0.0	80.0

Data Management

Chromatography software: Empower 3 FR 4

Results and Discussion

As shown in Figure 1B, 1 kb Plus DNA Ladder that consists of a series of dsDNA fragments ranging from 0.1 kbp to 10 kbp was separated on a Waters Protein-Pak Hi Res Q Strong Anion-Exchange Column. The ability of separating a wide range of different sizes of dsDNA is essential for the analysis of a restriction enzyme digestion.

The chromatographic separation is consistent with the agarose gel separation provided on the New England Biolabs website (Figure 1A and 1B). To help identify the size of the fragments easily, the amount of 1 kb and 3 kb fragments were intentionally made to be higher than other fragments in the 1 kb Plus dsDNA Ladder, which can be observed on the gel with the 1 kbp and the 3 kbp bands being thicker and brighter. Consistently, the peak areas of the 1 kbp and the 3 kbp fragments are also higher than those of other fragments on the anion-exchange chromatogram.

Since the number of negatively charged phosphodiester groups is proportional to the number of nucleotides, the DNA fragments should elute according to their size. Generally, this is true when the DNA fragments are short (<100 bp). As the DNA size increases, the composition of the DNA may have some impact on the AEX separation. For example, it has been shown that fragments having high A-T content elute later than expected based on their chain lengths.[2,3] In some cases, the shorter fragments having higher A-T content eluted later than the longer fragments

having lower A-T content.[4] This can be problematic for size assessment since the DNA does not elute in the order of its length. In these attempts, NaCl was used as the eluting salt in AEX separation. Interestingly, it has been shown that the melting temperature of the A-T rich DNA is higher than that of the G-C rich DNA in NaCl. The difference between A-T rich DNA and G-C rich DNA in the melting temperature is abolished when the experiments were carried out in tetramethylammonium chloride (TMAC). It has been hypothesized that TMAC can fit into the grooves at DNA structure and preferentially bind to A-T base pair, resulting in composition-independent melting temperature for DNAs.[5,6]

In our previous work, we showed that TMAC provides better separation power than NaCl as an eluting salt for analytes involving nucleic acid.[7,8] In the current study, TMAC is again experimented as the salt to separate various length of dsDNA fragments. Preliminary data from Multi-Angle Light Scattering (MALS) suggest that the molecular weights of the peaks in the chromatogram increases along with the retention time (data not shown). Although it is not completely clear why TMAC is able to separate DNA fragments based on their size independent of the fragment composition, it is assumed that TMAC plays an important role in eliminating the influence of the compositions in the dsDNA fragments on the final AEX separation outcome.

Traditionally, the size of the DNA fragments is assessed by running the fragment DNA on the agarose gel and comparing the position of the bands with that of the DNA Ladder that serves as a reference material run on the same gel under the same conditions. Similarly, we show here that in an AEX method, retention time can be used to assess the size of the DNA fragment by running it with the 1 kb Plus DNA Ladder under the same chromatographic conditions (Figure 1B and 1C). Figure 1D shows a plot of log(bp) versus retention time of the dsDNA fragments. The blue dots are the data points from the 1 kb Plus DNA Ladder, while the orange dots are those from the BstNI-digested pBR322 plasmid. The linear fit from the 1 kb Plus DNA Ladder indicates a strong correlation between the logarithm of the size and the retention time (R^2 = 0.981). Using this plot, the sizes of the restriction fragments are calculated from their retention times. The percent (%) error is calculated using the formula: {(calculated size – theoretical size)/theoretical size}. The measurement for most restriction fragments from pBR322 plasmid resulted in <11% error, which is highlighted by the orange dots that are located on or very close to the trendline in the plot. The % error for the first restriction fragment (0.121 kbp) is greater than that of the other DNA fragments. This deviation is likely attributed to the data point for the early eluting fragment in the 1 kb Plus DNA Ladder, 0.1 kbp, which also resides far from the trendline.

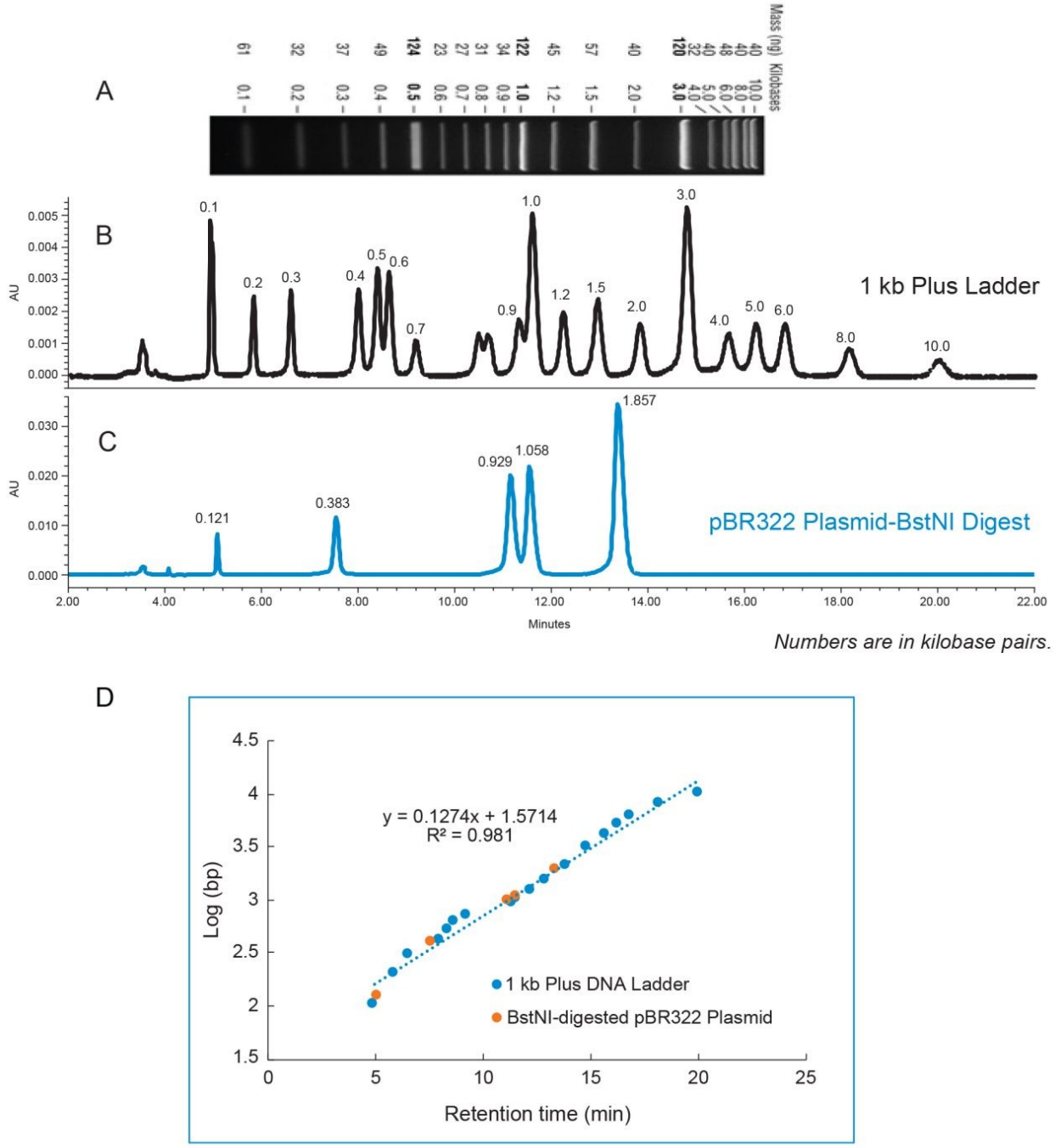

Figure 1. A) Agarose gel separation of 1 kb Plus DNA Ladder (reprinted from www.neb.com [2021] with permission from New England Biolabs); B) Anion-exchange separation of 1 kb Plus DNA Ladder on a Waters Protein-Pak Hi Res Q Column; C) Anion-exchange separation of DNA fragments resulted from BstNI-digested pBR322 plasmid on a Waters Protein-Pak Hi Res Q Column; D) A plot of log(bp) versus retention time of dsDNA fragments.

Conclusion

Anion-exchange chromatography is robust, reproducible, yields quantitative information, and is easy to be automated. We demonstrate here that a 1 kb Plus DNA Ladder, ranging from 0.1 kbp to 10.0 kbp dsDNA fragments, can be separated on a Waters Protein-Pak Hi Res Q Column. The AEX separation result is highly consistent with the agarose gel separation. The size of a dsDNA can be assessed by comparing the retention time of the dsDNA with that of the 1 kb Plus DNA Ladder. This method can be used, but not limited, to separate and assess the size of the DNA fragments obtained from the restriction enzyme digestion of a plasmid. Such an analysis can serve as an assay to demonstrate the presence and the identity of the plasmid throughout the manufacturing process of gene therapy products.

References

1. Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs), Guidance for Industry. US Food and Drug Administration (CBER), January 2020.
2. Kato, Y.; Sasaki, M.; Hashimoto, T. Separation of DNA Restriction Fragments by High-Performance Ion Exchange Chromatography. *J. Chromatogr*. 1983, 265, 342–346.
3. Kato, Y.; Yamasaki, Y.; Onaka, A.; Kitamura, T.; Hashimoto, T. Separation of DNA Restriction Fragments by High-Performance Ion Exchange Chromatography on a Non-Porous Ion Exchanger. *J. Chromatogr*. 1989, 478, 264–268.
4. Muller, W. Fractionation of DNA Restriction Fragments with Ion-Exchangers for High-Performance Liquid Chromatography. *Eur. J. Biochem*. 1986, 155, 203–212.
5. Shapiro, J. T.; Stannard, B. S.; Felsenfeld, G. Binding of Small Cations to Deoxyribonucleic Acid. Nucleotide Specificity. *Biochemistry* 1969, 8(8), 3233–3241.
6. Melchior, W. B. Jr.; Von Hippel, P. H. Alteration of the Relative Stability of dA-dT and dG-dC Base Pairs in DNA. *Proc. Nat. Acad. Sci. USA.* 1973, 70(2), 298–302.
7. Yang, H.; Koza, S.; Chen, W. Anion-Exchange Chromatography for Determining Empty and Full Capsid Contents in Adeno-Associated Virus. Waters Application Note, 720006825 < https://www.waters.com/nextgen/us/en/library/application-notes/2020/anion-exchange-chromatography-for-

determining-empty-and-full-capsid-contents-in-adeno-associated-virus.html> , 2021.

8. Yang, H.; Koza, S.; Chen, W. Plasmid Isoform Separation and Quantification by Anion-Exchange Chromatography (AEX). Waters Application Note, 720007207 < https://www.waters.com/nextgen/us/en/library/application-notes/2021/plasmid-isoform-separation-and-quantification-by-anion-exchange-chromatography-aex.html> , 2021.

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