Size Exclusion Chromatographic (SEC) Method for Oligonucleotides Using Heated Columns

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Introduction

Oligonucleotides are often analyzed by ion pairing reverse phase liquid chromatography (IPRP). While IPRP offers high resolution for most oligonucleotides, the identification of the peaks can be difficult as their elution times can be sequence or modification dependent, and secondary structures may exist depending on the method and column conditions. Herein, a size exclusion liquid chromatographic (SEC) method is presented as an alternate LC method for characterization studies. The difference of running small synthetic oligonucleotides and large mRNA samples at both 30°C and 80°C are shown. Possible controls and standards for oligonucleotide analysis are also presented.

Materials and Methods

Sample Preparation

The following vendors and corresponding samples were used: 1.) Phenomenex™: AL0-3042 and AL0-9253 containing Thyroglobulin, BSA, Myoglobin and Uridine 2.) Agilent®: DNA ladder standard part no. 5190-9029, RNA resolution standard part no. 5190-9028 3.) Thermo Scientific™: RiboRuler High Range RNA ladder part no. SM1821, RiboRuler Low Range RNA ladder part no. SM1831 and GeneRuler 100 bp Plus DNA Ladder SM0321 4.) IDT™: Single guide RNA samples (143 nt sg RNA) 10 nmol, reconstituted in 0.5 mL of RNase free water from Invitrogen part no. AM9937 5.) TriLink® Biologics: mRNA samples (EGFP mRNA 5moU, Fluc mRNA 5moU, beta gal mRNA 5moU and Cas9 mRNA 5moU), 100 µg were diluted 0.2x into RNase free water from Invitrogen part no. AM9937.

Figure 1. LC columns used in this study. (A) combo setup of Biozen™ dSEC-2 and dSEC-7 for screening studies, (B) dSEC-2 300 x 4.6 mm, (C) dSEC-7 300 x 4.6 mm, (D) Prototype column 50 x 2.1 mm column.

(A) Biozen™ dSEC-2 + Biozen™ dSEC-7
Part no.: 00F-4788-E0
150 x 4.6 mm, 3 µm, 200 Å 00F-4789-E0
150 x 4.6 mm, 3 µm, 700 Å
Surface Chemistries: Diol

(B) Biozen™ dSEC-2
Part no.: 00H-4788-E0
300 x 4.6, 3 µm 200 Å
Surface Chemistry: Diol

(C) Biozen™ dSEC-7
Part no.: 00H-4789-E0
300 x 4.6 mm, 3 µm 700 Å
Surface Chemistry: Diol

(E) Prototype column
50 x 2.1 mm, 3 µm

LC Conditions for SEC

Column: Biozen™ dSEC-2 or Biozen™ dSEC-7 or combo
Mobile Phase: 50 mM Ammonium Acetate or 2 x PBS
Flow rate: 0.35 or 0.5 mL/min
Injection Volume: 0.5 - 3 µL
Temperature: 30 or 80 °C
LC System: Thermo Vanquish Binary LC
Detection: UV 260 nm

LC Conditions for IPRP

Column: Prototype column
Mobile Phase: A1: 0.1 M TEAA pH 7
B1: 0.1 M TEAA in 50% Acetonitrile
Flow rate: 0.4 mL/min
Injection Volume: 0.5 – 3 µL
Temperature: 60 °C
LC System: Thermo Vanquish Binary LC
Detection: UV 260 nm

Results

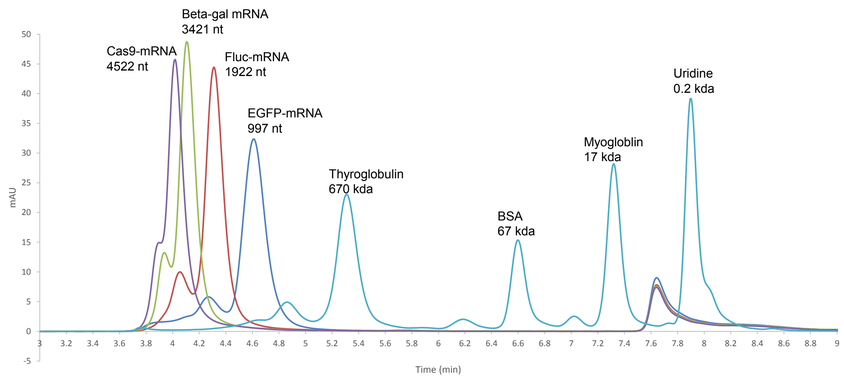


Figure 2. Size screening of mRNA samples utilizing a combo Biozen™ dSEC-2 + dSEC-7 column at 30 °C and a mobile phase of 2xPBS. Thyroglobulin, BSA, Myoglobin and Uridine were used as controls. The large aggregate of thyroglobulin was used as an indicator of the total exclusion volume (at 3.7 min). Uridine was used as an indicator of the total inclusion volume (at 7.9 min). Although EGFP-mRNA is ~350 kda, its hydrodynamic radius is larger than Thyroglobulin with a molecular weight of 670 kda. From this data, all further analysis of mRNA samples were performed on a 700 Å SEC column.

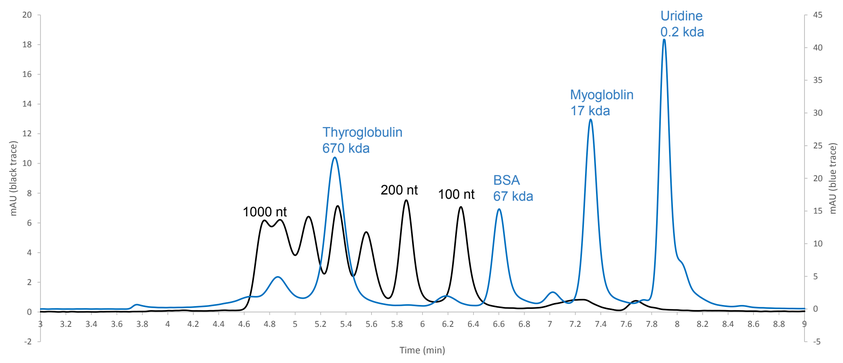


Figure 3. Size screening of oligonucleotides utilizing a combo Biozen™ dSEC-2 and dSEC-7 column at 30 °C and a mobile phase of 2xPBS. Analysis of the RiboRuler Low Range (Black) relative to protein standards (Blue) demonstrates that analysis of oligonucleotides smaller than 100 nt may be performed on a 200 Å SEC column.

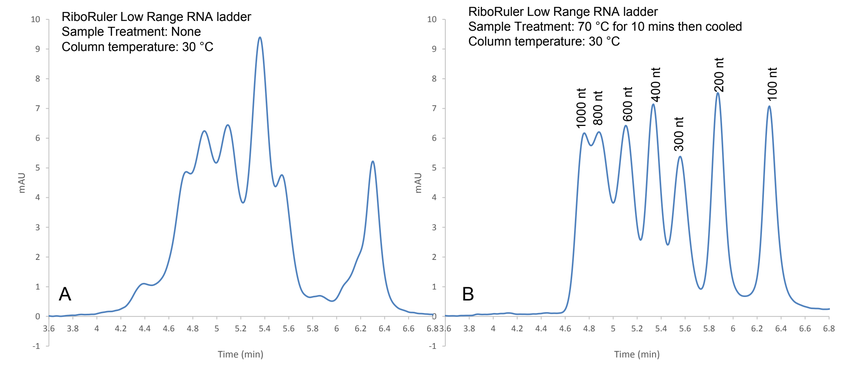


Figure 4. SEC chromatograms were obtained on a combo Biozen™ dSEC-2 + Biozen™ dSEC-7 column at 30 °C using a mobile phase of 2xPBS. When the RiboRuler Low Range RNA ladder sample was run without any treatment (A), the existence of various secondary and aggregated structures complicated the SEC chromatogram and the individual analytes in the ladder, e.g., 1000, 800, 600, 400, 200 and 100 nucleotides could not be identified. By pre-treating the RNA ladder at 70 °C for 10 min followed by cooling, the ladder became visible (B). This implied that the RNA ladders must be treated (heated and cooled) prior to SEC analysis.

Results

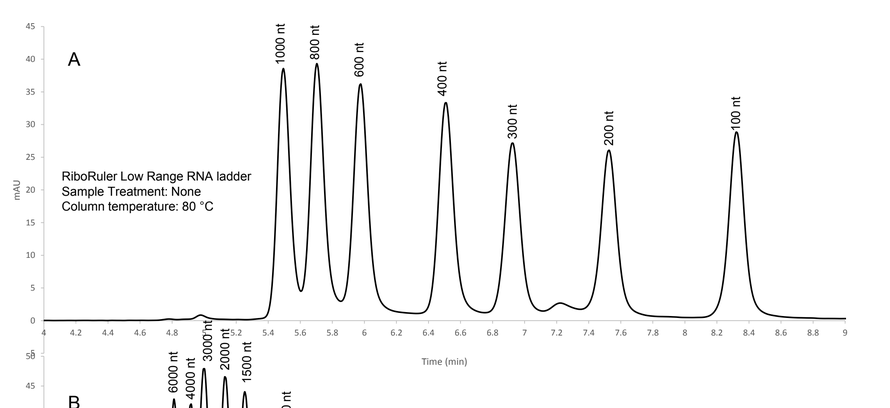


Figure 5. SEC chromatograms were obtained on a Biozen™ dSEC-7 at 80 °C using a mobile phase of 50 mM Ammonium Acetate pH 7. By running the SEC column at an elevated temperature, no sample pre-treatment was needed to allow the individual oligonucleotide species to be seen. This temperature was found to be sufficient for both the RiboRuler Low (A) and High (B) Range RNA ladders.

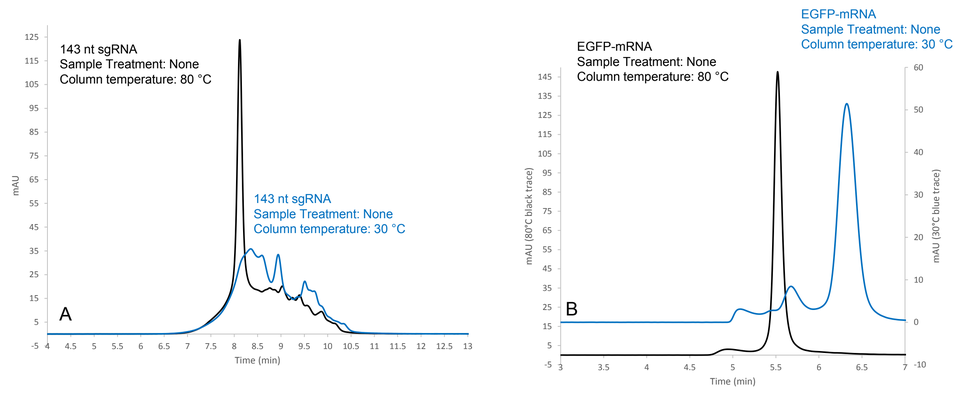
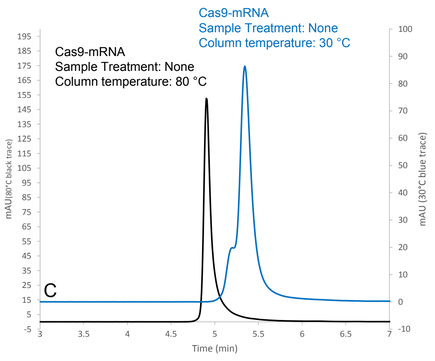


Figure 6. SEC chromatograms were obtained on a Biozen™ dSEC-7 using a mobile phase of 50 mM Ammonium Acetate pH 7. (A) 143 nucleotide single guide RNA sample was analyzed at two column temperatures 80 °C (black) and 30 °C (blue). (B) EGFP-mRNA sample was analyzed at two column temperatures 80 °C (black) and 30 °C (blue). (C) Cas9-mRNA sample was analyzed at two column temperatures 80 °C (black) and 30 °C (blue). For all samples, at 30 °C, there are more aggregates and secondary structures observed. At 80 °C, these forms appear to collapse into a primary dominant monomeric form.



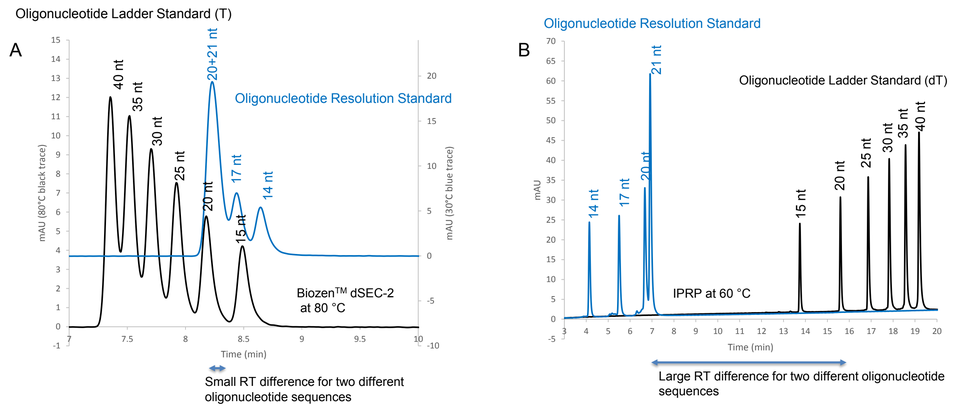


Figure 7. Comparison of Oligonucleotide Ladder Standard (T) and Oligonucleotide Resolution Standard shows that by SEC (A), the retention time is less sequence dependent than by IPRP (B).

Discussion and Conclusion

For the SEC analysis of oligonucleotides, the temperature was found to be an important factor in both sample pre-treatment and on-column analysis as shown in **Figures 4, 5** and **6**. Heating the SEC column reduces the need for offline sample pre-treatment and greatly reduces the assumptions regarding the secondary structures the analytes may take on during the cooling process. Due to the negative backbone of the oligonucleotides, low buffering capacity and low salt mobile phases may be used on the diol-based Biozen™ dSEC-2 and dSEC-7 SEC columns to achieve high efficiency separations. Comparison of **Figures 2** and **6** demonstrates that the size variants observed in both EGFP-mRNA and Cas9-mRNA are similar in both 2xPBS and 50 mM Ammonium Acetate mobile phases. The use of 50 mM Ammonium Acetate buffer (**Figures 5, 6** and **7**) may be interfaced directly with a mass spectrometer for an additional layer of identification and quantitative analysis. The reduced sequence and modification dependence by SEC as shown in **Figure 7** also offers an additional modality for identification purposes to support IPRP studies. When high buffering and high salt mobile phases are used, well characterized protein standards and controls may be used to assist in analytical method development. Additionally, RNA ladders (developed for gel analysis) may also be used but they must either be pre-treated prior to SEC analysis or run at an elevated column temperature to reduce the amount of secondary and aggregate structures they may acquire from manufacturing, storage or transport.

This study demonstrated oligonucleotide and mRNA sample analysis at both 30 and 80 °C on the Biozen™ dSEC-2 and dSEC-7 columns. Stability and life-time studies are in-progress at these novel conditions. Current experiments, spanning approximately 1 month, and >100 injections have shown good reproducibility in both peak area recovery and retention time.

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