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Size and Purity Assessment of Single-Guide RNAs by Anion-Exchange Chromatography (AEX)

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Abstract

Single-guide RNA (sgRNA) is a critical element in the CRISPR/Cas9 Technology for gene editing, the size of which usually ranges from 100 to 150 bases. In this application note, we show that the size of several sgRNAs could be estimated by comparison to a Low Range ssRNA Ladder (50–500 bases) using an optimized anion-exchange method developed on a Waters Protein-Pak Hi Res Q Column. In addition, the purity of the sgRNA samples can be assessed using the same anion exchange method, providing an informative and non-complex method for sgRNA product consistency.

Benefits

- Waters Protein-Pak Hi Res Q Column separation of a Low Range ssRNA Ladder with the size ranging from 50 to 500 bases
- Waters Protein-Pak Hi Res Q Column separation of ssRNAs and their impurities
- Size and purity estimation of ssRNAs having a size range of 100–150 mer under the same gradient conditions using the AEX method on Waters Protein-Pak Hi Res Q Column

Introduction

The discovery of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) bacterial immunity systems and the rapid adaptation of RNA guided CRISPR/CRISPR Associated Protein 9 (Cas9) Technology to mammalian cells have had a significant impact in the field of gene editing.[1–3] The Cas9 protein, a non-specific endonuclease, is directed to a specific DNA site by a guide RNA (gRNA), where it makes a double-strand break of the DNA of interest. The gRNA consists of two parts: CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The crRNA is usually a 17–20 nucleotide sequence complementary to the target DNA, and the tracrRNA serves as a binding scaffold for the Cas9 nuclease. While crRNAs and tracrRNAs exist as two separate RNA molecules in nature, the single-guide RNA (sgRNA), which combines both the crRNA sequence and the tracrRNA sequence into a single RNA molecule, has become a commonly used format. The length of a sgRNA is in the range of 100–150 nucleotides. It is critical to characterize the sgRNA, as it is the core of the CRISPR/Cas9 technology.

Anion-exchange chromatography (AEX) separates molecules based on their differences in negative surface charges. This analytical technique can be robust, reproducible, and quantitative. It is also easy to automate, requires small amounts of sample, and allows for the isolation of fractions for further analysis. AEX has been utilized in multiple areas related to gene therapy, including adeno-associated virus empty and full capsid separation, plasmid isoform separation, and dsDNA fragment separation.[4–6] Since the sgRNAs are negatively charged due to the phosphate groups on the backbone, we investigated AEX for size and purity assessment of sgRNAs.

In this application note, we show that using a Waters Protein-Pak Hi Res Q strong Anion-Exchange Column on an ACQUITY UPLC H-Class Bio System, a single-stranded RNA (ssRNA) ladder ranging from 50 to 500 bases can be separated and used for estimating the size of ssRNAs in the approximate range of 100–150 bases, including the sgRNAs for CRISPR/Cas9 System. Moreover, the purity of these ssRNAs can be estimated with the same gradient conditions.

Experimental

Sample Description

HPRT (purified and crude) is a pre-designed CRISPR/Cas9 sgRNA (Hs.Cas9.HPRT1.1AA, 100 mer). GUAC is a customized ssRNA (150 mer), which contains repeats of GUAC sequence. HPRT sgRNA and GUAC ssRNA were purchased from Integrated DNA Technologies (IDT). Rosa26 and Scrambled #2 are both pre-designed CRISPR/Cas9 sgRNAs purchased from Synthego (100 mer). Low Range ssRNA Ladder was purchased from New England Biolabs (N0364S).

Method Conditions

LC Conditions

LC system:	ACQUITY UPLC H-Class Bio
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.:	60 °C
Sample temp.:	10 °C
Injection volume:	1–10 µL
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 (an AutoBlend Plus Method, Henderson-Hasselbalch derived).

Time (min)	Flow (mL/min)	pH	Salt (mM)	Salt curve
0	0.4	9.0	0	
5	0.4	9.0	1400	11
9	0.4	9.0	1400	11
29	0.4	9.0	2100	6
31	0.4	9.0	2400	6
33	0.4	9.0	2400	6
33.1	0.4	9.0	0	11
45	0	9.0	0	11

In the above gradient table, the buffer is 20 mM Tris pH 9.0. The initial salt concentration is set to 0 mM to ensure all the analytes are strongly bound onto the column. After 5 mins, the salt concentration is increased to 1400 mM where most of the impurities will elute, based on prior investigation. After 4 mins equilibration, the separation gradient starts. The salt concentration increases linearly from 1400 m to 2100 mM in 20 mins for the Low Range ssRNA Ladder separation, as well as individual ssRNAs. Then it is ramped up to 2400 mM 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	3.3	16.7	0.0	80.0
5	3.3	16.7	46.7	33.3
9	3.3	16.7	46.7	33.3
29	3.3	16.7	70.0	10.0
31	3.3	16.7	80.0	0.0
33	3.3	16.7	80.0	0.0
33.1	3.3	16.7	0.0	80.0
45	3.3	16.7	0.0	80.0

Data Management

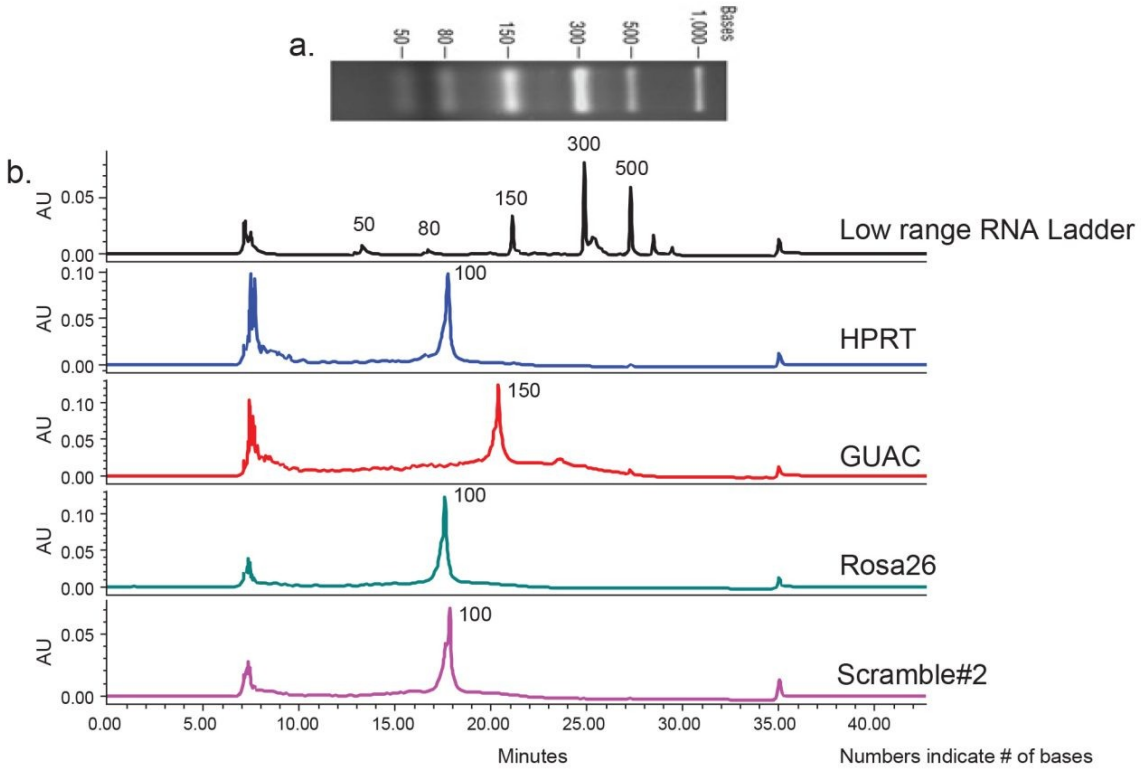
Chromatography software: Empower 3 (FR 4)

Results and Discussion

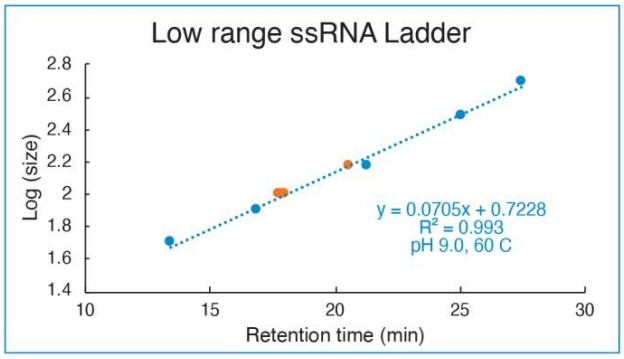
Size Assessment

Various mobile phase conditions were tested using a Low Range ssRNA Ladder for size assessment of the ssRNAs, including pH (7.4 and 9.0), column temperature (30 °C and 60 °C) and salt (NaCl and TMAC).

The results from the optimal conditions are shown in Figure 1B. Using a pH 9.0 Tris buffer with 60 °C column temperature and a TMAC salt gradient, the Low Range ssRNA Ladder (50–500 bases) along with four pre-made sgRNAs (100 mer), and one customized ssRNA (150 mer) were separated on a Waters Protein-Pak Hi Res Q Column. The separation for the Low Range ssRNA Ladder on this strong anion exchange column was very similar to that on an agarose gel, as shown in Figure 1A. A calibration curve was constructed based on the retention time and the logarithm of the number of bases of each ssRNA in the ladder (Figure 1C, blue dots). The linear fit from the Low Range ssRNA Ladder indicates a strong correlation between the logarithm of the size and the retention time (R^2=0.993). Using this plot, the size of the ssRNAs was calculated from their individual retention time. The percent error is calculated using the formula {(calculated size – theoretical size)/theoretical size}. The percent error was less than 6% for all the RNAs tested (Figure 1d), as evidenced by the orange data points residing on or very closely to the trendline of the calibration curve. Notice that small percent error was obtained from four pre-made sgRNAs from two different manufacturers and a customized ssRNA with an artificial sequence. Although ssRNAs with shorter than 100 bases and larger than 150 bases were not tested, it is possible that this method can be used for the ssRNAs size assessment in the range of 50–500 bases.



c.



d.

	Theoretical size (mer)	Calculated size (mer)	%error
HPRT	100	97	-3.28%
GUAC	150	149	-0.99%
Rosa26	100	94	-5.82%
Scramble#2	100	98	-1.85%

Figure 1A. Agarose gel separation of Low Range ssRNA Ladder (Reprinted from www.neb.com (2021) with

permission from New England Biolabs); 1B. Anion-exchange separation of Low Range ssRNA Ladder and ssRNAs on a Waters Protein-Pak Hi Res Q Column; 1C. A plot of log(size) vs. retention time of Low Range ssRNA Ladder (blue dots) and individual ssRNAs (orange dots); 1D. Size estimation of individual ssRNAs based on retention time and calibration curve. Small percent error was obtained for all ssRNAs.

It is noteworthy that a mobile phase condition with pH 7.4 Tris buffer, 60 °C column temperature and a TMAC salt gradient also resulted in good size estimation with percent error <5% for all pre-made sgRNAs (100 mer) and ~12% for the artificially made GUAC ssRNA (150 mer). Overall, 60 °C column temperature resulted in one single peak for each ssRNA which is needed to determine the retention time of the peak for size assessment. 30 °C column temperature resulted in more than one major peaks, which are presumably the isomers of the ssRNAs. Multiple peaks were also observed when using NaCl as the salt, regardless of the pH and column temperature.

Purity Assessment

Purified and crude HPRT sgRNA was separated on the Protein-Pak Hi Res Q Column (Figure 2) using the same gradient conditions for size assessment. The relative purities of the crude and purified samples were measured as 37.4% and 88.0%, respectively, based on the peak areas indicated. The majority of the impurities eluted prior to 50 bases although lower abundance impurities appear to be present up to the size of the HPRT sgRNA.

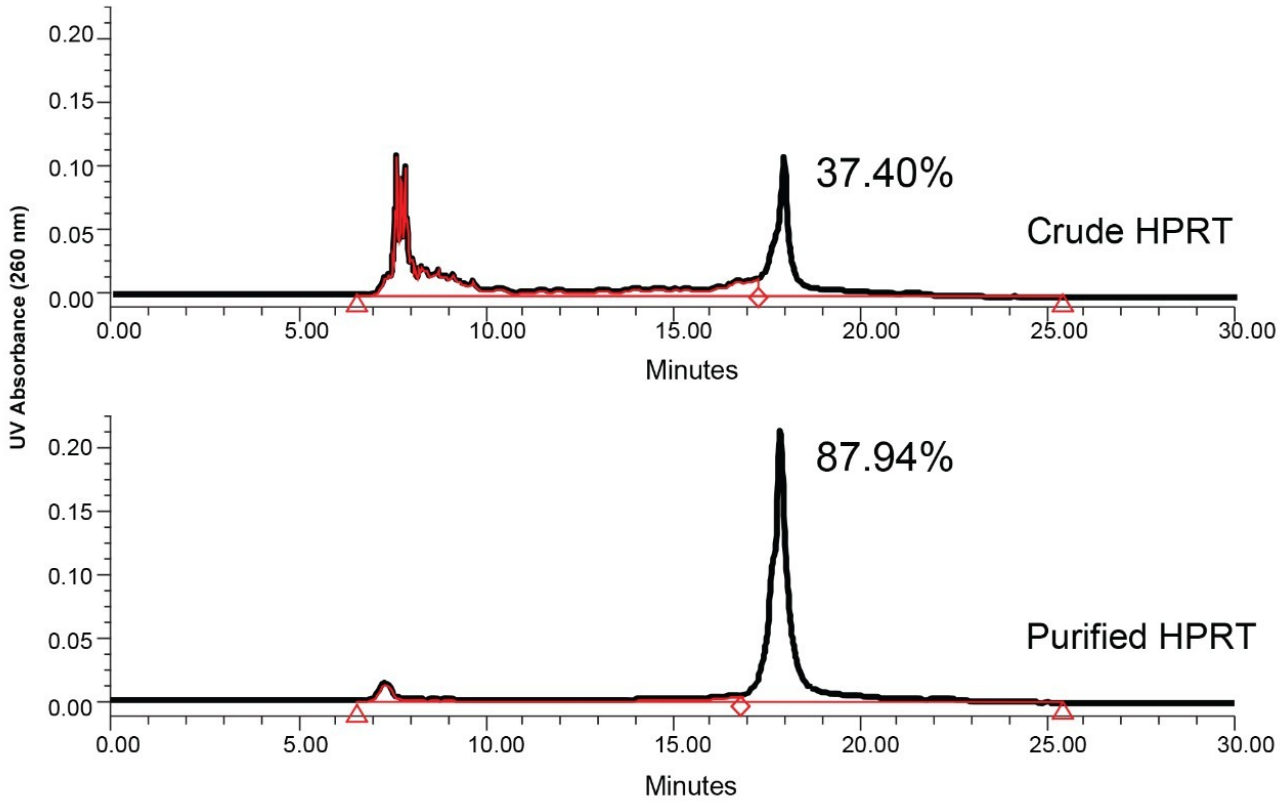


Figure 2. Crude and purified HPRT sgRNA for CRISPR/Cas 9 System were separated on a Waters Protein-Pak Hi Res Q Column using the same conditions as in Figure 1B (see Experimental for details).

Conclusion

Anion-exchange chromatography is robust, reproducible, easy to automate, yields quantitative information, and requires a small amount of sample. We demonstrate here that the components of a Low Range ssRNA Ladder, ranging from 50 to 500 bases, can be separated on a Waters Protein-Pak Hi Res Q Column with a linear correlation between the log of base-number and observed retention time when TMAC is used as an elution salt. The size of ssRNAs ranging from 100 to 150 bases can be estimated by comparing the retention time of the ssRNAs with that of the Low Range ssRNA Ladder. In addition, the purity of a sgRNAs may also be observed from the same chromatographic separation. This method can potentially be applied to the analysis of sgRNAs

which are the key element for CRISPR/Cas9 gene editing technology.

References

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