High Throughput Analysis of CRISPR sgRNA using Ultrashort Ion Pairing Reversed Phase Column Chromatography and High-Resolution Mass Spectrometry

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Background & Introduction

In the age of FDA approved CRISPR based therapeutics, high throughput (HT) and reliable analytical methods are more important than ever. CRISPR gene editing requires two components, a CRISPR Associated Protein (Cas) and a single guide RNA (sgRNA), which together form a ribonucleoprotein (RNP) complex. These RNPs are used to edit genomes by targeting sequences of DNA, making a single strand cut, and inserting a new sequence. Methods for analyzing RNA by ion-pairing reversed-phase liquid chromatography (IP-RPLC) with high-resolution mass spectrometry (HRMS) exist but tend to use analytical columns that are ≥ 100 mm and require ≥ 30 min run times. We developed an IP-RPLC-HRMS method by scaling a published 30-min method to a HT 5-min method. We utilized hexafluoroisopropanol (HFIP) and N,N-diisopropylethylamine (DIPEA) our preferred mobile phase additives.

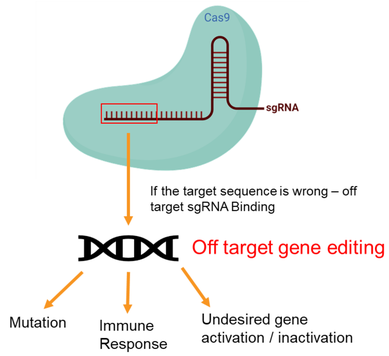

Figure 1 (Left). CRISPR requires a Cas enzyme and sgRNA component to form an RNP and edit genes. sgRNA forms a complex with Cas and guides the complex to the target gene. The Cas enzyme cuts the target DNA to enable gene editing. Rapid analysis of the sgRNA is important to prevent off target sgRNA binding and downstream off target gene editing.

Figure 1. CRISPR Complexes and off target gene editing.

Methods

Table 1. Initial Method Parameters for IP-RPLC

System	Waters ACQUITY™ H-Class Bio System equipped with Flow-Through Needle (FTN) and Phodotiode Array (PDA)		
Columns	ACQUITY Premier Oligonucleotide BEH C18 Column 2.1 × 100 mm, P/N: 186010540 ACQUITY Premier Oligonucleotide BEH C18 Column 2.1 × 20 mm, P/N: 186011021		
High Resolution Mass Spectrometer (HRMS)	Waters Xevo™ G2 XS QTof Mass Spectrometer		
Mobile Phase A	0.1% DIPEA, 1% HFIP in Water		
Mobile Phase B	0.0375% DIPEA, 0.075% HFIP in 65:35 ACN:Water (v:v)		
Gradient	Time (min)	%A	%B
	0	97	3
	13	45	55
	15	5	95
	17	97	3
	30	97	3

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Ion-Pairing Reverse Phase Liquid Chromatography (IP-RPLC)

IP-RPLC using HFIP as an ion pair agent and DIPEA to achieve a slightly basic pH enables effective separation of highly negatively charged nucleic acids. Both HFIP and DIPEA are volatile additives and allow for detection by mass spectrometry. **Table 1** shows initial parameters for IP-RPLC (prior to scale-down and optimization).

Scale-down from 100-mm to 20-mm UHPLC column

Durations for gradients were reduced five-fold as a starting point and then further optimized. A systematic approach was taken: (1) directly transferring the method from the 100- to the 20-mm column, (2) scaling the reequilibration times, (3) scaling duration for all gradient steps and (4) optimizing chromatography.

High Resolution Mass Spectrometry. An ESI source was used in negative mode. Data were collected from *m/z* 500 to 7,000. Collision energy of 6 eV, cone voltage of 40 V, capillary voltage of 2 kV were used. The source temp was 100°C, desolvation temp 650°C, cone gas flow 50 L/h and desolvation gas flow 1,200 L/h.

Forced Degradation. Samples were degraded with 0.1 N HCl to determine the suitability of applying ultrashort columns and HT methods for stability-indicating measurements.

Results – Scale-down, Forced Degradation, & HRMS

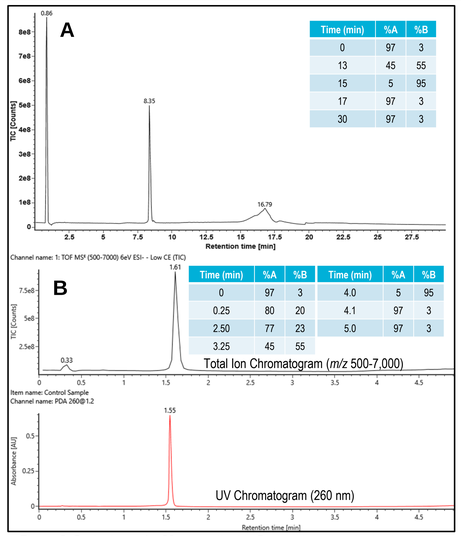


Figure 2. Scale-down of a 30 min method to 5 min using ultra-short columns.

Figure 2 (left) shows the systematic approach used for scaling to a HT method.

(A) The original method, run on a 100-mm column, employs gradient elution at a rate of 4%B per min. The 100mer sgRNA elutes ~8.4 min in the middle of the gradient, with approximately 37% mobile phase B. The gradient is held at 95% mobile phase B for 2 minutes, and the column is reequilibrated to initial conditions for 13 minutes.

(B). Final scaled gradient program. The gradient is rapidly ramped from 3 to 20% B over 0.25 minutes, enabling a slower ramp rate of 1.3% B per min during the elution window of the same 100mer sgRNA used for development throughout the project. With this high-throughput method, the sample elutes at ~22% mobile phase B.

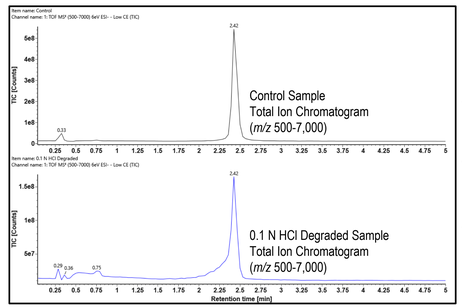


Figure 3. Comparison of control and degraded samples.

Figure 3 (left) shows representative total ion chromatograms (TICs) for control and degraded sgRNA methods. A decrease in relative intensity in the main peak is observed, as well as multiple degradants eluting ahead of the main peak.

Figure 4 shows representative HRMS data. (A) shows a raw spectrum collected from the main sgRNA peak from *m/z* 500-7,000. (B) shows the spectrum deconvoluted using MaxEnt1 to give the average neutral mass of the intact sgRNA.

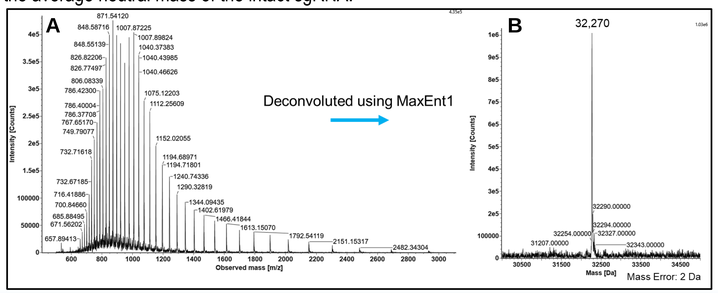


Figure 4. Representative HRMS raw and deconvoluted spectra for intact sgRNA.

Results – Comparison of Cleavage Chemistries for IVT

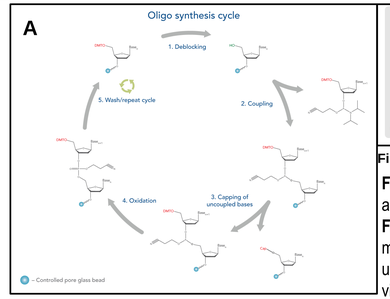
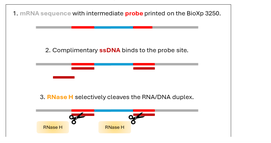


Figure 5. Methods for producing oligonucleotides.

Figure 5 shows **(A)** the cycle for chemical oligo synthesis and **(B)** in vitro transcription (IVT).

Figure 6 (below) shows two methods of IVT-synthesized mRNA cleavage to form sgRNA. The 5-min method can be used to evaluate purity of sgRNA synthesized and cleaved via different methods.

Method 1: RNase H cleaves mRNA synthesized via IVT at sites where ssDNA probe is bound



COMPONENTS	100 µl REACTION
RNA:DNA duplex	2 µg
RNase H Reaction Buffer(10X)	10 µl (1X)
RNase H	1 µl (5 units)
Nuclease-free H_2O (NEB#S1500S)	up to 100 µl

Method 2: mRNA synthesized via IVT is self-cleaved at specific sites by self-cleaving ribozyme

Gao, Y.; Zhao, Y. Self-Processing of Ribozyme-Flanked RNAs into Guide RNAs in Vitro and in Vivo for CRISPR-Mediated Genome Editing. *Journal of Integrative Plant Biology* **2014**, *56* (4), 343–349. https://doi.org/10.1111/jipb.12152.

https://www.neb.com/en-us/protocols/2019/07/30/protocol-for-cleavage-with-rnase-h-neb-m0297?pdf=true

Figure 6. Methods for cleavage of IVT-synthesized mRNA to form sgRNA for CRISPR applications.

Figure 7 shows **(A)** overlaid TICs for comparison of two methods for cleavage chemistry of mRNA to generate sgRNA and **(B)** comparison of deconvoluted spectra for sgRNAs from (A)

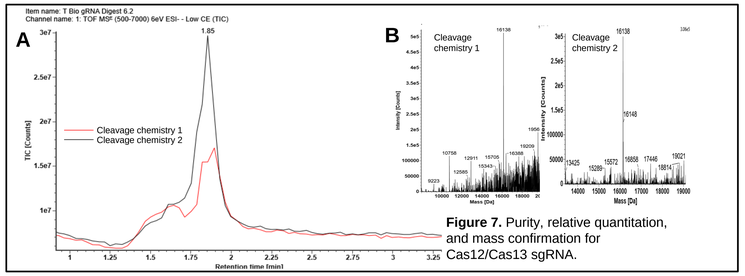


Figure 7. Purity, relative quantitation, and mass confirmation for Cas12/Cas13 sgRNA.

Conclusions

- Our lab has demonstrated the feasibility of rapidly obtaining information about sgRNA quality using IP-RPLC with HRMS, a readily adoptable approach that can be achieved by scaling existing methods and potentially be applied as a stability-indicating assay.
- We have shown the utility of our method for comparing IVT methods for sgRNA synthesis.
- Continued development of rapid chromatographic separations will be important for decreasing manufacturing time and cost for CRISPR-edited gene and cell therapies.

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