INVESTIGATING CYCLIC ION MOBILITY AS A MEANS TO BOOST COVERAGE IN THE TOP-DOWN SEQUENCING OF SYNTHETIC GUIDE RNAS

Catherine Tremblay[1], Chris Knowles[2], Michael Ruehl[3], Christopher Gawlig[3], James I. Langridge[2], Dale A. Cooper-Shepherd[2]
1. Waters Corporation, 34 Maple Drive, Milford, MA, USA. 2. Waters Corporation, Stamford Ave, Altrincham Road, Wilmslow, SK9 4AX, UK. 3. BioSpring GmbH, Alt-Fechenheim 34, 60386 Frankfurt am Main, DE.

OVERVIEW

- **We present investigations into the utility of ion mobility separation in the top-down sequencing of synthetic guide RNAs (sgRNAs)**
- **sgRNAs are oligonucleotide products of significant interest in the cell and gene therapy field**
- **Sequencing can be laborious and rely on endonuclease digestion and long LC-MS experiments for mapping**
- **Top-down sequencing offers the potential of a rapid product characterization without the need for lengthy sample preparation**
- **The SELECT SERIES™ Cyclic™ IMS instrument has a unique geometry enabling ion mobility separation of top-down product ions which has been shown to increase spectrum resolution and increase sequence coverage for proteins**
- **Here we demonstrate that cyclic IMS boosts sequence coverage in top-down sequencing of 100mer sgRNA**

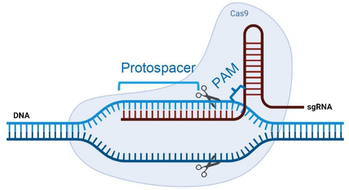


Figure 1. A schematic representation of a CRISPR-Cas9 ribonucleoprotein complex with a guide RNA (sgRNA) bound to both the complementary DNA and the Cas9 protein. Created with BioRender.com

The arrival of CRISPR-based therapies is causing significant investment in oligonucleotide research and development. Guide RNAs (gRNAs) are central to the CRISPR-Cas9 system and act to recruit the Cas9 nuclease to cut at specific, gRNA-directed DNA sequences for the purposes of gene editing. 'Single' or 'synthetic' guide RNAs (sgRNAs) combine the crRNA and tracrRNA portions of gRNA into a single sequence which can be produced by solid phase synthesis. Like any therapeutic-related product, the sgRNA structure must be characterized, in this case by virtue of the nucleotide sequence.

Oligo-mapping is the current go-to method for confirming nucleotide sequences, but top-down sequencing is of interest due to its rapid nature and its minimal sample preparation requirements.

Here we investigate the use of cyclic ion mobility separation of top-down fragmented RNA product ions to boost sequence coverage of sgRNAs. This is a principle that has already been demonstrated for proteins fragmented by CID[1] and ECD[2]. The unique geometry of the SELECT SERIES Cyclic IMS instrument, the high performance of the ion mobility device and the superior resolving power of the time-of-flight analyzer (100,000 FWHM) offer exciting potential for this application

METHODS

Four different RNAs were studied in this work, the full length sgRNA (100mer) and three shorter oligos consisting of the 5' end 25, 50 and 75 nucleotides, (25mer, 50mer and 75mer).

All experiments were performed on a SELECT SERIES Cyclic IMS mass spectrometer in single pass mode. Test RNA samples were separated using an ACQUITY™ UPLC™ Premier chromatography system equipped with an ACQUITY UPLC Oligonucleotide BEH™, 130 Å, C18 1.7 µm 2.1 × 100 mm column operated at 60 °C. Mobile phase A was 7 mM triethylamine (TEA) and 80 mM hexafluoroisopropanol (HFIP) in water, mobile phase B was 3.5 mM TEA and 40 mM HFIP in 50/50 methanol:water. Experiments were performed in MS or HDMSMS mode at various trap collision voltages to assess the information content obtained. Ion mobility-MS data were manually extracted using Driftscope™ v3.0 and imported into a development version of the CONFIRM sequence app in the waters_connect™ suite of software. The sequencing data shown are from direct infusion HDMSMS experiments of a single charge state precursor as indicated. Trap CEs were ramped 20-27 V for the 25- and 50mer and 20-30 V and 25-37 V for the 75– and 100mer, respectively. CONFIRM parameters were as follows—mass error, 15 ppm; intensity cutoff, 10 counts; isotope intensity cutoff, 70 %; isotope similarity cutoff, 70 %. **Only terminal product ions were matched during data processing, no internal fragments were included.**

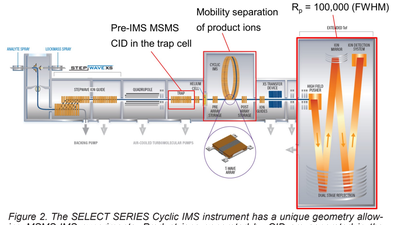


Figure 2. The SELECT SERIES Cyclic IMS instrument has a unique geometry allowing MSMS-IMS experiments. Product ions generated by CID are separated in the cyclic device to decrease spectrum crowding and aiding in assignment.

HIGH RESOLUTION INTACT MS USING W MODE

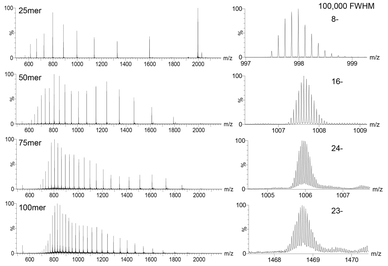


Figure 3. Intact spectra of the four oligos acquired by IP-RPLC-MS using W mode. The 25mer, 50mer, 75mer, and 100mer. The spectra demonstrate the high resolving power capability of the SELECT SERIES Cyclic IMS instrument at 100,000 FWHM.

UNIQUE INSTRUMENT GEOMETRY TO PERFORM TOP-DOWN MS

Top-down fragmentation spectra are highly congested. As a result there has been a desire for higher resolving power mass analyzers, namely FTICR. That said, FT-based analyzers can be prohibitively expensive and slow. Rapid ion mobility experiments are therefore an attractive option to potentially increase resolving power, decreasing spectrum congestion by separating product ions by virtue of their ion mobilities. The principle of using ion mobility to increase coverage in top-down protein sequencing has been demonstrated several times over and for both CID[1] and ECD[2] data for protein sequencing.

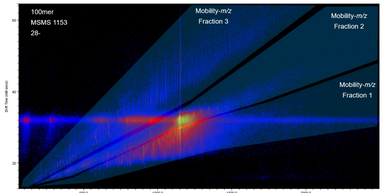


Figure 4. Ion mobility product ion spectrum for the 28– charge state of the 100mer sgRNA. The product ions separate according to their mobility into charge series. These series are referred to as fractions, which can be extracted and processed individually to determine the coverage.

Ion mobility mass spectra for all oligos were acquired in single pass ion mobility mode. Two charge states were assessed per oligo and the best spectrum taken forward. The ion mobility-mass spectra from the MSMS experiments were divided into 3-5 mobility fractions (Figure 4) and processed in a development version of CONFIRM sequence. The congestion of the spectrum for the 100mer can be seen in Figure 5. The individual mobility fractions have much fewer ions, reducing signal overlap and making assignment easier.

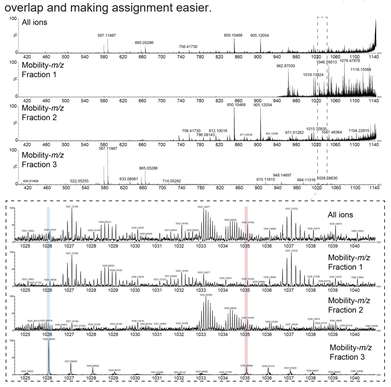


Figure 5. Top—spectrum of all product ions for the 28– precursor of the 100mer sgRNA. Lower 3 panels show spectra from the individual mobility fractions. Bottom (dashed line box) - zoomed region as indicated showing reduction in spectrum congestion in the mobility fractions relative to the 'all ions' spectrum.

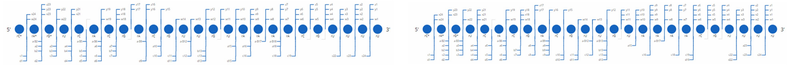
IMPROVED SEQUENCE COVERAGE USING ION MOBILITY

The coverage maps for the 25-, 50-, 75– and 100mer oligos are shown in Figure 6. **Note that terminal fragments only are matched in these data**. The maps on the left are for data processed without the mobility dimension. The maps on the right are the combined coverage for all mobility fractions for each RNA. For the 25mer, the shortest RNA, the number of matched terminal product ions increased from 101 without mobility to 111 with mobility. 100 % coverage was obtained in both cases. This is unsurprising since the 25mer generates a smaller number of potential fragments relative to larger RNAs, meaning spectrum congestion is low, and mobility does not add a large benefit. For the 50mer the number of matched product ions increases from 125 without mobility to 146 with mobility. The coverage increases from **96 % to 100 %** showing the benefit of adding mobility, but albeit with a modest improvement. For the 75mer, the number of matched product ions increases from 135 without mobility to 186 with mobility, increasing the coverage from **67 % to 96 %, a very significant improvement**. For the 100mer, a modest 50 % coverage was obtained with 134 ions without mobility. **With mobility the coverage increased to 74 %** another significant improvement.

These data clearly demonstrate the benefit of ion mobility separation of product ions for increasing coverage in top-down sequencing of sgRNA. Moreover, it is clear that the largest improvement in coverage is observed for the longer RNAs, indicating that the more congested the spectrum, the greater the benefit of mobility.

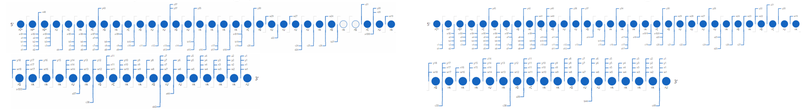
25mer 10-

MS only – **100 % coverage, 101 ions** | IMS on – **100 % coverage, 111 ions**



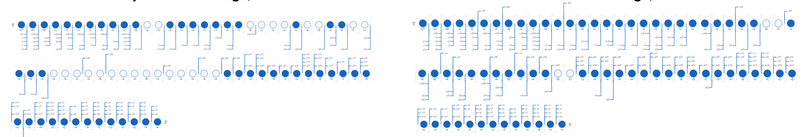
50mer 19-

MS only – **96 % coverage, 125 ions** | IMS on – **100 % coverage, 146 ions**



75mer 25-

MS only – **67 % coverage, 135 ions** | IMS on – **95 % coverage, 186 ions**



100mer 28-

MS only – **50 % coverage, 134 ions** | IMS on – **74 % coverage, 172 ions**

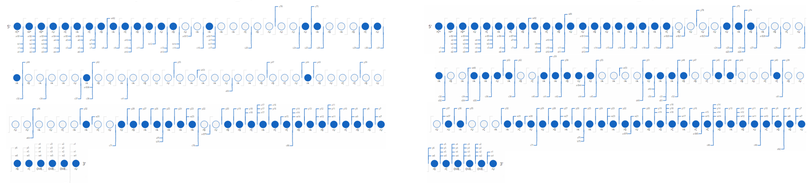


Figure 6. Coverage maps from the CONFIRM sequence application in waters_connect. Left—results from MS only data processing. Right—results from data processing with prior mobility fractionation.

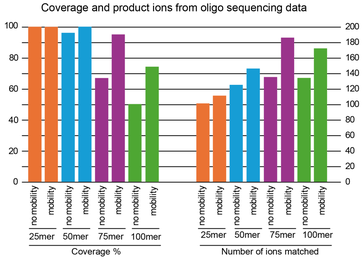


Figure 7. Bar graph showing the increase in the coverage and number of matched terminal ions for the different lengths of RNA.

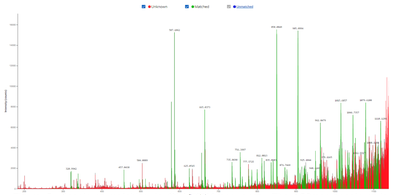


Figure 8. Spectrum matching in CONFIRM sequence. The 100mer spectrum is shown. Matches are done on the m/z spectrum directly. Green signals are those that are matched. Red are those unmatched, potentially internal fragments which could further increase coverage.

CONCLUSION

- **Ion mobility is shown to increase the sequence coverage obtained in top-down fragmentation experiments of RNAs**
- **Ion mobility reduces spectrum congestion making assignments easier for processing software**
- **Ion mobility has the greatest benefit with larger RNAs where spectrum congestion is most significant**
- **The unique geometry of the SELECT SERIES Cyclic IMS mass spectrometer (with quadrupole prior to IMS) enables these powerful experiments**
- **The high performing time-of-flight mass analyzer capable of R_p = 100,000 FWHM further adds to the excellent data quality obtained**
- **Utilizing internal fragment matches in the future could further boost sequence coverage of sgRNA products**

SELECT SERIES, Cyclic, ACQUITY, UPLC, BEH, Driftscope, and waters_connect are all trademarks of Waters Technologies Corporation.

References

1. Avtonomov, D. M. Polasky, D. A. Ruotolo, B. T. Nesvizhskii, A. I. *Anal. Chem.* **2017**, 90, 3, 2369-2375
2. Shaw, J. B. Cooper-Shepherd, D. A. Hewitt, D. Wildgoose, J. L. Beckman, J. S. Langridge, J. I. Voinov, V. G. *Anal. Chem.* **2022**, 94, 9, 3888-3896.

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS