

Development of a Rapid and Automated, Compliance Ready Workflow for Full Sequence Coverage of RNAi Therapeutics

CASSS Mass Spec 2023

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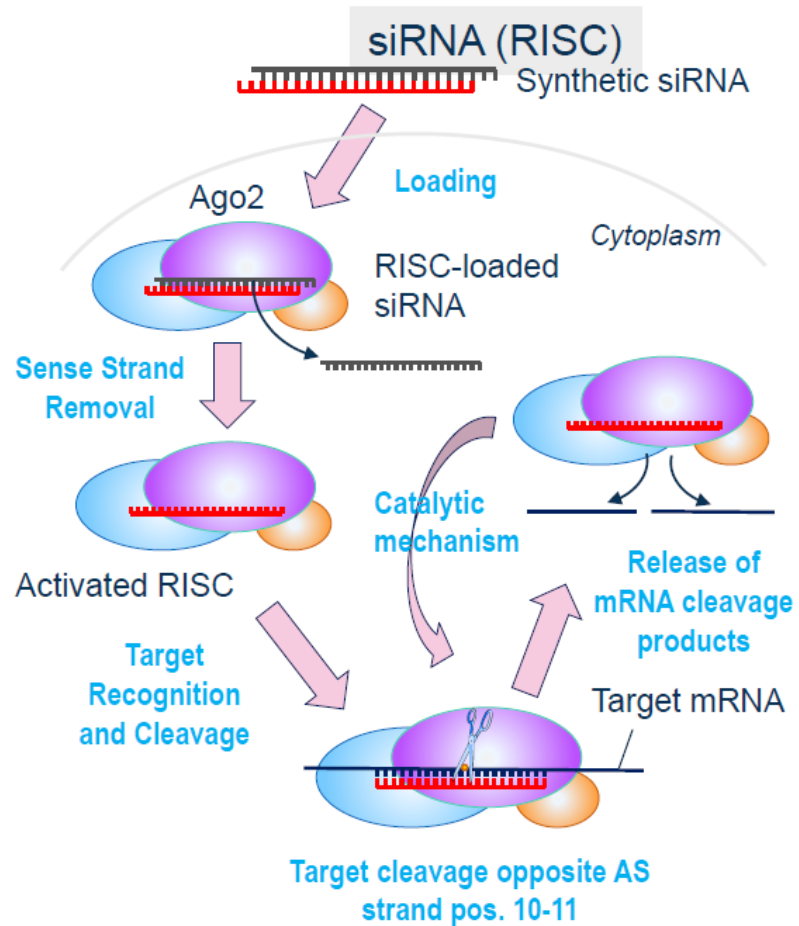
|| Agenda

- Background
 - Antisense and RNAi
 - ICH (and CMC) Specifications and Considerations
 - Current methods and workflows
- Software and Data Processing Options
- Method Considerations
- Considerations for Implementation into GMP / QC Environment
- Summary and Future Work

RNAi Pathway and siRNA Evolution

From the Liver to the CNS

	siRNA
Mechanism of Action	RISC
Target	mRNA
Location of Engagement	Cytoplasm
Nature of Interaction	Catalytic

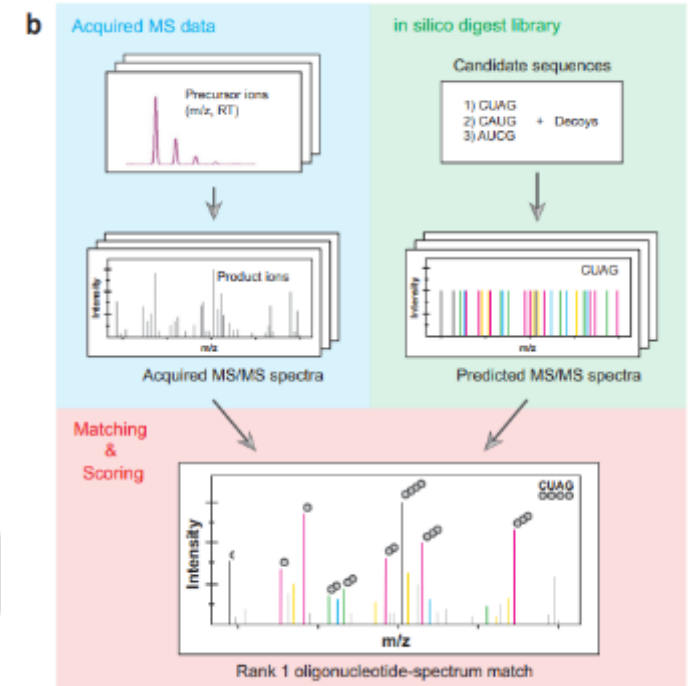
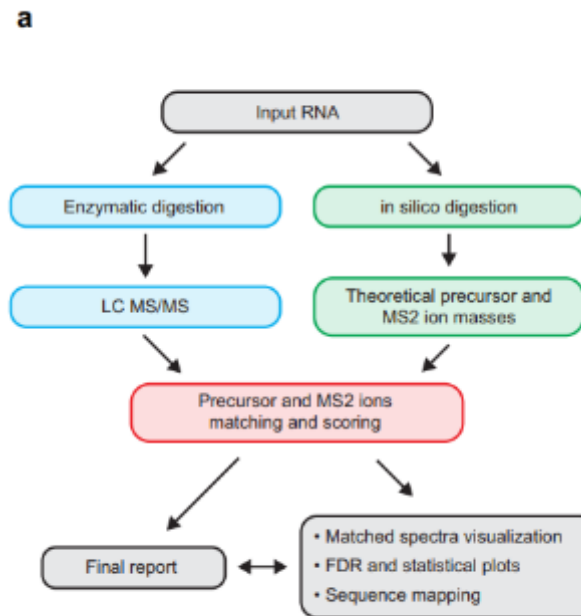
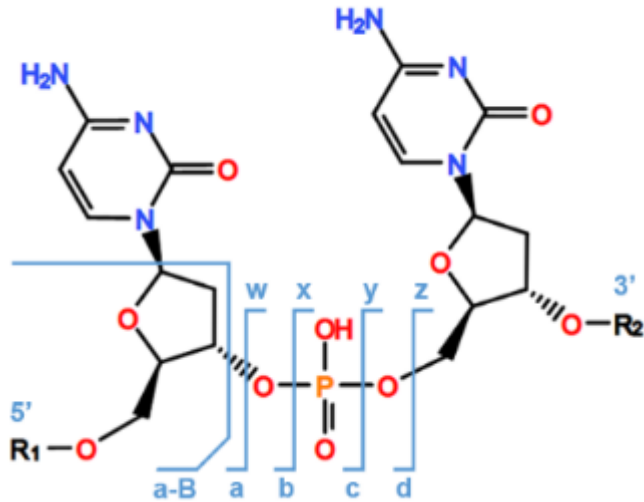


Identification by Sequence Confirmation

Referenced Figures from D'Ascenzo, LI, et al. *Nature Communications* (2022), and Waters CONFIRM Sequence Application

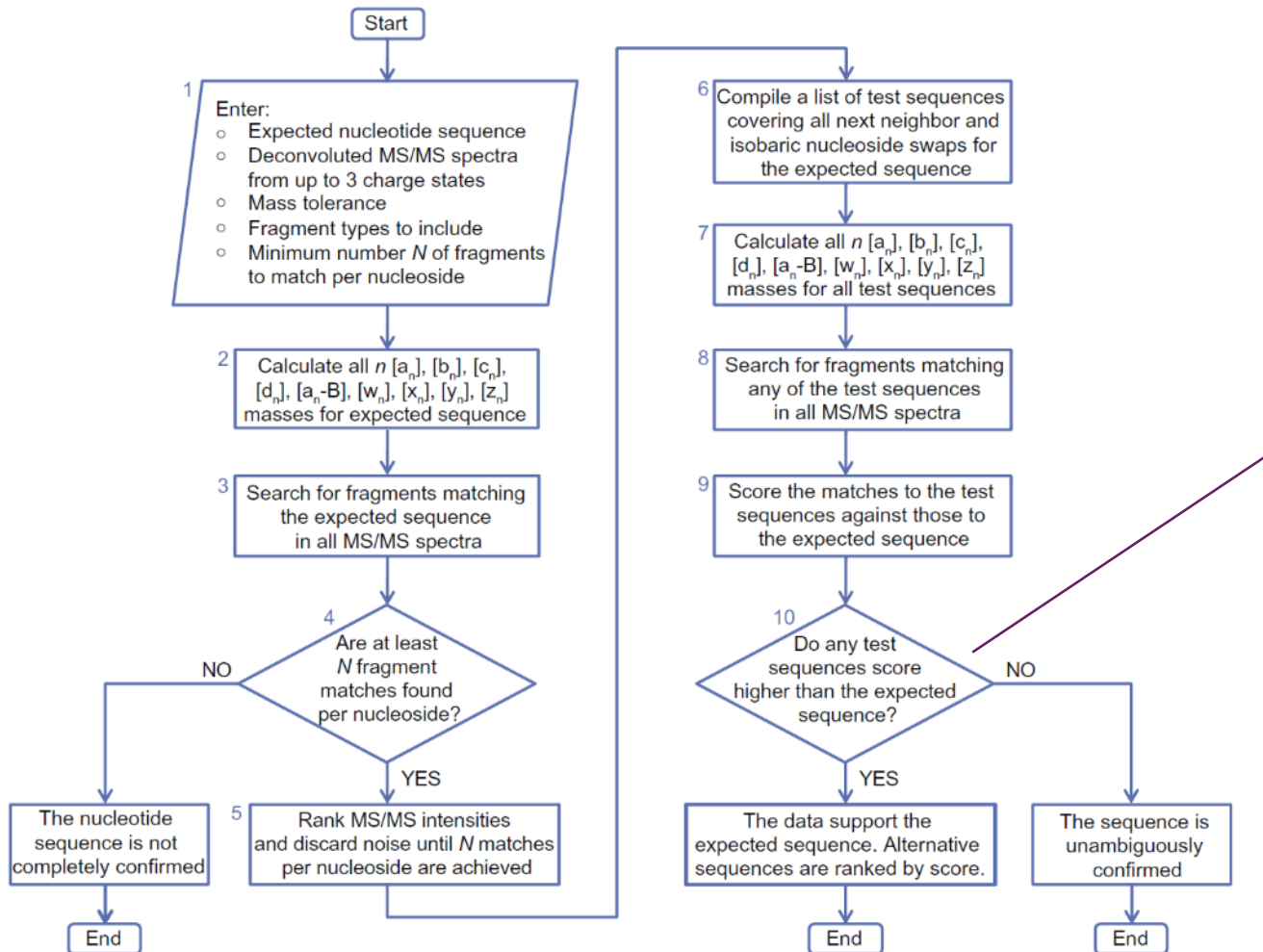
- All sequence confirmation methods verify the sequence of the oligonucleotide based on the common McLuckey notation (forward and reverse ion fragments) and use either fragment precursors or deconvoluted masses to identify monomers.

McLuckey notation shown on a section of a DNA oligonucleotide



Methods – Deconvoluted MS Fragment Matching

Referenced Figure from M. Kretschmer et al. *Analytical Biochemistry* (2010)



Scoring systems vary between different software / processing methods and need to be validated for use in GMP and QC environments.

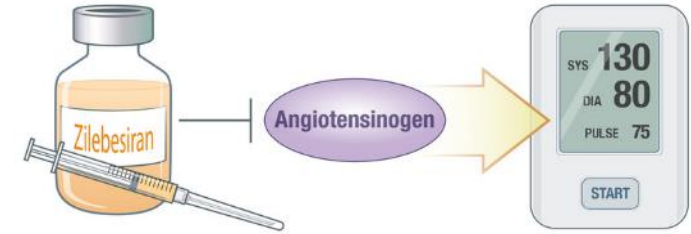
Swap sequences (synthesized or in silico) are implemented for validation.

Software for Sequence Confirmation

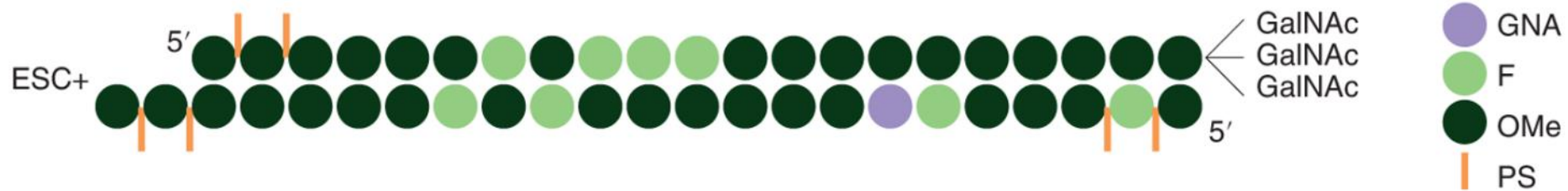
- Instrument companies now have many different software available for semi-automated or automated sequence confirmation.
- Here, we evaluated one software (CONFIRM sequence) to assess the ease of use for GMP compliance.
- We assess this software's capability in:
 - 100% sequence coverage of the FLP
 - Sequence coverage not confirmed / less than 100%, or with a lower score, for swap sequences (synthesized or in silico)

Using Zilebesiran as an Example

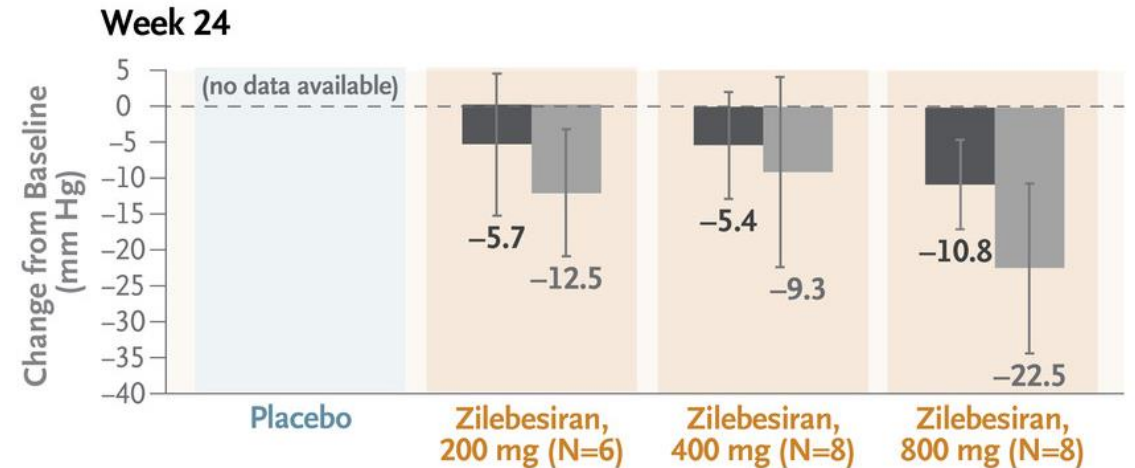
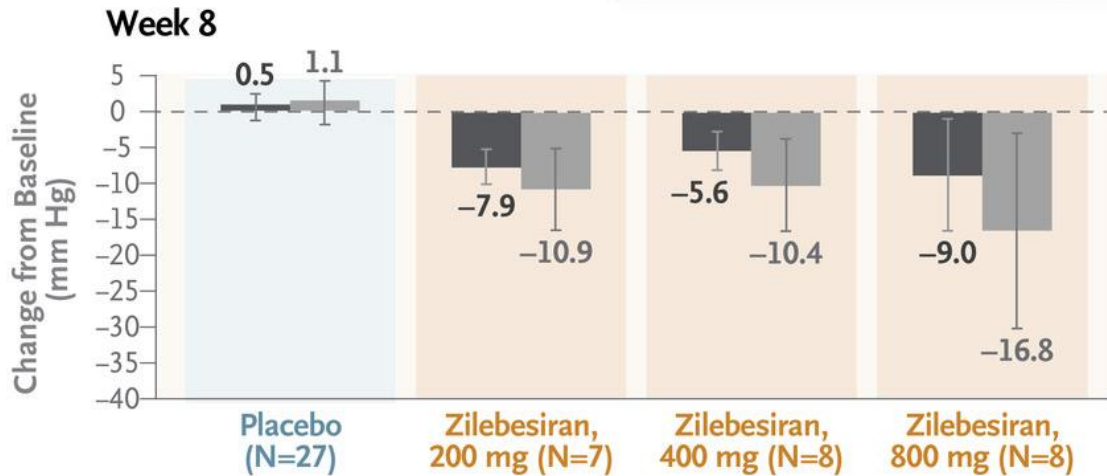
RNAi Therapeutic for Hypertension, Targeting AGT



*Partnership with Roche



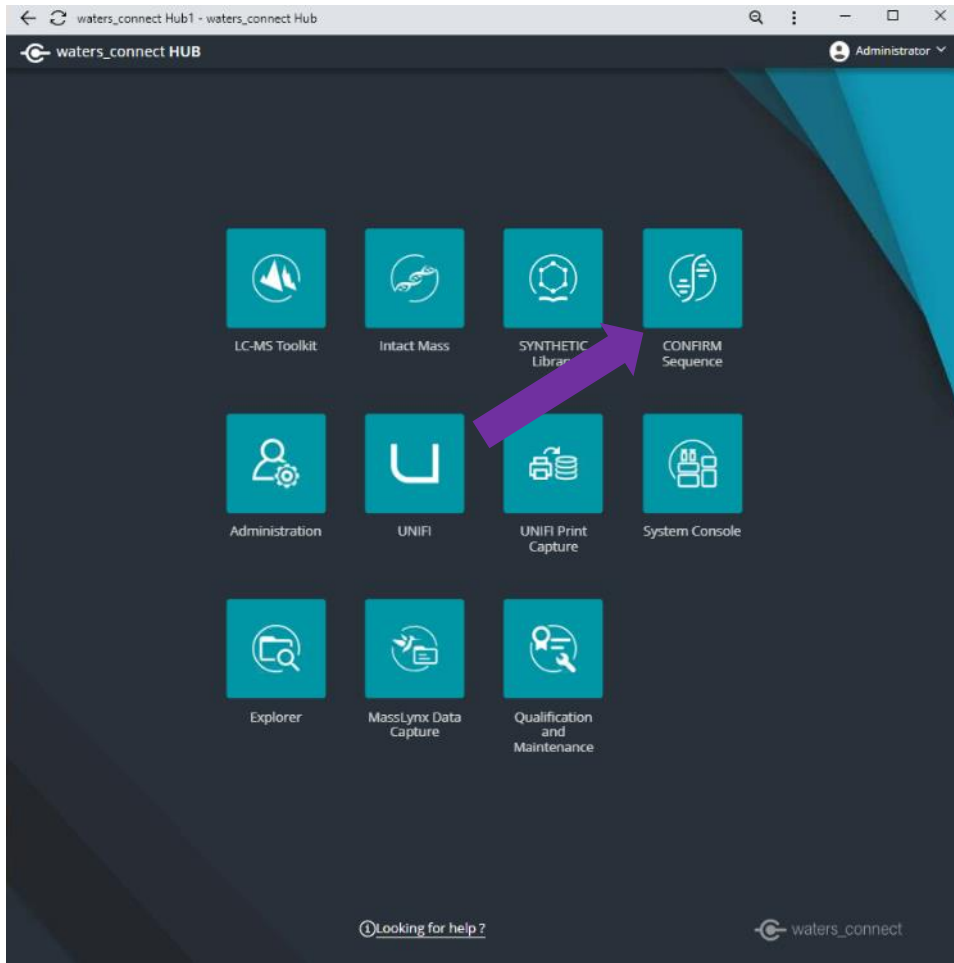
■ Diastolic blood pressure ■ Systolic blood pressure



CONFIRM Software and Processing Method

Figures from CONFIRM Software, Developed by Waters Corporation

More “semi-automated” as data still needs to be imported to CONFIRM.



Create New Method

Name

Please supply a name

Description

Mass error (ppm)

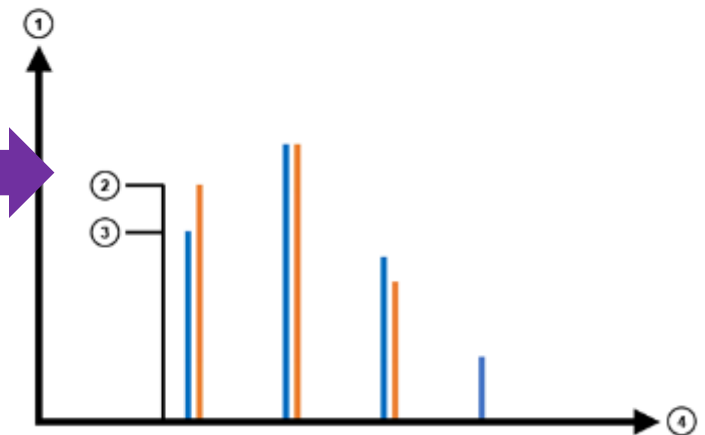
Cross-spectrum confirmation

Intensity cutoff (counts)

Isotope intensity ratio cutoff (%)

Isotope similarity cutoff (%)

The intersection and union for an isotope peak match

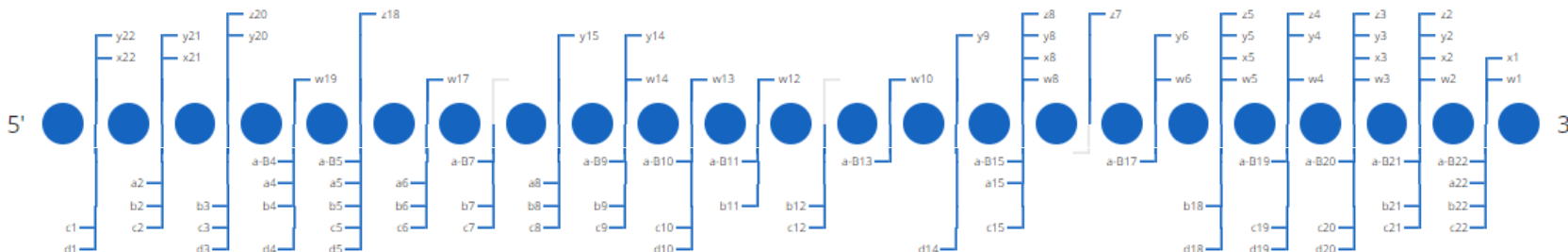


Zilebesiran Antisense (AS) Sequence Confirmation

100% Sequence Coverage



Sequence coverage: 100.00% (3/3 spectra selected)



MSMS spectra from different collisions are used for sequence confirmation

Spectrum	Retention time window (min)	Acquisition details	Precursor observed mass (Da)	Charge state	% Precursor	% Coverage	Dotmap
QCN AGT01 AS modified method Jun 02, 2022 AGT01 AS LIMS 9097 1087.3 1: TOF MSMS 1087.2>(200-2500) 26eV ESI-	3.35 - 3.65	MS/MS Jun 02, 2022 15:30:51 (+00:00)	7,614.1263	7-	32.25	69.57	●●●●●●●●○●●●●●●●●○●●●●●●●●○●●●●●●●●
QCN AGT01 AS modified method Jun 02, 2022 AGT01 AS LIMS 9097 1268.1 1: TOF MSMS 1268.6>(200-2500) 30eV ESI-	3.35 - 3.65	MS/MS Jun 02, 2022 15:41:40 (+00:00)	7,614.1264	6-	39.46	78.26	●●●●●●●●●●●●●●○●●●●○●●●●●●●●
QCN AGT01 AS modified method Jun 02, 2022 AGT01 AS LIMS 9097 1522.2 1: TOF MSMS 1522.5>(200-2500) 35eV ESI-	3.35 - 3.65	MS/MS Jun 02, 2022 16:02:36 (+00:00)	7,614.1251	4- 5- 6- 7-	46.04	91.30	●●●●●●●●●●●●○●●●●●●●●●●●●●●

Zilebesiran AS Swap Sequences

Synthesized Swap Sequences

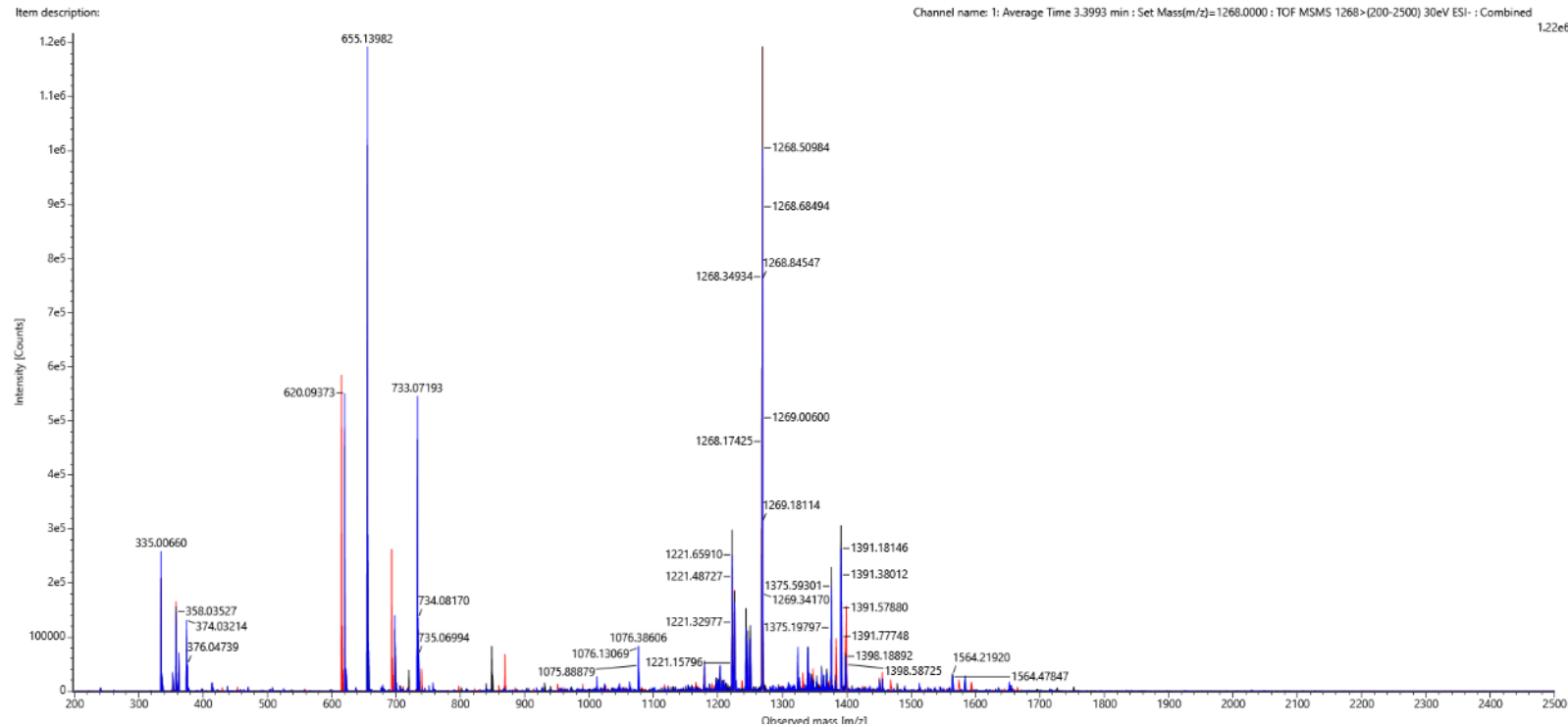
Zilebesiran AS



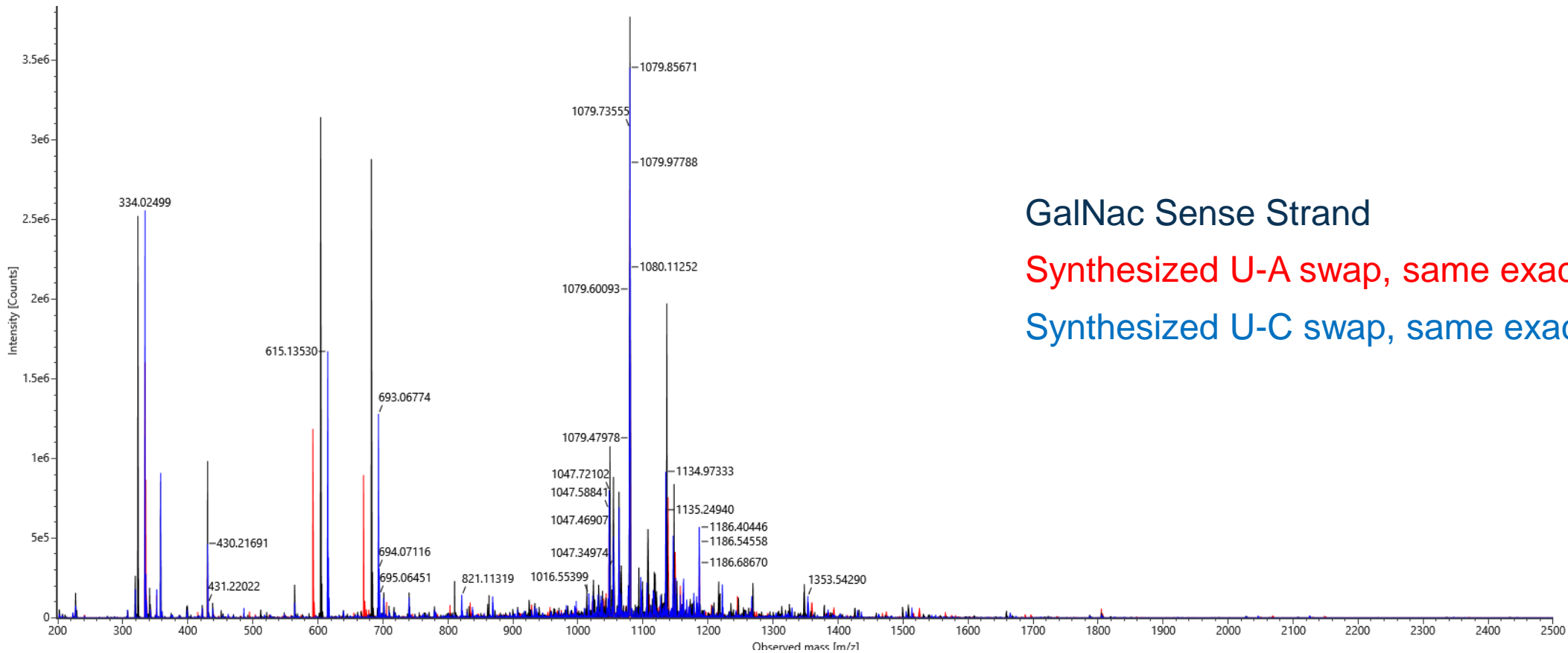
Swap 1 synthesized:



Swap 2 synthesized:



GalNac-Conjugated Sense Strand A

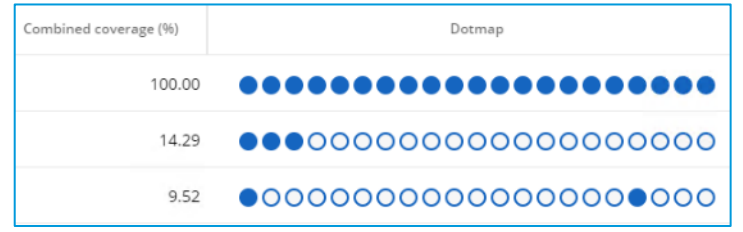
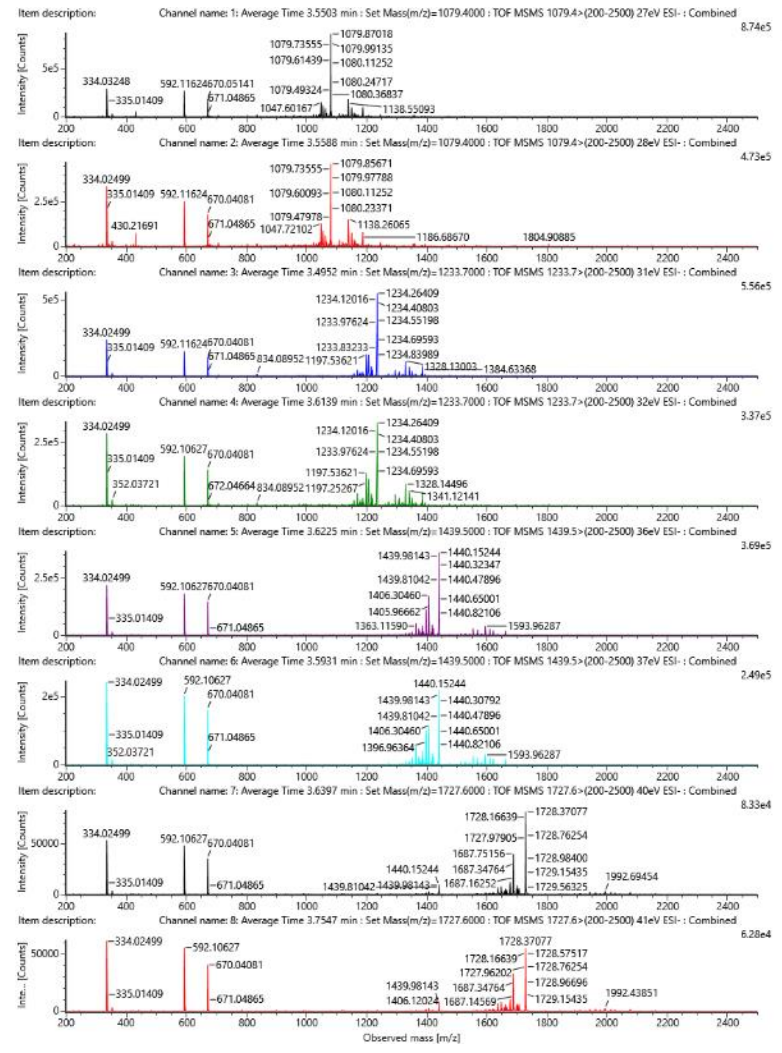
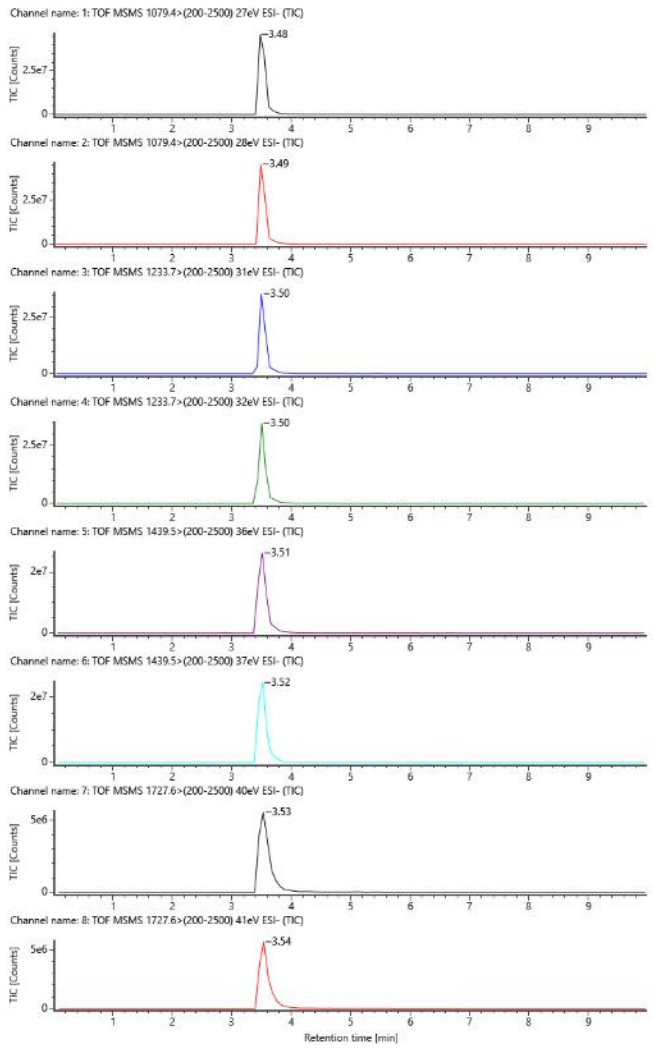


GalNac Sense Strand

Synthesized U-A swap, same exact mass

Synthesized U-C swap, same exact mass

Multiple Collision Energies from 1 Injection



* Native oligo as tested by MS/MS (data from figures to left)

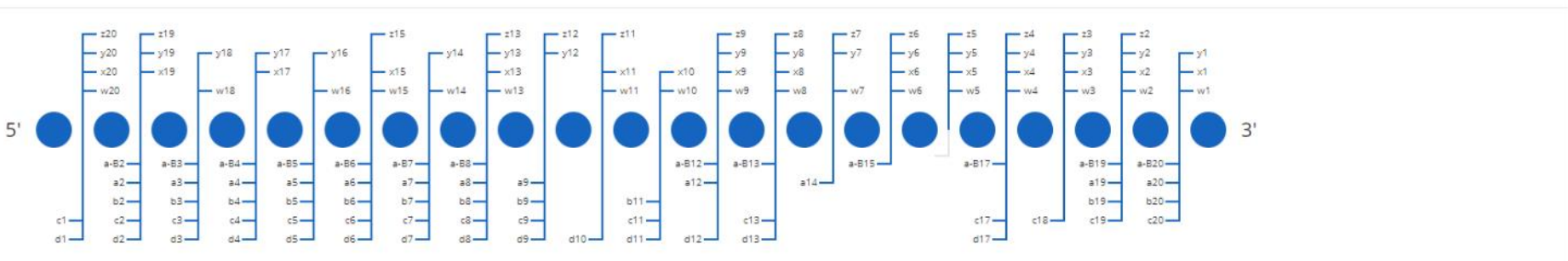
** Assignment challenged with in silico swapped sequences

Data analysis completed in < 10 min

Multiple Collision Energies from Direct Infusion

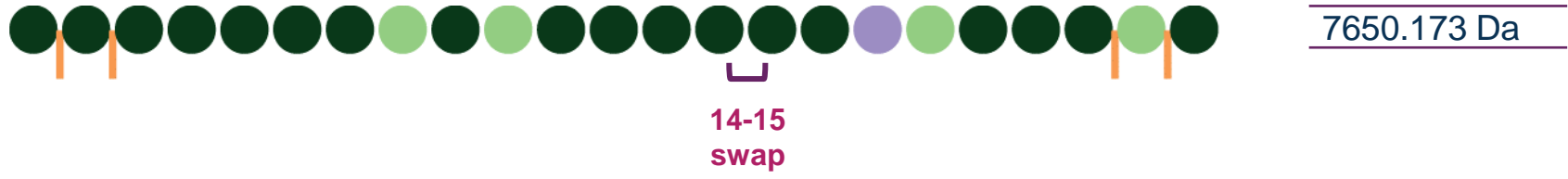
Completed in < 3 min

Sequence coverage: 100.00% (9/9 spectra selected)



Spectrum	Retention time window (min)	Acquisition details	Precursor observed mass (Da)	Charge state	% Precursor	% Coverage	Dotmap
SOD1 SS MSMS Function Table Infusion 23Sep22 FunctionTable 1 1: TOF MSMS 1413>(200-2500) 32eV ESI-	2.00 - 8.00	MS/MS Sep 23, 2022 23:42:38 (+00:00)	7,067.2648	5-	27.41	100.00	
SOD1 SS MSMS Function Table Infusion 23Sep22 FunctionTable 1 2: TOF MSMS 1413>(200-2500) 33eV ESI-	2.00 - 8.00	MS/MS Sep 23, 2022 23:42:38 (+00:00)	7,067.2623	5-	20.14	100.00	
SOD1 SS MSMS Function Table Infusion 23Sep22 FunctionTable 1 3: TOF MSMS 1413>(200-2500) 31eV ESI-	2.00 - 8.00	MS/MS Sep 23, 2022 23:42:38 (+00:00)	7,067.2632	5-	36.08	100.00	

Tricky Example 1 – Next Neighbor Amidite Swap



Intensity cutoff at 1500,
Isotope settings at 70

Monoisotopic mass (Da)	Combined coverage (%)	Dotmap
7,650.1723	82.61	●●●●●●●●○●●●●●●●●○●●●●●●●●
7,650.1723	73.91	●●●●●●●●○●●●●●●○●●●●●●○●●●●●●●●

Native sequence
Simulated swap

Intensity cutoff at 1000,
Isotope settings at 65

Monoisotopic mass (Da)	Combined coverage (%)	Dotmap
7,650.1723	100.00	●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●
7,650.1723	91.30	●●●●●●●●●●●●●●●●●●○●●●●●●●●●●●●●●

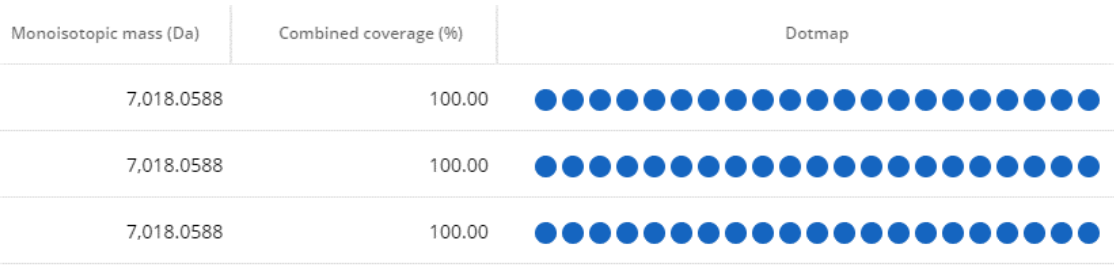
Native sequence
Simulated swap

Tricky Example 2: Next Neighbor Amidite Swap

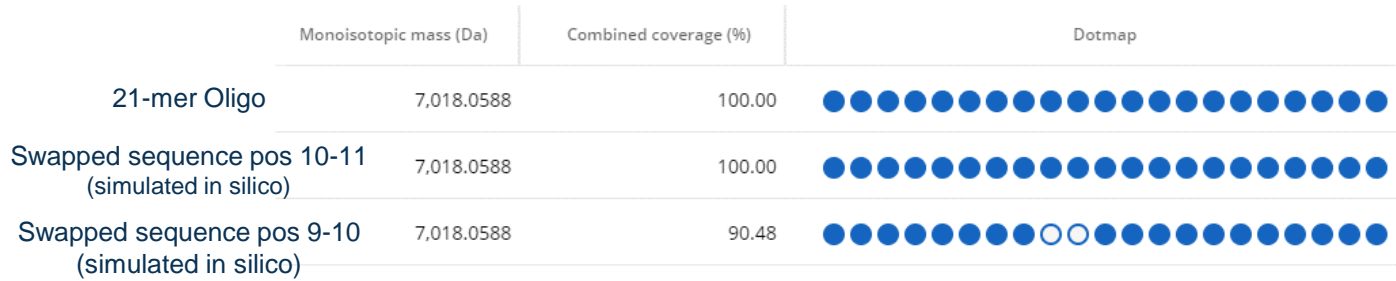
21-mer Oligo

21-mer Oligo	7018.059
Swapped sequence pos 9-10	7018.059
Swapped sequence pos 10-11	7018.059

Isotope similarity cutoff at 70%



Isotope similarity cutoff at 72%



||| Conclusions and Future Work

- Software such as CONFIRM Sequence and alternative vendor software can be used to streamline sequence confirmation.
 - Able to confirm 100% sequence coverage
 - Able to distinguish and confirm a sequence against its in silico swap sequences
- Multiple collision energies are necessary for full sequence coverage.
- Processing settings such as mass tolerance must be fine tuned.
- Vendor software could be improved to be “fully” automated (import data directly into CONFIRM).

|| Acknowledgements

Analytical Development

- Matthias Kretschmer
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- Roche
- And many more!

| | Thank you!