Improved sensitivity for the quantification of oligonucleotides in plasma using microflow LC and accurate mass spectrometry

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Presentation outline

IMPROVED SENSITIVITY FOR THE QUANTIFICATION OF OLIGONUCLEOTIDES IN PLASMA USING MICROFLOW LC AND ACCURATE MASS SPECTROMETRY

- Background
- QTOF duty cycle & Zeno trapping on the ZenoTOF 7600 system
- Microflow LC
- Quantification of oligonucleotides in rat plasma
- Non-targeted impurity analysis
- Conclusions

Oligonucleotide therapeutics

BACKGROUND AND INTRODUCTION

- Typically, short strings of synthetic nucleotides
- Alter, suppress, or restore expression of target genes associated with disease or health
- Increasing numbers of companies are currently investigating oligonucleotide therapeutics to treat diseases
  - High specificity
  - Ability to reach formerly undruggable targets
- Two major therapeutic approaches:
  - Antisense oligonucleotides (ASOs)
  - short interfering RNA (siRNA)

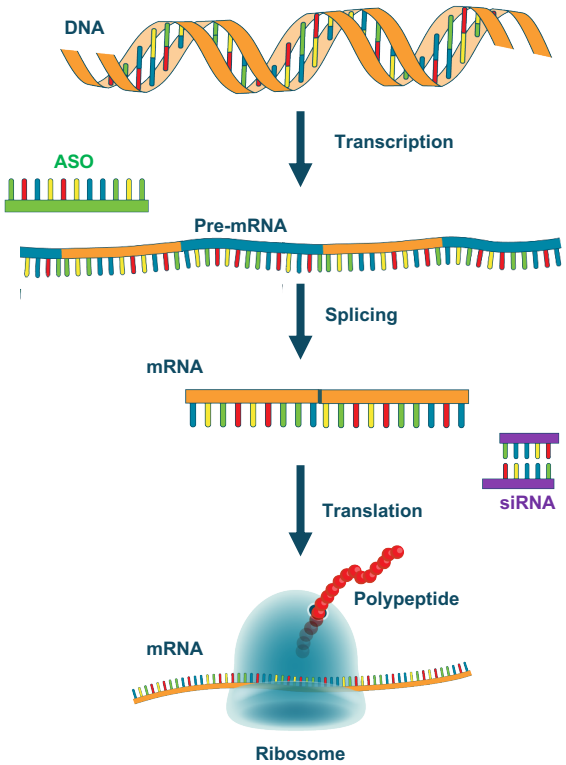
Summary of some of the clinically approved oligonucleotides

Brand name [generic name]	Type of treatment	Target	Disease	Year of approval
Vitravene [formivirsen]	ASO	mRNA encoding IE2	CMV retinitis	1998
Macugen [pegaptanib]	Aptamer	VEGF165	AMD of the retina	2004
Kynamro [mipomersen]	ASO	ApoB-100 mRNA	Homozygous familial hypercholesterolemia	2013
Exondys 51 [eteplirsen]	SSO	DMD 001-gene (exon 51 target site)	Duchenne muscular dystrophy	2016
Spinraza [nusinersen]	ASO	SMN2 mRNA	Type 1, 2, and 3 spinal muscular atrophy	2016
Inotersen [tegsedi]	ASO	Transthyretin	Hereditary transthyretin amyloidosis	2018
Givosiran [Givlaari]	siRNA	Aminolevulinate synthase 1	Acute hepatic porphyria	2019

Oligonucleotide therapeutics

TYPES OF OLIGONUCLEOTIDE DRUGS

Targeted mRNA splice switching



Targeted mRNA degradation

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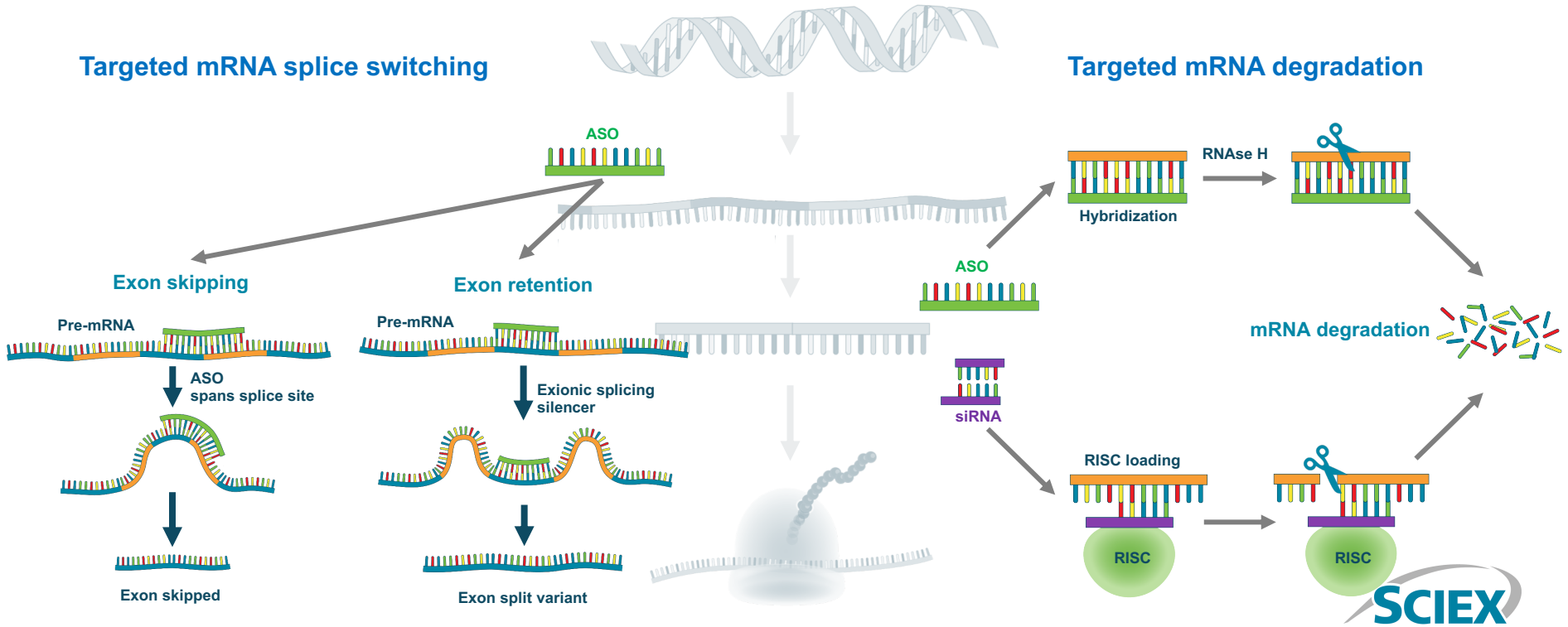
TYPES OF OLIGONUCLEOTIDE DRUGS



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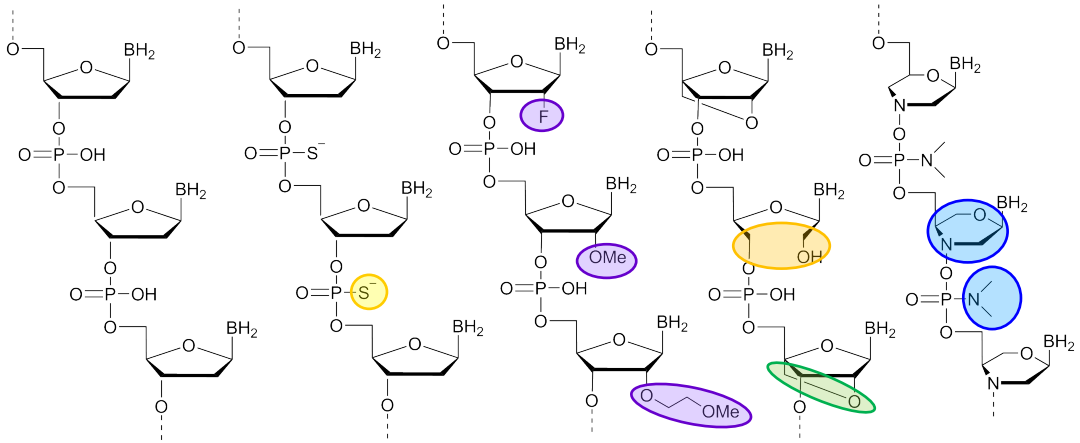
Oligonucleotide therapeutics

OLIGONUCLEOTIDE MODIFICATIONS

Types of chemical modifications to therapeutic oligonucleotides



DNA with phosphodiester (PO)

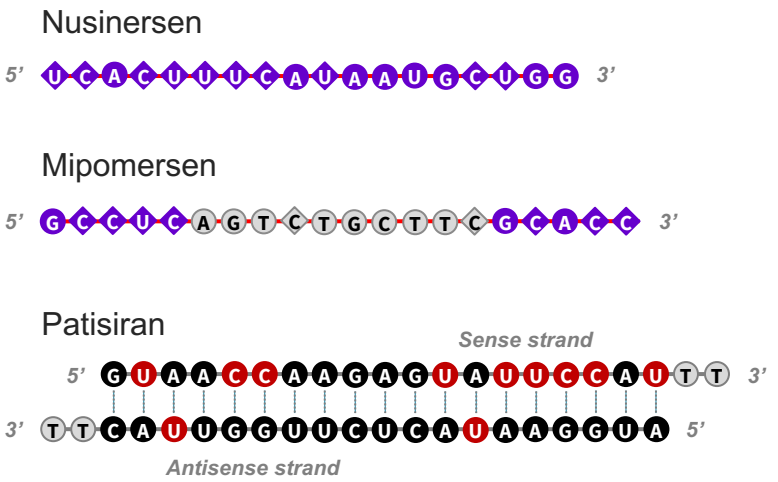
Phosphorothioate (PS)

2’ modifications

Ribose modifications

Morpholino

Examples of these modifications in approved therapeutic oligos



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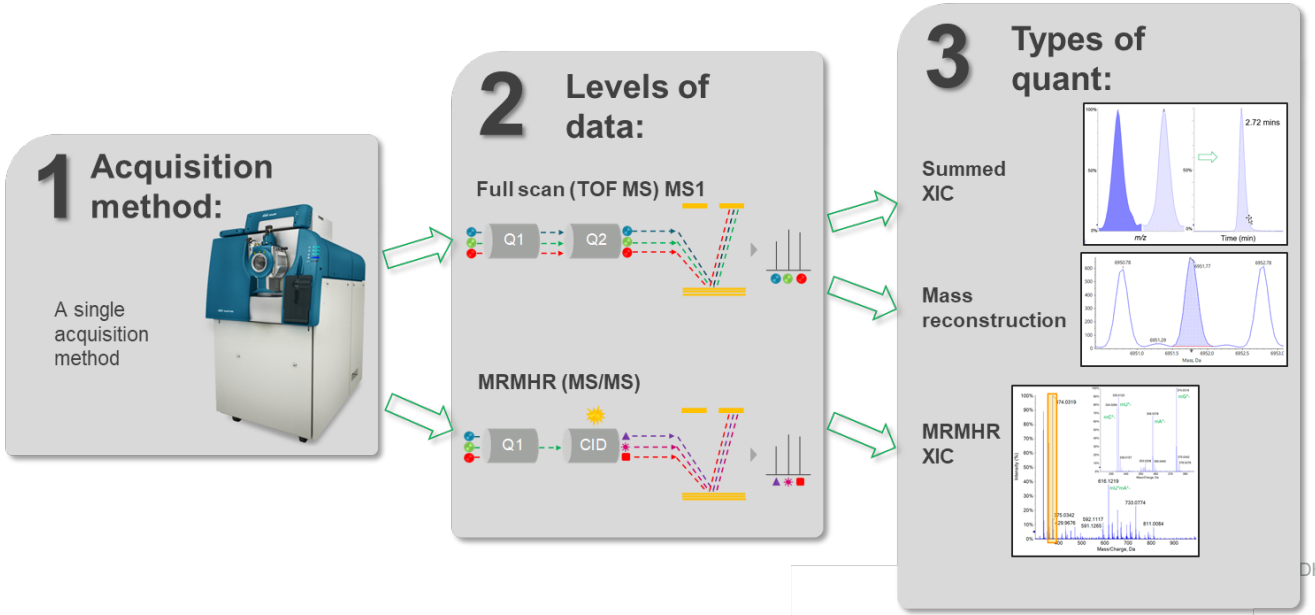
Quantification of oligonucleotide therapeutics

DIFFERENT OPTIONS FOR OLIGONUCLEOTIDE QUANTIFICATION

- Hybridization Assay (ELISA): good sensitivity but limited selectivity & linear dynamic range
- LC-MS MRM : better selectivity & LDR, but lower sensitivity
- LC-HRMS : Good for characterization, but limited sensitivity for quantification because of low duty cycle



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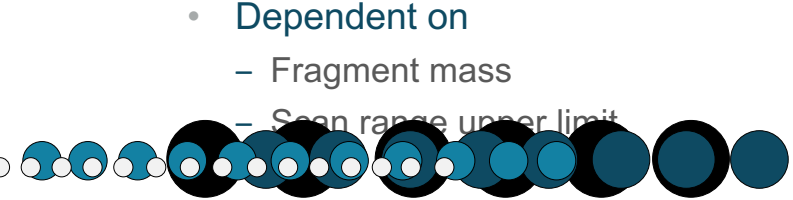
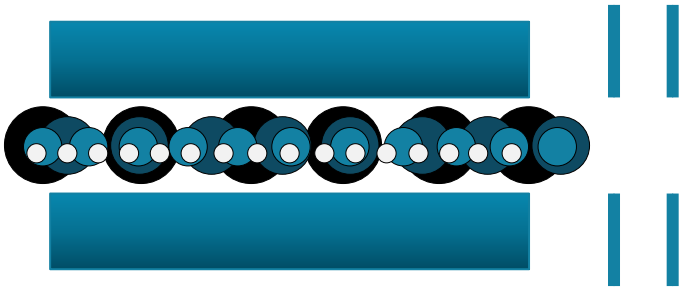
What is duty cycle?

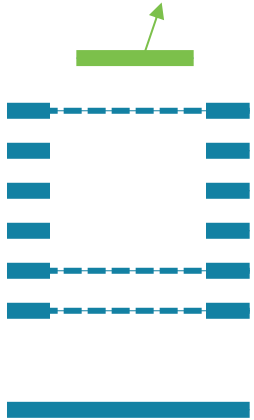
… AND WHY IS DUTY CYCLE IMPORTANT?

- What is duty cycle?
  - % of ions injected into the TOF
  - Typically, ~5-25%
    - Dependent on
      - Fragment mass
      - Scan range upper limit
- Why is duty cycle not 100%?
  - Ion losses occur when combining:
    - Pulsed measurement technique
      - TOF
    - Continuous ion beam
      - Quadrupole



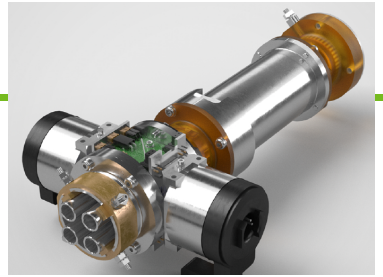


Quadrupole region
Continuous ion beam



TOF accelerator
Ion pulsing

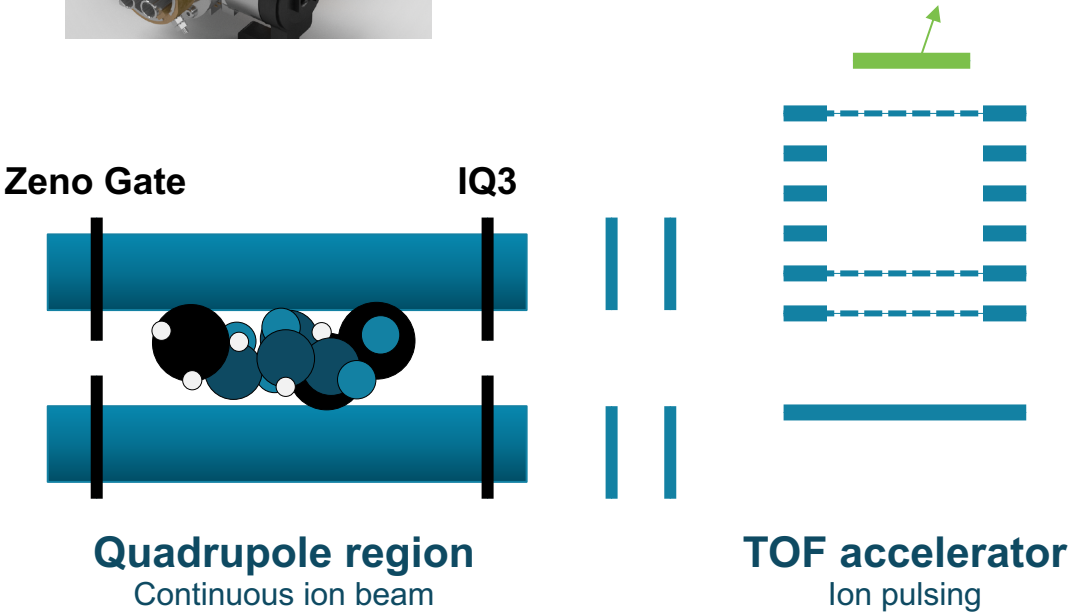
Operation of the Zeno trap



J. Am. Soc. Mass Spectrom. (2017) **28**: 2143-2150)

FOR SENSITIVITY GAINS IN MS/MS

- The Zeno trap on the ZenoTOF 7600 system addresses the duty cycle problems with QTOFs by providing control of the ion beam from the collision cell into the accelerator
- Ions are gated then released from the Zeno trap in an ordered fashion based on potential energy
  - Generally, higher m/z ions are released first, followed by lower m/z ions
  - A wide range of ions now arrive in the accelerator to be pushed during the same pulse



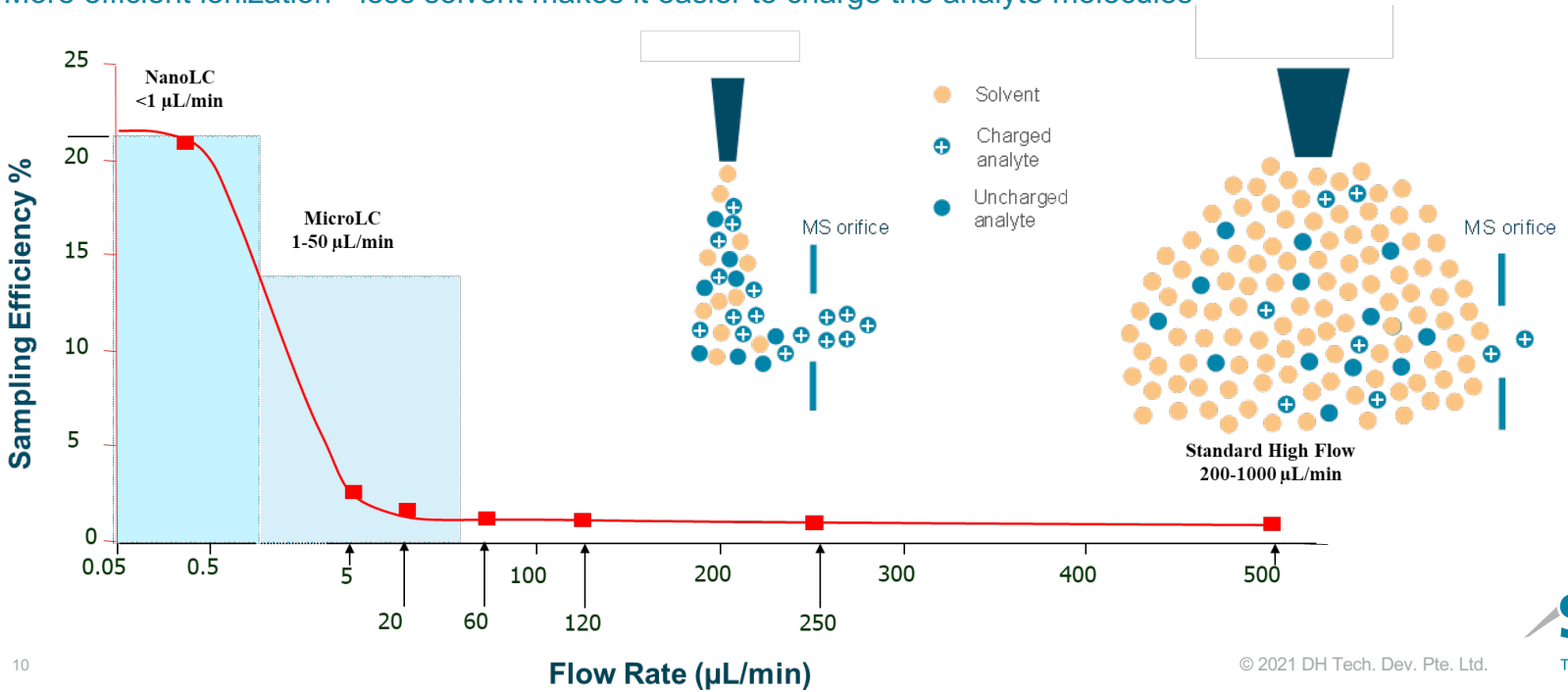
➢ This results in improved MS/MS sensitivity across the entire mass range, with ≥ 90% of all ions injected into TOF region

Microflow LC

Sampling efficiency = fraction of ionized molecules in the liquid flow entering the MS

More confined spray with higher concentration of analytes – easier to pull ions into the MS

More efficient ionization - less solvent makes it easier to charge the analyte molecules



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Trap and elute workflow

USING TRAP AND ELUTE TO LOAD LARGE VOLUMES WITH HIGH THROUGHPUT

High flow:
Load / analyze 30 µL sample @ 250 µL/min

Microflow direct inject:
Load 30 µL sample @ 5 µL/min, analyze @ 5 µL/min

Microflow trap & elute:
Load 30 µL sample @ 35 µL/min, analyze @ 5 µL/min

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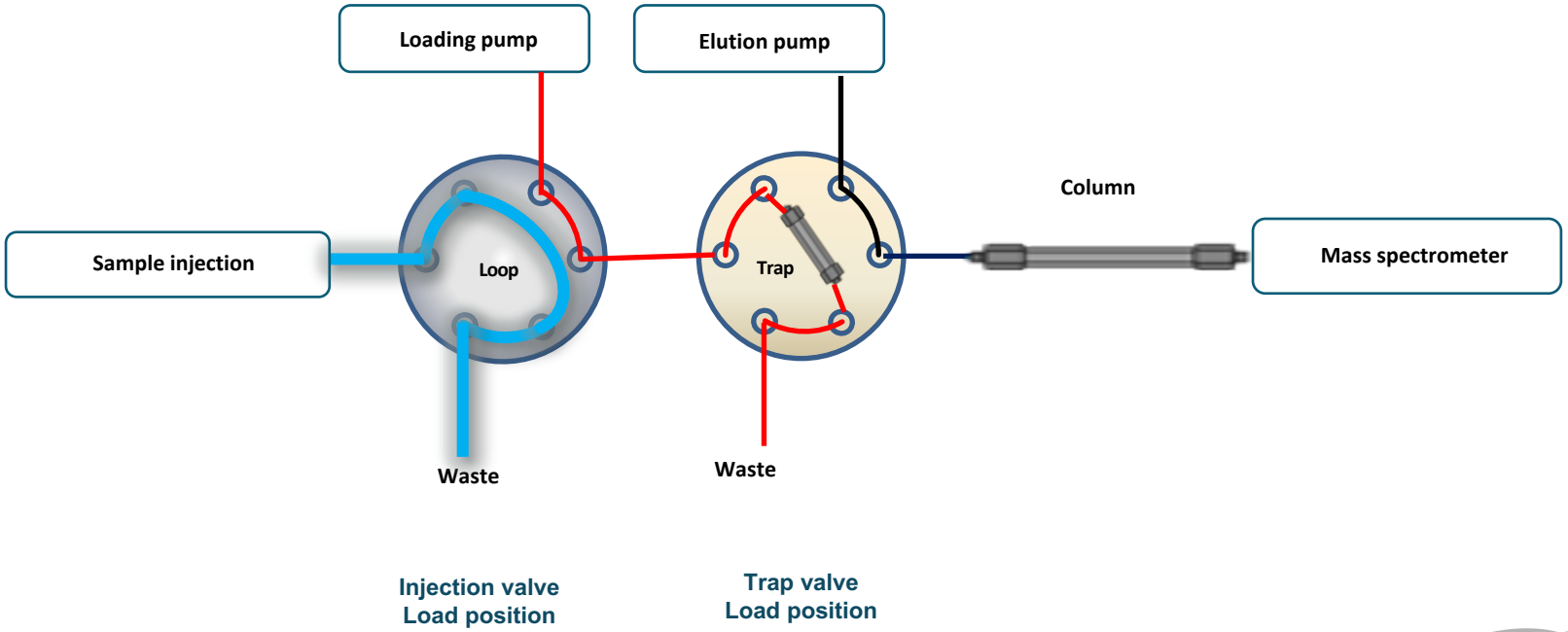
Step 1 in trap and elute workflow

SAMPLE IS INJECTED INTO THE SAMPLE LOOP



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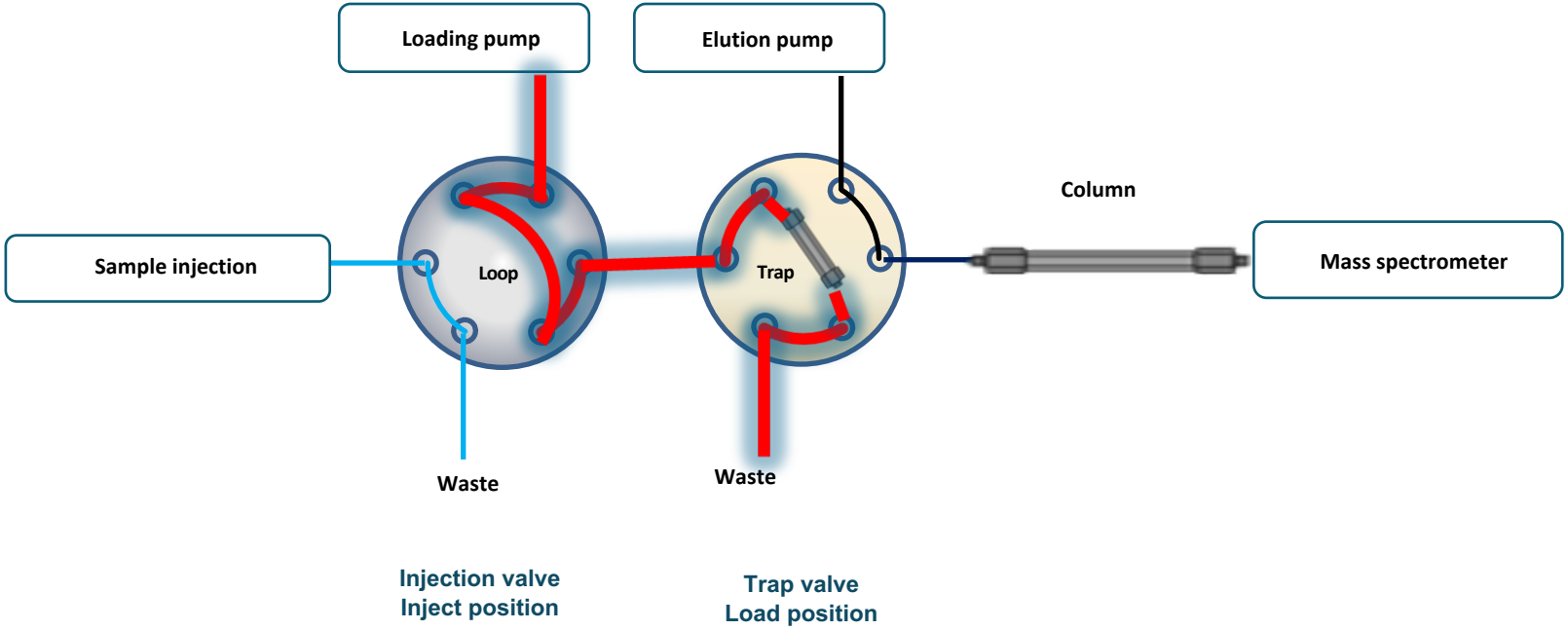
Step 2 in trap and elute workflow

SAMPLE IS LOADED ONTO THE TRAP COLUMN



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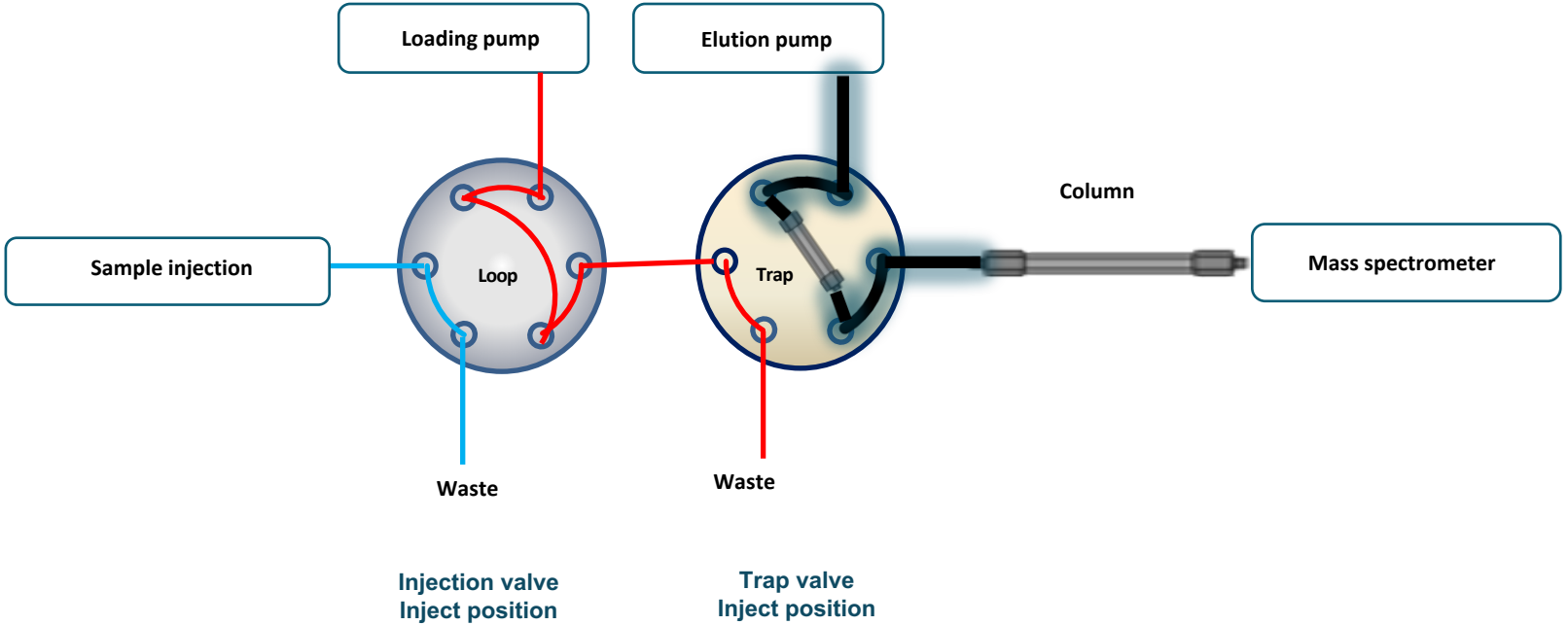
Step 3 in trap and elute workflow

SAMPLE IS ELUTED FROM THE TRAP COLUMN ONTO THE ANALYTICAL COLUMN AND ANALYZED



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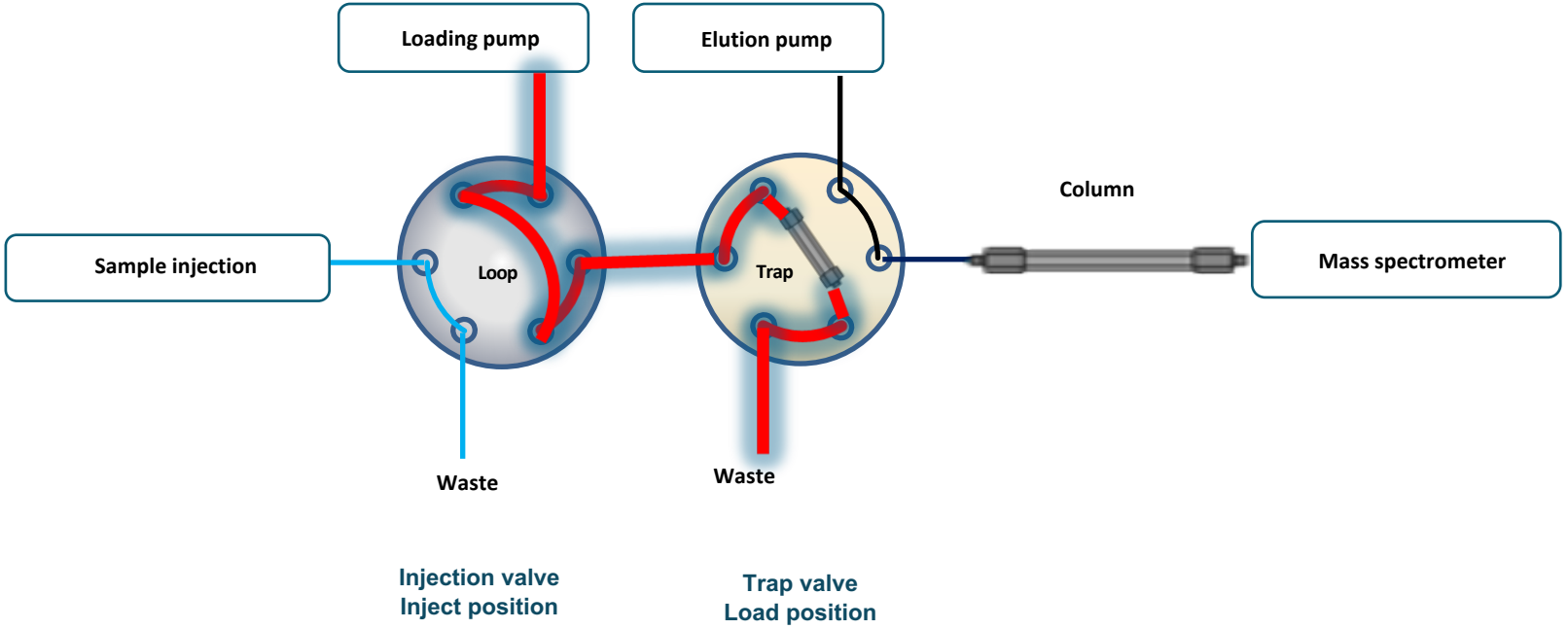
Step 4 in trap and elute workflow

TRAP IS WASHED USING THE LOADING PUMP



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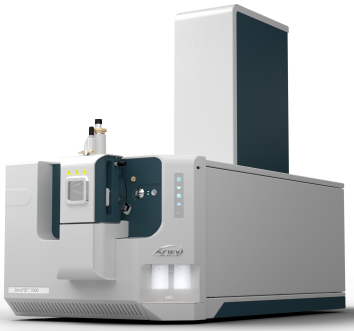
A case study: quantification of oligonucleotides in rat plasma

- Objective: determine the quantification sensitivity for 4 antisense oligonucleotides
  - 3 Phosphorothioated 18- to-33-mer drug structures (fomivirsen, nusinersen and eluforsen)
  - 1 Phosphorothioated and 2'-O-methylated 20-mer ("methyl oligo")
- Rat plasma was prepared using 3 mL Clarity OTX cartridges (Phenomenex), following the Phenomenex protocol for oligonucleotides
- Calibration curves were spiked into the prepared plasma
- A 23-mer standard DNA oligonucleotide was used as an internal standard (IS)
- Samples were analysed using trap & elute microflow LC on the ZenoTOF 7600 system, using MRM^{HR} with Zeno trapping



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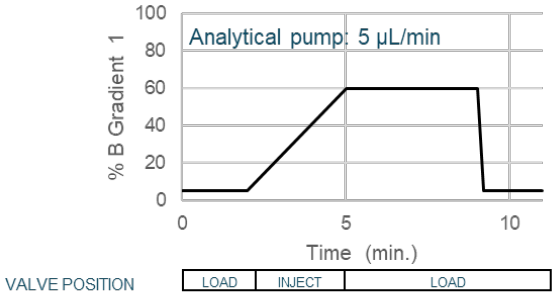
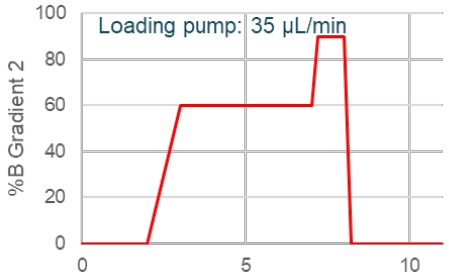
LC method conditions

MICROFLOW LC WORKFLOW

LC system:	M5 MicroLC
Mobile phase A:	15 mM N,N-diisopropylethylamine, 35 mM hexafluoroisopropanol in water
Mobile phase B:	15 mM N,N-diisopropylethylamine, 35 mM hexafluoroisopropanol in 90/10 methanol/water
Analytical gradient:	5-60% B in 3 min at 5 µL/min
Loading:	0% B for 2 min at 35 µL/min
Injection volume:	30 µL
Trap:	0.3 x 5 mm YMC-Triart 3 µm C18 @ 80ºC
Analytical column:	0.3 x 50 mm YMC-Triart 3 µm C18 @ 80ºC





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MS method conditions

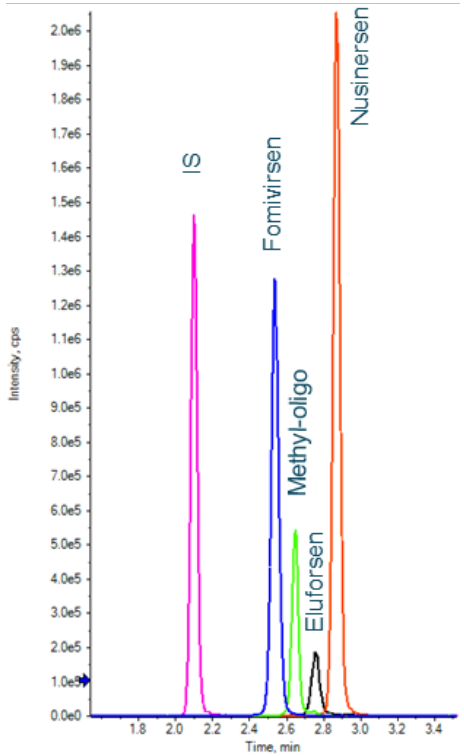
- Mass spectrometer: ZenoTOF 7600 system
- Source: OptiFlow Turbo V ion source with a low micro electrode
- Negative ionization
- MRM^{HR} mode
- Charge state that provided the most intense fragments selected for each analyte
- CE and DP optimized for best S/N on the selected fragment
- Accumulation time 0.03 sec (cycle time 0.4 sec)
- Q1 resolution LOW (for best S/N)

ID	Q1 mass (m/z)	Fragment (m/z)	DP (V)	CE (V)
Fomivirsen	*741.4*	*319.02*	*-125*	*-33*
Methyl-oligo	*694.2*	*374.03*	*-135*	*-39*
Nusinersen	*790.9*	*393.05*	*-135*	*-40*
Eluforsen	*715.8*	*358.04*	*-135*	*-39*
IS	*786.3*	*303.04*	*-135*	*-49*

Parameter	MS	MS/MS
Scan mode	*TOF MS*	*MRM^{HR}*
Polarity	*negative*	
Gas 1	*20 psi*	
Gas 2	*40 psi*	
Curtain gas	*32 psi*	
Source temperature	*100°C*	
Ion spray voltage	*-3000 V*	
Declustering potential	*-80 V*	
CAD gas	*12*	
Start mass	*500 m/z*	*100 m/z*
Stop mass	*2,000 m/z*	*2,000 m/z*
Q1 resolution	*NA*	*Low*
Accumulation time	*0.1 s*	*0.03 s*
Collision energy	*-10 V*	*See Table 4*
CE spread	*0 V*	*0 V*
Zeno trap	*NA*	*ON*
ZOD threshold (CID)	*NA*	*20,000 cps*
Time bins to sum	*6*	*12*
QJet ion guide RF amplitude	*190 V*	*190 V*

Chromatography

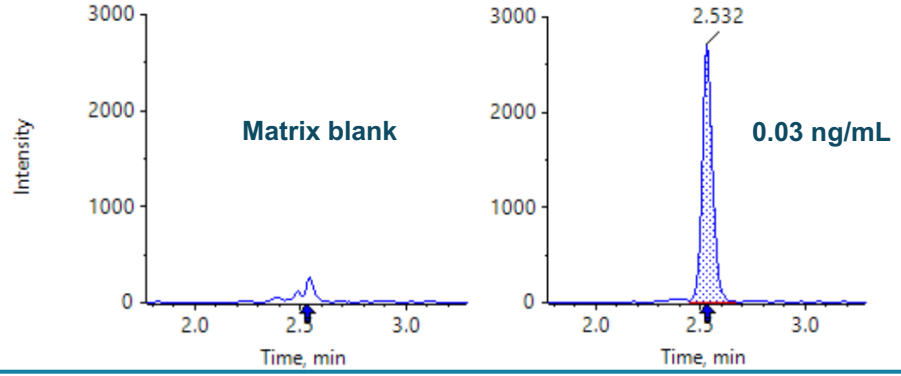
TRAP-AND-ELUTE MICROFLOW METHOD



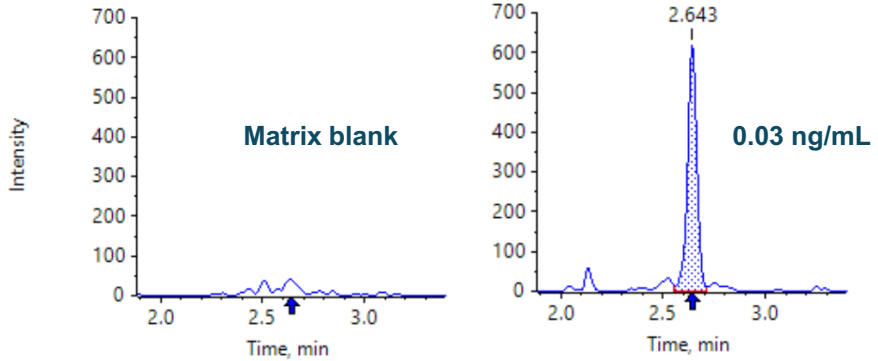
- TOF MS XIC for 4 target oligonucleotides plus internal standard (IS)
- All oligonucleotides are baseline separated
  - Baseline separation is important because overlap amongst precursors can occur due to the large number of charge states of each oligonucleotide

Blank and LLOQ for all analytes

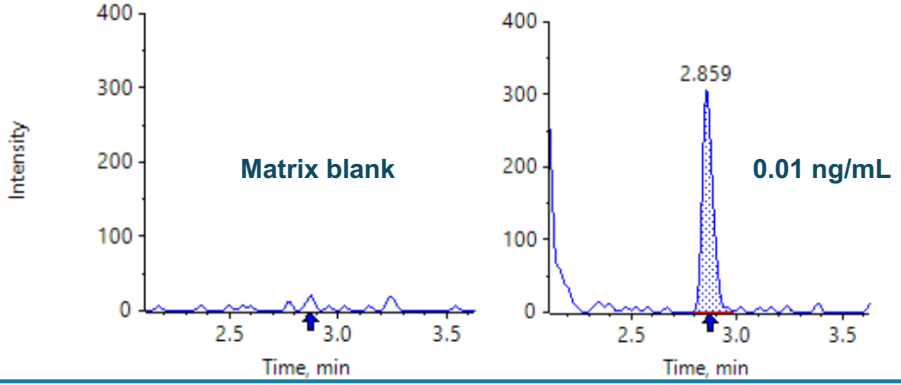
Fomivirsen:



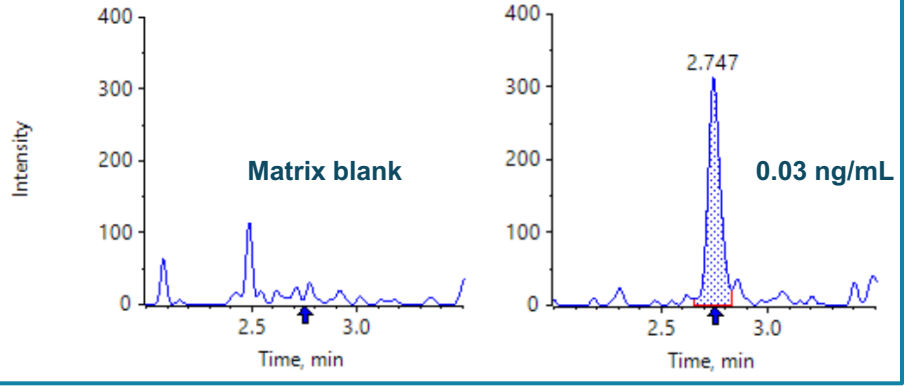
Methyl-oligo:



Nusinersen:



Eluforsen:

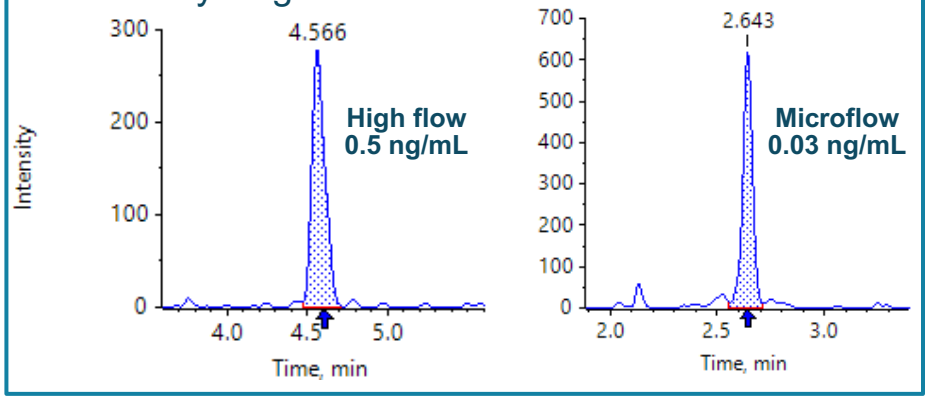


Comparison of high flow and microflow

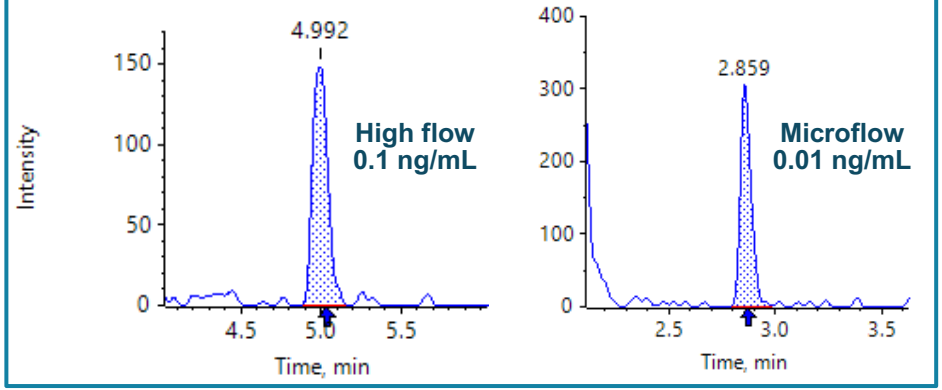
LLOQ VS. HIGH FLOW (0.2 mL/min)

- Similar S/N at 10 -16x higher LLOQs

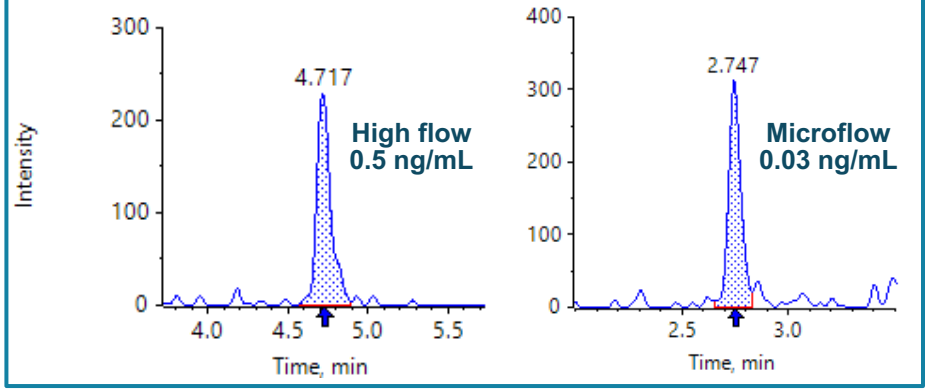
Methyl-oligo:



Nusinersen:



Eluforsen:

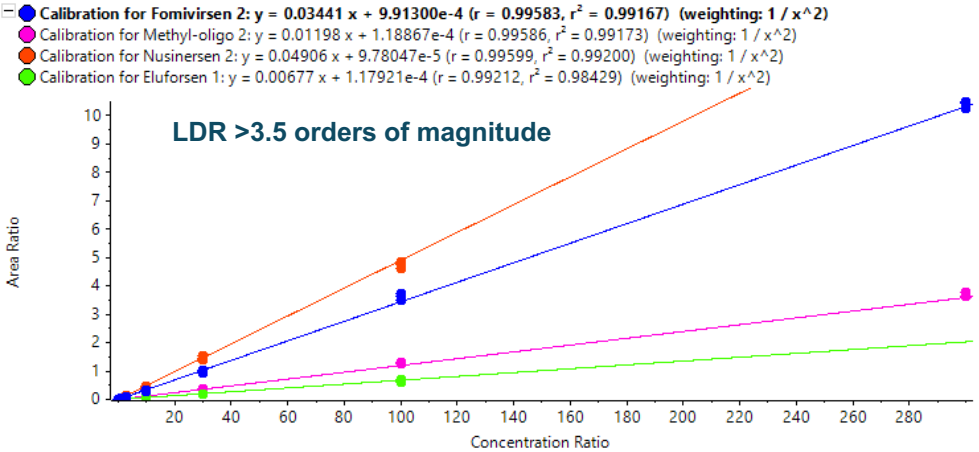


LLOQs and linear range

QUANTITATIVE PERFORMANCE

- **LLOQ criteria:**
  - LLOQ: CV < 20% and accuracy between 80% and 120%
  - Any point above the LLOQ: CV < 15% and accuracy between 85% and 115%

Analyte	LLOQ (ng/mL)	ULOQ (ng/mL)	Linearity (orders)	CV at LLOQ (%)	Accuracy at LLOQ (%)
20-mer phosphorothioated and 2'-O-methylated antisense oligonucleotide (ASO)	0.03	300	4	16.2	98.3
Fomivirsen	0.03	300	4	11.2	96.3
Nusinersen	0.01	100	4	3.7	96.0
Eluforsen	0.03	100	3.5	18.7	95.7



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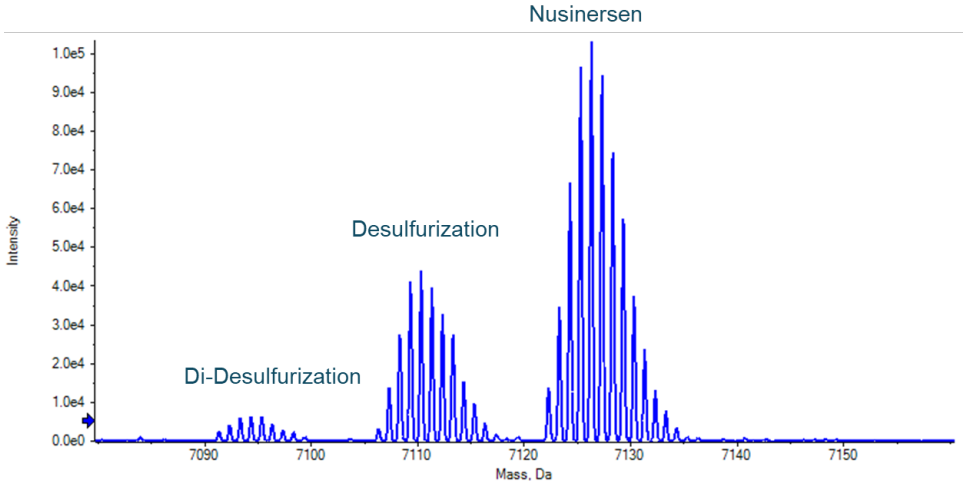
Non-targeted impurity analysis

IDENTIFICATION AND RELATIVE QUANTIFICATION

- Reconstructed TOF-MS spectrum of nusinersen chromatographic peak
- Based on the accurate mass and isotope pattern, the SCIEX Molecule Profiler software identified the additional species as desulfurization and di-desulfurization products
  - Common impurities with one or two sulfur atoms exchanged with an oxygen
  - Likely from synthesis or storage. No evidence of in-source fragmentation (desulfurization products were chromatographically separated from the main product)



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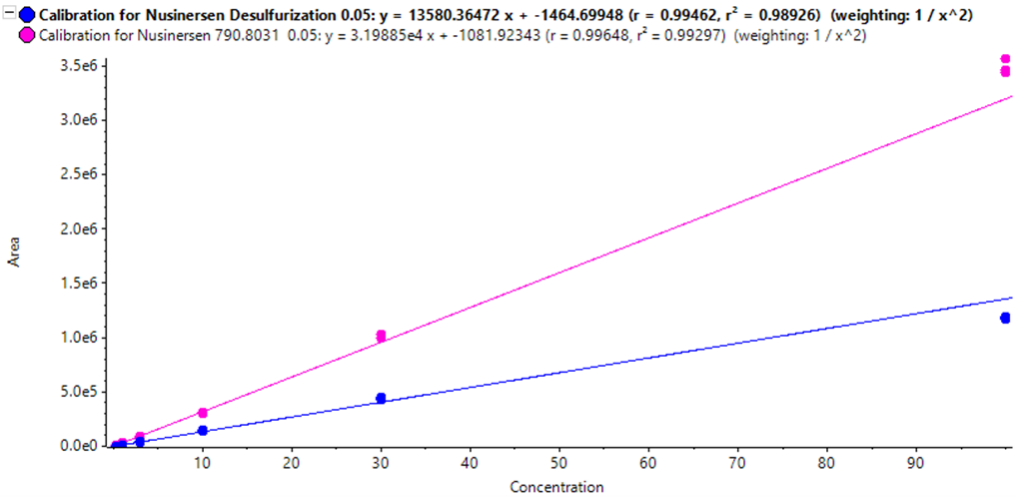
Non-targeted impurity analysis

IDENTIFICATION AND RELATIVE QUANTIFICATION

- Whilst no MRM^{HR} data was acquired for the desulfurization products, the relative amount versus the main product could be determined using the TOF-MS data
- The peak areas vs concentration were linear for both nusinersen and the desulfurization product
  - Using the most intense isotope of the TOF MS spectrum
  - On average, ratio of calculated area was 41% with a CV of 3.2%



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Conclusions

- Low pg/mL LLOQs were achieved for ASOs in rat plasma using MRM^{HR} mode, the Zeno trap and microflow LC
- The TOF-MS data acquired as part of the MRM^{HR} workflow can be used for the identification and relative quantification of additional impurities/metabolites
- Analysis time using trap-and-elute microflow LC method was comparable to that of using an analytical flow LC method, resulting in similar sample throughput
- Reduced consumption of LC-MS grade ion-pairing reagents for microflow LC provides significant cost savings and increases LC-MS system robustness

Acknowledgements

- Xin Zhang
- Eshani Nandita
- Remco van Soest

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Thank you for your attention

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Questions and answers

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