QUANTITATIVE ANALYSIS AND METABOLITE PROFILING OF OLIGONUCLEOTIDES

High-resolution mass spectrometry for Oligonucleotides/ Liam Moran, PhD/ Ashland, OH/ November 1, 2021

TOPICS COVERED

1 ANALYTICAL CHALLENGES FOR OLIGONUCLEOTIDE (OGN) DEVELOPMENT

2 EXPLORIS™ 240 PROGRESS

3 METABOLITE IDENTIFICATION

4 CONCLUSIONS AND FUTURE DIRECTIONS

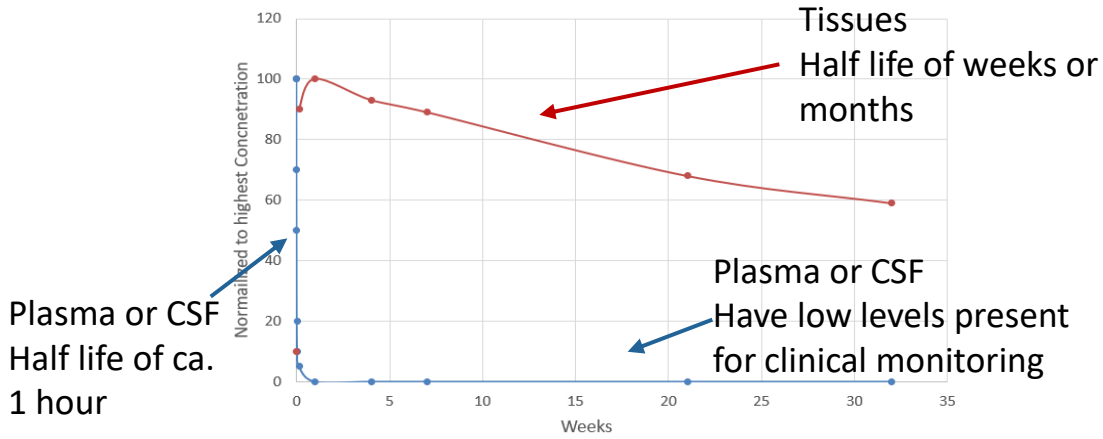
OLIGONUCLEOTIDE DEVELOPMENT

Analytical challenges

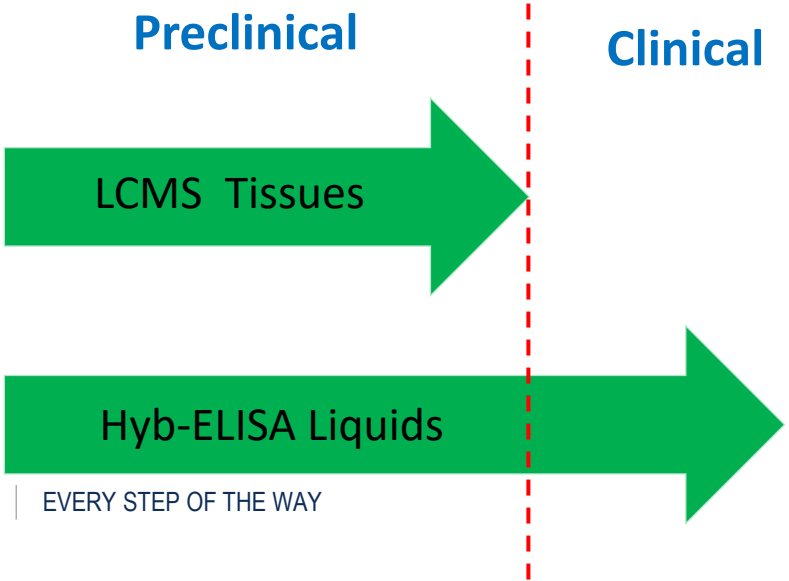
ANALYTICAL CHALLENGES FOR OLIGONUCLEOTIDE DEVELOPMENT

Therapies based on oligonucleotides (OGN) often require multiple bioanalytical assay formats.

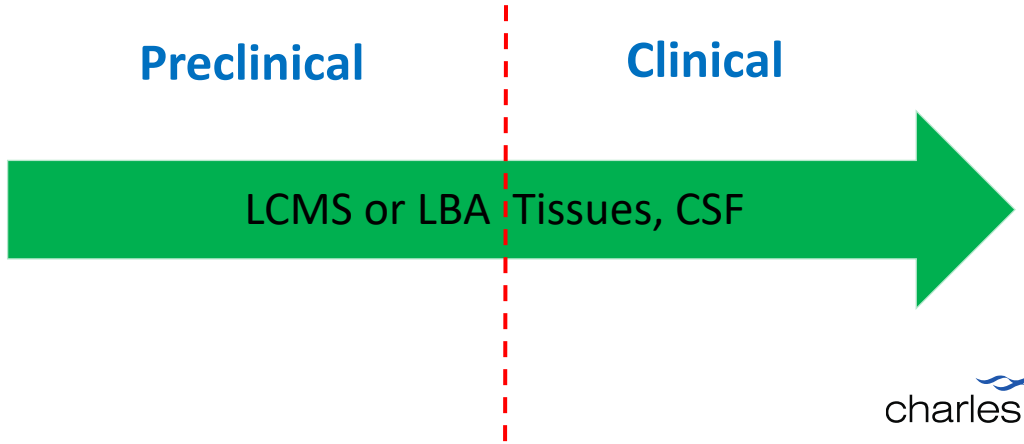
- Hybridization ELISA (Hyb-ELISA) is typically used to monitor the levels of liquid matrices (CSF, or plasma) in preclinal and clinical studies. Mass spec might not have the sensitivity to detect plasma/CSF levels.
- The levels of drug in tissue is very high relative to plasma and mass spec can be used. Moreover, the specificity of mass spec allows for more rapid development times than LBA methods in complex tissue homogenates.



Typical analytical scheme



Less common analytical scheme



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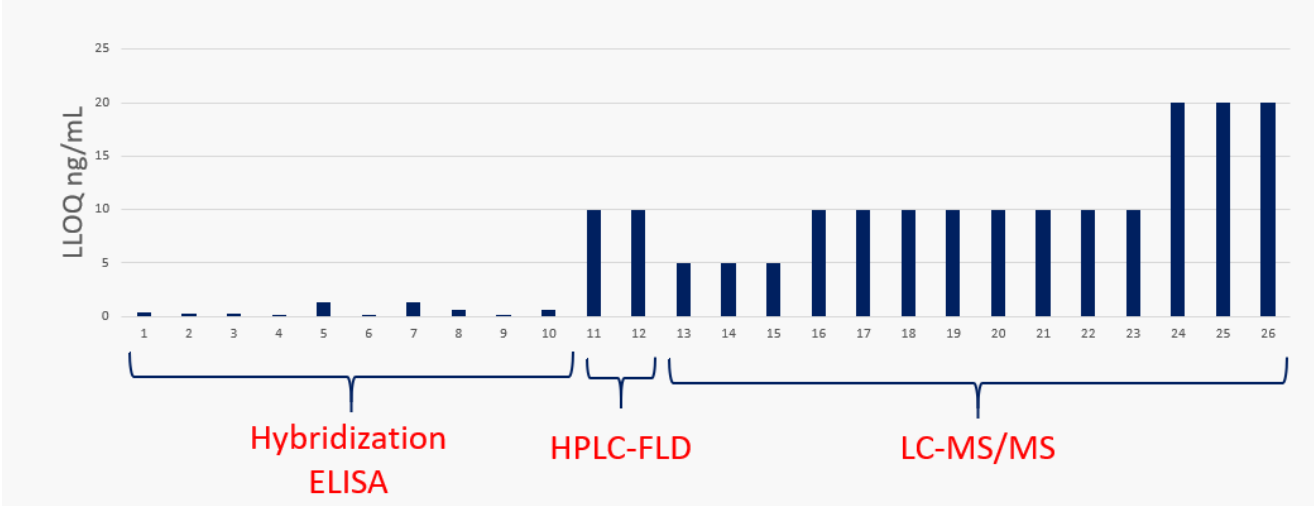
ANALYTICAL CHALLENGES FOR OLIGONUCLEOTIDE DEVELOPMENT

- Hybridization ELISA has lower limits of quantitation (LLOQ) of approximately 100-200 pg/mL. The development time, including getting reagents made, is 12-14 weeks. This method cannot distinguish full run length OGNs from shorter catabolites.
- Hybridization with FLD detection has an LLOQ of 2-10 ng/ml. The development time, including getting reagents made, is 12-14 weeks.
- LC-MS/MS has an LLOQ of 5-20 ng/mL and has a development time of 1-2 weeks. This method can quantitate full run length OGNs and a limited number of shortmers or conjugated forms.
- LC-HRMS (high resolution mass spectrometry) has an LLOQ of 5-20 ng/mL and has a development time of 1-2 weeks. This method can measure many different metabolite and/or conjugated forms of OGNs.

MS is preferred for discovery and lead optimization and Hyb-ELISA is more suited for preclinical and clinical development.

The sensitivity of different assay formats is shown in the Graph below



Sampling of LLOQs from different assay formats
Assay formats: LBA is 100 X more sensitive than LCMS

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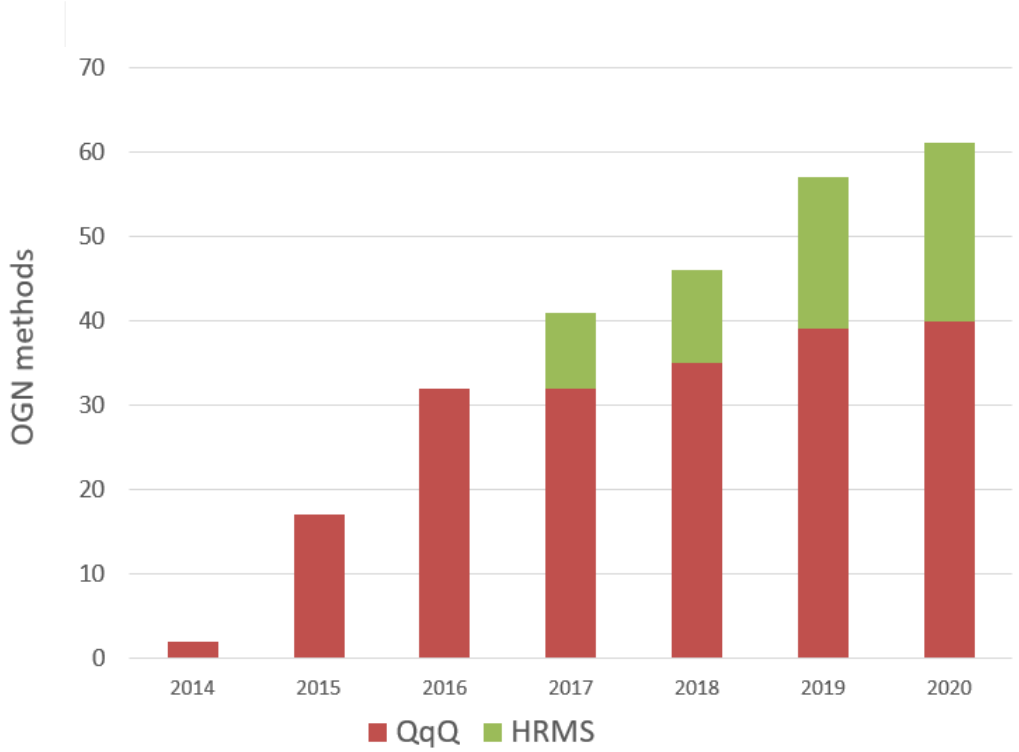
HRMS IS NEEDED FOR COMPLEX OGN THERAPEUTICS

Therapeutic complexity increasing

Drivers for HRMS on Antisense Oligonucleotides (ASOs)

1. Therapies have more unique strands
   - Many therapies have at least sense and antisense
   - Multivalent therapies have up to 6 unique strands
2. Therapies have modifications that need to be monitored for toxicokinetics (TK)
   - Glyco forms
   - Peptide complexes
   - ASO MAb complexes
   - Aliphatic forms
3. Lipid-coated nanoparticles
   1. Novel excipients require TK
   2. Some of these components have polydisperse molecular weight distributions and are better served by HRMS
4. Low level metabolites in matrices like CSF or Plasma



Number of studies run by LC-MS/MS vs LC-HRMS in CRL Ashland

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HRMS IS NEEDED FOR COMPLEX OGN THERAPEUTICS

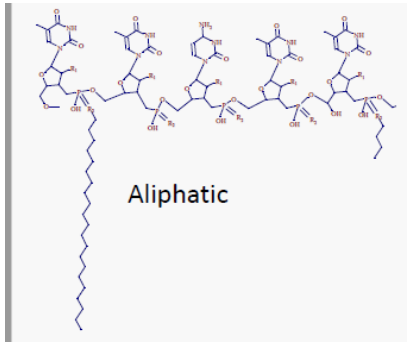
Therapeutic complexity increasing

Conjugated OGNs

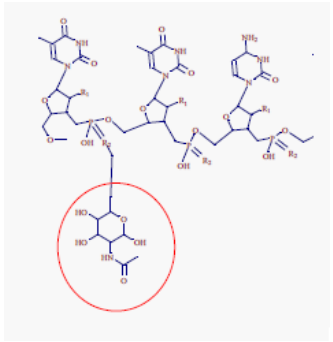
Antibody-OGN

Peptide-OGN

Aliphatic-OGN

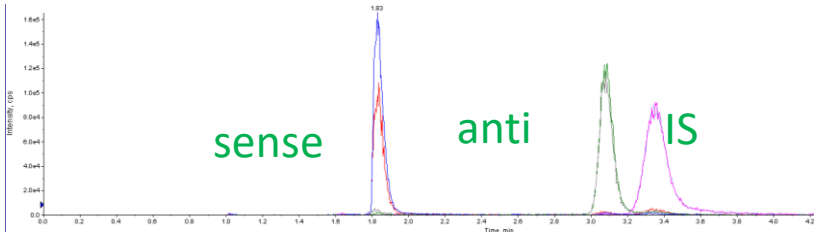


Glyco-OGN

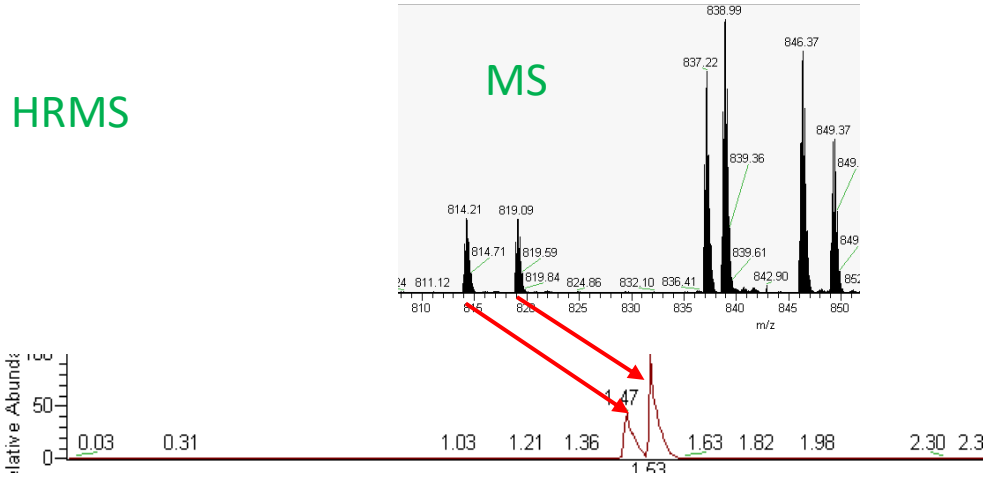


Multi-stranded therapies

QqQ Method 1 in 300



HRMS



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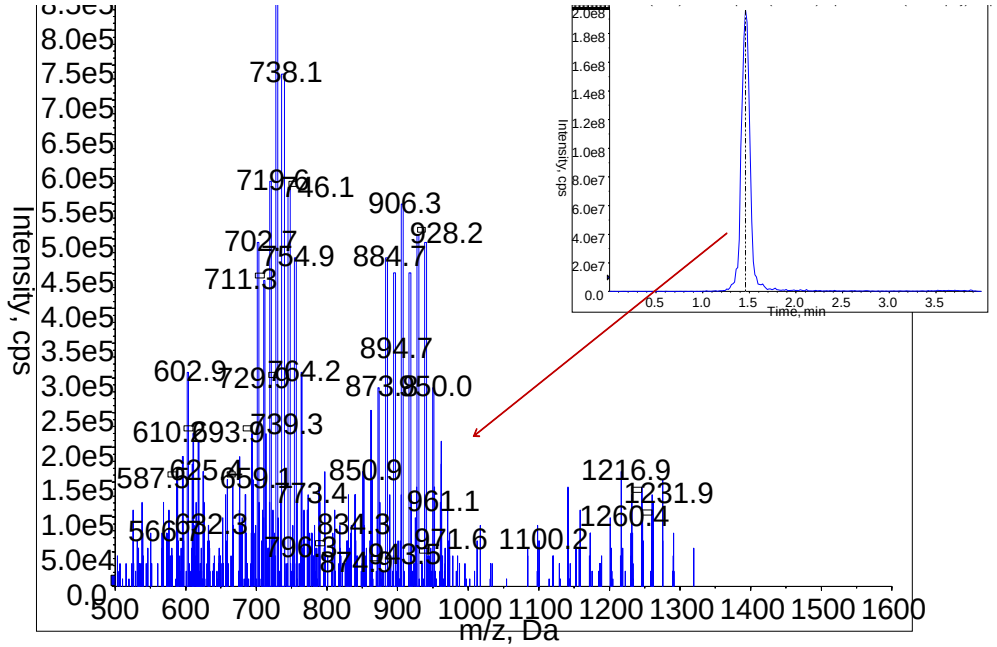
HRMS IS NEEDED FOR COMPLEX OGN THERAPEUTICS

Therapeutic complexity increasing

Lipid-encapsulated OGNs



- Many ASO therapies are formulated in lipid nanoparticles.
- All non-GRAS status excipients usually require TK monitoring in development.
- Formulations can have as many as seven components that need to be measured. It can be advantageous to use HRMS for complicated mixtures.
- Excipients with polydisperse weight distributions often work better by HRMS than SRM as multiple components can be summed.

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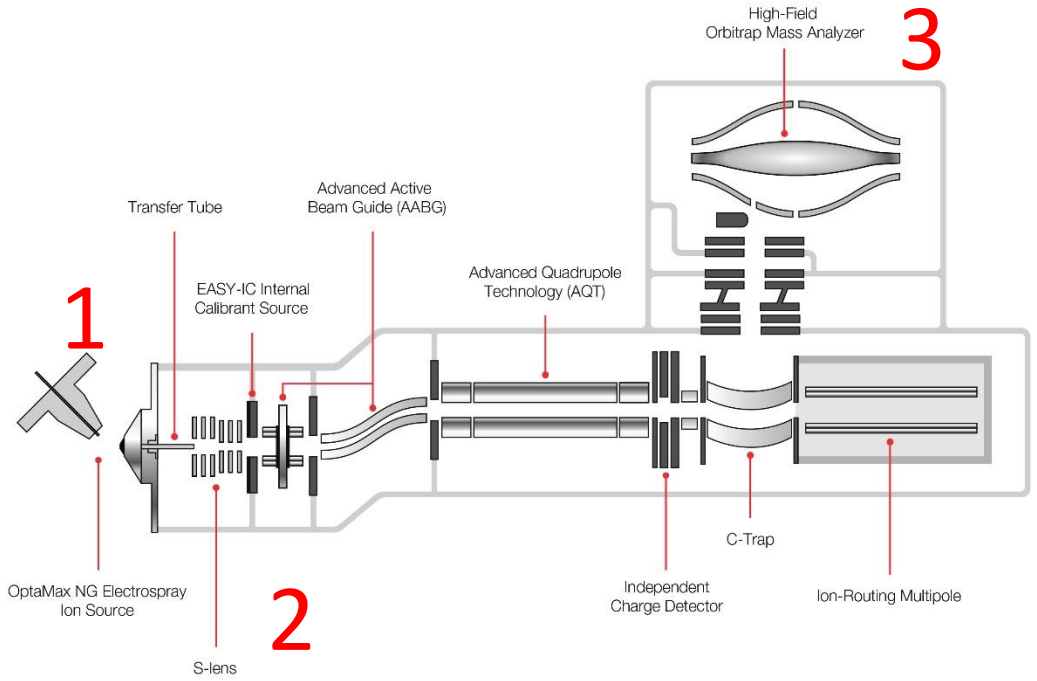
EXPLORIS™ 240

Progress

OVERVIEW OF THERMO EXPLORIS™ 240

Hardware

- The Exploris has improvements in three key areas over previous generations of QqHRMS systems (Q-exactive).
- They are described on the next slide.



Exploris™ 240

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OVERVIEW OF THERMO EXPLORIS™ 240

Differences with previous generations

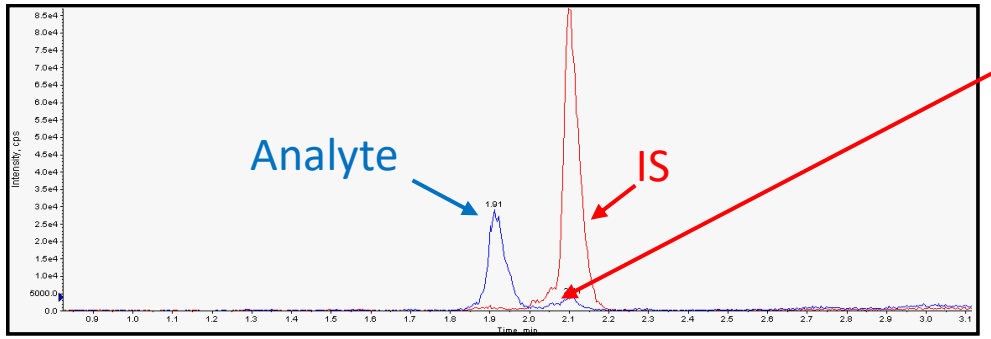
What are the differences with previous generations?

1. The ion source has an internal lock mass generation.
    - The calibrant is introduced inside the vacuum space.
    - The scan function of an orbitrap is a well-defined polynomial so only one mass is needed to correct the whole spectra.
    - This feature is useful for some qualitative applications but is not necessary for quantitation (discussed below).
2. Ion Source: Q-Exactive has 3 turns in the beam line but Exploris 240 has 4 turns in the beam line.
    - The increase in beamline complexity likely provides more robustness.
    - The Q-Exactive generation has adequate robustness (vacuum is not broken between 6-month PM/OQ visits).
    - Other vendor platforms that are line of site to Q1 require frequent venting/cleaning.
3. Mass analyzer driven at a higher voltage/frequency twice the resolution or speed.
    1. This feature gives the system significantly high full-scan detection capabilities.
    2. This resolution is useable on a chromatography time scale > 2Hz.
4. Data system and LIMs environment.

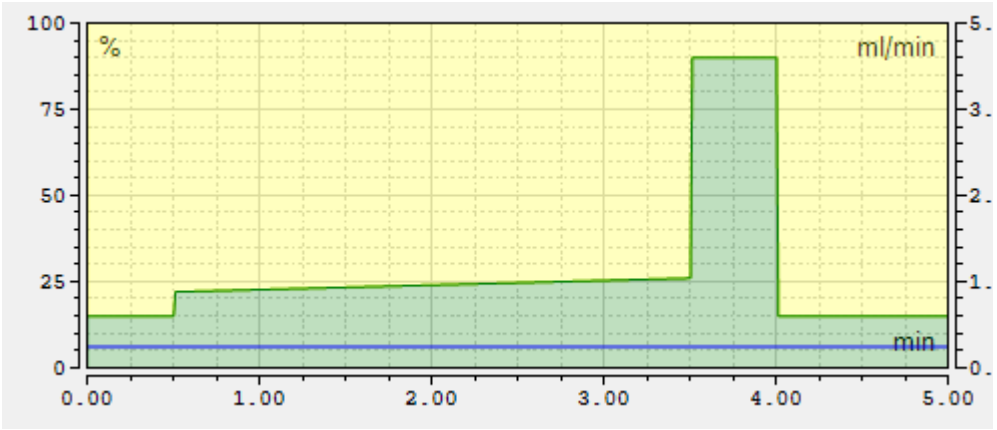
WHY CAN HRMS MEASURE MORE COMPONENTS THAN LC-MS/MS

Therapeutic complexity increasing

Triple quadrupole SRM

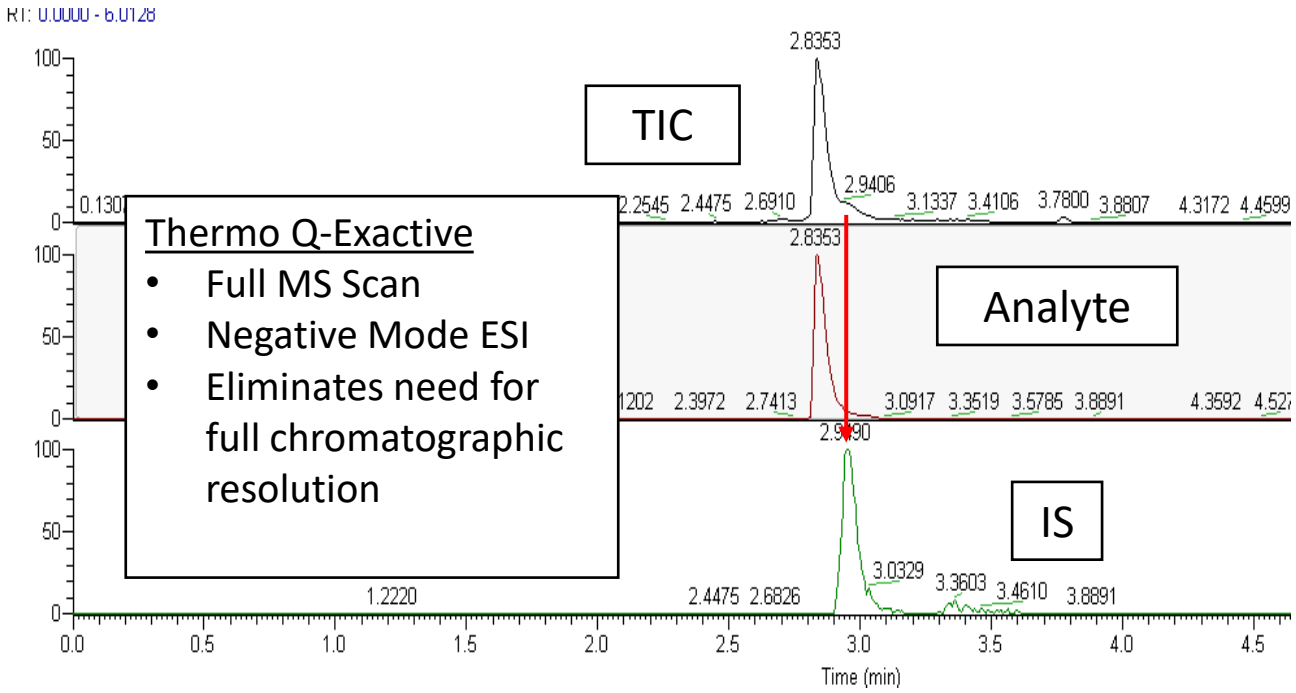


Chromatographic resolution of the IS and analyte is required as there is cross talk in the MRM transitions



The limited "real estate" in the chromatogram precludes multiple analytes for most methods

High Resolution mass spec



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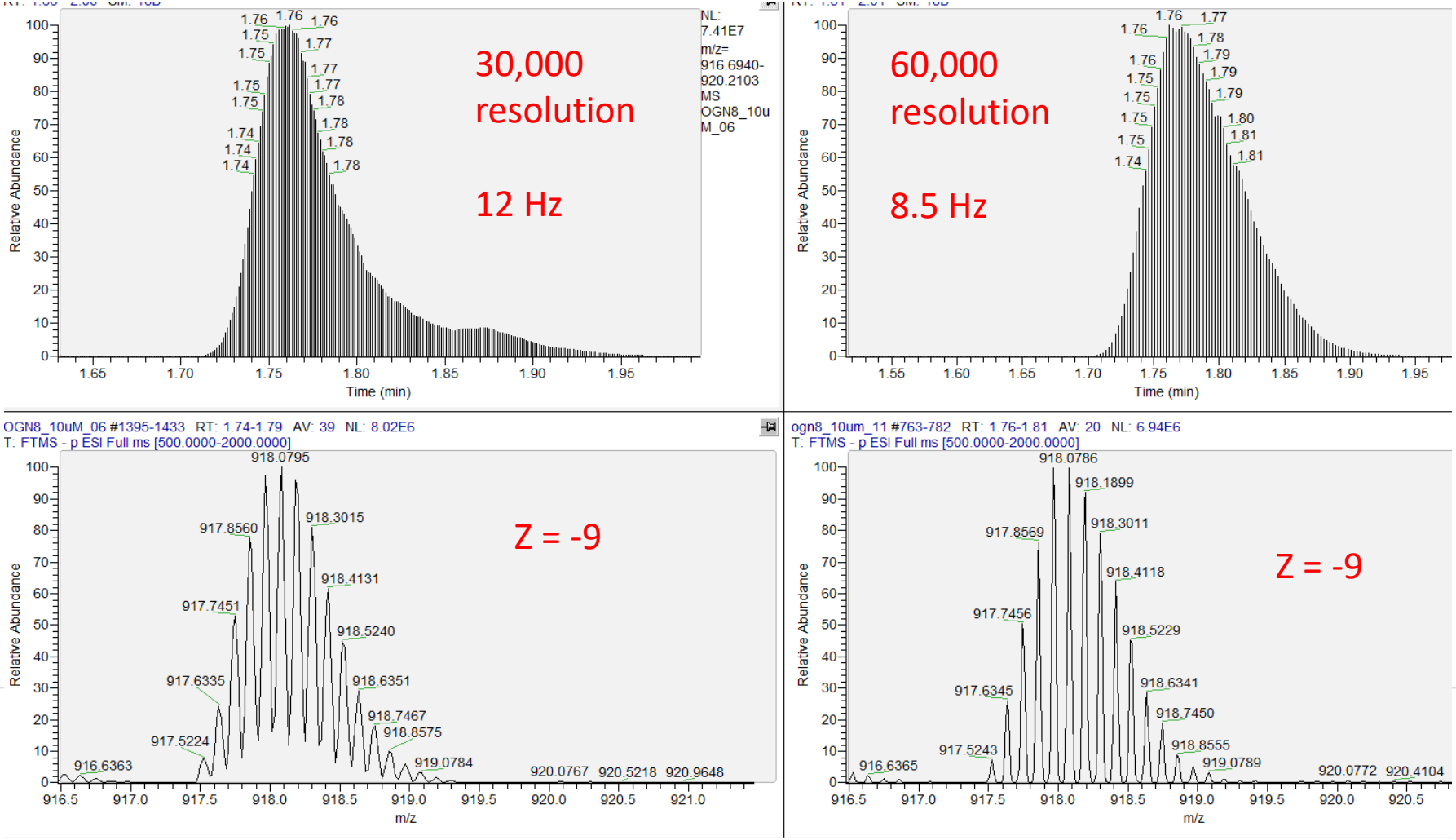
SCAN DENSITY VS RESOLUTION ON THE EXPLORIS™240



The scan density is high at lower resolutions and micro scans should be used to limit file size.

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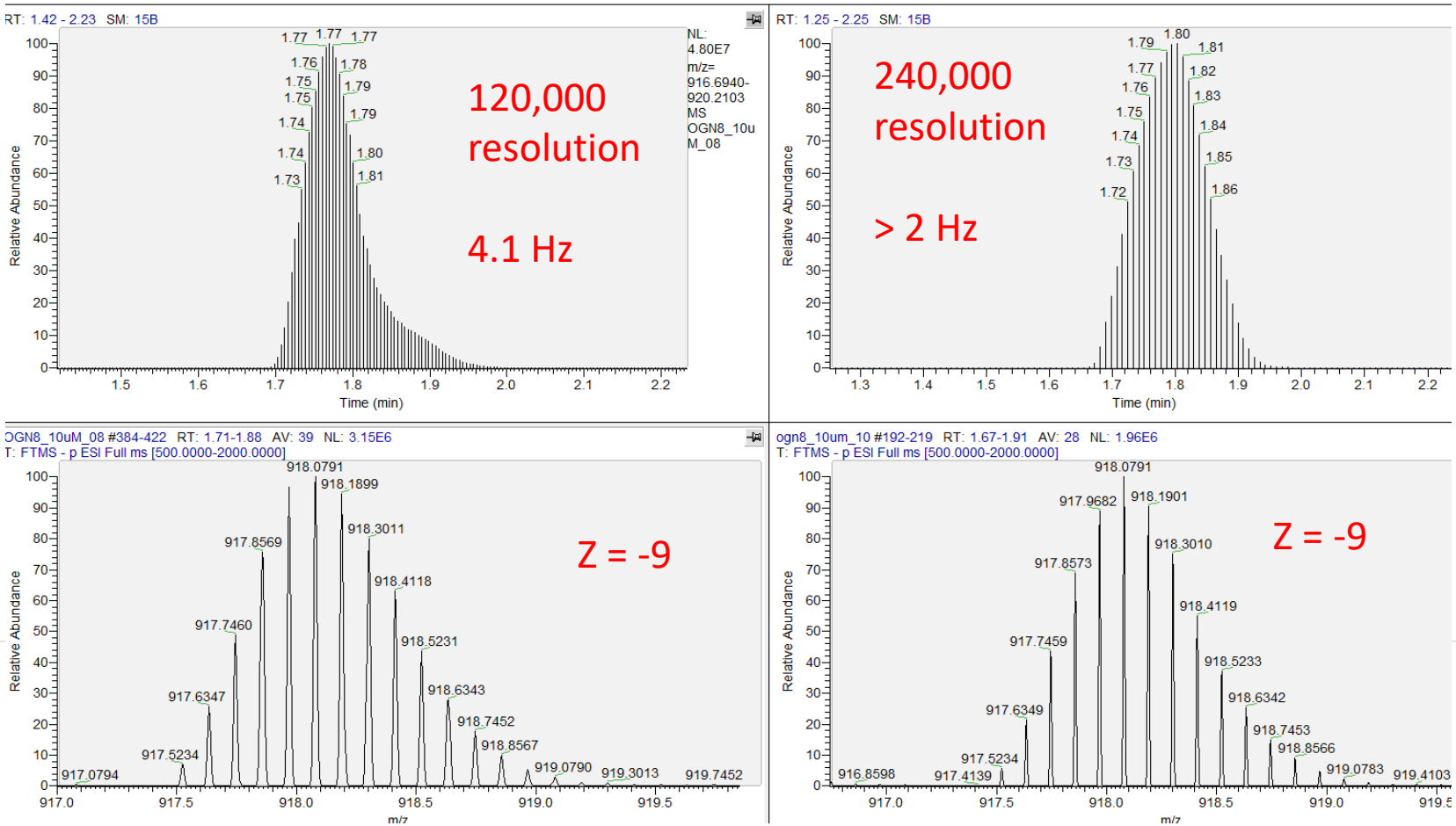
SCAN DENSITY VS RESOLUTION ON THE EXPLORIS™240



The 240,000 resolution scan mode is acceptable on a chromatographic time scale.

Approximately 30 points across the peak are achieved.

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COMPARISON OF QUANTITATIVE DATA BETWEEN Q-EXACTIVE AND EXPLORIS™ SIM

Exploris 240

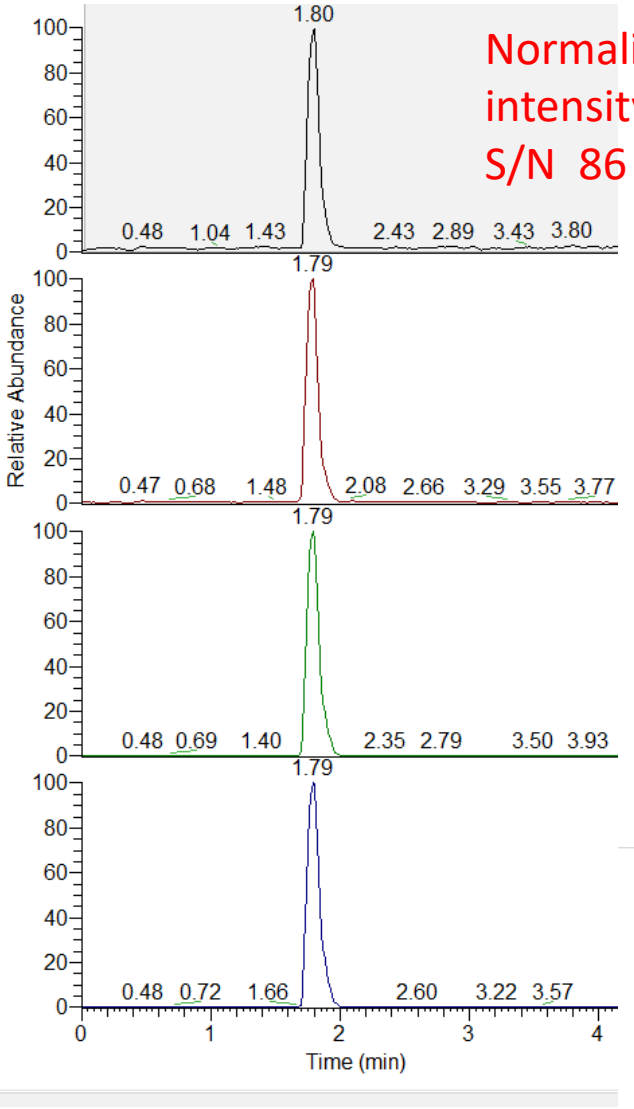
Q-Exactive

2.5 ng/ml

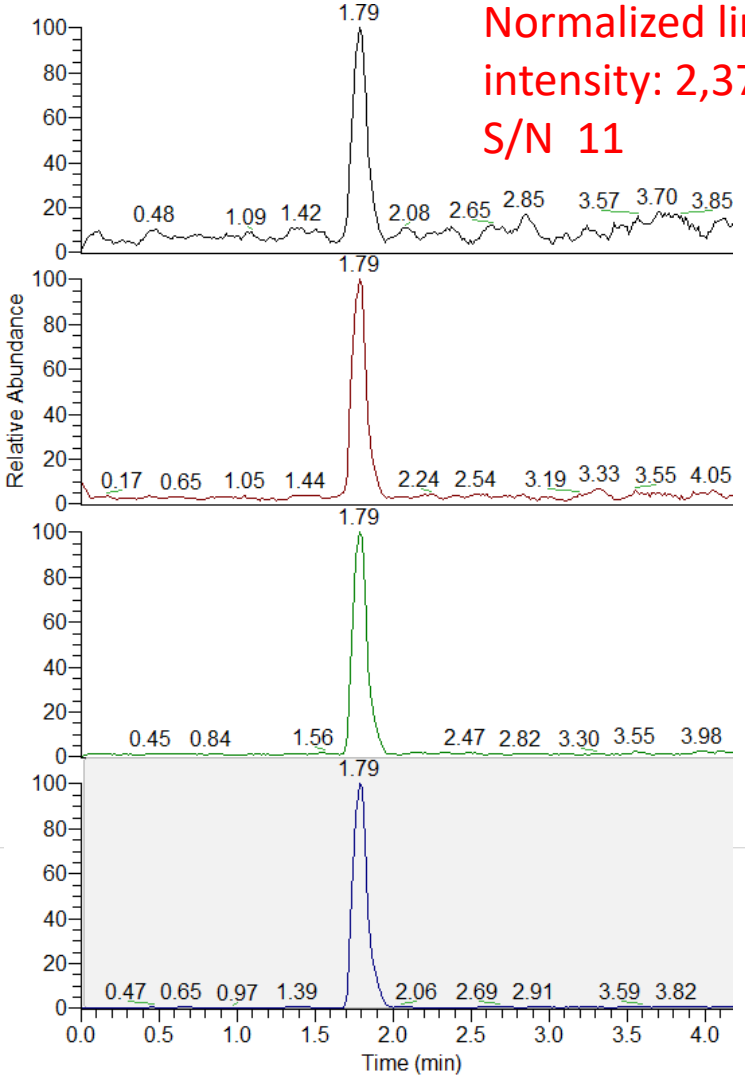
5 ng/ml

10 ng/ml

20 ng/ml



Normalized line intensity: 14,500
S/N 86



Normalized line intensity: 2,370
S/N 11

Standard in rat plasma

QE run in 70,000 resolution

Exploris run in 120,000 Resolution

Peak height six times higher

Signal to noise 8 times better

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COMPARISON OF QUANTITATIVE DATA BETWEEN Q-EXACTIVE AND EXPLORIS™ SIM

Exploris 240

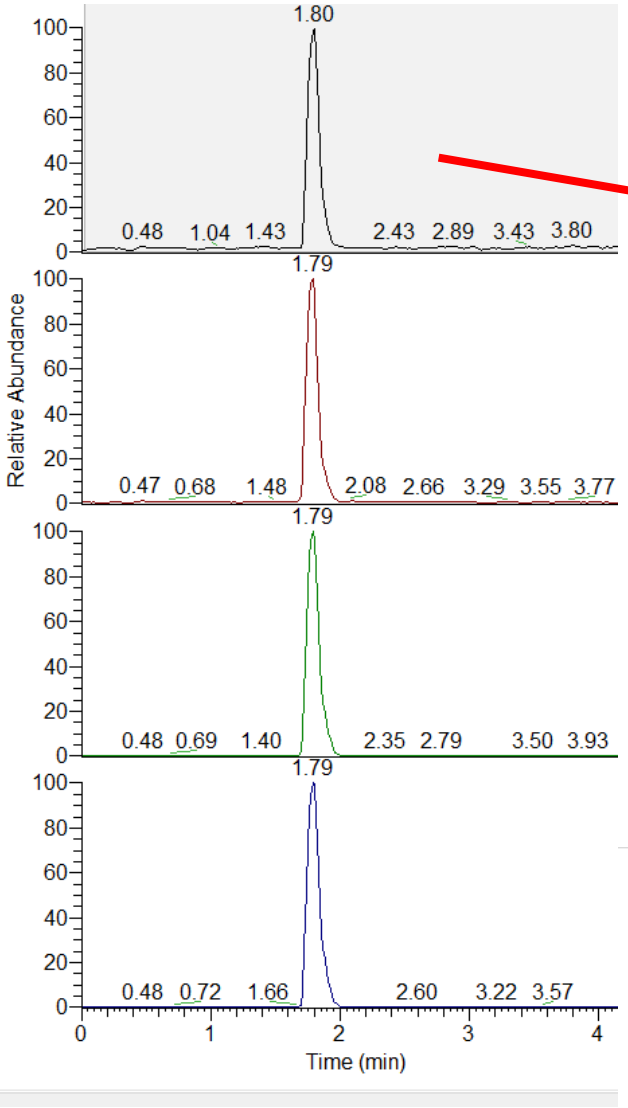
Q-Exactive

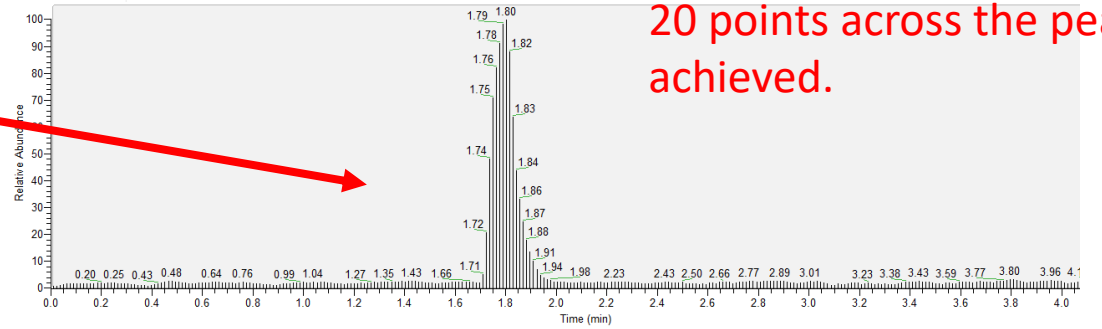
2.5 ng/ml

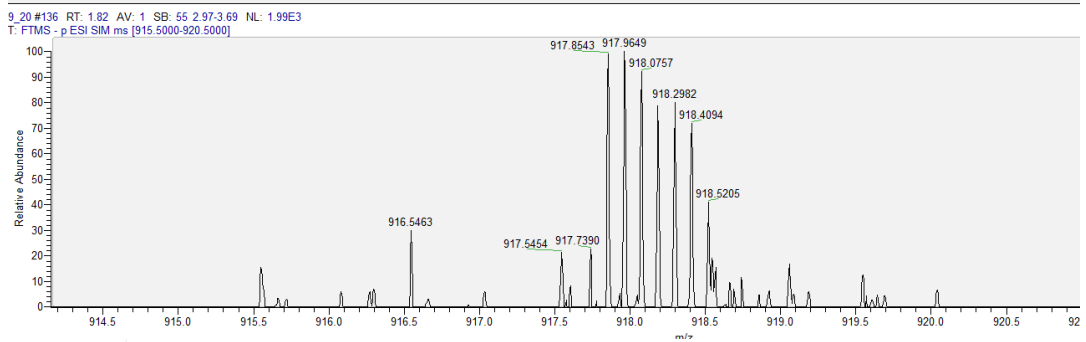
5 ng/ml

10 ng/ml

20 ng/ml







At lower levels, maximum inject time is reached but 20 points across the peak achieved.

The extra resolution begins to separate out noise components, lowering background.

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COMPARISON OF QUANTITATIVE DATA BETWEEN Q-EXACTIVE AND EXPLORIS™ SRM

Exploris 240

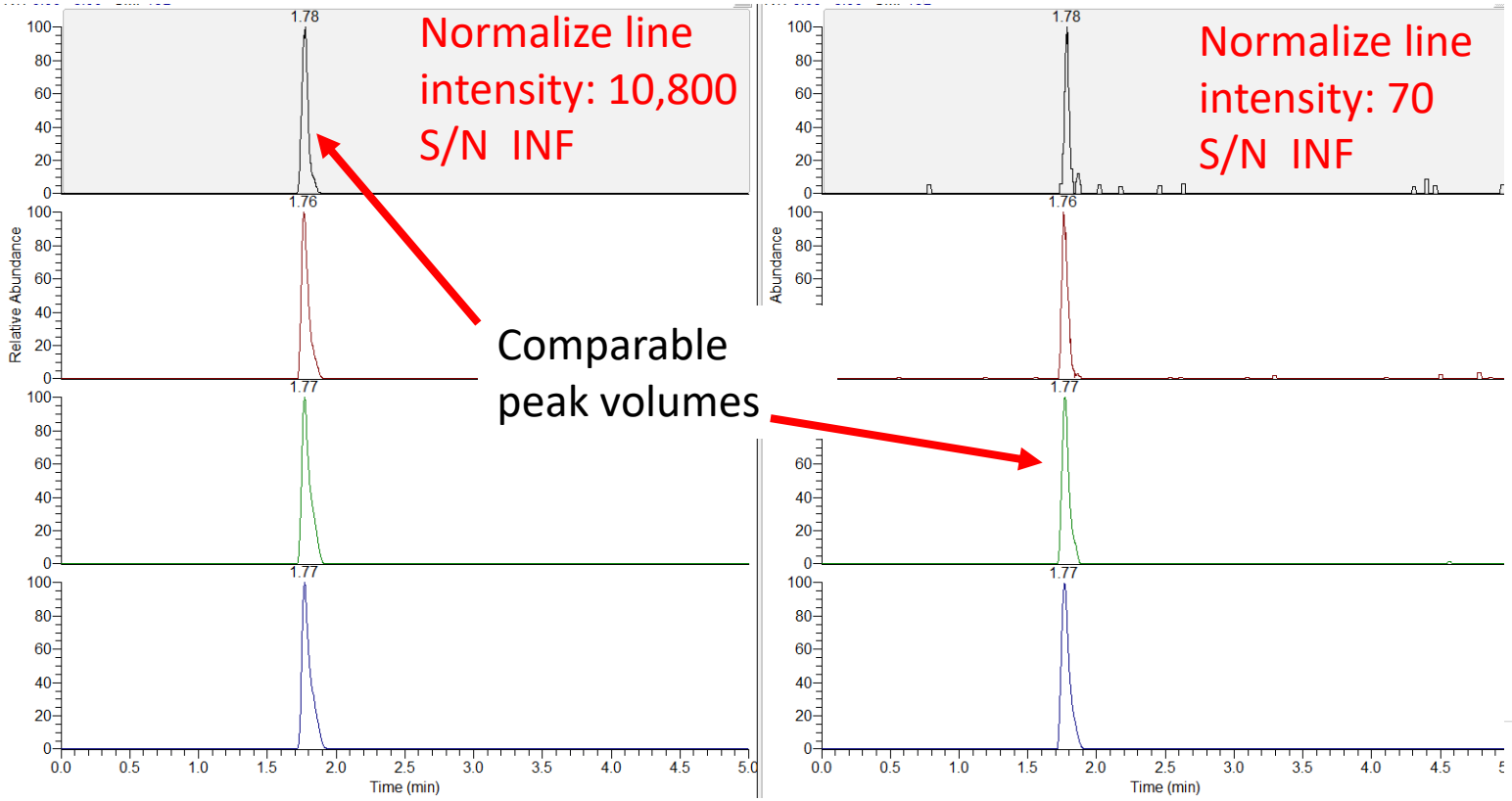
Q-Exactive

2.5 ng/ml

5 ng/ml

10 ng/ml

20 ng/ml



LLOQs are not set based on SN on orbitraps

The signal needs to be about 5,000 for adequate image current

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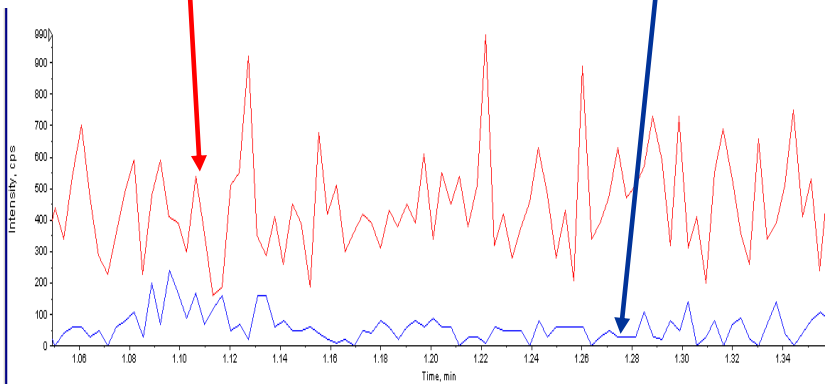
EFFECT OF LOW PURITY HFIP ON MS TRACES

Triple quadrupole

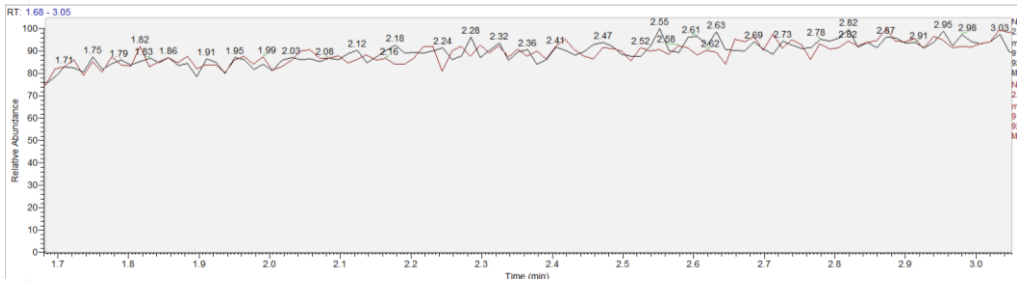
Low Purity HFIP

High purity HFIP

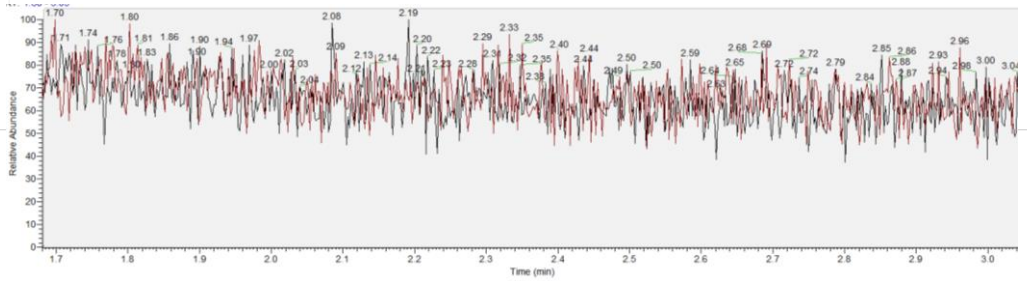


Exploris 240

SIM



PRM



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METABOLITE IDENTIFICATION

Full scan data from Exploris™ 240

METABOLITE IDENTIFICATION OF OGNS

The search for shortmers

- Oligonucleotides with modified backbone chemistries are extremely stable. They have half lives of weeks or months in tissues. Dosing approximately every three months.
- During lead optimization, it is usually not practical to assess stability with *in-vitro* testing as a cell-based test system cannot be kept viable.
- Unlike small molecules, this is not a search for known unknowns (the usual suspects -14, +16, +28, +32, +176, +305) and unknown unknowns.
- The main biotransformation products are shortmers, or incrementally decreased bases. The kinetics of the appearance of short run length OGNs is a key differentiator for lead optimization.
- What is the in-life study design? Dose and wait.

ACGTCACTAATC
CGTCACTAATC
GTCACTAATC
TCACTAATC
CACTAATC

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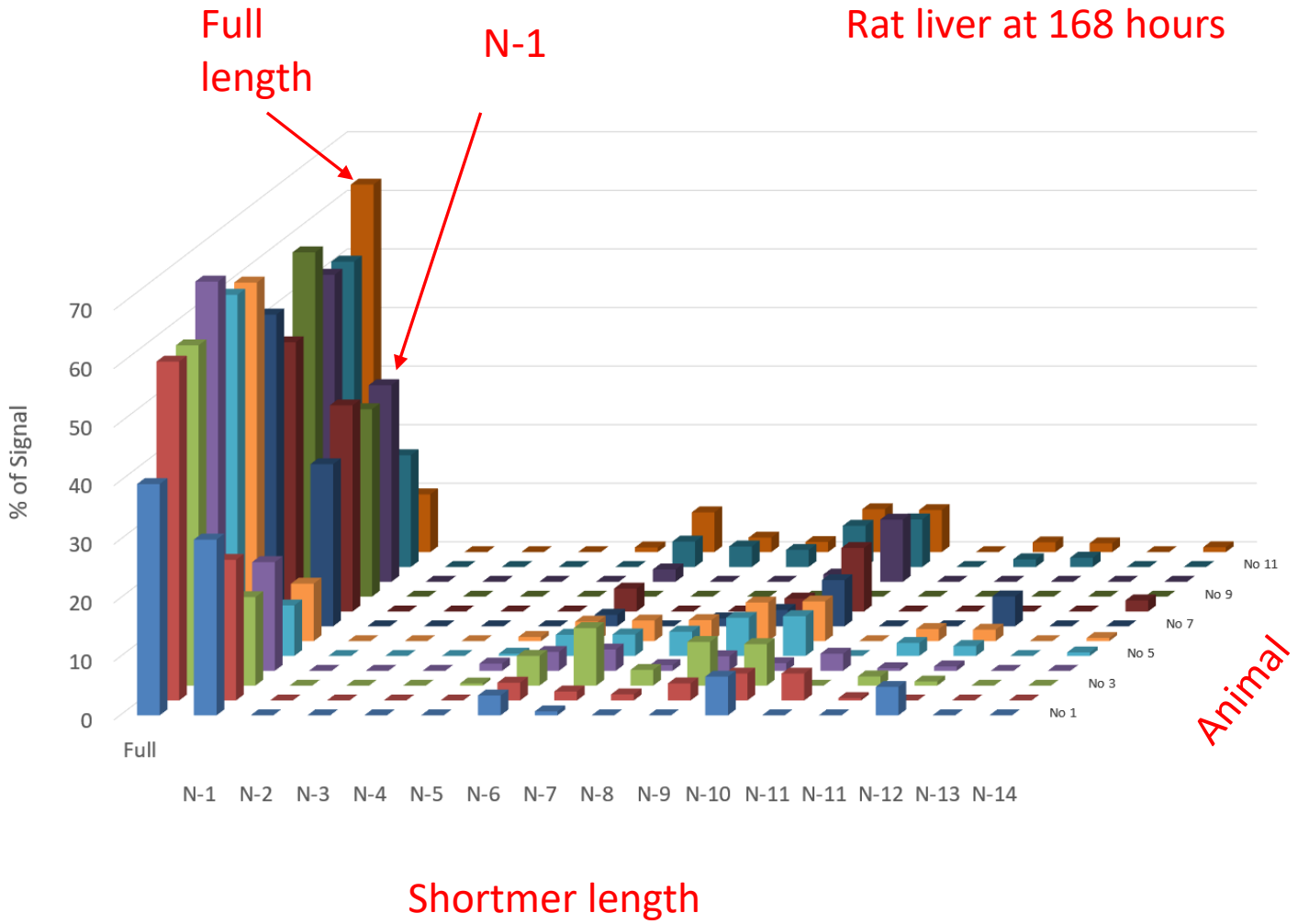
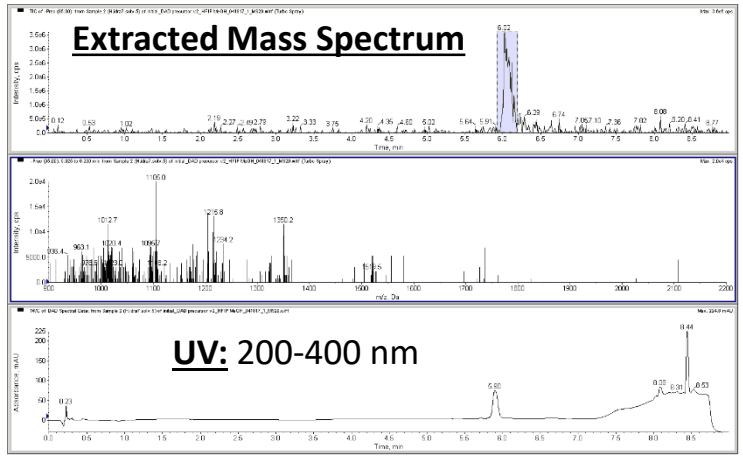
METABOLITE PROFILE



- The percentages of the OGN-related material are estimated by MS signal.
- Some shortmers can be detected by UV but the levels are generally too low to cover all shortmers.



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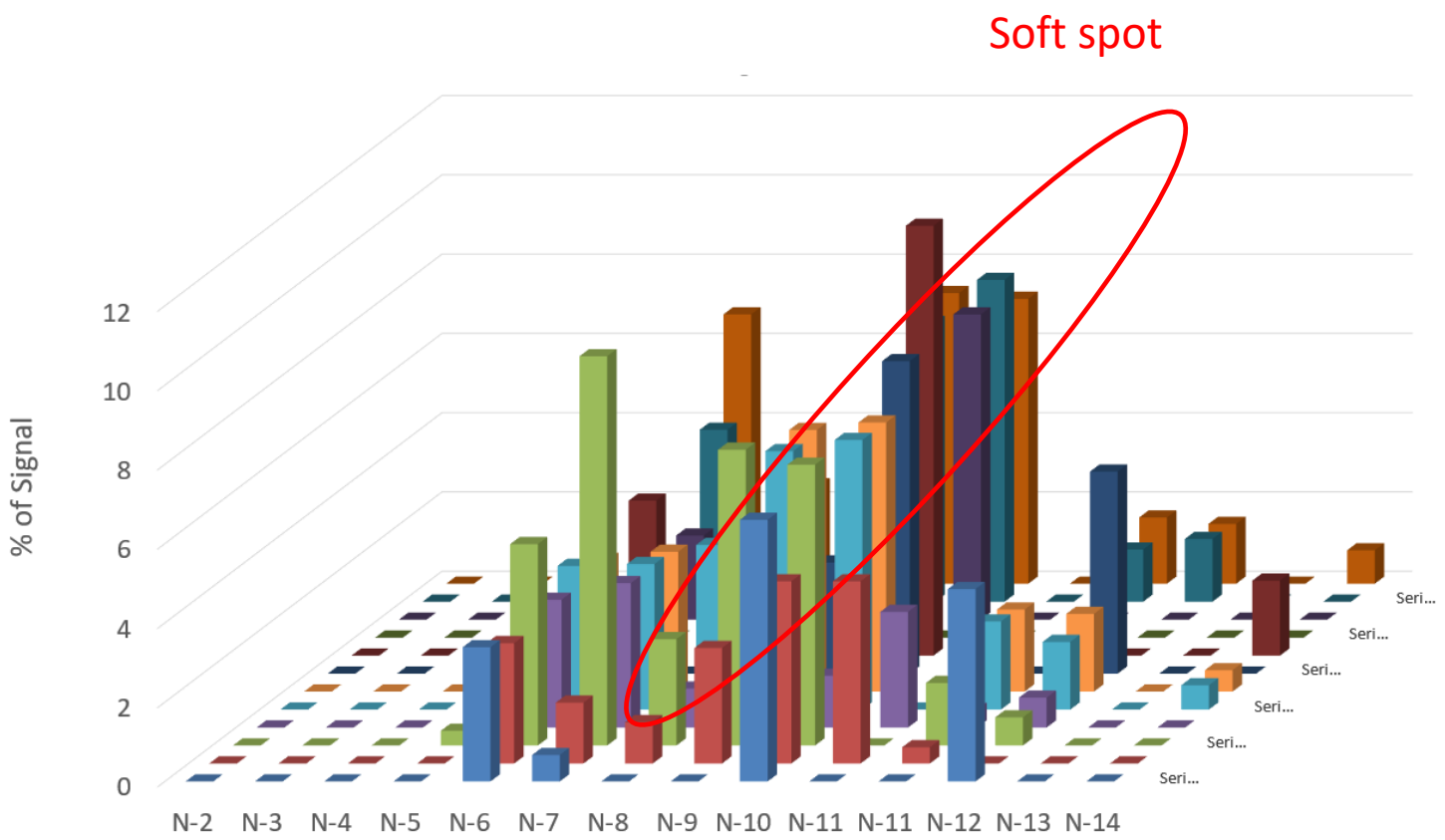
METABOLITE PROFILE



- Previous plot with the Full run length and n-1 removed.
- The smaller shortmers are low potency and will not contribute to pharmacology.
- The key driver to shorten the length of time in-life is full scan sensitivity to find known masses.

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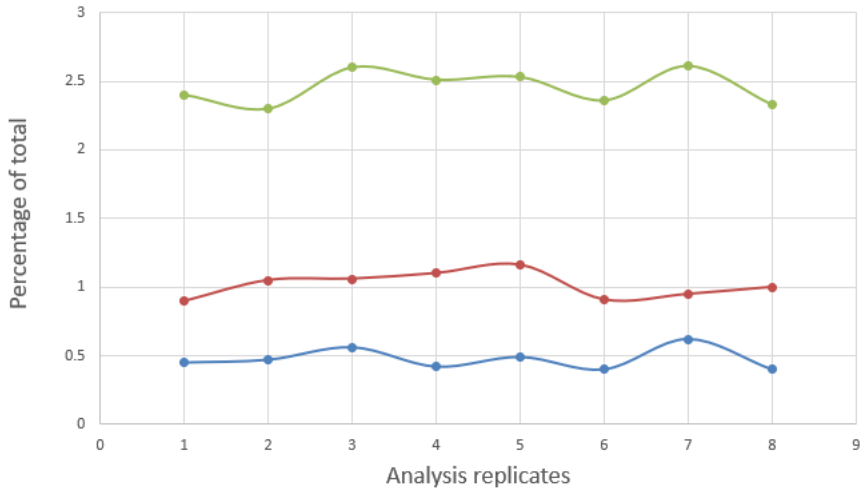
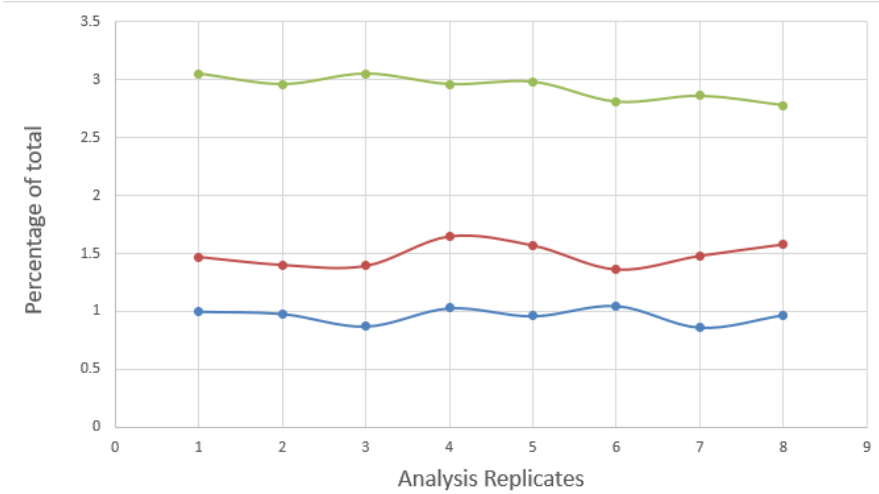
LOW LEVEL OGN ANALYSIS REPLICATES



N-5



N-10

- Analysis replicates of shortmers spiked at low levels: 0.5, 1 and 2.5 %
- The CV is < 20% and this is lower than inter-animal variability
- The full scan sensitivity/ion statistics is approximately 3X better than previous platforms

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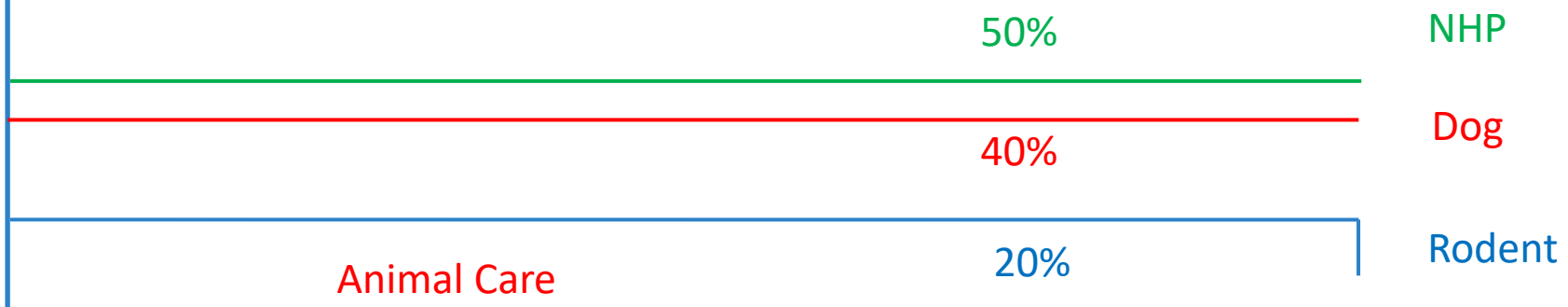
IMPACT ON TIMING AND COST

Perhaps the biggest benefit the Exploris 240 will have in OGN development is shrinking the time and cost associated with running in-vivo metabolite profiling. Extending a study long enough for a less sensitive platform can add 20-50% more cost and 4X more time.

Initial study cost
1) Animals
2) Protocol development
3) Dosing
4) Documentation
5) Animal care



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CONCLUSIONS AND FUTURE DIRECTION

CONCLUSIONS AND FUTURE DIRECTIONS

- The Thermo Exploris has significant gains over previous generations of Orbitrap instruments.
- The improvement in targeted quantitation is approximately 3-fold; however, this is not enough to compete with hyb-ELISA for low level assessments of Oligos in CSF and Plasma.
- The full scan sensitivity and the enhanced resolution will permit compressed metabolite profiling studies.
- The overall data system and engineering greatly enhance the production aspects of the system.
- Ruggedness is TBD but this might take years to assess relative to a Q-Exactive.
- Clinically relevant sensitivity should be achievable with antisense affinity capture:
    - Capture with antisense (magnetic bead or MSIA DART).
    - Low flow to enhance sensitivity (column capacity will work with clean sample).
    - This would be as expensive/time consuming as hyb-ELISA but would provide specificity when needed.

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