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Aliri's Results for Oligonucleotide Ring Trial Study Prove Hybridization LC-MS Approach Superior to LBA in Achieving Lower Detection Limits and Higher Specificity

Troy Voelker, Ph.D.

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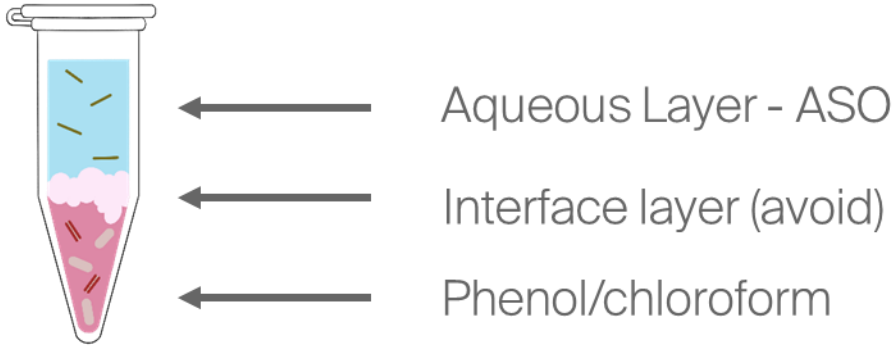
Objectives of the AAPS Oligo Ring Trial

- In this first-of-its kind study, 10 labs joined together to compare the effectiveness of LC-MS, LBA, and qPCR in quantifying concentrations of oligonucleotides in biological samples.
- Aliri facilitated the LC-MS method development of the PMO (Viltolarsen), which was analyzed using three mass spectrometry platforms: a QExactive, a time-of-flight (TOF), and a triple quadrupole instrument.
- Gain insights into hybridization LCMS methods used for the quantification of 3 oligonucleotide molecules.
- Compare data from hybridization LCMS methods to other platforms such as LBA for quantitation of oligonucleotides.
- Apply these concepts to their drug programs with these case study examples.

Improving Extraction Specificity/Selectivity

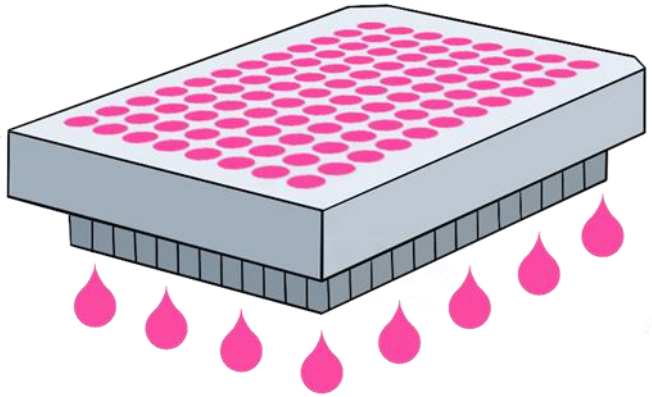
Liqid/Liquid Exchange with Phenol/Chloroform (ASO)

- Three layers, aqueous, interphase, and organic

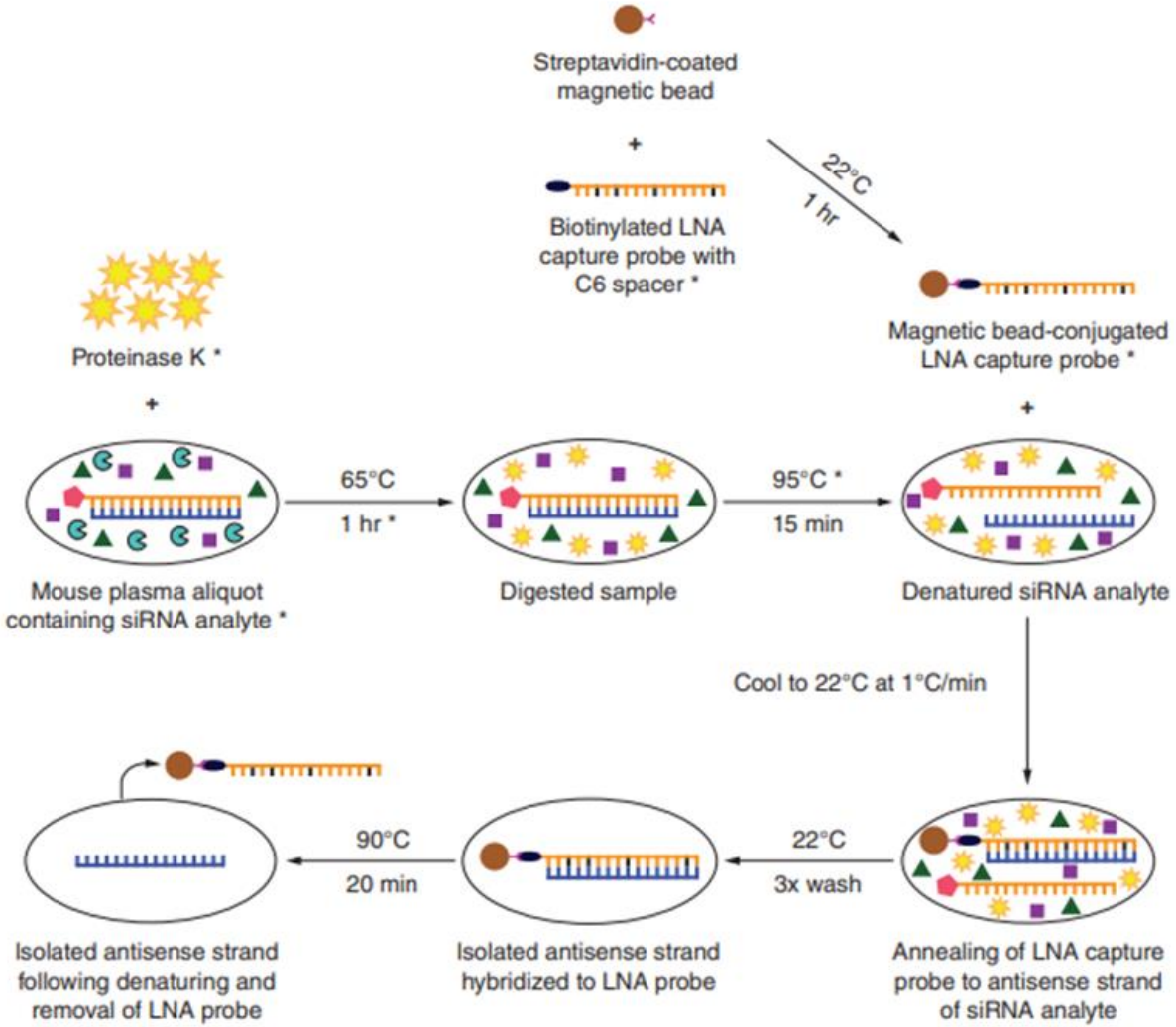


Solid Phase Extraction (siRNA/GalNAc-ASO)

- Phenomenex Clarity SPE
- WAX SPE



Improving Extraction Specificity/Selectivity



Karan Agrawal*, Lijuan Kang, Shaofei Ji, Jennyfer Tena, and Wenying Jian (2023) Evaluating the use of locked nucleic acid capture probes in hybrid LC-MS/MS analysis of siRNA analytes. *Bioanalysis (V15, N18).*

Considerations for OGN LC/MS Hybridization Extraction Approach

- Probe design
- Order of extraction events
- Post extraction Non-specific binding

Probe Design

LNA, PNA, or DNA

- All have been reported to work in the hybridization methodology
- PNA and LNA have a higher binding affinity and may be advantageous with siRNA
- Full length complementary strand is better than a partial length to achieve high recovery
- No reported difference in biotinylating either the 3’ or 5’ end of the probe
- Cross-checking the self-hybridization score and Tm is an important step in early method development
- Using longer length probes or changing the backbone chemistry can help avoid chromatographic interference

Order of Extraction Events

Hybridization order of events

- Hybridize to probe first or hybridize to ASO first?

When to add the internal standard

- Either take it through the hybridization or just add it to the final plate
    - If added before, will there be competition with the ASO if it is present in very high concentrations
    - If added before, should it be a different sequence or a matching sequence of longer length
    - If added after, will the hybridization process be efficient enough to afford good precision

Post Extraction non-specific binding

- What is the impact of using just a LoBind plate
- If the Internal Standard is added post-hybridization, will it take up the binding sites without impacting precision
- Is the use of plasma better than the use of a surfactant
- DNA Lobind plates with ion-pairing based reconstitution solvent results it significant adsorption. If the ion-pairing reagents are necessary, Protein Lobind plates work better.
- EDTA buffered with ammonium hydroxide has proven to be effective to minimize adsorption

LC/MS Platforms

- Triple quadrupole mass spectrometer Sciex 6500+
- High resolution mass spectrometer TOF Sciex 7600
- High resolution mass spectrometer Thermo Scientific Orbital Trap

LC/MS Qualification Criteria

- One accuracy and precision run with 15/20% Mean/CV acceptance criteria
- Matrix effects at the Low and High concentration QC levels with six individual lots at n=3
- Dilution QC at n=6 at 15% Mean/CV
- Stability tests were not performed

Viltolarsen (PMO) Method Development in Rat Plasma

Assay:	Hybridization LC-MS/MS
Analyte(s):	Viltolarsen
Matrix:	Rat Plasma
Range:	0.100 - 100 ng/mL
Internal Standard (IS):	Golodirsen
Extraction Type:	Hybridization
Capture probe:	Biotinylated full-length complementary LNA probe
Sample volume:	25.0 µL
Regression:	Linear $1/x^2$
LC-MS platform:	Shimadzu Nexera X2 UHPLC & SCIEX 7500 Triple Quadrapole Mass Spectrometer

Extraction procedure:

- Aliquot 25.0 µL of rat plasma samples in a KingFisher deep 96-well plate
- Add Clarity OTX Lysis Buffer
- Add Proteinase K (2hr 60C)
- Add LNA Probe
- Incubate the plates (Heat to 95C and cool)
- Add streptavidin beads
- Incubate the plates (35C for 30min)
- Process the sample plates on the KingFisher Flex:
    1. Wash# 1: 500 µL of Wash Buffer
    2. Wash# 2: 500 µL of Wash Buffer
    3. Elution: 150 µL of Elution Solution with working IS
- Store the plate at 4°C until injection

Method Summary:

Rat plasma samples (25µL) spiked with Viltolarsen were digested first with Proteinase K, then extracted using Biotinylated full-length complementary capture probe coupled to streptavidin-coated magnetic beads. The hybridization complex was then purified using the KingFisher Flex (Thermo) magnetic sample processor, followed by heat denaturation to release the ASO in the elution solution fortified with the internal standard for LC-MS analysis. Samples were injected using a Shimadzu LC-30AD Nexera X2 UHPLC coupled to a SCIEX API7500 triple quad mass spectrometer.

Viltolarsen Platform Analytical Ranges in Rat Plasma

LC-MS/MS – 770.4 > 852.6 Curve Range: 0.1 -- 100 ng/mL Linear 1/x2 weighting					
Cal/QC Level	Nominal Conc (ng/mL)	Mean Observed Conc (ng/mL)	SD	%Accuracy	%CV
LLOQ QC	0.1	0.109	0.0166	109.0	15.2
LQC	0.3	0.270	0.0139	90.0	5.1
MQC	40	39.7	5.27	99.3	13.3
HQC	80	79.1	4.18	98.9	5.3
DQC (25x)	2000	TBD	TBD	TBD	TBD

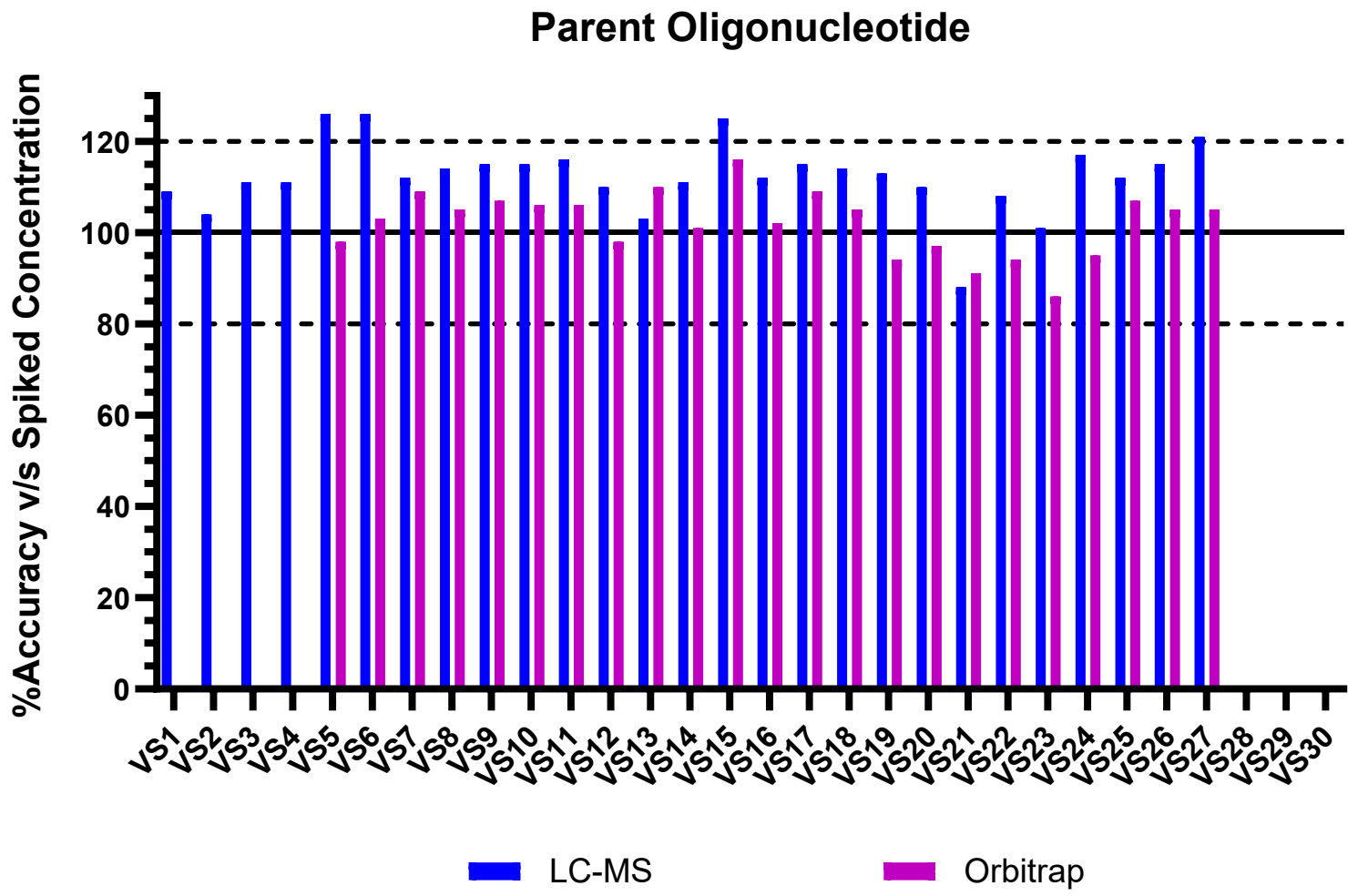
LC-Trap-MS -- Sum of 990.2010, 990.3425, 990.0599, 990.4844, 989.9183, 1155.0664, 1155.2330, 1154.9003 Curve Range: 5 -- 500 ng/mL Linear 1/x2 weighting					
Cal/QC Level	Nominal Conc (ng/mL)	Mean Observed Conc (ng/mL)	SD	%Accuracy	%CV
LLOQ QC	5	4.89	0.295	97.8	6.0
LQC	15	14.1	1.18	94.0	8.4
MQC	80	74.7	4.00	93.4	5.4
HQC	400	426	36.4	106.5	8.5
DQC (5x)	10000	TBD	TBD	TBD	TBD

LC-TOF-MS -- TBD					
Cal/QC Level	Nominal Conc (ng/mL)	Mean Observed Conc (ng/mL)	SD	%Accuracy	%CV
LLOQ QC	TBD	TBD	TBD	TBD	TBD
LQC	TBD	TBD	TBD	TBD	TBD
MQC	TBD	TBD	TBD	TBD	TBD
HQC	TBD	TBD	TBD	TBD	TBD
DQC (100x)	TBD	TBD	TBD	TBD	TBD

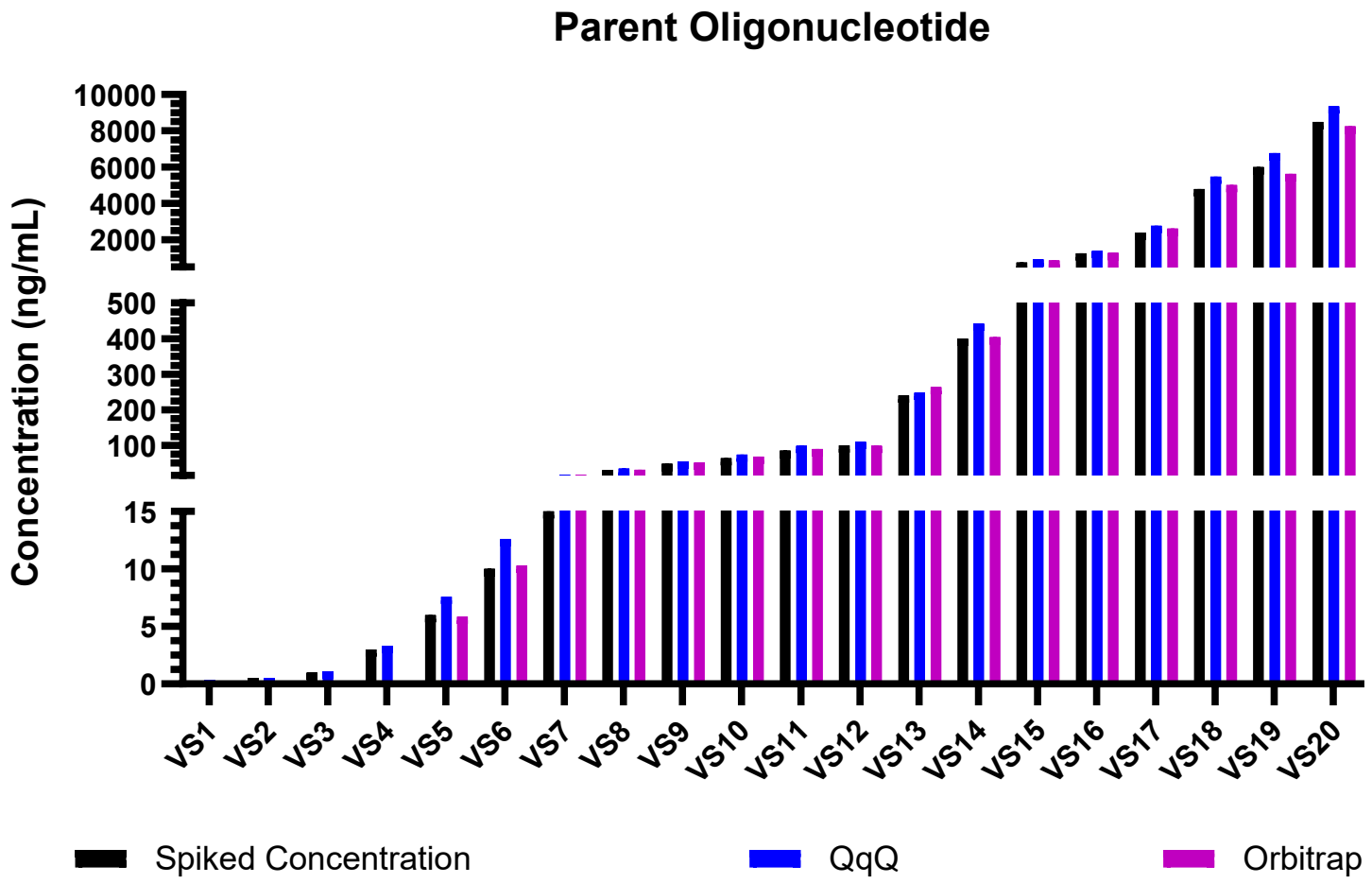
Results for LC/MS Platforms Rivals LBA Sensitivity and Specificity

- Achieved higher sensitivity (0.1 ng/mL LLOQ) on the triple quadrupole
- Established higher specificity on all LC/MS platforms
- Attained unmatched accuracy on the QExactive
- No apparent interference was observed
- Achieved wide dynamic range & high throughput on all LC/MS platforms
- Sample data between the TOF and QQQ were in tight agreement for MS/MS data

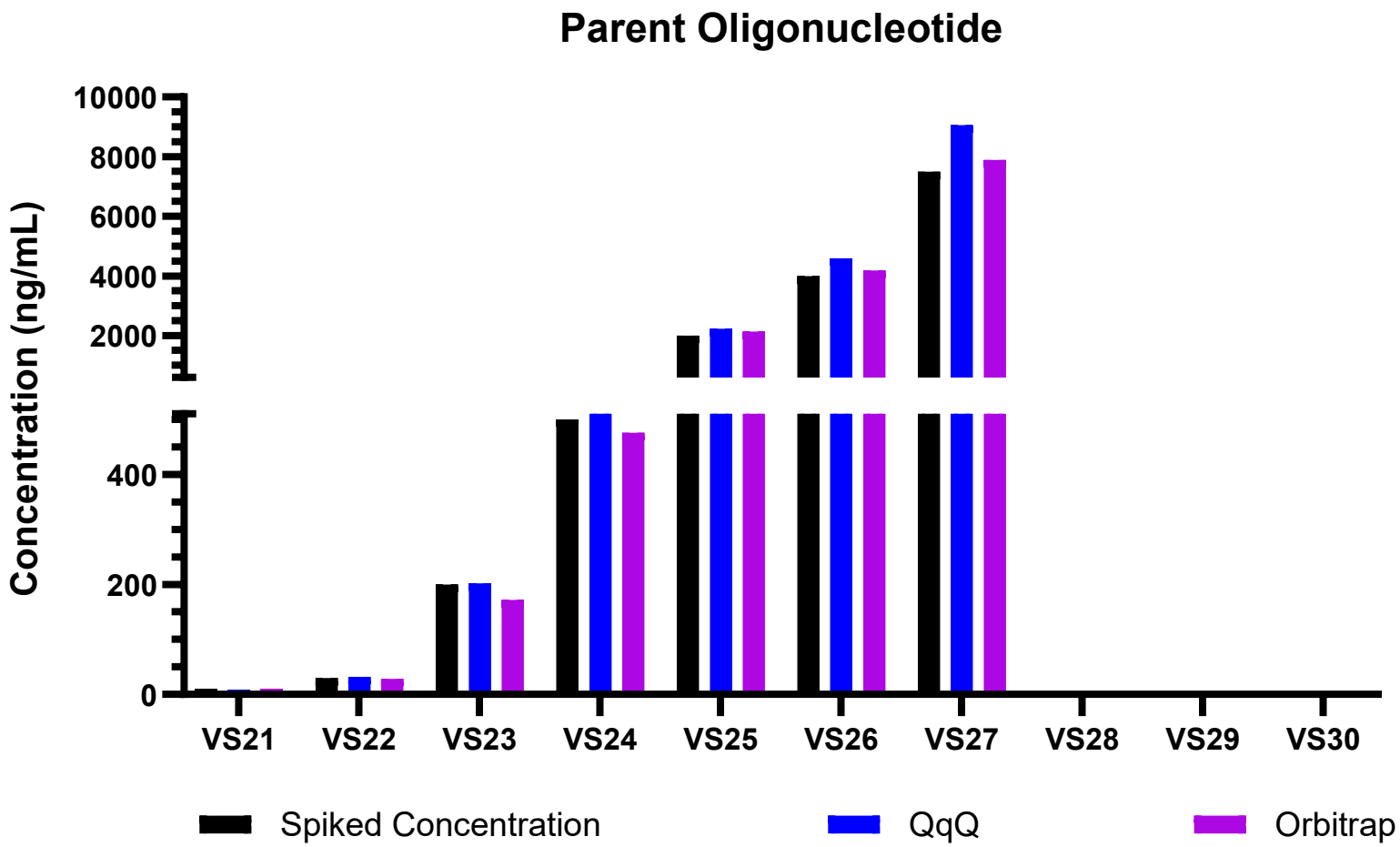
Viltolarsen Accuracy



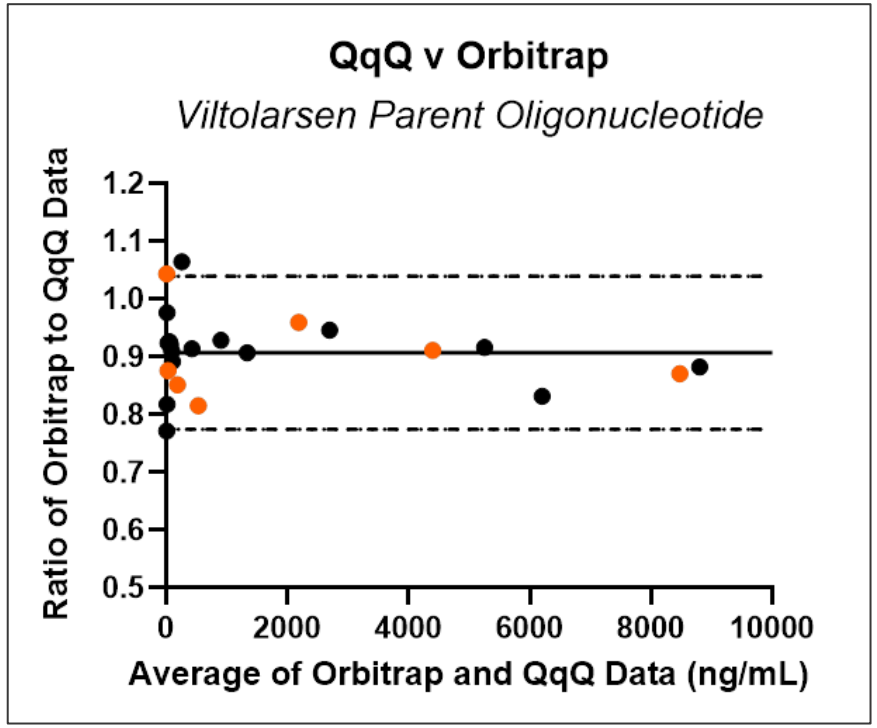
Viltolarsen Calculated Concentrations

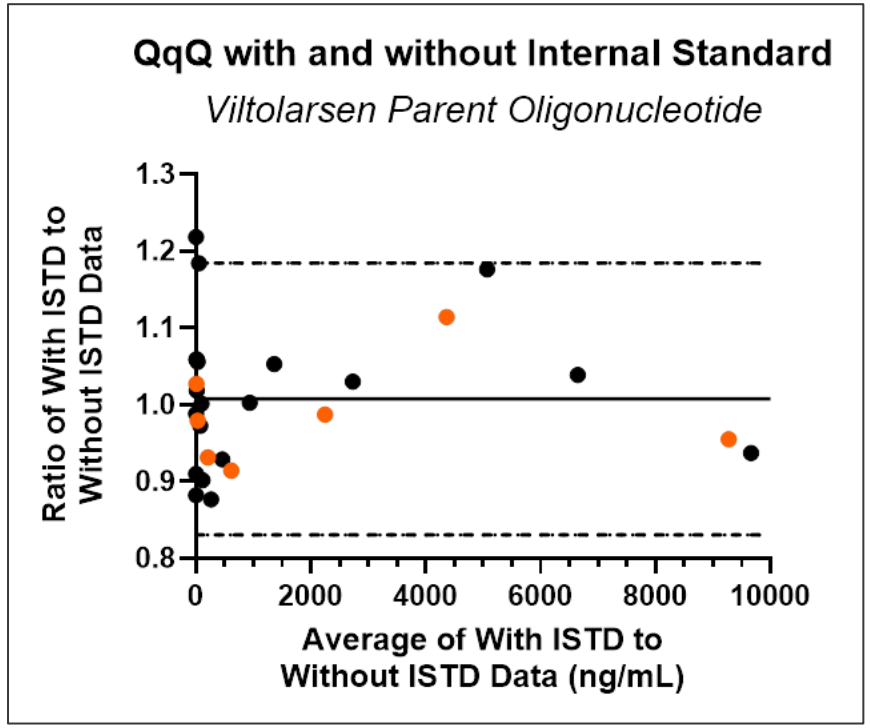


Viltolarsen Calculated Concentrations



Viltolarsen Bland-Altman Plots

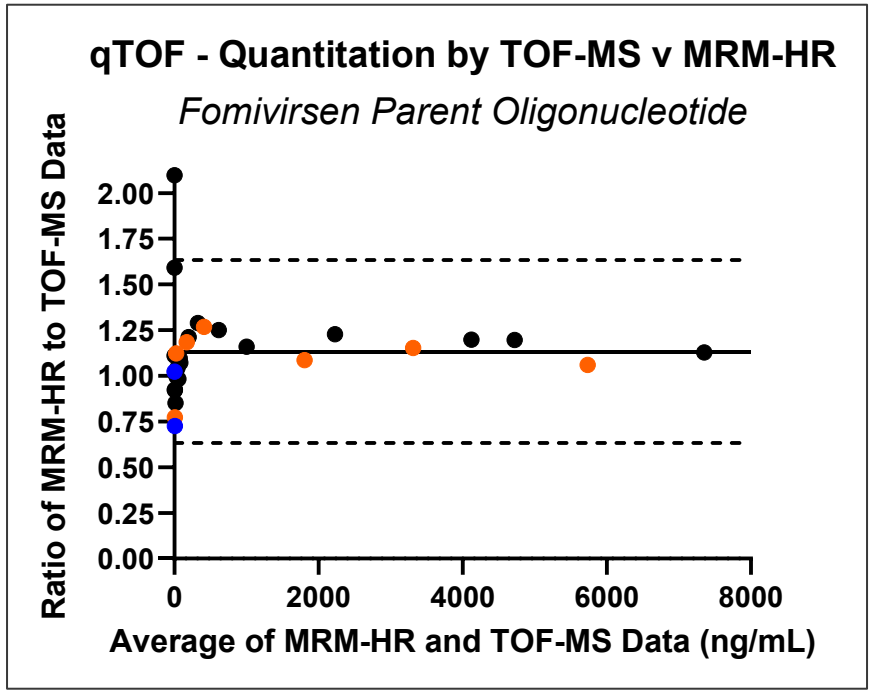


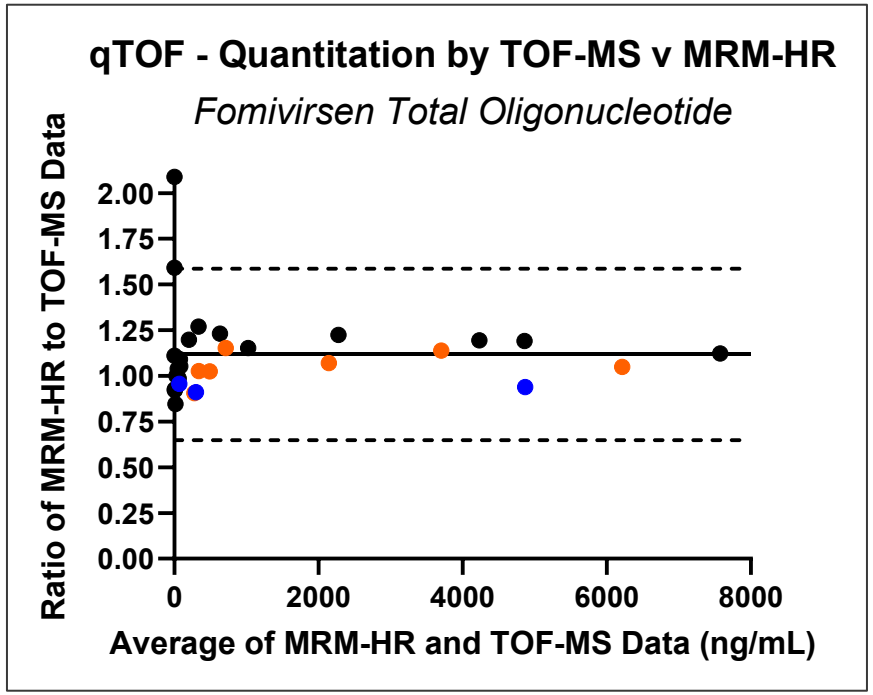


Samples 1 – 20 (Parent Oligo Only)

Samples 21 – 27 (Parent + Metabolite)

Viltolarsen Bland-Altman Plots





Samples 1 – 20 (Parent Oligo Only)
Samples 21 – 27 (Parent + Metabolite)
Samples 28 – 30 (Metabolite Only)

Notes of interest

- There wasn’t any reported workflow about the extraction order that didn’t produce good precision
- Due to limitations of producing the PMO metabolite, it is not truly representative of what we would expect to observe in vivo

About the Scientist

- Troy Voelker, Ph. D., Sr. Lab Director at Aliri Bioanalysis, has worked in the CRO industry for over 19 years and has led method development for over 10 years.
- He has worked with oligonucleotides for over 15 years and currently leads the AAPS Oligonucleotide Bioanalysis discussion group
- *Contact: troy.voelker@aliribio.com*

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info@aliribio.com