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High-throughput LC-MS/MS method for therapeutic oligonucleotides

Method for supporting pre-clinical studies

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11/29/23

OLIGONUCLEOTIDE DRUGS APPROVED

EXONDYS 51 (eteplirsen) Injection

Duchenne muscular dystrophy

CMV infection

Familial hypercholesterolemia

Tegsedi® (inotersen) injection 284 mg/1.5 mL

TTR Polyneuropathy

VYONDYS 53 (golodirsen) Injection

Duchenne muscular dystrophy

waylivra® (volanesorsen) injection 285 mg/1.5 mL

Familial chylomicronemia

SPINRAZA® (nusinersen) injection 12 mg/5 mL

Spinal muscular atrophy

Batten disease

Viltepso™ (viltolarsen) injection

Duchenne muscular dystrophy

onpattro® (patisiran) lipid complex injection 10 mg/5 mL

TTR polyneuropathy

GIVLAARI® (givosiran) injection for subcutaneous use 189 mg/mL

Acute hepatic porphyria

OXLUMO™ (lumasiran) for injection 94.5 mg/0.5 mL

Primary hyperoxaluria type 1

HEPLISAV-B® [Hepatitis B Vaccine (Recombinant) Adjuvanted]

CpG 1018 Hepatitis B vaccine

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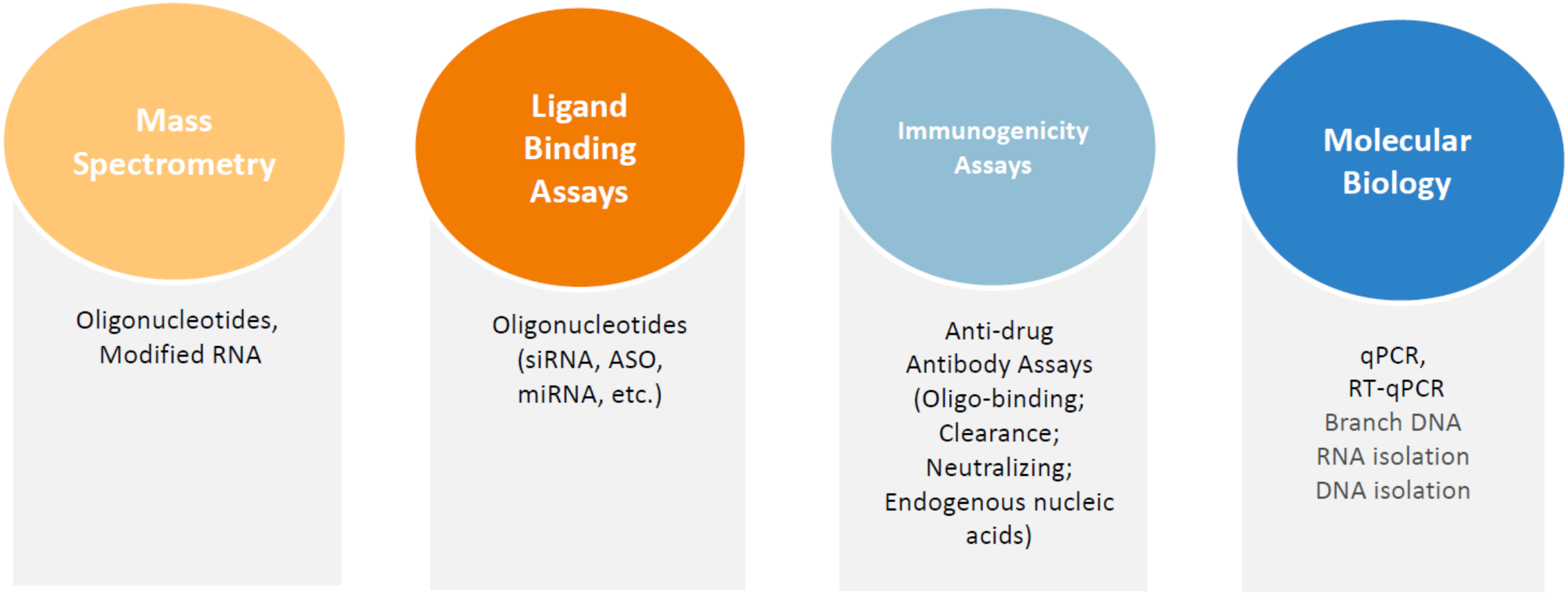
ANALYTICAL METHODOLOGY AT CRL

Consistent and global experience across multiple modalities



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HIGH-THROUGHPUT QUANTIFICATION OF OLIGONUCLEOTIDES FOR PRECLINICAL STUDIES

Transporter substrate assays

- Working Range requirement: 0.1-1,000 nM
- Matrices: HBSS, KH pH 7.4, cell extracts
- Sample number per study: <1000

Need for speed

- Low cost
- Rapid turnaround time
- Method development: Fit-For-Purpose
  Method Validation: in 1-2 days
- Sample analysis and report: in 1-2 days

Fast, cheap and good

Can or cannot I have the best of them all?

OLIGO THERAPEUTICS - TWO PRIMARY ASSAY TYPES

Both Play a Role in Oligo Development Programs

Hybridization ELISA	LC-MS
Expensive custom probes required (capture, detector, cutting, etc. at $2000 - $10,000 each)	Internal standard required (off-the-shelf oligo may be used, $500)
Moderate selectivity Assay reagents typically cross-react with catabolites / shortmers	Excellent selectivity Identify exact species measured and can monitor catabolites / shortmers
Throughput slightly less than average ELISA because of increased # of assay steps	Throughput average to slightly < average if multiple extractions required
No instrument issues	Harsh instrument conditions results in extra cleaning and downtime
Superior sensitivity 0.05 to 2 ng/mL LLOQ in plasma	Less sensitive 1 to 100 ng/mL LLOQ in plasma
Smaller dynamic range (2+ order of magnitude)	Larger dynamic range (3+ order of magnitude)
Minimal variation in assay procedure once format selected for a given drug platform, but skill of analysts very important	Minimal variation in sample extractions and LC conditions from analyte-to-analyte results in quick method development

Fast, cheap and good

Can or cannot I have the best of them all?

TYPICAL SENSITIVITY OF HYBRIDIZATION ASSAYS

Biological Matrix	Format			
	Competitive Hybridization	Dual Hybridization	Hybridization Ligation	Nuclease-Dependent Cutting ELISA
Plasma (µL sample)	10 to 100 ng/mL (10 µL)	10 to 100 pg/mL (10-100 µL)	0.2 to 2 ng/mL (100-200 µL)	0.5 to 3 ng/mL (25-100 µL)
Tissues with Extraction	No Data	1 to 10 ng/g	5 to 30 ng/g	15 ng/g
Tissues without Extraction	No Data	10 ng/g	1 to 10 ng/g	15 ng/g

LC-MS of oligos

ION-PAIRING

- TEA-HFIP
- Apolar phases

ESI-MS

- SIM or MRM

RP

- NH_4OAc / NH_4O_2CH
- Apolar phases

HILIC

- NH_4OAc / NH_4O_2CH
- HILIC phases

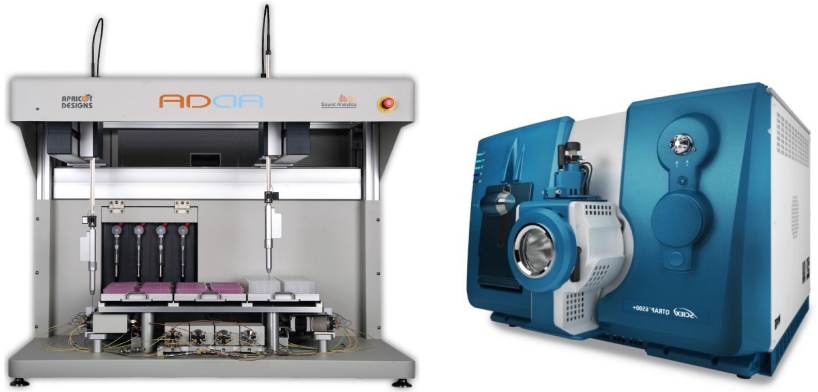
HOW TO BOOST ANALYTICAL CAPACITY?

BY HAVING MORE LC-MS

KPI		Capacity increase
Throughput	(+)	2x
Uptime	(+)	2x
Maintenance	(-)	2x
Cost	(-)	2x
Mass Spectrometer Utilization	(-)	1x

BY HAVING HIGH THROUGHPUT LC-MS

KPI		Capacity increase
Throughput	(+)	2-16x
Mass Spectrometer Utilization	(+)	2-16x
Cost	(+)	2x
Maintenance	(+)	1x
Uptime	(-)	1x



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Theoretical plate hight small if:
1. Small particle size
2. Low flow rate
3. Low eluent viscosity
4. High temperature
5. Small molecule

High Throughput Approach

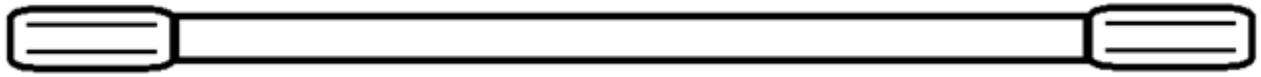
Make a choice

- Resolution: Only for critical component(s)
- Plate Number: Less important for gradient separation.
- Peak capacity: Less important for MRM detection, matrix effect?
- Retention: Desalting, control of matrix effect
- Selectivity: Only for critical analytes

$$N = \left(a\sqrt{\frac{1000\eta\varepsilon_T}{t_o \Delta P}} + b\frac{1000\eta\varepsilon_T}{\delta^2 \Delta P} + c\frac{\delta^2}{t_o} \right)^{-1}$$

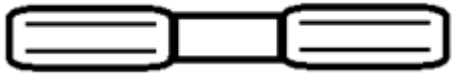
$$n_{C,G} \approx 1 + \frac{1}{4}\cdot\sqrt{N}\cdot\frac{10}{1+10\frac{t_o}{t_G}}$$

100 x 2.1 mm, 3um, 1 ml/min, tg: 2.2 min



t0: 0.22 min
Cycle time: 3.96 min
Peak capacity ~ 126

10 x 2.1 mm, 3um, 1 ml/min, tg: 0.2 min



t0: 0.02 min
Cycle time: 0.36 min
Peak capacity ~ 40

Cycle time: tg + 3 x t0 +5x t0

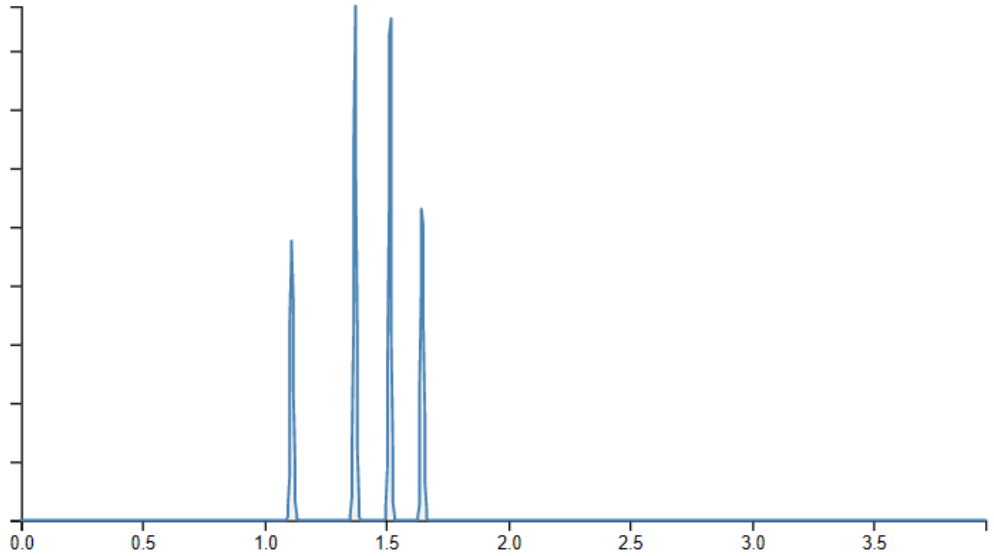
Chromatographer has to choose either high efficiency or high speed (or rely on very high selectivity).

High Throughput Approach

Make a choice

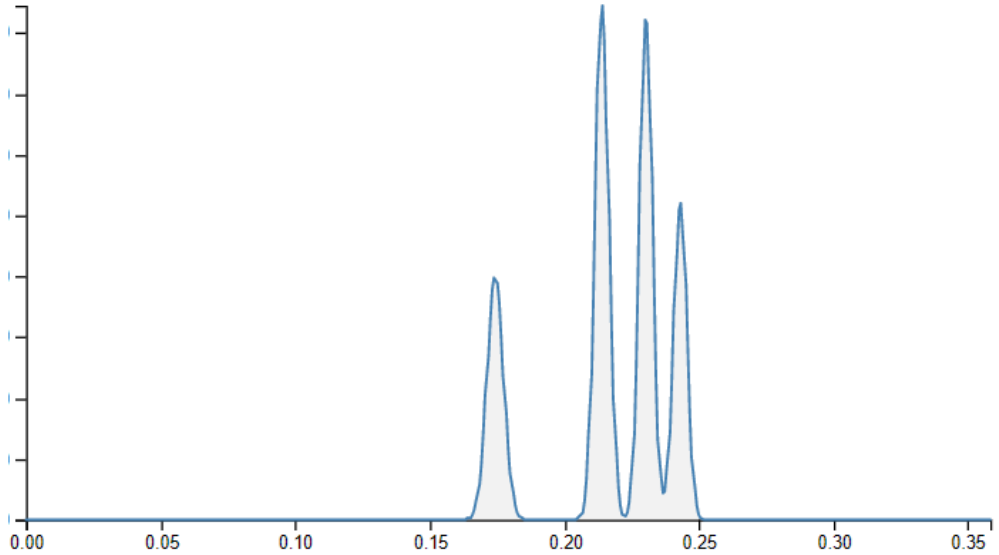
100 x 2.1 mm, 3um, 1 ml/min, tg: 2.2 min

t0: 0.22 min
Cycle time: 3.96 min
Peak capacity ~ 126



10 x 2.1 mm, 3um, 1 ml/min, tg: 0.2 min

t0: 0.02 min
Cycle time: 0.36 min
Peak capacity ~ 20 (40)



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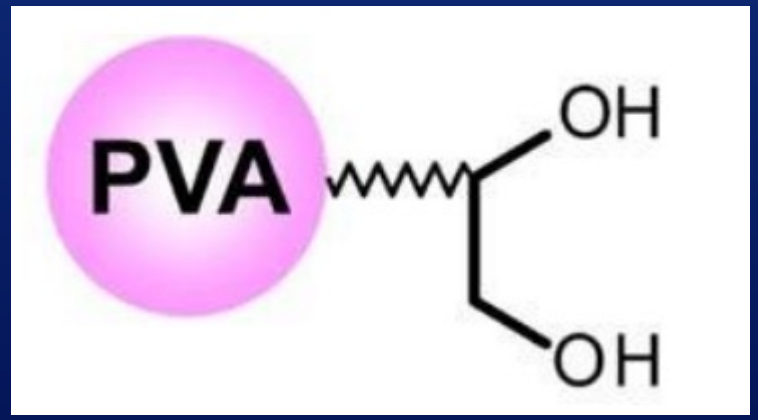
HT-LC-MS/MS OF OLIGOS

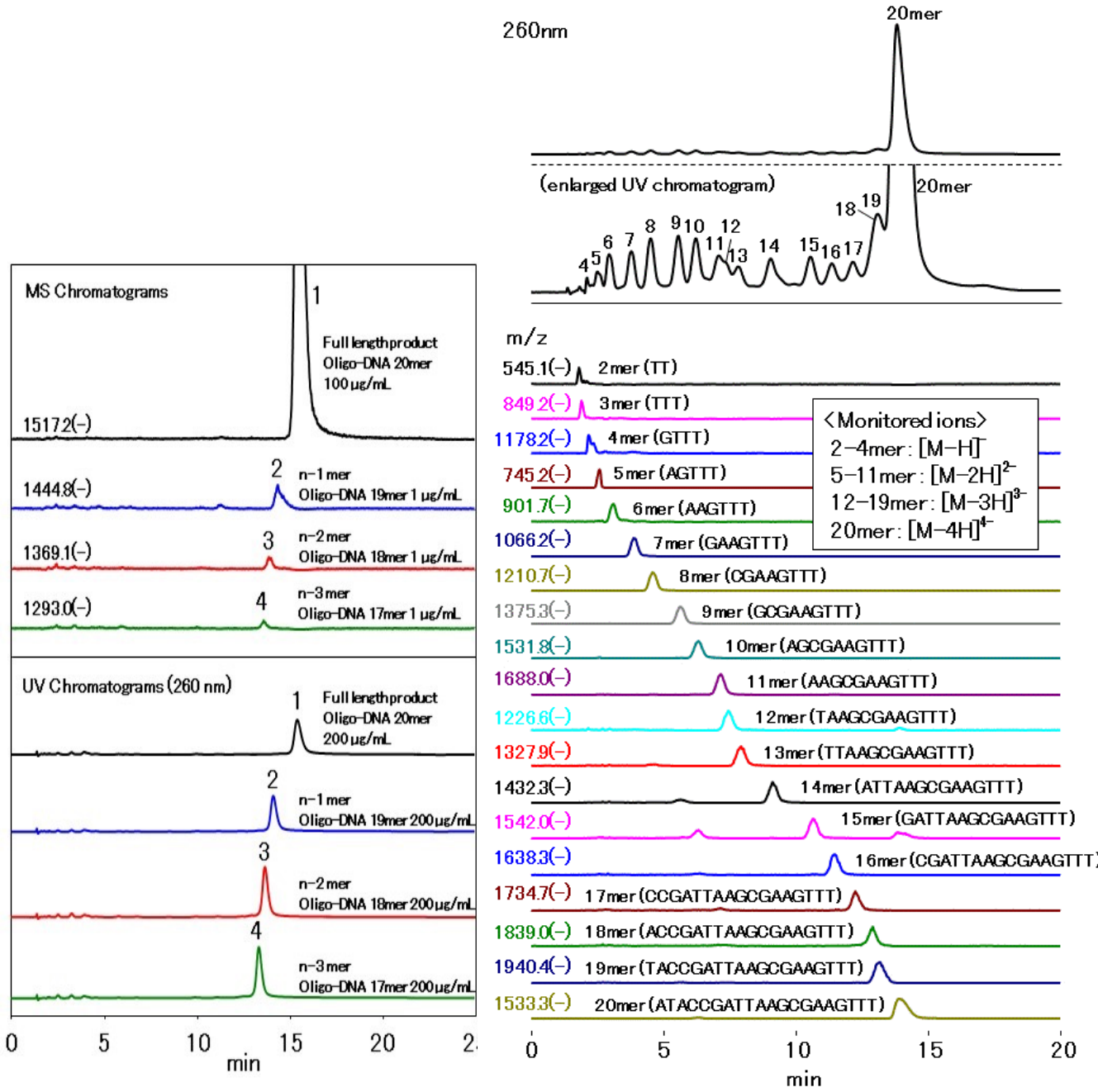
1. **HILIC-NH_4OAc**
2. RP-NH_4OAc
3. TRP BUFFERS

HILIC WITH NH_4OAc

- HILIC-MS analysis of oligonucleotides (Easter 2010) without the use of ion-pair reagents (Loube/MacNeil 2019)
- Applicable to oligodeoxy(ribo)nucleotides and phosphorothioates (PS)
- Improved LC-MS sensitivity over ion-pair reverse phase methods
- Additional chromatographic developments required to match UV-based PS methods

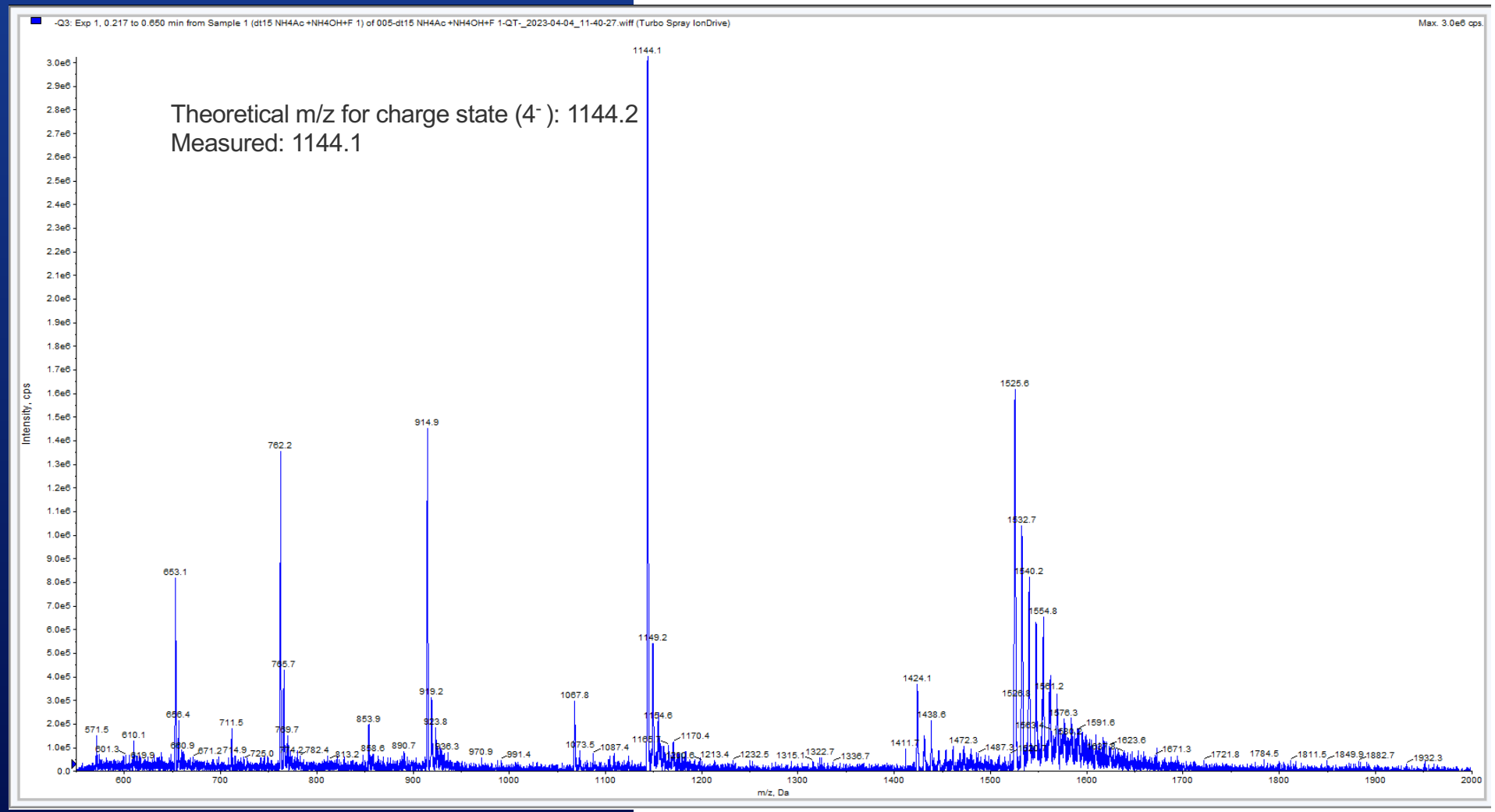


ShodexTM Technical Article No.4;
LC/MS Analysis of Oligonucleotides
Using a Polymer-Based Diol Column - Shodex™ HILICpak™ VN-50 2D



Mass Spectrum (after HILIC)

$(pdT)_{15}$, NH_4OAc in $MeCN/H_2O$, Shodex HILIC



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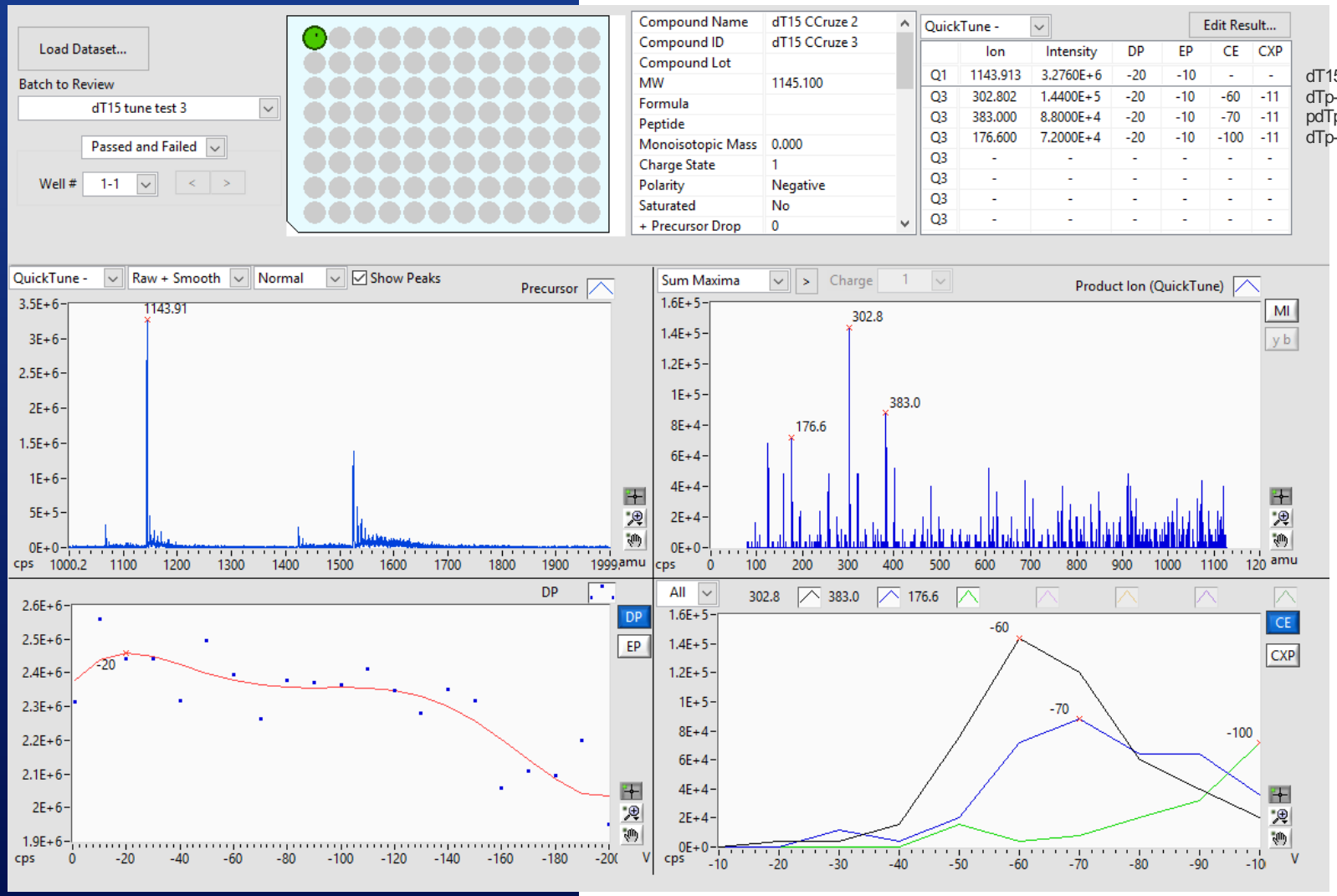
MRM Optimization

$(pdT)_{15}$ in H_2O



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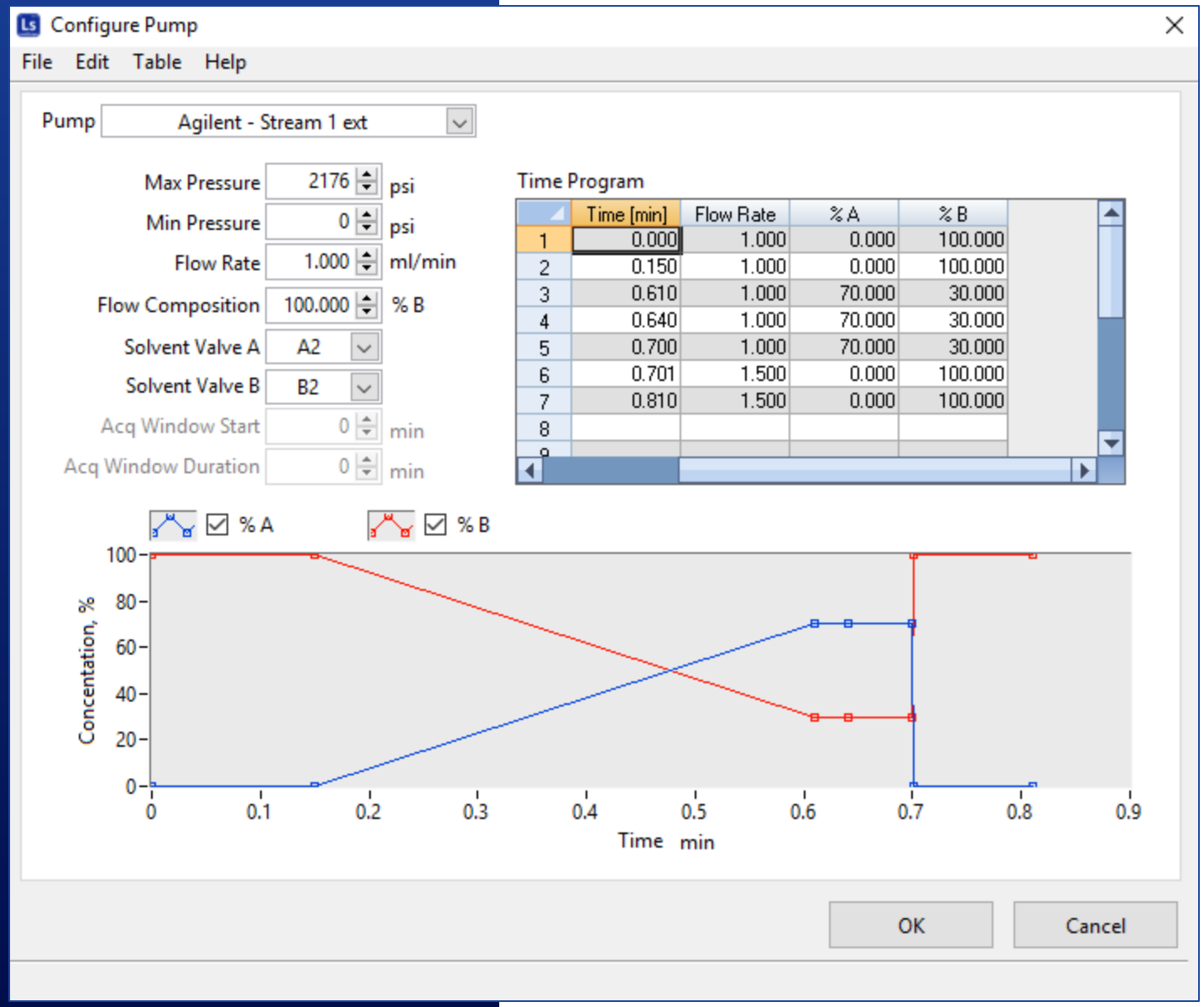
HPLC GRADIENT

$(pdT)_{15}$ in H_2O



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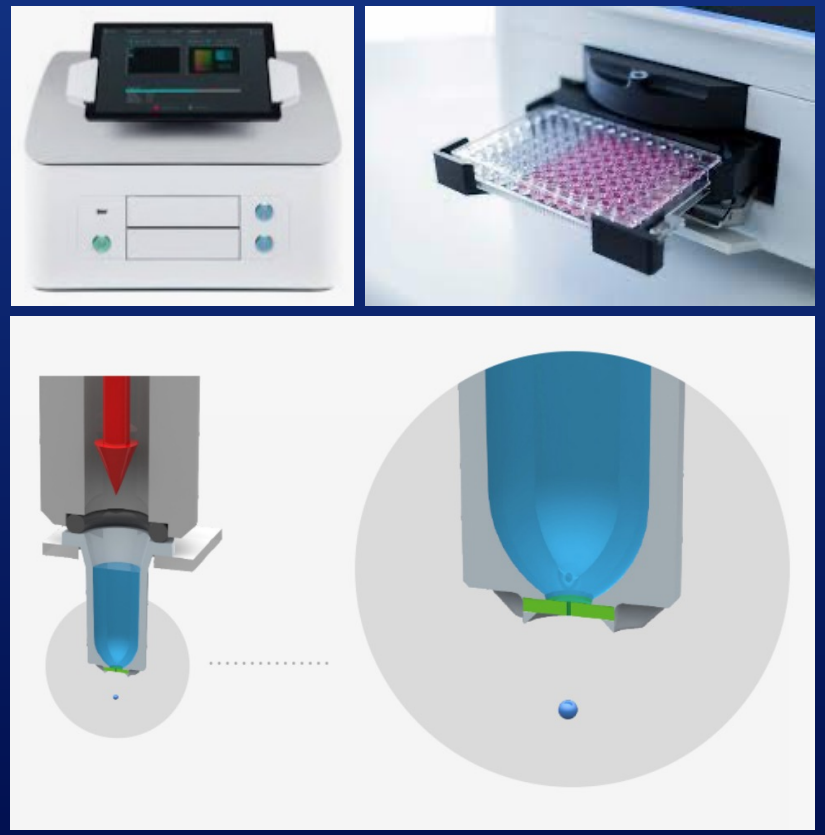
CALIBRATION SAMPLE PREPARATION

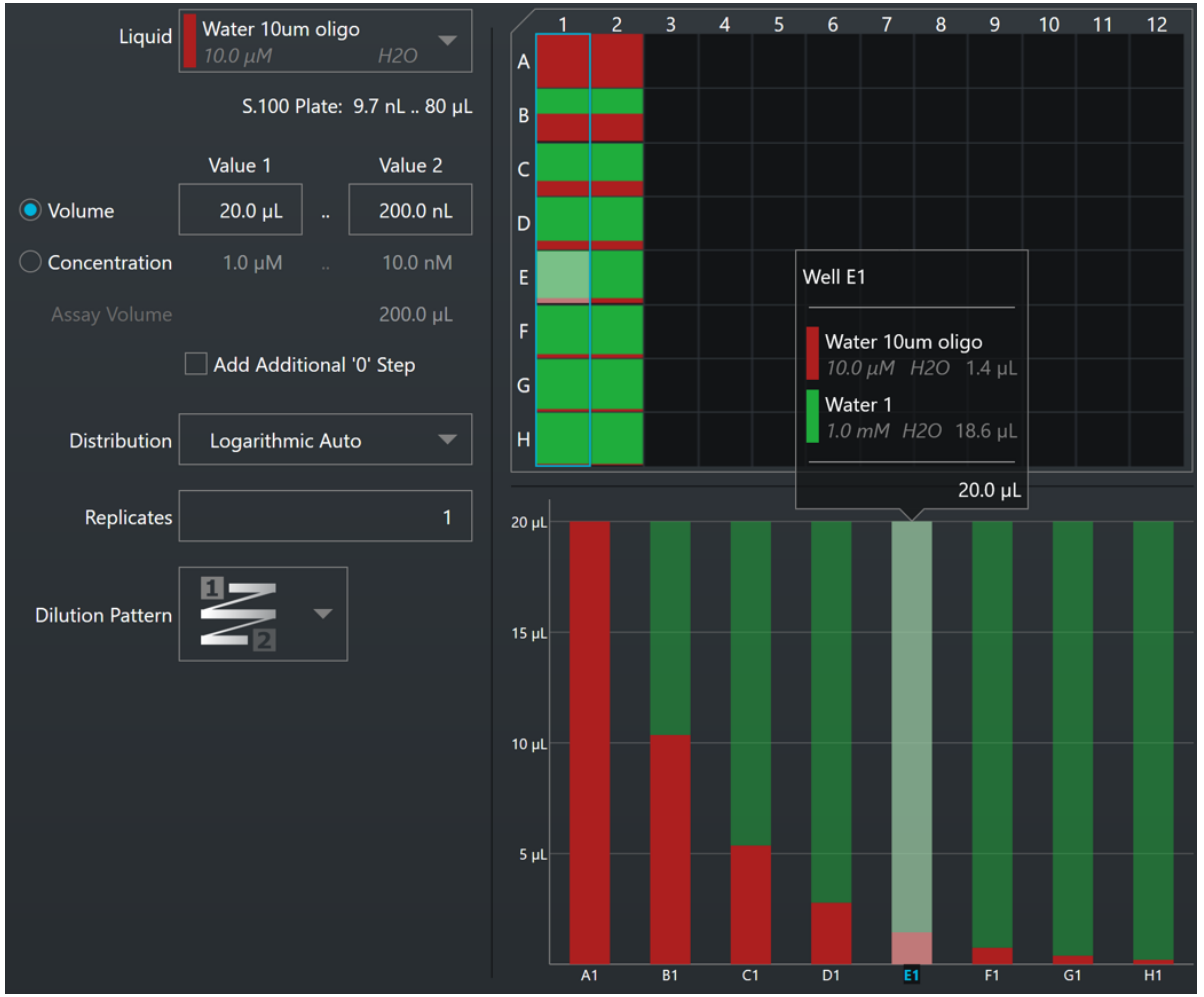
$(pdT)_{15}$ in H_2O or buffer

Instrument: iDOT non-contact liquid handler

QC preparation separately

Time needed: <1min/96 well





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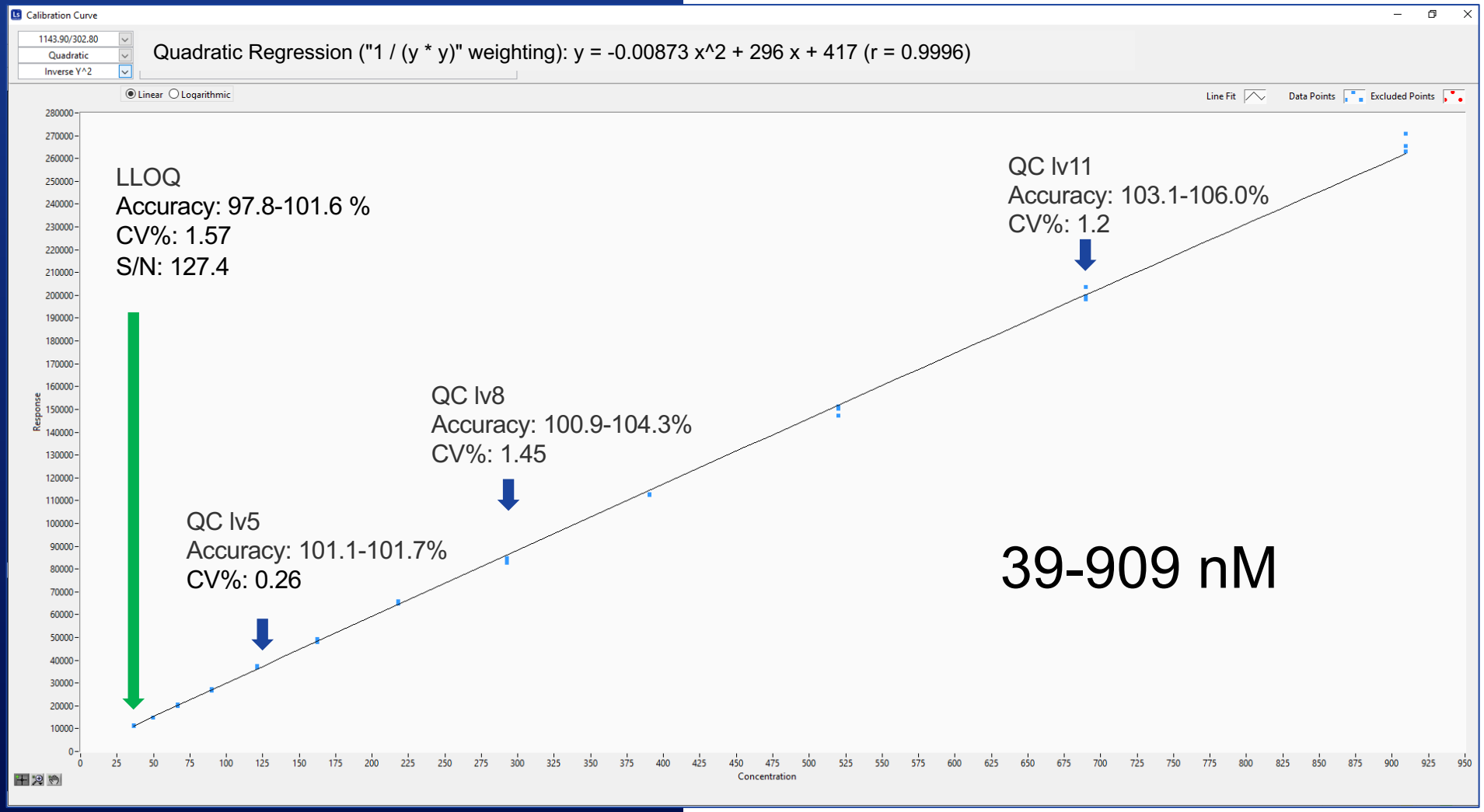
CALIBRATION

$(pdT)_{15}$ in H_2O, NH_4OAc in $MeCN/H_2O$, Shodex HILIC

The benefits of HILIC for oligos (R. MacNeil et al., Bioanalysis (2019) 11(12), 1155–1167):

- less signal suppression and variability
- less instrumental downtime for maintenance and cleaning
- less frequent analytical column changes
- no need for exotic flushing processes to switch over of instrumentation for other methods
- smoother workflows and better efficiency



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HILIC CALIBRATIONS OF 12 DNA PRIMERS

18-25mers in H_2O, NH_4OAc in MeCN/H_2O, Shodex HILIC

	Target	R/F	Sequence	Mass	Charge	Transition	S/N	LLOQ nM	ULOQ nM	QC 1 (99nM)	QC 1 (99nM)	QC 2 (244nM)	QC 2 (244nM)	QC3 (593nM)	QC3 (593nM)
1	hOATP4C1 R	R	CTACAATACCTTGCGTGACGGC	6695	-5	1337.65/1307.40	11.9	39	909	105.30%	104.10%	102.60%	104.70%	103.30%	102.70%
2	SVCT2 (SLC23A2) R	R	AAGCTAGGAGCCCAGGATCA	6160	-4	1538.40/1501.10	54.2	39	909	96.00%	92.90%	97.40%	95.10%	104.60%	98.30%
3	SVCT1 (SLC23A1) R	R	TAACCATGTGCTGGTCGTGG	6164	-4	1539.54/1501.31	56.8	39	909	96.00%	100.50%	98.60%	102.40%	103.40%	102.60%
4	pig Bcrp (Abcg2) R	R	CTGCTGAAACACTGGTTGGTT	6452	-5	1289.13/624.73	34.2	39	909	101.60%	98.20%	103.80%	103.00%	101.80%	105.00%
5	cyMDR1 R	R	ACAGTGTCAGTTGCCAACCA	6086	-4	1520.02/1486.30	42.9	39	909	109.10%	95.50%	99.20%	100.70%	101.70%	99.00%
6	hOATP4C1 F	F	AAATCGAAGTCTCTGCCTTGTCCTC	7568	-5	1512.13/1074.15	31.8	39	909	103.50%	109.30%	101.10%	104.30%	101.00%	96.00%
7	SVCT2 (SLC23A2) F	F	GCTGCAGCCAGCTAGGTC	5501	-4	1373.59/1336.00	79.0	39	909	104.40%	96.90%	103.00%	103.50%	100.10%	99.80%
8	SVCT1 (SLC23A1) F	F	GGCCTTTGTCAAGTCATCCC	6044	-4	1509.53/1472.06	32.1	39	909	96.30%	100.20%	99.30%	99.70%	98.30%	96.40%
9	SNAT1 (SLC38A1) F	F	GCTTTGGTTAAAGAGCGGGC	6213	-5	1241.25/1211.17	15.1	39	909	101.00%	97.10%	107.40%	108.30%	103.00%	100.80%
10	SNAT2 (SLC38A2) F	F	AATAGAGACCACCGAGGCCG	6145	-5	1227.71/1200.69	18.8	39	909	108.70%	91.70%	100.40%	93.80%	101.40%	103.60%
11	ATB0,+ (SLC6A14) F	F	TTGGGGTGGCTTAGTTGCTC	6186	-5	1235.99/1205.56	23.7	39	909	97.00%	101.30%	106.60%	103.10%	95.20%	98.20%
12	ASCT1 (SLC1A4) F	F	TCTCCTCGCCTTTCTCGCAC	5931	-4	1481.29/769.90	74.2	39	909	101.80%	97.50%	106.20%	105.80%	97.20%	95.10%

WORKS FROM WATER

- Matrix: H_2O → SPE for any other matrices

- ADDA – Sciex 6500+ Triple Quadrupole MS
- Negative MRM charge state of parent: -4 or -5

- Working Range 39-909 nM
- Signal-to-noise at LLOQ ≥ 11.9
- Accuracy of low QC (99 nM) 91.7-109.3%

Quant
Quadratic Regression ("1 / (y * y)" weighting): y = 0.0119 x^2 + 45.7 x + -36 (r = 0.9982)
Quadratic Regression ("1 / (y * y)" weighting): y = 0.00876 x^2 + 130 x + -744 (r = 0.9984)
Quadratic Regression ("1 / (y * y)" weighting): y = 0.0154 x^2 + 189 x + -823 (r = 0.9988)
Quadratic Regression ("1 / (y * y)" weighting): y = 0.00686 x^2 + 29.8 x + 136 (r = 0.9973)
Quadratic Regression ("1 / (y * y)" weighting): y = 0.0236 x^2 + 193 x + -443 (r = 0.9987)
Quadratic Regression ("1 / (y * y)" weighting): y = 0.00919 x^2 + 68.5 x + 86 (r = 0.9995)
Quadratic Regression ("1 / (y * y)" weighting): y = 0.0202 x^2 + 173 x + -791 (r = 0.9992)
Quadratic Regression ("1 / (y * y)" weighting): y = 0.0102 x^2 + 131 x + -63.1 (r = 0.9990)
Quadratic Regression ("1 / (y * y)" weighting): y = 0.0262 x^2 + 91.9 x + 400 (r = 0.9972)
Quadratic Regression ("1 / (y * y)" weighting): y = 0.00991 x^2 + 33 x + 31.1 (r = 0.9952)
Quadratic Regression ("1 / (y * y)" weighting): y = 0.00396 x^2 + 81.3 x + -259 (r = 0.9932)
Quadratic Regression ("1 / (y * y)" weighting): y = 0.0866 x^2 + 218 x + 1.46e+003 (r = 0.9987)

- ASO, ssDNA, ssRNA, thioates up to ~25mers, longer run time for siRNA, dsDNA
- MS limitations – mass range and CID optimization

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SPE OF OLIGOS

High-Throughput

Clarity® OTX™ was designed with the fast-paced DMPK/ADME environment in mind. It is a simple, rapid, and reproducible solution that efficiently extracts oligos from biological matrices and can be easily automated to eliminate sample backlogs and meet critical deadlines. By eliminating the need for LLE (liquid-liquid extraction), providing a 96-well plate format for liquid handler compatibility, and specifically targeting synthetic oligo therapeutics chemistries, Clarity OTX delivers a 15-minute extraction procedure.

Suitable for Most Oligo Therapeutics & Samples

Oligo Types:	Sample Types:
DNA	Plasma
Aptamers	Serum
RNAi/siRNA	Urine
Thioates	Tears
Lipid-conjugates	Saliva
Liposome encapsulated	Tissue

SPE OF OLIGOS

Oasis Cartridges and 96-Well Plates

Product Description

The Oasis WAX 96-well plate contains the Oasis WAX sorbent, which is a polymeric reversed-phase, weak anion exchange mixed-mode sorbent that has been optimized for fast, simple, and highly selective sample preparation of strong acidic compounds. The unique balance of hydrophobicity and water-wettability of the Oasis WAX sorbent means you will never have to worry about poor results if individual wells of the 96-well plate dry out during the critical steps prior to sample loading. The Oasis WAX 96-well plate is designed to be used on many manifold configurations and most robotic liquid handling systems.

Optimized sample preparation

The solid-phase extraction (SPE) of RM1 and the analog internal standard VA1 from human plasma was performed as follows. The SPE sorbent was Waters Oasis® WAX, 10 mg, a mixed-mode phase with cation exchange and reversed-phase moieties, in 96-well format. The 96-well 1 ml collection plates were regular inert polypropylene from Porvair (Wrexham, UK). Each step where liquid was applied was performed with the minimal aid of positive pressure to help percolation and passage of the liquid sample through the sorbent bed. The positive pressure manifold was from Agilent Technologies (DE, USA)

The analog internal standard VA1, in 1:1, v:v, acetonitrile:water, at 2500 nM, was added in 20 µl aliquots to 100 µl plasma within 1.5 ml regular polypropylene tubes. This resulted in an internal standard concentration of 500 nM in matrix. Then, a two-second vortex of each tube took place. This was followed by the addition of 225 µl 4.5% H_3PO_4 (aq) to each sample and another vortex step.

An oligonucleotide bioanalytical LC–SRM methodology entirely liberated from ion-pairing

Robert MacNeill*,[1], Tisha Hutchinson[1], Vishva Acharya[1], Ryan Stromeyer[1] & Susan Ohorodnik[1]

[1]At time of publication: Covance – Bioanalysis, PO Box 2360 Mettlers Road, East Millstone, NJ 08875-2360, USA
Note: At time of writing: Envigo CRS, PO Box 2360 Mettlers Road, East Millstone, NJ 08875-2360, USA
*Author for correspondence: Tel.: + 1 732 873 2550; Fax: +1 732 873 3992; robert.macneill@covance.com

HT-LC-MS/MS OF OLIGOS

1. HILIC-NH_4OAc
2. **RP-NH_4OAc**
3. TRP BUFFERS

RP WITH NH_4OAc

NH_4OAc vs. Ion Pair agents

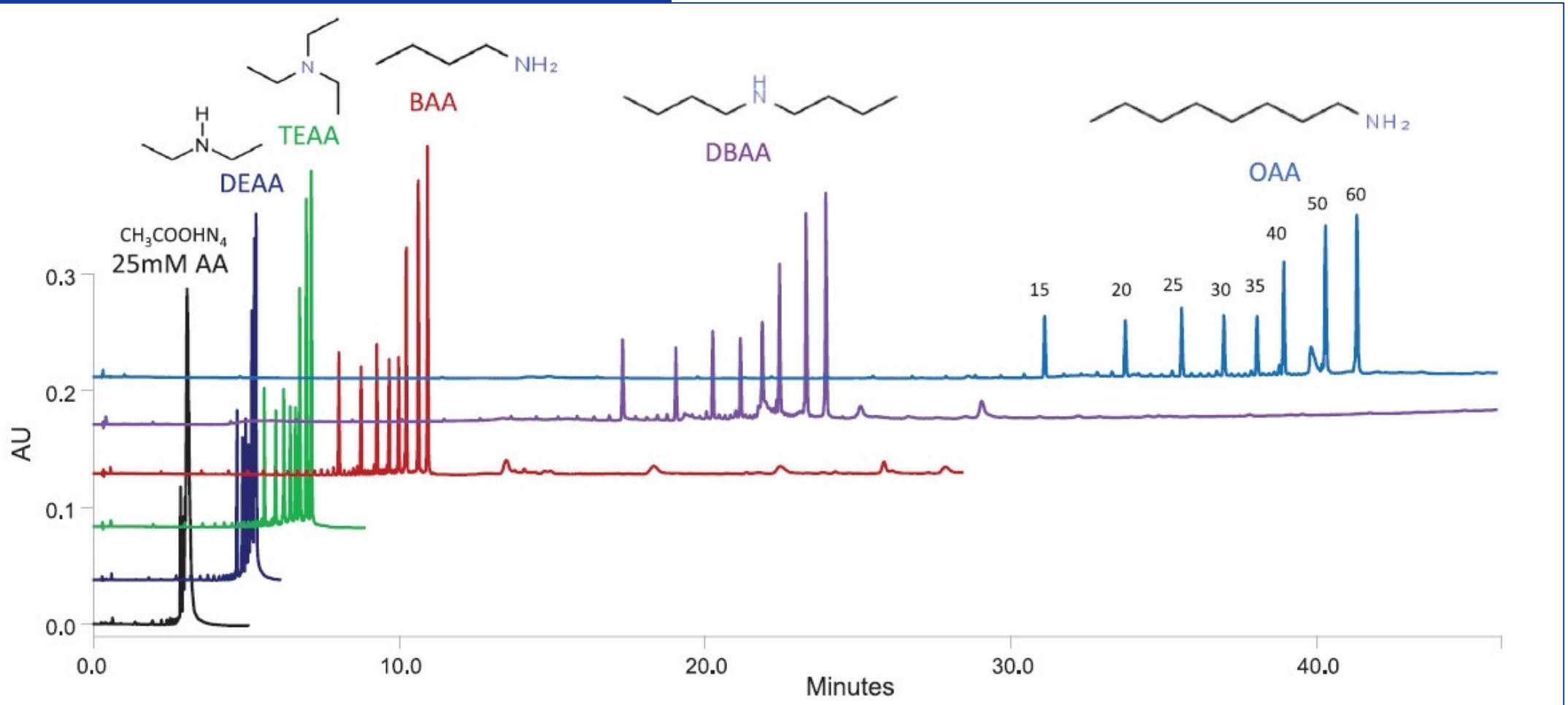

Fig. 2. Separation of 15, 20 25, 30, 35, 40, 50, and 60 mer oligodeoxythymidines using selected 100 mM ion-pairing buffers. The data are compared to non-ion-pairing 25 mM ammonium acetate buffer. Retention and peak capacity data are listed in Table 4. For separation conditions see Experiment 2 in Table 3.

Michael Donegan, Jennifer M. Nguyen, Martin Gilar (Waters), J. Chrom. A, https://doi.org/10.1016/j.chroma.2022.462860

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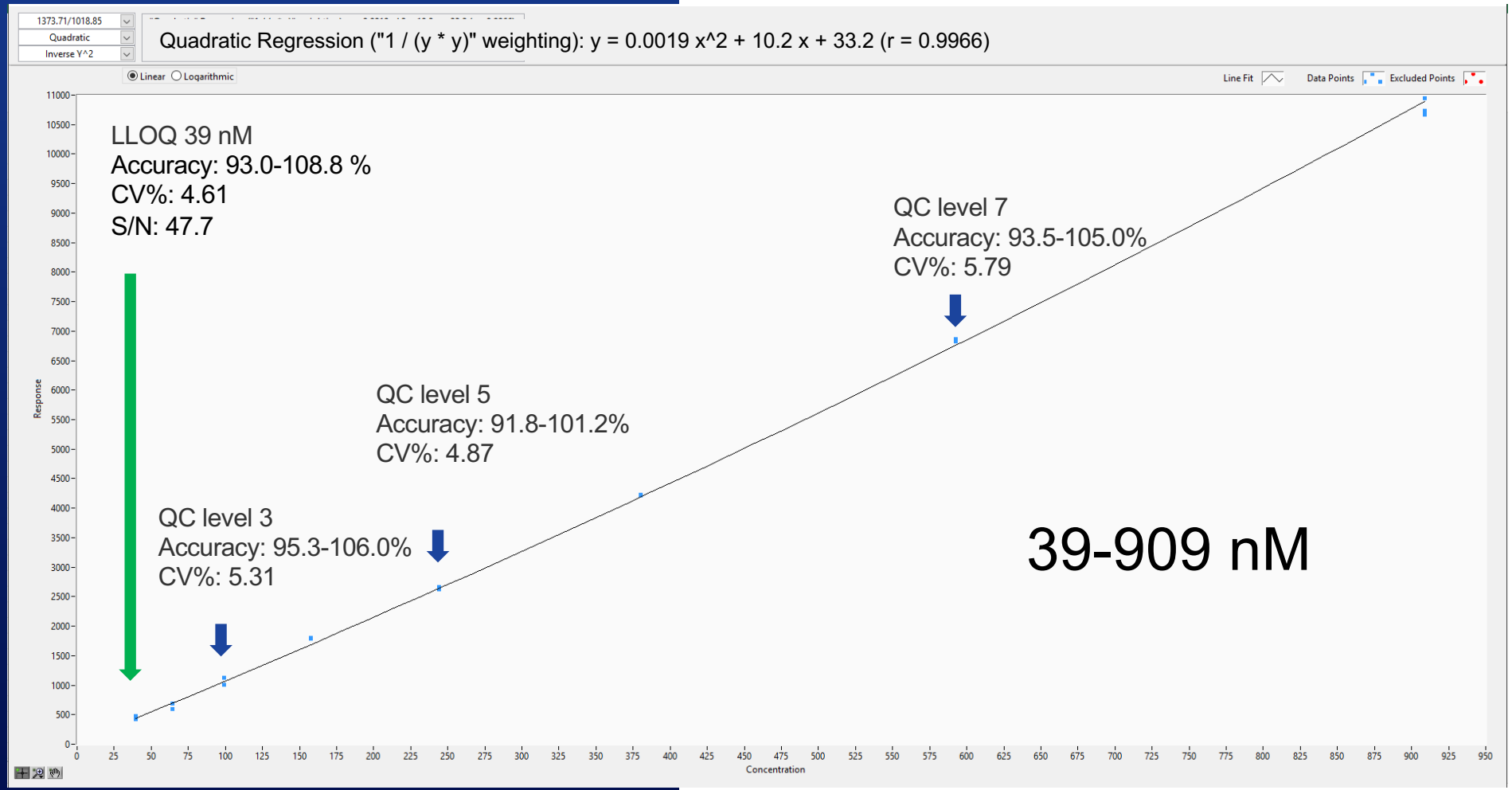
CALIBRATION

- GCTGCAGCCAGCTAGGTC (SVCT2 F) in HBSS, NH_4OAc in H_2O/MeCN, Hypersil Gold C18, 10*2.1mm, 3um
- ADDA-6500+
- MRM (4-)
- S/N 47.7



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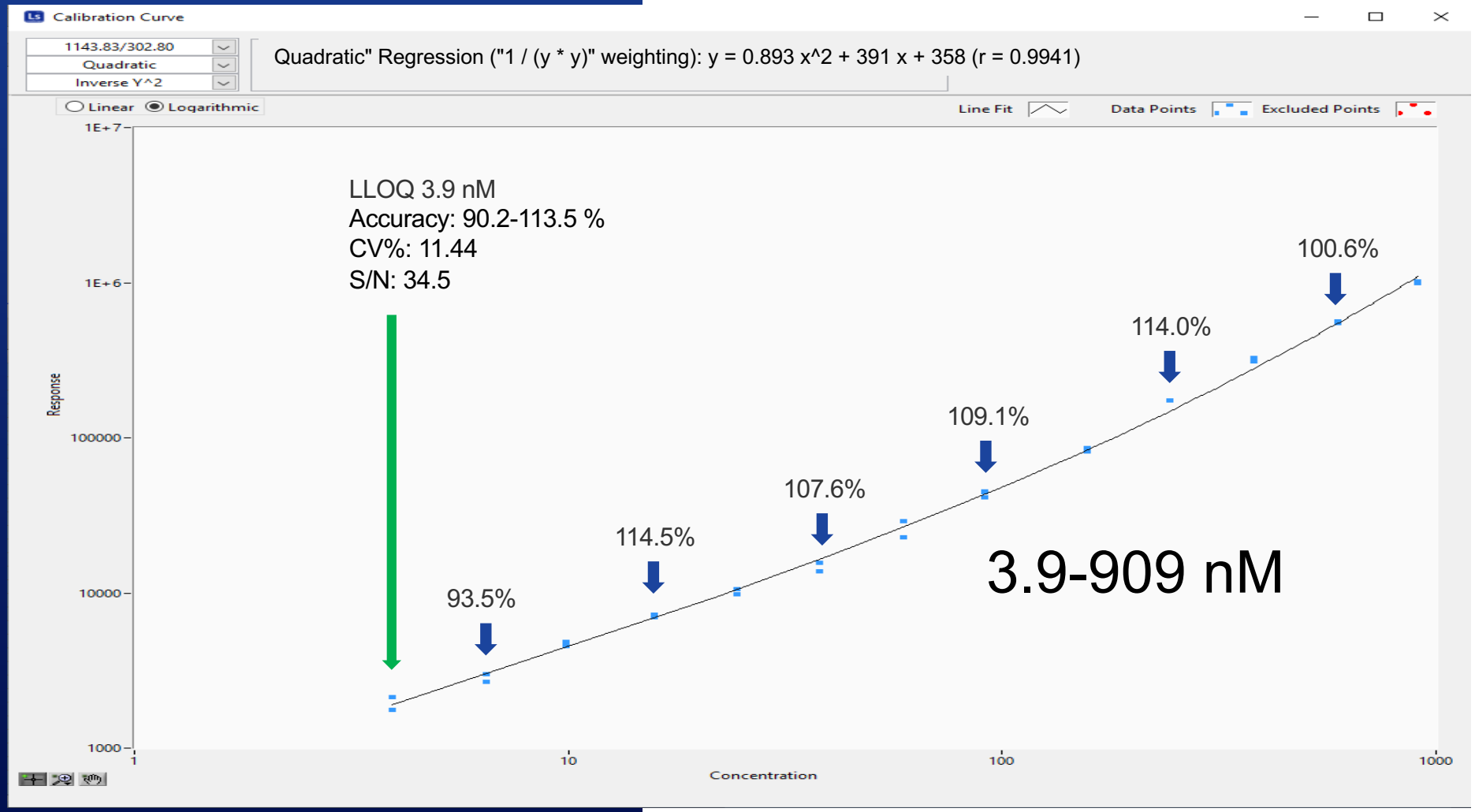
CALIBRATION (Inj.: 50 µL)

- $(pdT)_{15}$ in HBSS, NH_4OAc in H_2O/MeCN, Hypersil Gold C18, 10*2.1mm, 3 µm
- ADDA-6500+
- MRM (4-)
- S/N 34.5



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HT-LC-MS/MS OF OLIGOS

1. HILIC
2. **NH_4OAc**
3. TRP BUFFERS

CALIBRATION (inj: 50 µL)

- GCTGCAGCCAGCTAGGTC (SVCT2 F) in BUFFER, NH_4OAc in H_2O/MeCN, Hypersil Gold C18, 10*2.1mm, 3 µm
- ADDA-6500+ MRM (4-)
- 10-1,000 nM

Buffer	QC1 (37.3 nM)		QC2 (139 nM)		QC3 (518 nM)		Analyte Quant
HBSS	99.70%	95.80%	91.80%	92.70%	94.10%	99.90%	Quadratic Regression ("1 / (y * y)" weighting): y = 0.118 x^2 + 140 x + 96.2 (r = 0.9964)
KH	88.00%	97.00%	89.70%	96.00%	100.80%	102.10%	Quadratic Regression ("1 / (y * y)" weighting): y = 0.118 x^2 + 143 x + 904 (r = 0.9969)
SM	111.50%	107.40%	100.50%	94.40%	96.10%	103.60%	Quadratic Regression ("1 / (y * y)" weighting): y = 0.109 x^2 + 204 x + 559 (r = 0.9974)
BSEP	108.30%	105.70%	107.80%	98.70%	98.00%	99.90%	Quadratic Regression ("1 / (y * y)" weighting): y = 0.159 x^2 + 44.6 x + 145 (r = 0.9928)

HBSS: Hank's balanced salt solution
KH: Krebs-Henseleit Buffer pH 7.4
SM: Start mix
BSEP: Bile Salt Export Pump

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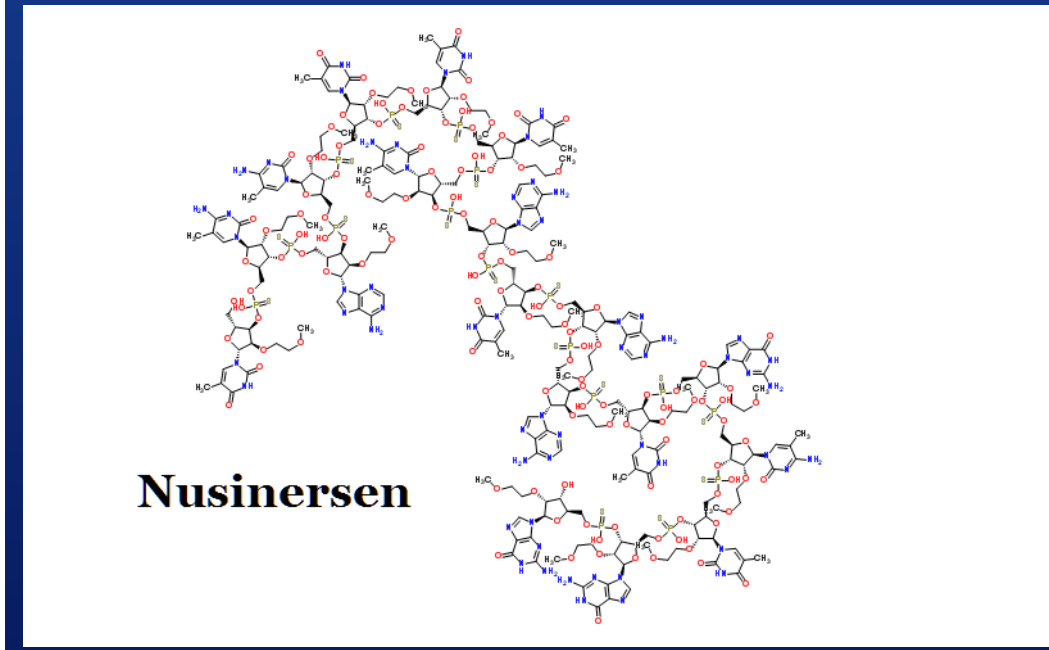
Buffer TEST

1 μM 18mer (SVCT2), 100 nM $(pdT)_{15}$ IS

	TA/IS			Mean	Stdev	CV%
H2O	1.09	1.08	1.01	1.06	0.04	3.45%
HBSS	3.86	3.72	3.70	3.76	0.07	1.83%
KH	3.72	3.87	4.00	3.86	0.11	2.94%
SM	4.69	4.87	4.78	4.78	0.07	1.49%
BSEP	3.42	3.53	3.74	3.56	0.13	3.73%
	Peak Area			Mean	Stdev	RSD%
H2O	26337	32698	27822	28952	2717	9.38%
HBSS	25238	28471	28293	27334	1484	5.43%
KH	22378	24408	26135	24307	1535	6.32%
SM	20427	22305	24999	22577	1876	8.31%
BSEP	15352	21027	21544	19308	2805	14.53%

Uptake transporter assay with HT-LC-MS/MS



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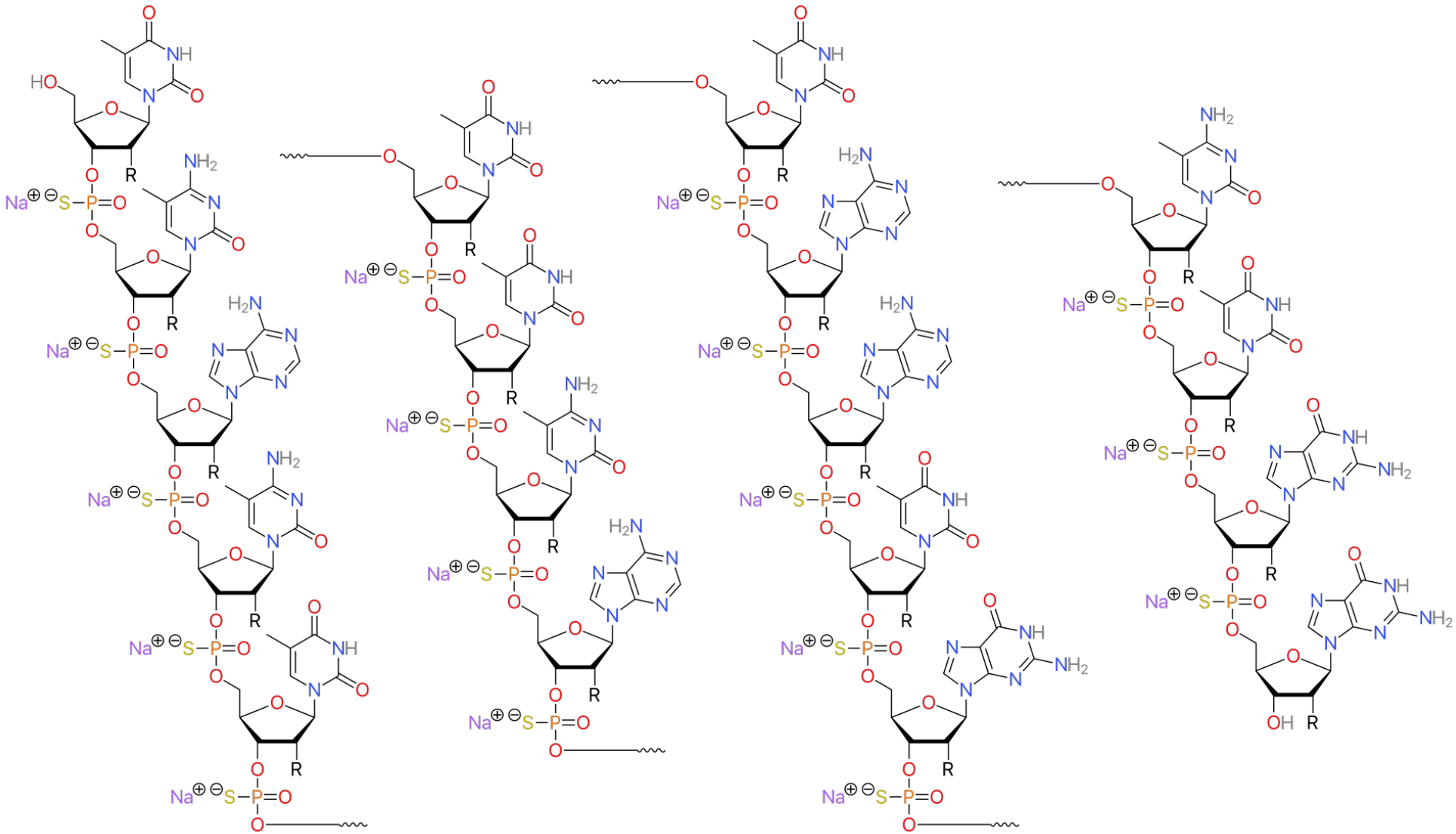
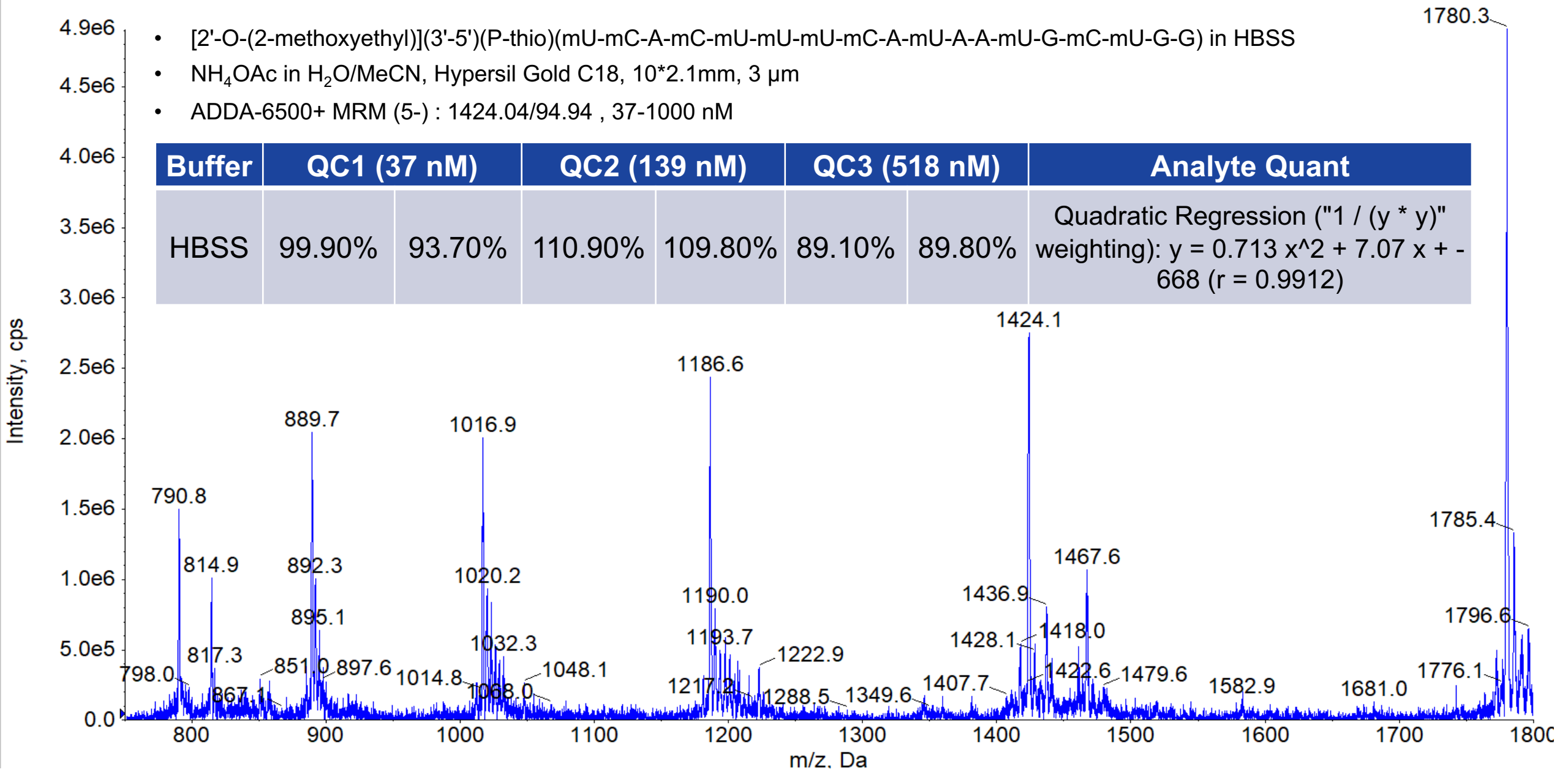


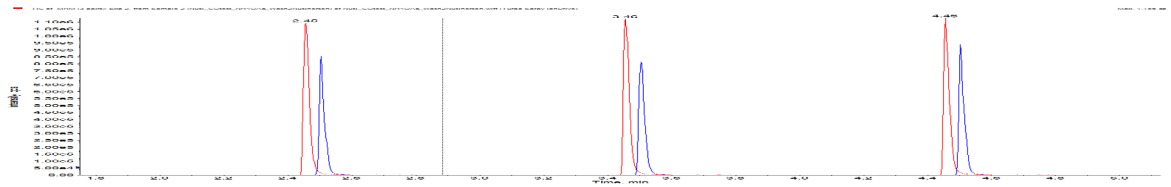
CALIBRATION

- [2'-O-(2-methoxyethyl)](3'-5')(P-thio)(mU-mC-A-mC-mU-mU-mU-mC-A-mU-A-A-mU-G-mC-mU-G-G) in HBSS
- NH_4OAc in H_2O/MeCN, Hypersil Gold C18, 10*2.1mm, 3 µm
- ADDA-6500+ MRM (5-) : 1424.04/94.94 , 37-1000 nM

Buffer	QC1 (37 nM)		QC2 (139 nM)		QC3 (518 nM)		Analyte Quant
HBSS	99.90%	93.70%	110.90%	109.80%	89.10%	89.80%	Quadratic Regression ("1 / (y * y)" weighting): y = 0.713 x^2 + 7.07 x + -668 (r = 0.9912)



Summary

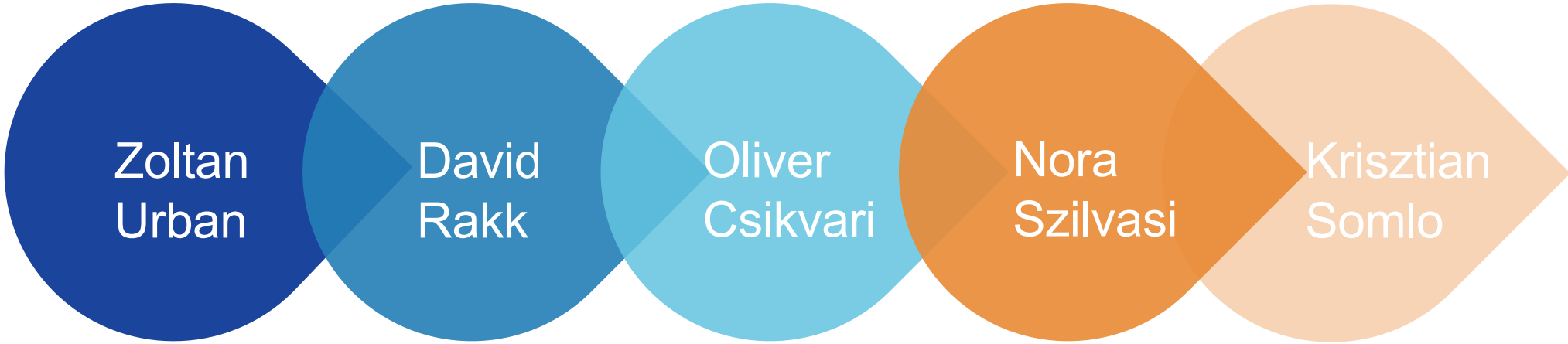


High-throughput quantitative measurement of 15-25 mer ssDNA from preclinical *in vitro* buffers with 1 minute cycle time without strong ion-pair reagents was performed applicable for small therapeutic ASOs.

Method	Matrix	Sample preparation	Benefits	Disadvantages
IP-RP-MS	MIC	OE	• Generic • Great separation	• Signal suppression • Variability • Contamination related MS down-time, maintenance, cleaning, long switch procedure • Long acquisition • HPLC column degradation
HILIC-MS	Water	SPE	• Easy switch bw methods • Fast • Good separation	• Matrix effect
RP-MS	MIC	Simple	• Low LLOQ • Easy switch bw methods • Fast	• LC selectivity • ds oligos separation

Future perspectives: evaluation of method performance for larger and duplex DNA targets and other matrices. Continuous improvement on detection limit.

Acknowledgement



- Screen group lead
- Senior scientist
- Scientist
- RnD Scientist
- Assistant

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