Considerations and Experiences in Developing Bioanalytical Assays for the Quantitative Determination of Oligonucleotides

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Overview

- Introduction
  - Growing interest in therapeutic oligonucleotides
  - Mechanism of action
- Structural considerations
- Approaches to unique analytical challenges
  - Detection
  - Chromatography
  - Sample Extraction
- Validation results
- Future work

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Introduction

- Synthetic nucleic acid-based drug candidates
  - Typically 15-35 nucleotides in length
- Inhibit gene expression
  - Block the production of harmful proteins
  - Diseases with genetic backgrounds
  - Highly target specific
- Antisense oligonucleotides (AS-OGN)
  - Single stranded DNA or RNA
  - Specifically bind to the complementary sequence of target mRNA
- Short interfering RNA (si-RNA)
  - Double stranded RNA
  - Gene silencing by RNA interference pathway

Introduction

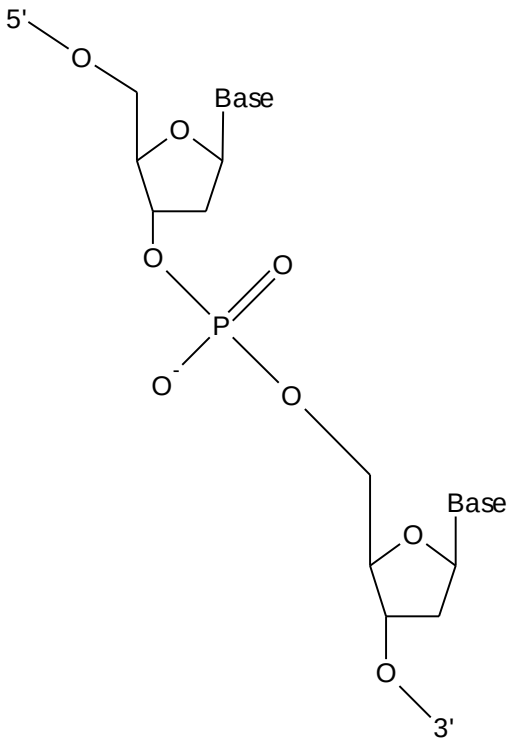
- Currently two drugs on the market
  - Vitravene® for cytomegalovirus infection
  - Macugen® for wet macular degeneration
- Exponential growth in activity
- 2009
  - 231 therapeutic programs
  - 80-90 oligonucleotide drugs in the clinic
  - 4 in phase III
- Highly challenging from an analytical perspective

Bioanalysis of Oligonucleotides

- Method of choice has been hybridization-based ligand-binding assay (ELISA)
  - High sensitivity (25 pg/mL)
  - Low selectivity (unable to distinguish from metabolites)
- LC-MS/MS
  - High selectivity
  - Poor sensitivity (only 1 ng/mL achieved in the literature)
- Can we achieve ELISA sensitivity with LC-MS/MS selectivity?

Structure

- Acidic proton at each phosphodiester bond
- Highly charged poly-anionic backbone
- Extremely polar
- DNA or RNA based
- Phosphorothioate linkage
  - Non-bridging oxygen replaced by a sulphur
  - More resistant to nuclease digestion



LC-MS/MS analysis of oligonucleotides has many technical challenges associated with the size and chemistry of the molecules that are not encountered with small molecule bioanalysis

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Test Compounds

- DNA chemistry
- Phosphorothioate linkage
- 15 nucleotides long

- Oligo1

AGA-TCG-GTC-ATG-ACC

MW=4577

- Oligo2

ACT-ACA-TCC-ATG-ACC

MW=4481

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Development of an LC-MS/MS assay

- Validate a bioanalytical method for Oligo1
  - Human plasma
  - Sample volume 200 µL
  - Oligo2 used as internal standard
- Achieve maximum sensitivity
  - Obtain sub ng/mL levels?

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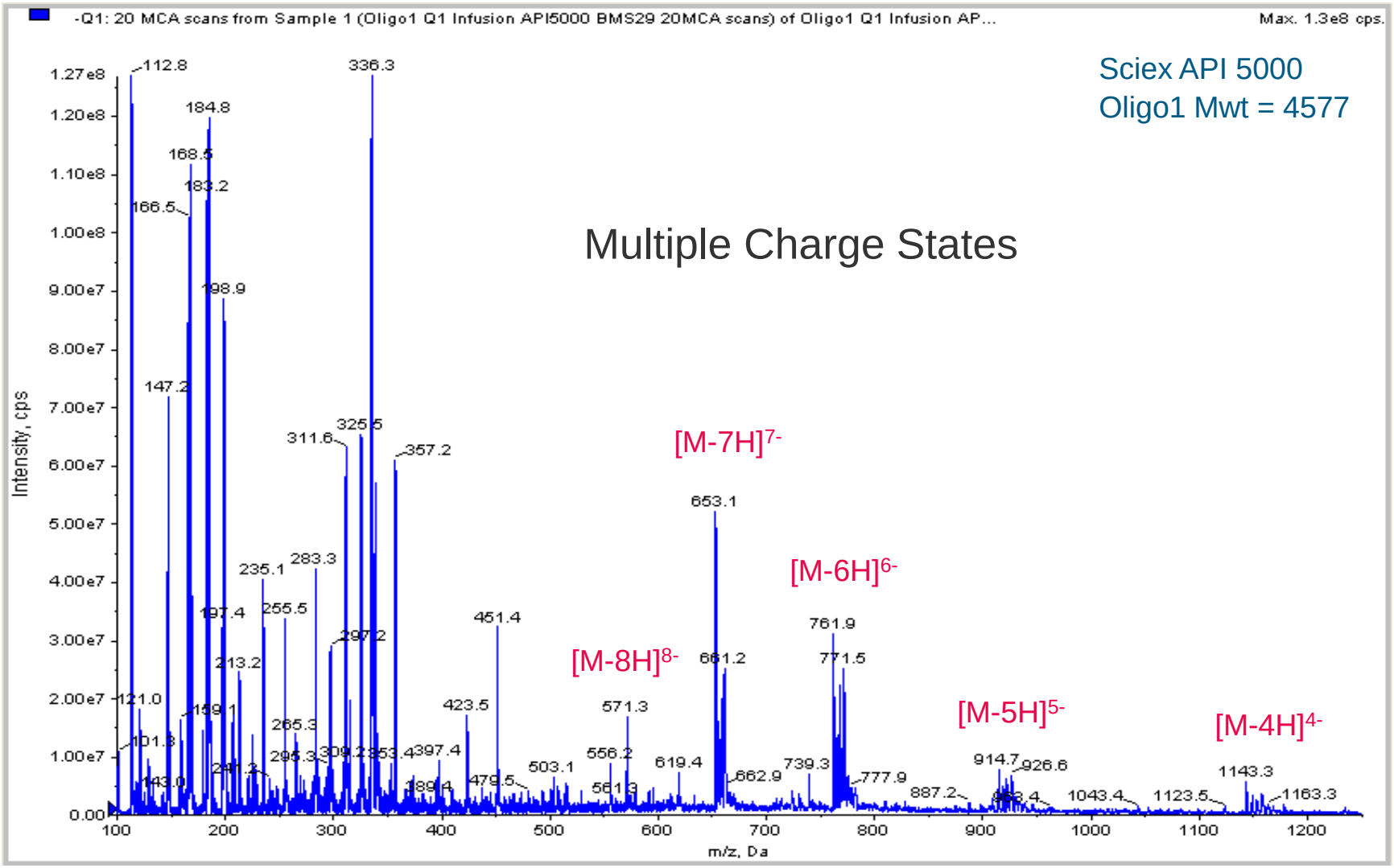
Detection (Q1 Scan of Oligo1)



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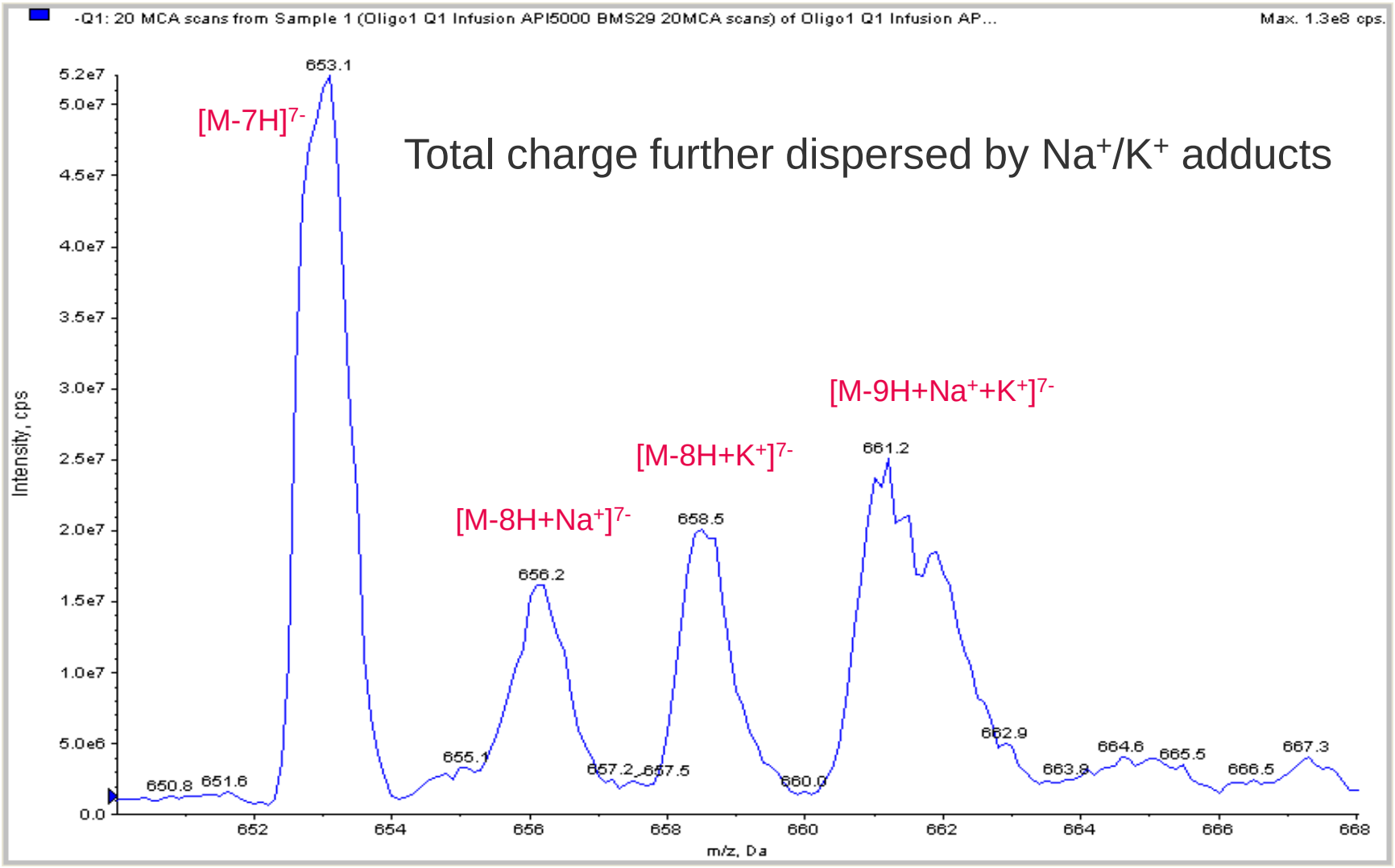
Detection (Q1 Scan of Oligo1)



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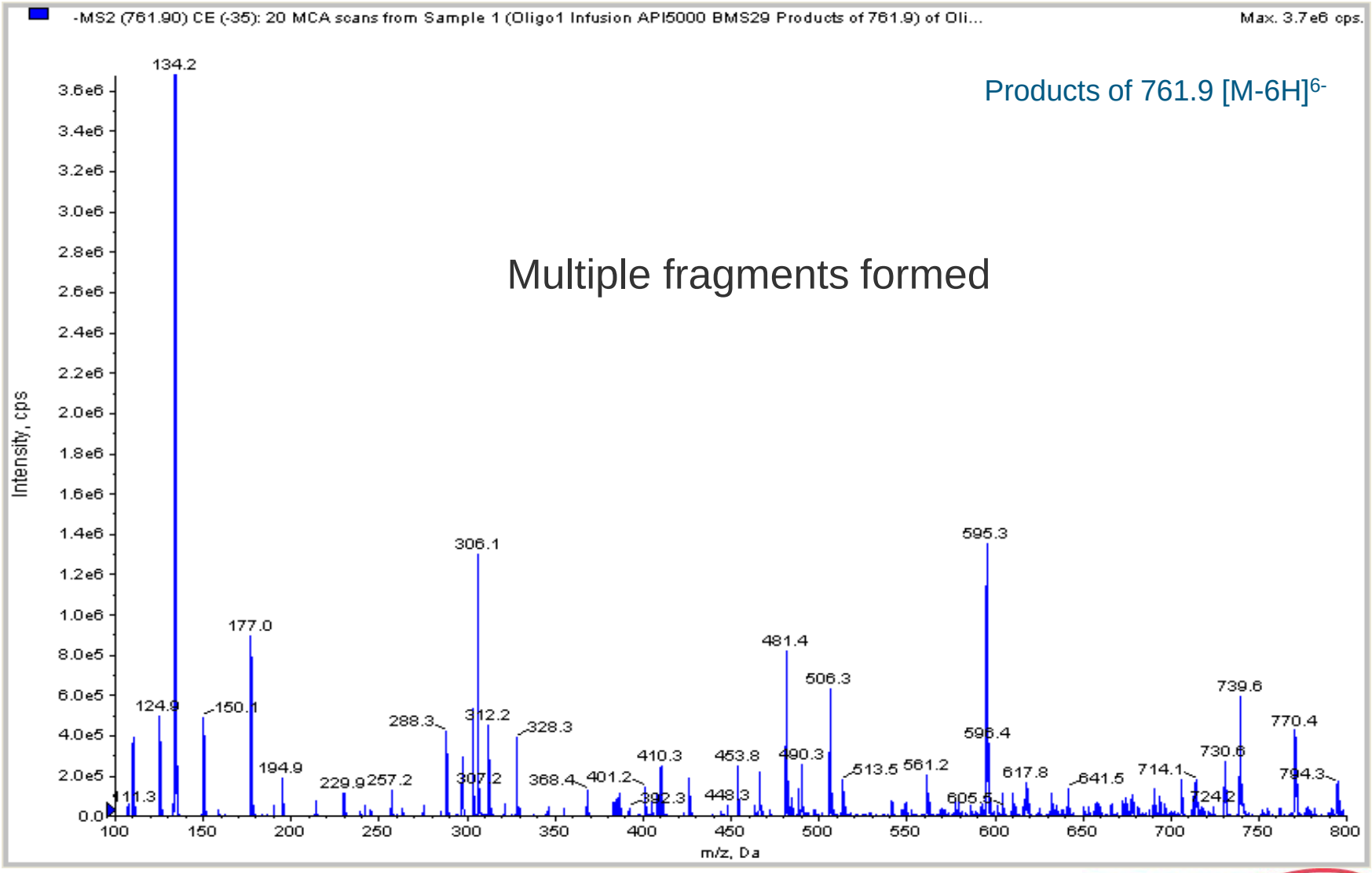
Detection (Fragmentation of Oligo1)



Products of 761.9 $[M-6H]^{6-}$

Multiple fragments formed

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Detection

- Summing of MRM transitions
  - Enhanced MRM detection sensitivity
  - Decreased assay variability caused by the dynamic change of charge states during LC/MS analysis
- 5 transitions summed for Oligo1
- 3 transitions summed for Oligo2
- Dwell time 50 ms
- Turbo IonSpray interface
  - Significant decrease in response above 600 °C (>80 %)
  - Thermally labile

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Chromatography

- No retention on standard reversed phase
- Solution based on chemical structure
  - Ion-exchange chromatography
  - Ion-pair reversed phase chromatography
- Ion-pair reversed phase is the system of choice for LC-MS/MS analysis
  - Triethylamine (TEA)
  - Hexafluoroisopropanol (HFIP) as the buffering acid
- TEA improves chromatographic performance, but causes signal suppression
  - HFIP improves droplet volatility and increases desorption of the oligonucleotide into the gas phase

Chromatography

- UPLC
- Ion-pair, reversed-phase chromatography
- OST C_{18} column (50 x 2.1 mm, 1.7 µm, Waters)

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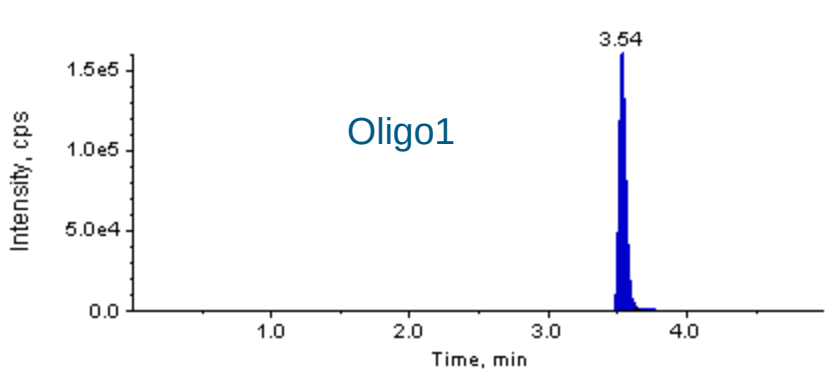
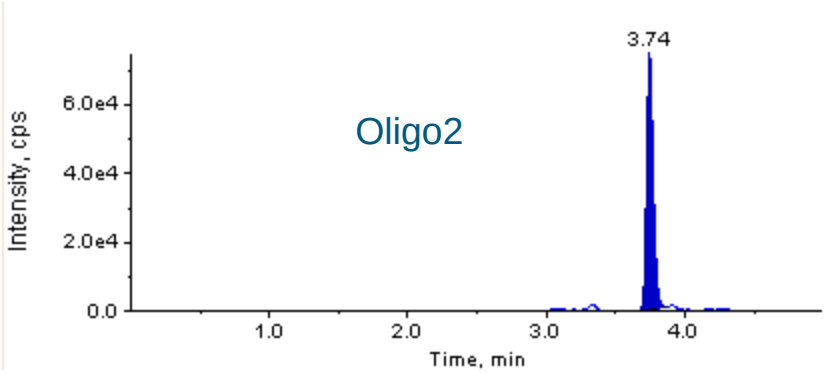
Chromatography

- Gradient system
  - Mobile phase A: 15 mM TEA + 400 mM HFIP
  - Mobile phase B: (50:50, v/v) 15 mM TEA + 400 mM HFIP: Methanol
- Mobile phase B: 25 % → 50 % over 5 minutes
- Column temperature 60 °C





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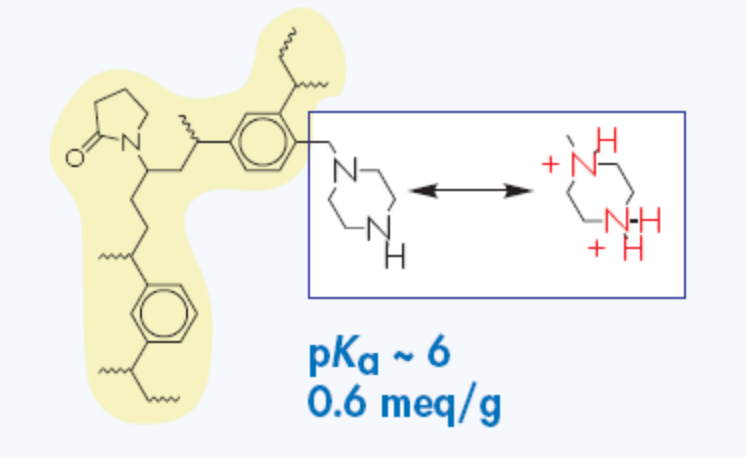
Sample Extraction

- Simple approaches used for small molecules are not applicable
  - Protein precipitation
    - » Oligonucleotides are highly protein bound and are precipitated out with this method
  - Generic solid phase extraction (HLB)
    - » Difficult to suppress ionisation of oligonucleotide due to low pKa leading to poor recoveries
- Answer to the problem lies in the chemical structure of oligonucleotides

Sample Extraction

- Utilise negative charges on OGN backbone
- Orthogonal to chromatographic system
- Oasis WAX
  - Mixed mode weak anion exchange
  - Retain and release strong acids



Retain OGN by ionizing sorbent

Elute OGN by neutralising sorbent

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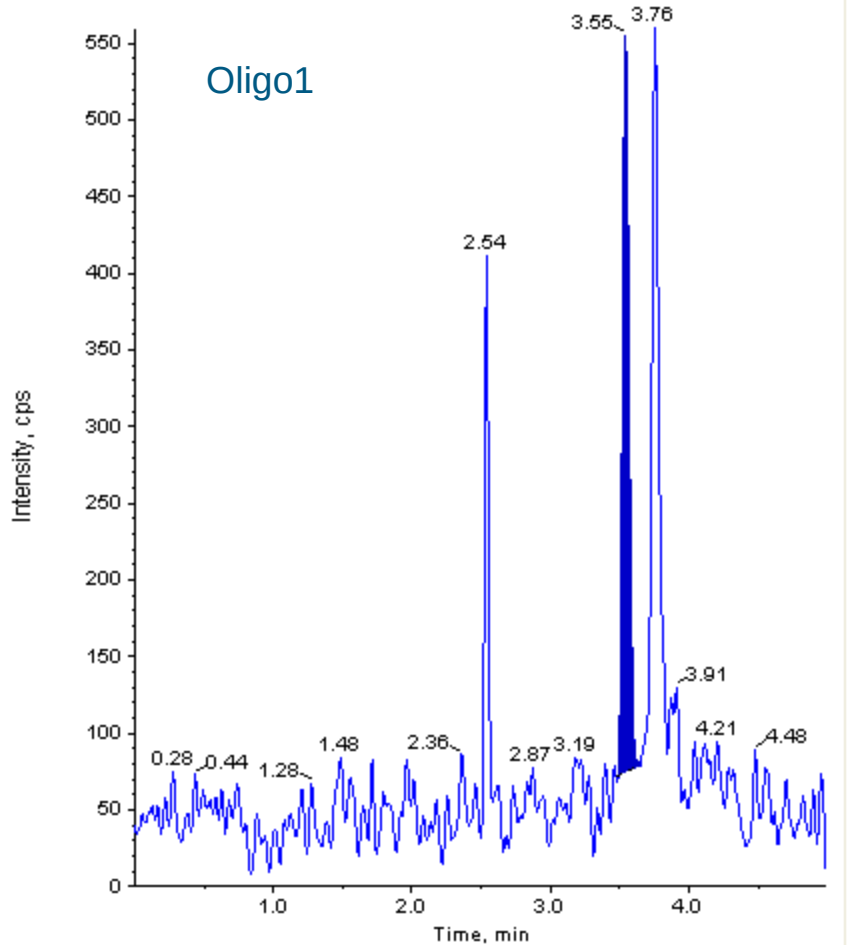
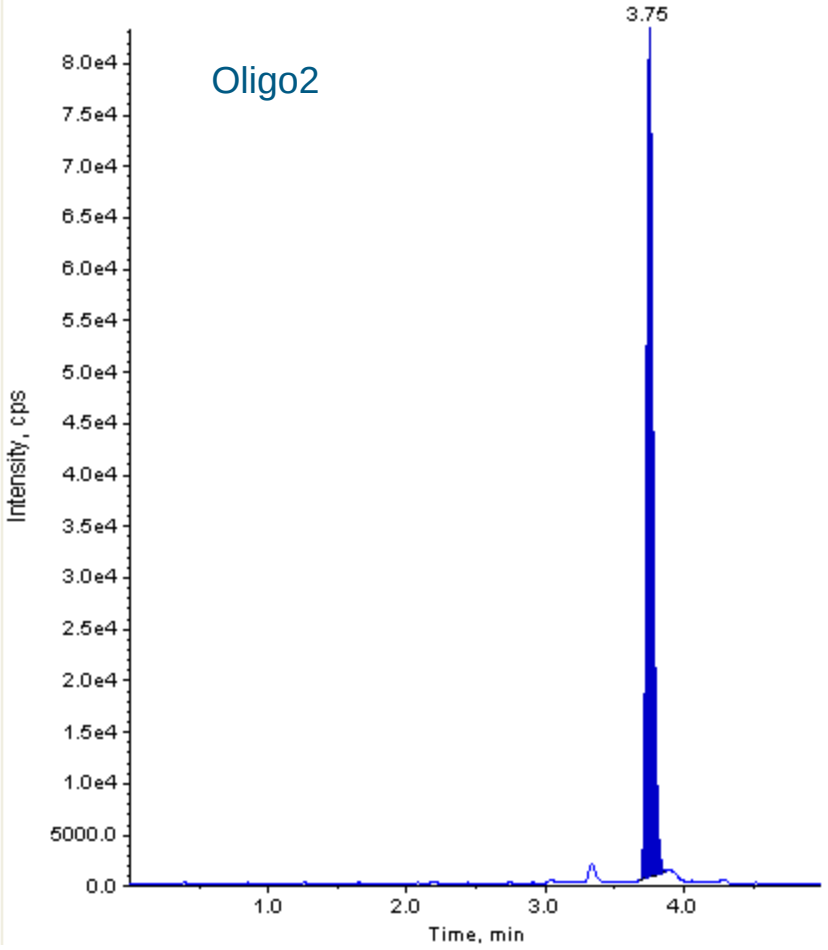
Sample Extraction

- Sample pre-treated with phosphoric acid
  - Reduced protein binding and improved recovery
  - Ionizing of SPE sorbent
- Wash to remove neutral/cationic species retained by hydrophobic interaction
  - High percentage of methanol
- Elute
  - (70:30:2, v/v/v) 15 mM TEA + 400 mM HFIP: MeOH: ammonia
  - Optimised for Oligo1 and Oligo2
  - More hydrophobic anionic species not eluted
- Partial evaporation step
  - Possible adsorption issues with complete drying
  - Add (85:15, v/v) 15 mM TEA + 400 mM HFIP: MeOH

LLOQ (0.025 nM = 0.1 ng/mL)





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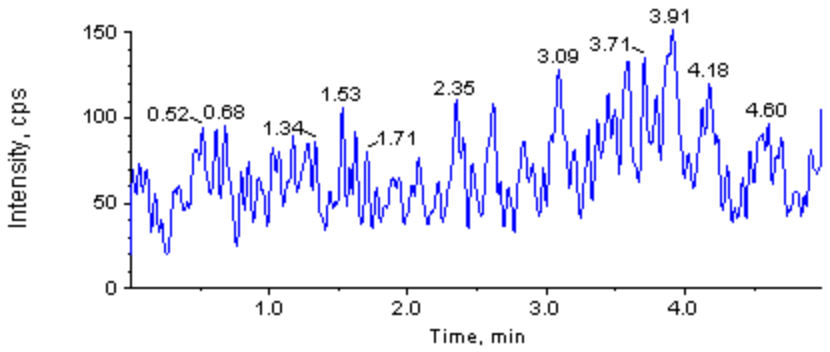
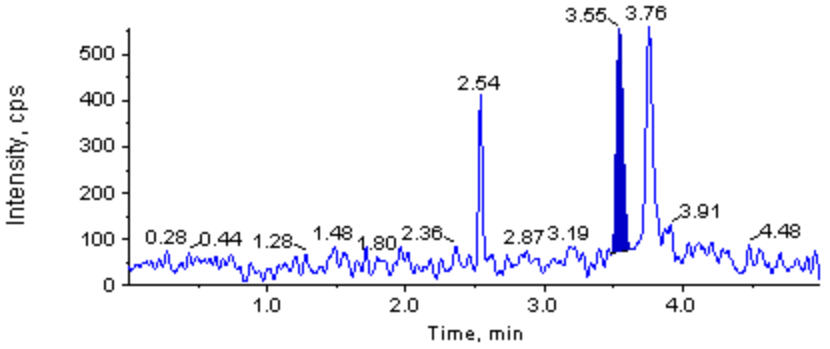
Blank Human Plasma



Blank Plasma



Cal 0.025 nM

- No interfering peaks in chromatographic region of interest
- Six individual human plasma samples investigated

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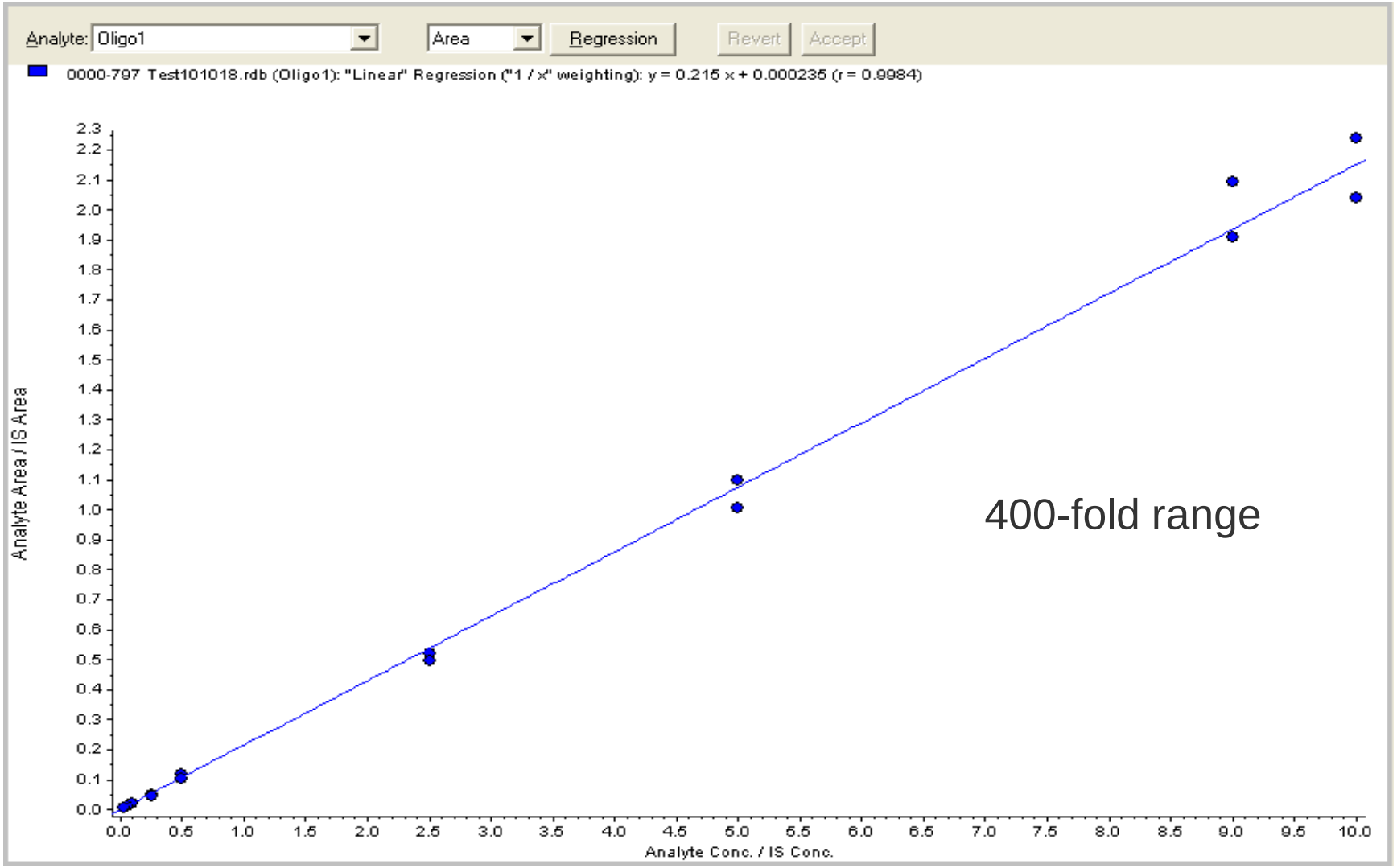
Calibration Line (0.025 – 10 nM)



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Inter-day Validation Data

Batch	Replicate	QC 0.025 nM (LLOQ QC) Observed concentration (nM)	QC 0.125 nM (LoQC) Observed concentration (nM)	QC 2 nM (MeQC) Observed concentration (nM)	QC 8 nM (HiQC) Observed concentration (nM)
Val001	1	0.0282	0.142	2.01	8.03
	2	0.0328	0.132	2.02	8.78
	3	0.0281	0.135	2.03	8.82
	4	0.0289	0.132	1.97	7.76
	5	0.0275	0.144	2.12	8.19
	6	0.0264	0.133	2.26	9.25
Val002	1	0.0322	0.148	1.86	7.83
	2	0.0261	0.137	1.85	7.55
	3	0.0216	0.133	1.81	7.39
	4	0.0253	0.131	1.97	7.50
	5	0.0272	0.136	1.87	7.30
	6	0.0293	0.157	1.95	8.30
Val003	1	0.0251	0.130	2.12	8.30
	2	0.0225	0.151	1.96	8.21
	3	0.0221	0.146	1.99	8.10
	4	0.0245	0.135	2.06	7.97
	5	0.0320	0.130	2.11	8.20
	6	0.0253	0.155	2.25	9.10
Mean (nM)		0.0270	0.139	2.01	8.14
Standard deviation (n-1)		0.00332	0.00884	0.127	0.563
RSD (%)		12.3	6.4	6.3	6.9
Accuracy (%)		108.0	111.2	100.5	101.8
n		18	18	18	18

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Individual Plasma Samples at MeQC

Quality control level (nM)	Matrix sample number	Observed concentration (nM)	Accuracy (%)
2	1	2.23	111.5
	2	2.19	109.6
	3	2.02	100.9
	4	2.17	108.6
	5	2.26	113.1
	6	2.13	106.5

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Matrix Effect – Oligo1

Quality control level (nM)	Peak area of pure standard	Mean peak area of pure standard (RSD%)	Matrix sample number	Peak area of sample spiked post extraction	Percentage modification
2	152229.6	148067.4	1	164019.5	10.8
	147606.7	(2.7)	2	151799.5	2.5
	144365.8		3	169420.6	14.4
			4	172683.5	16.6
			5	157449.4	6.3
			6	166392.8	12.4

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Recovery – Oligo1

Quality control level (nM)	Replicate	Peak area of extracted QC	Mean peak area (RSD%)	Peak area of reference QC	Mean peak area (RSD%)	Recovery (%)
0.125	1	7900.2	7989.1	9980.3	10674.5	74.8
	2	9091.3	(7.8)	11944.2	(10.3)	
	3	8098.1		10099.1		
	4	7447.3				
	5	7339.0				
	6	8058.8				
2	1	121471.9	112768.3	152936.5	147268.5	76.6
	2	106055.9	(4.8)	149326.6	(4.7)	
	3	111027.0		139542.4		
	4	116255.6				
	5	112307.6				
	6	109491.9				
8	1	452011.5	451077.1	722376.1	614275.0	73.4
	2	467510.9	(7.8)	591137.3	(16.0)	
	3	508410.4		529311.6		
	4	448653.1				
	5	413439.1				
	6	416437.5				
Mean recovery (%)						74.9
Range (%)						3.2

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24 Hour Room Temperature Matrix Stability

Replicate	QC 0.125 nM (LoQC)	QC 8 nM (HiQC)
	Observed concentration (nM)	Observed concentration (nM)
1	0.110	7.36
2	0.113	7.61
3	0.113	7.54
4	0.116	7.45
5	0.112	7.65
6	0.120	7.21
Mean (nM)	0.114	7.47
Standard deviation (n-1)	0.00352	0.166
RSD (%)	3.1	2.2
Accuracy (%)	91.2	93.4

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Freeze/Thaw Stability (3 Cycles)

Replicate	QC 0.125 nM (LoQC)	QC 8 nM (HiQC)
	Observed concentration (nM)	Observed concentration (nM)
1	0.116	7.55
2	0.120	7.71
3	0.117	8.41
4	0.141	8.31
5	0.120	8.42
6	0.126	8.00
Mean (nM)	0.123	8.07
Standard deviation (n-1)	0.00933	0.374
RSD (%)	7.6	4.6
Accuracy (%)	98.4	100.9

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Method Summary

- 15-mer Oligonucleotide (4.5 kDa)
- Human plasma assay
  - 200 µL sample volume
- Solid-phase extraction
  - weak anion exchange mechanism
- UPLC chromatography
  - ion-pair reverse phase system
- Sciex API 5000 detection
  - 5 MRM transitions summed
- LLOQ of 0.1 ng/mL
  - 400-fold linear range
  - Recovery of 75 %
  - 3 F/T cycles and 24 hour room temperature matrix stability

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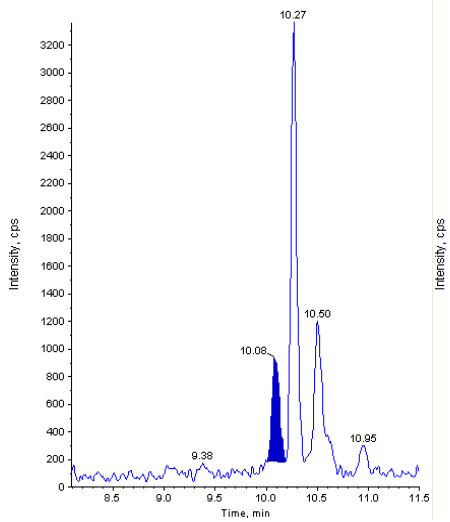
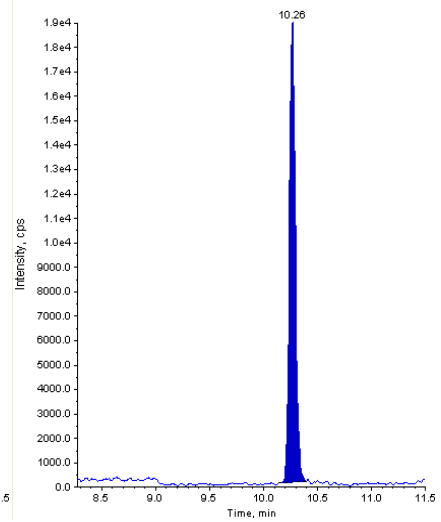
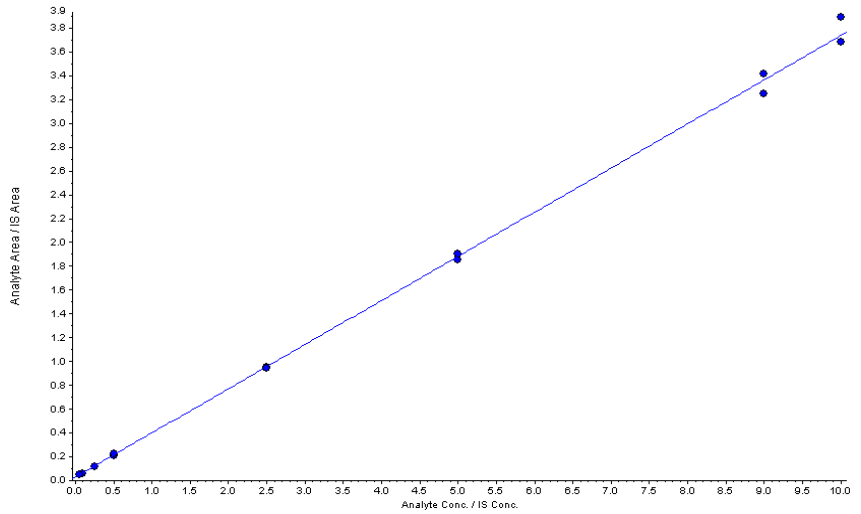
Larger Oligonucleotides?

- 25-mer (7.6 kDa)
- Modified chromatography and sample extraction
- More challenging to resolve from IS
- LLOQ is 0.4 ng/mL
- Initial results promising
- Still to be validated







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Future Work

- Addition of Symbiosis on-line SPE
  - Currently inject 30-40 µL from a total of 200 µL
  - Load entire 200 µL onto on-line C_{18} cartridge
  - Elute to MS with mobile phase gradient
  - Approach routinely used for peptide analysis
- Evaluate HILIC system
  - Sensitivity gains from removal of TEA?

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