NovaBioAssays

Innovative Mass Spectrometric Solutions

Quantitative Bioanalysis of Oligonucleotides

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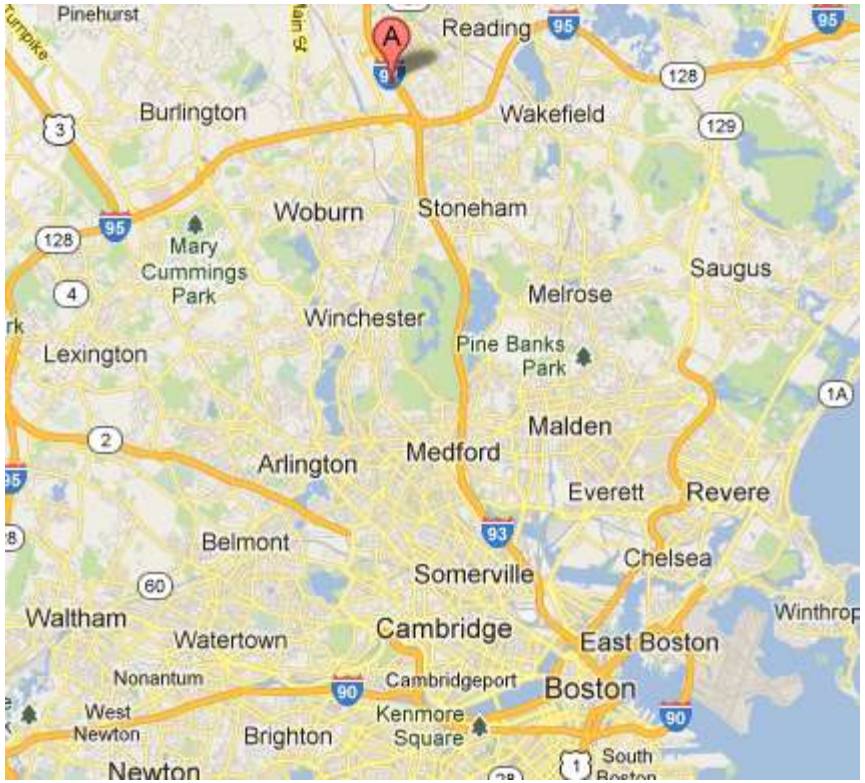
May 24, 2016
Thermo BioSeparations Workshop

Outline

- Overview
  - Thermo instruments at NovaBioAssays
  - Bioanalysis of oligonucleotide therapeutics
- DNApac PA200 columns for hybridization based LC-fluorescence assays
- DNApac RP columns for ion-pair LC-MS/MS and LC-HRAM analysis
  - Full Scan, target-SIM and PRM/DIA analysis on Q-Exactive
- Q & A

Where Are We



52 Dragon Court, Suite 3B
Woburn, MA 01801, USA
Phone: (781)933-3480

What Do We Have (1/3)

AB SCIEX 5000 LC/MS/MS
(X2)

Thermo TSQ Vantage
(nano and ESI source)

Thermo Q Exactive MS (Plus)
(x2)

Bruker MicroTOFQ II
(nano and ESI source)
(x2)

Waters Acquity UPLC
Waters nanoAcquity UPLC
(x7)

Accela 1250 and CTC PAL

Thermo Easy nLC

What Do We Have (2/3)

Thermo Vanquish Flex UHPLC System
With Vanquish™ Fluorescence
Detector F (4 Excitation/Emission)

Waters Acquity UPLC with PDA
And Fluorescence Detectors

What Do We Have (3/3)

TomTec Quadra 3 SPE
Automated Liquid Handler
(x2)

PPE, LLE, SPE

Thermo Versette™
Automated Liquid Handler

Thermo KingFiser Flex 96
Magnetic Particle Processor

Affinity/Immunoaffinity Purification
Mass Spectrometric Immunoassay (MSIA)

What Can We Do?

Quantitative Bioanalysis (API5000 or Q-Exactive or FD/PDA)

- Protein bioanalysis using LC-MS/MS or LC-HRAM
- Lipids/Polymers using LC-MS/MS or LC-HRAM
- Small molecule analysis using LC-MS or LC-HRAM
- Oligonucleotides bioanalysis using
  - LC-MS/MS or LC-HRAM
  - Hybridization UPLC fluorescence
  - UPLC-UV

Qualitative analysis (Q-Exactive and Q-tof)

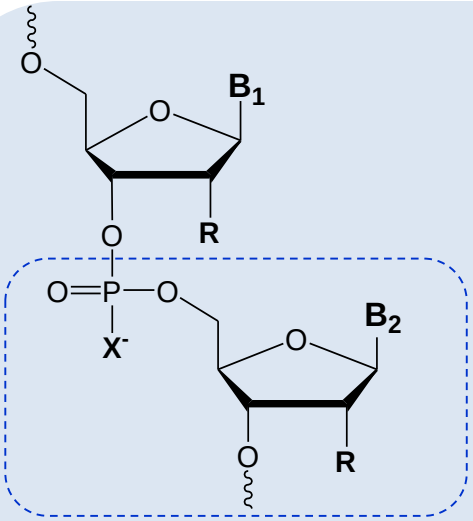
- Primary structural characterization using LC-HRAM and LC-MS/MS
- Tertiary structural characterization using H/D exchange LC-HRAM and LC-MS/MS
- Quant-Qual analysis using LC-Q Exactive

In Vitro Protein Binding and Stability/Metabolite ID

- Rapid Equilibrium Dialysis(RED) and Ultracentrifugation
- Microsomal/Plasma/Whole blood stability
- Hepatocyte incubation

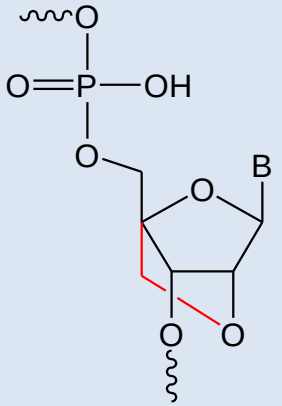
Quantitative Bioanalysis of Oligonucleotides

Chemical Structures of Oligonucleotides

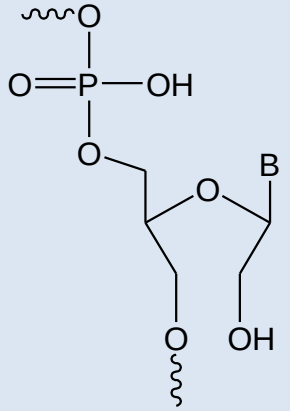


X = O (Phosphodiester)
S (Phosphorothioate)

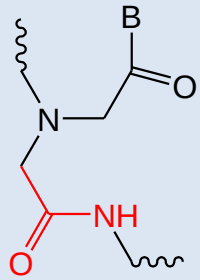
R = -H (DNA)
-OH (RNA)
-OCH_3 (2'-Methyl)
-F (2'-Fluoro)
-$OCH_2CH_2OCH_3$ (2'-MOE)



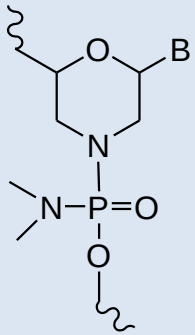
LNA



UNA



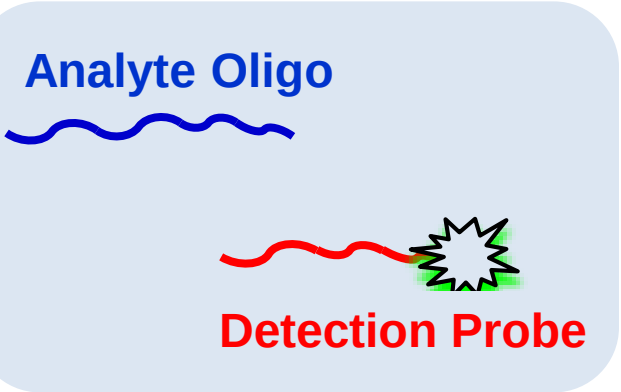
PNA



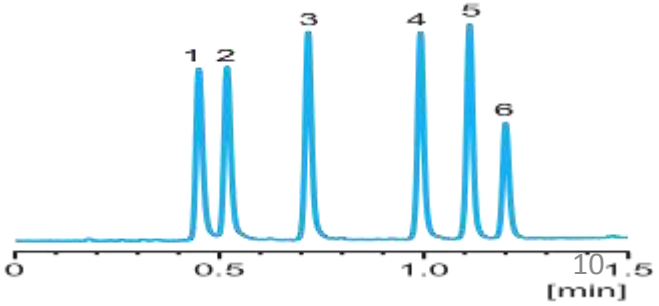
PMO

Quantitative Bioanalysis of Oligonucleotides/mRNA

- Hybridization-based assay (indirect analysis)
  - Northern blot/*in situ* hybridization
  - Microarray
  - Deep sequencing (NGS)
  - RT-qPCR
  - Hybridization ELISA
    - Direct ELISA, Sandwich ELISA, etc
    - Branched DNA (bDNA) signal amplification technology (QuantiGene)



- Chromatographic-based assay (direction analysis)
  - HPLC-UV/Fluorescence
  - Hybridization-based LC-fluorescence
  - LC-MS/MS and LC-HRAM



Quantitative Bioanalysis of Oligonucleotides/mRNA

- Hybridization-based assay (indirect analysis)
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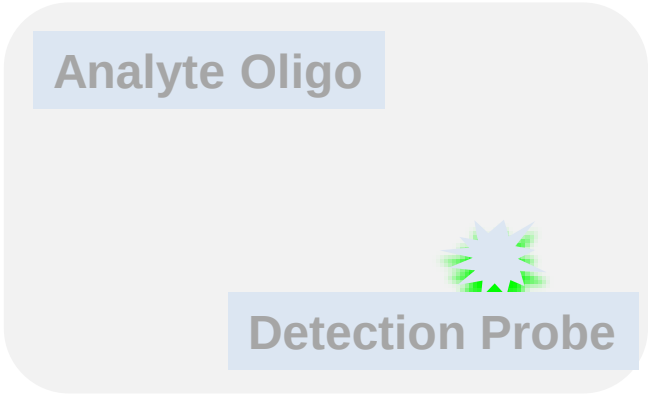

- Chromatographic-based assay (direction analysis)
  - HPLC-UV/Fluorescence
  - Hybridization-based LC-fluorescence
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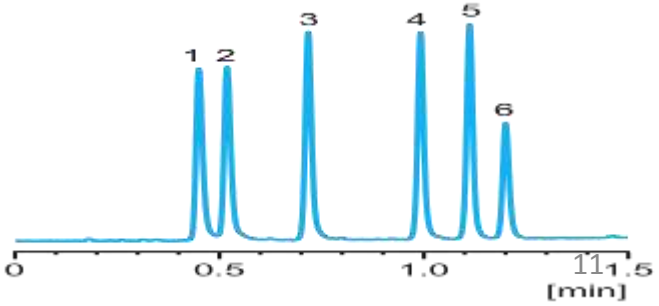
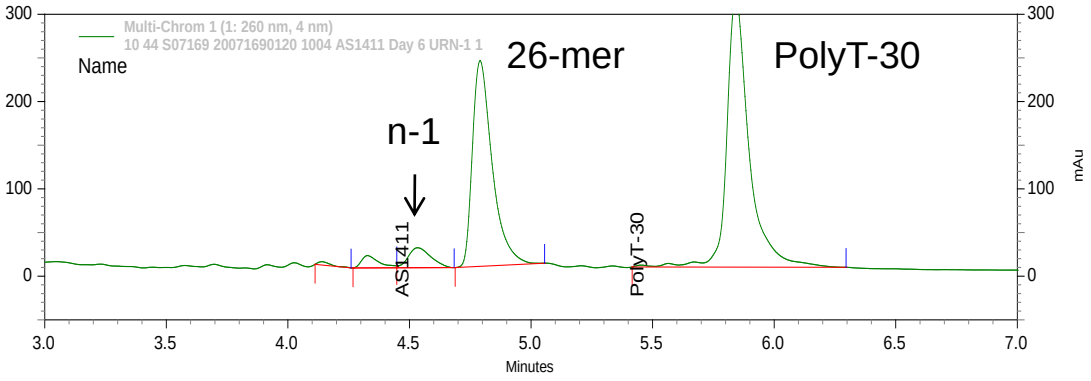


Ion-Pair (IP)-UPLC-UV Assays

- Simple sample preparation (single LLE or SPE)
- High reproducibility (15/20% acceptance criteria)
- Relatively selective (primarily by length/charge)
- Estimation of metabolite concentrations
- Reasonable run time (<10 minutes/sample)
- Low sensitivity (~100 ng/mL LLOQ)

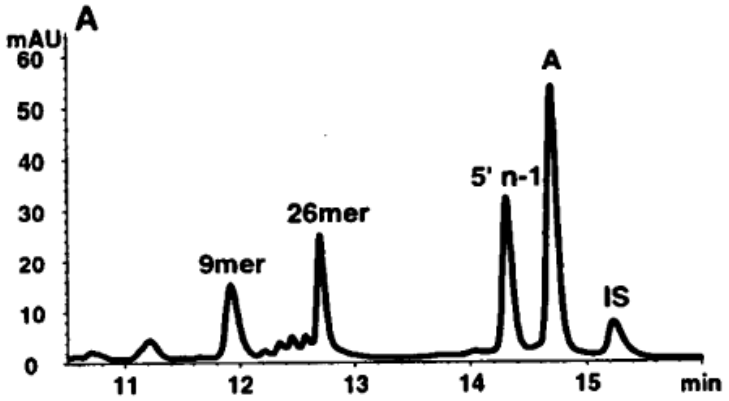


Mobile Phase:
20 mM tetrabutylammonium sulfate in water/MeCN
pH=9

Jian Chen, et. al., "Quantitative determination of AS1411 oligonucleotide in monkey plasma using UPLC-PDA," *ISSX, 2008*

Strong Anion Exchange (SAX)-HPLC-UV

- Relatively selective (primarily by length/charge)
- High reproducibility (15/20% acceptance criteria)
- Simple sample preparation (single LLE or protease k digest)
- Estimation of metabolite concentrations
- Low sensitivity (~200 ng/mL LLOQ)
- Only works for charged oligos
- Long run time (20-35 min)

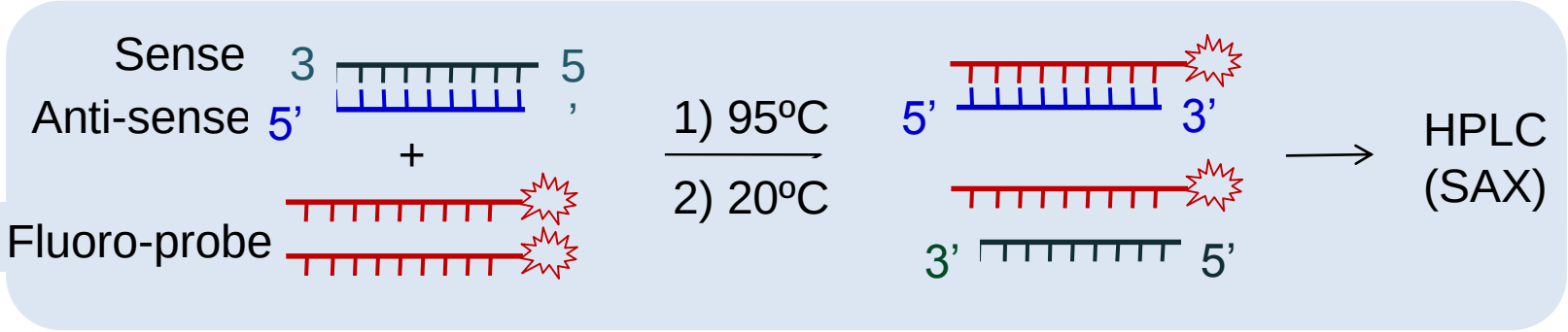


Analyte: 35-mer modified ribozyme RNA
IS: 43-mer analog RNA
LC column: DNAPac® PA100
MP: (50 mM Tris pH=8.0)/Ethanol 80/20 (v/v) with 50-250 mM $NaClO_4$

Sandberg, et al. *Antisense & Nucleic Acid Drug Development 2000;* 10:153-162.

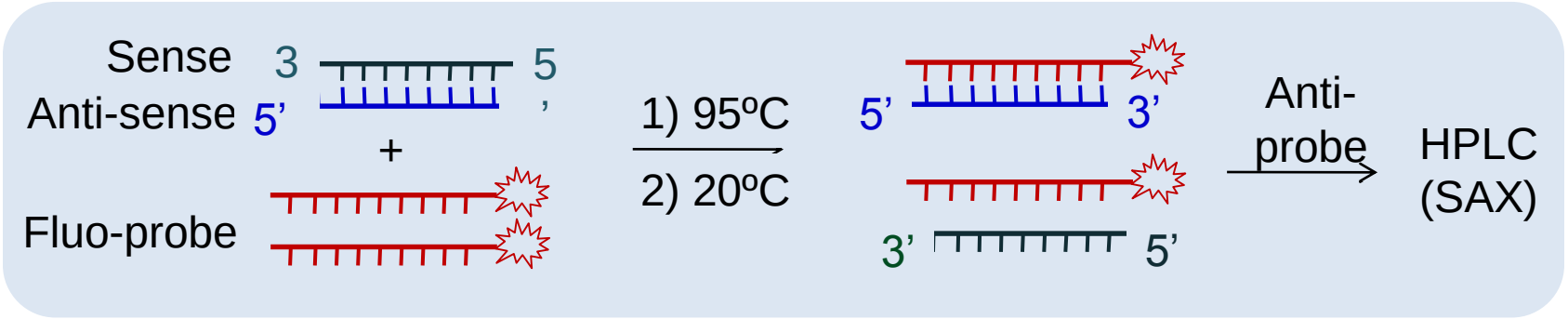
Hybridization-based HPLC-Fluorescence Assays

- High sensitivity (~ 1 ng/mL LLOQ)
- Good specificity (Hybridization and LC separation)
- Large dynamic range (1000x)
- Simple sample preparation (protein digestion or LLE)



- Good reproducibility (± 15% accuracy/precision)
- Tolerance to structure modifications
  - Base, sugar and phosphate backbone modifications

Quantify siRNA in HuPl Using Fluorescence Labeled DNA Probe (1.00 – 500 ng/mL)

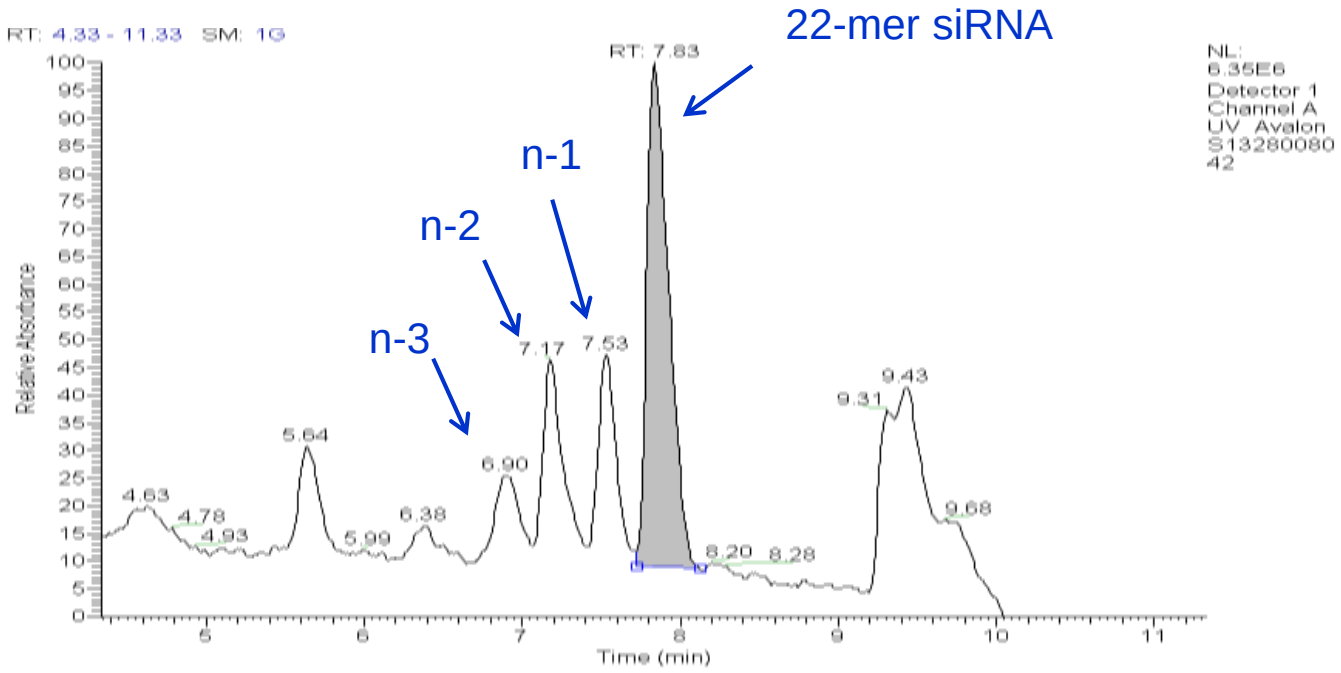


Analyte: 21-mer siRNA
Probe: 21-mer Atto dye labeled DNA oligo
Anti-probe: Cholesterol conjugated DNA oligo

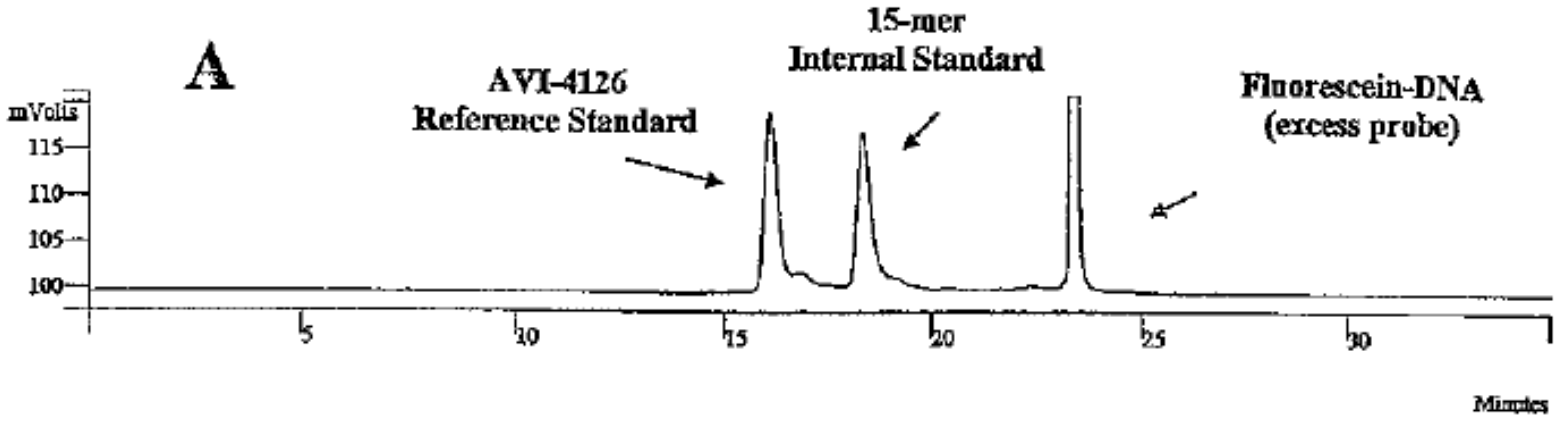
Sample Prep: Protease K digestion
Column: DNA Pac-PA-200, 4x250 mm
MPA: 1 mM EDTA in 25 mM Tris-HCl (pH 8.0) with 10% MeCN
MPB: 1 mM EDTA and with 800 mM NaClO4 in 25 mM Tris-HCl with 10% MeCN

Hutabarat R, et al. AAPS Annual Meeting 2011, poster presentation.

Typical Chromatogram of 22-mer siRNA in HuPl Samples (>24 Hours)



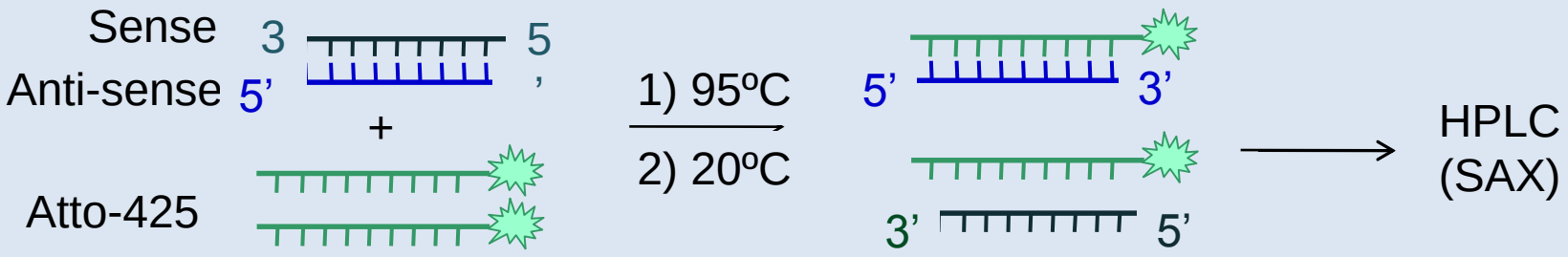
Quantification of a Neutral 20-mer PMO Using a 15-mer Fluorescein-DNA (40.0-4,000 ng/mL)



AVI-4126:	5'-ACGTTGAGGGGCATCGTCGC-3' PMO (Phosphorodiamidate Morpholino Oligomer)
LC column:	Dionex DNA Pac PA-100 (4 x 250 mm)
Mobile Phase:	A) 25 mM Tris pH=8.0; B) 25 mM Tris pH=8.0 with 1.0 M NaCl

Arora V, et al. J of Pharmaceutical Sciences 2002; 91(4):1009-1018.

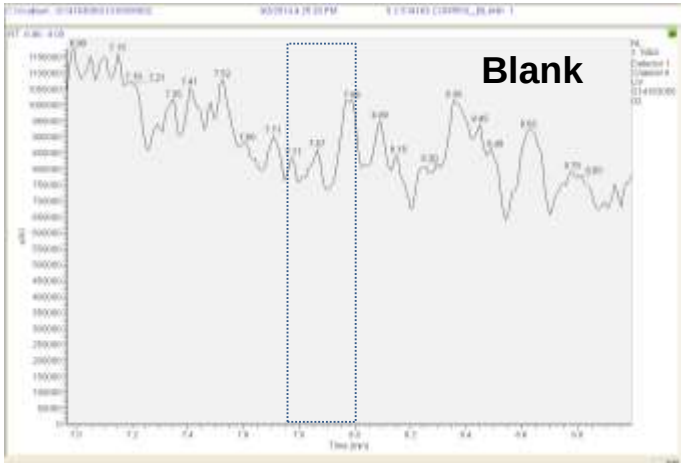
Quantification of siRNA in HuPl Using Fluorescence-labeled PNA probe (1.00-1000 ng/mL)

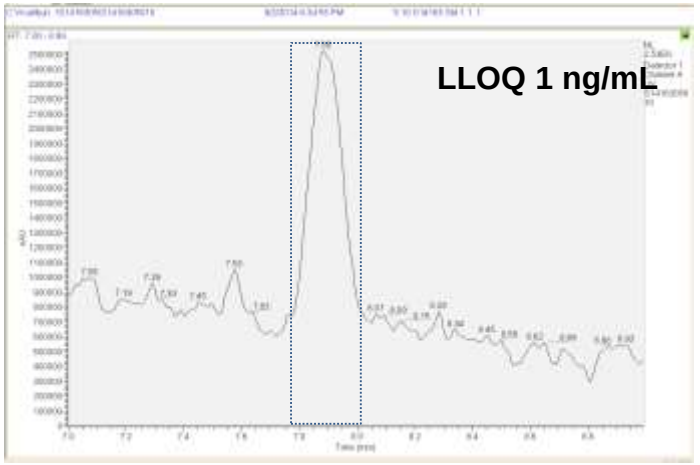


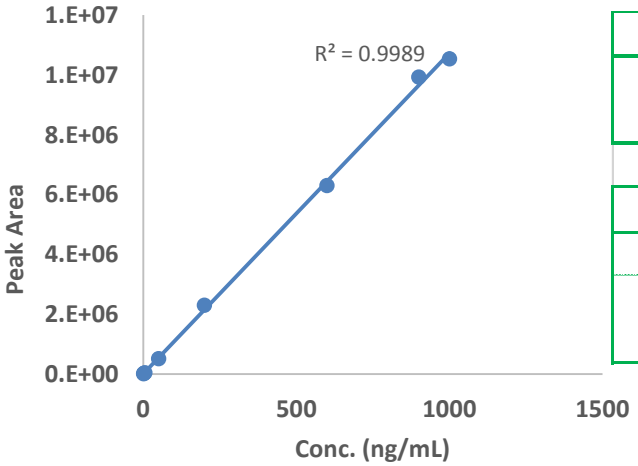
Analyte: 21-mer siRNA
Probe: 18-mer Atto dye labeled PNA oligo

Sample Prep: Protease K digestion
Column: DNA Pac-PA-200, 4x250 mm

Method Performance of Quantitative Determination of a siRNA in Rat Plasma using PNA Assay







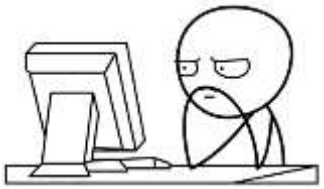
Quality Control Samples		
QC (10 Runs)	Precesion (CV%)	Accuracy (%Bias)
	2.9%~11.2%	-0.7%~2.5%

Incurred Sample Reanalysis			
ISR Samples	20%Difference from Orginal	%Difference from original	ISR samples
45	45 (100% pass)	< ±10%	43
		±10% ~ ±15%	2

arrowhead pharmaceuticals

Pros and Cons of Hybridization-based HPLC-Fluorescence Assays

- High sensitivity (~ 1 ng/mL LLOQ)
- Good specificity (Hybridization and LC separation)
- Large dynamic range (1000x)
- Simple sample preparation
- Internal standard is not necessary
- Good reproducibility (± 15% accuracy/precision)
- Tolerance to structure modifications
  - Base, sugar and phosphate backbone modifications
- Long run time (15-30 minutes/sample)
- One analyte/fluorophore at a time



Fast and Mutiple Analyte Hybridizaion based LC-Fluorescence Assays

DNApac PA200: (PEEK)	8 µm, 4.6 x 250 mm
DNApac PA200 RS: (Peek -lined Stainless Steel)	**4 µm, 4.6 x 150 mm 4.6 x 50 m**

Thermo Vanquish Focused UHPLC System
With Vanquish™ Fluorescence Detector F (4 Excitation/Emission)
Chromeleon 7.2 Chromatography Data System

Data to be shared soon…

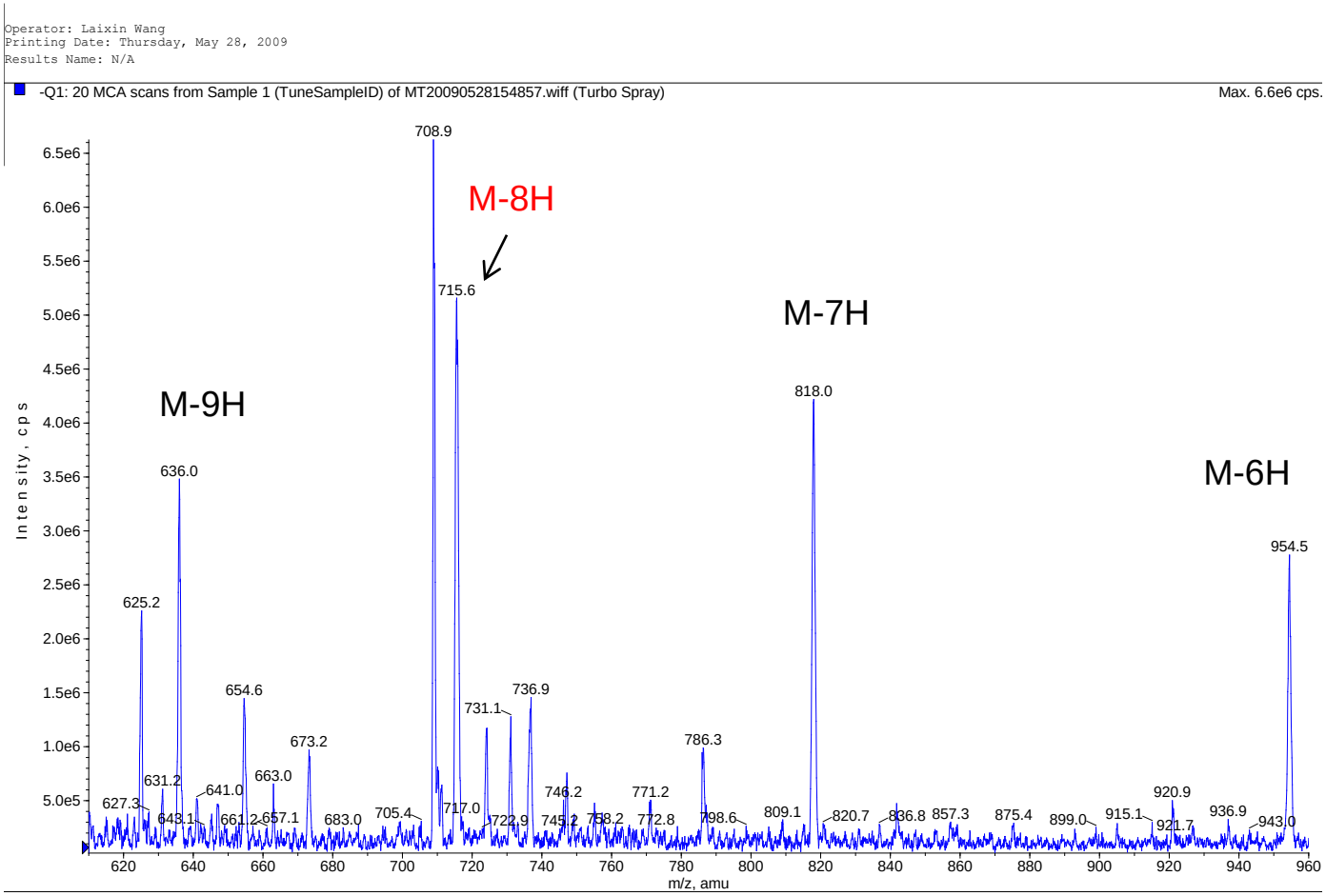
LC-MS/MS & LC-HRAM Assays

- Highest specificity
  - Molecular weight identification
  - Product ion characterization
  - LC Separation
- High throughput (~5 minutes/sample)
- Wide dynamic range (1000x)
- Good sensitivity (< 5 ng/mL LLOQ)
- No enzymes or special reagents needed
- Good reproducibility (±15/20% acceptance criteria)
- Need high performance mass spectrometer
- Potentially challenging assay development

Challenges & Solutions of Developing LC-MS/MS Assays

- Non-specific adsorption (stickiness)
  - Add disruption reagent(s) in extraction buffer
  - Avoid extensive drying down of samples
  - Minimize use of polypropylene container
  - Keep stock solution at high concentrations.
- Stability in matrix
  - Use K_2EDTA as anticoagulant
  - Add CHAPS or BSA to urine samples.
- Short column life time and carryover
  - Clean-up sample “appropriately”
  - Re-generate the column after each injection (back flush the column).
- Charge distribution and metal ion adducts in mass spectrum
  - HFIP and TEA/DIPEA buffered water and MeOH/MeCN as mobile phase.

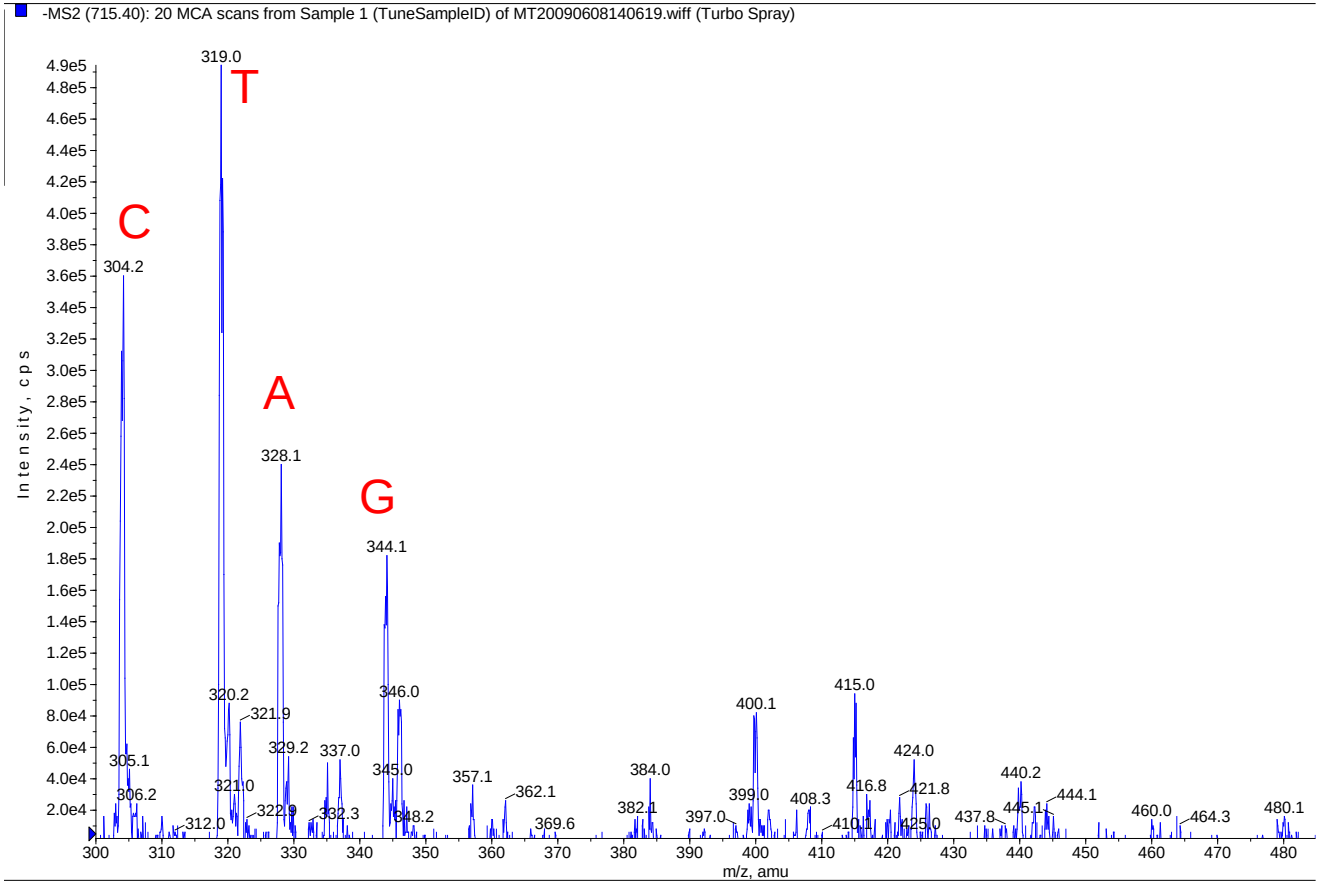
Q1 MS Scan of a 18-mer PS-ODN on a Triple Quad



Calculated *m/z*	
M	5731.6
M-6H	954.3
M-7H	817.8
M-8H	715.5
M-9H	636.8

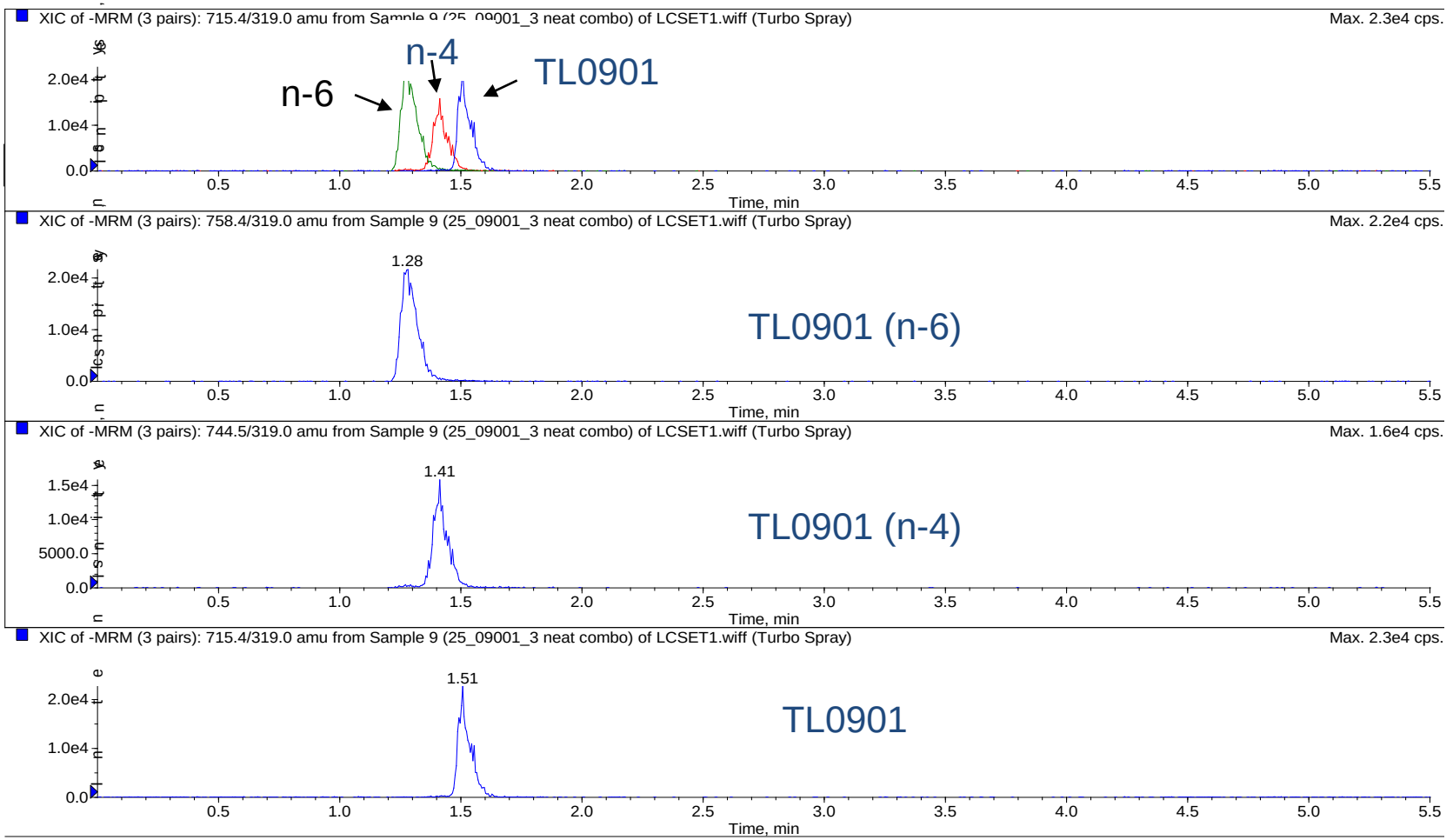
Laixin Wang, et. al., *AAPS Annual Meeting and Exposition,* November 2009, Los Angeles, CA,

Product Ion Scan of a 18-mer PS-ODN on a Triple Quad

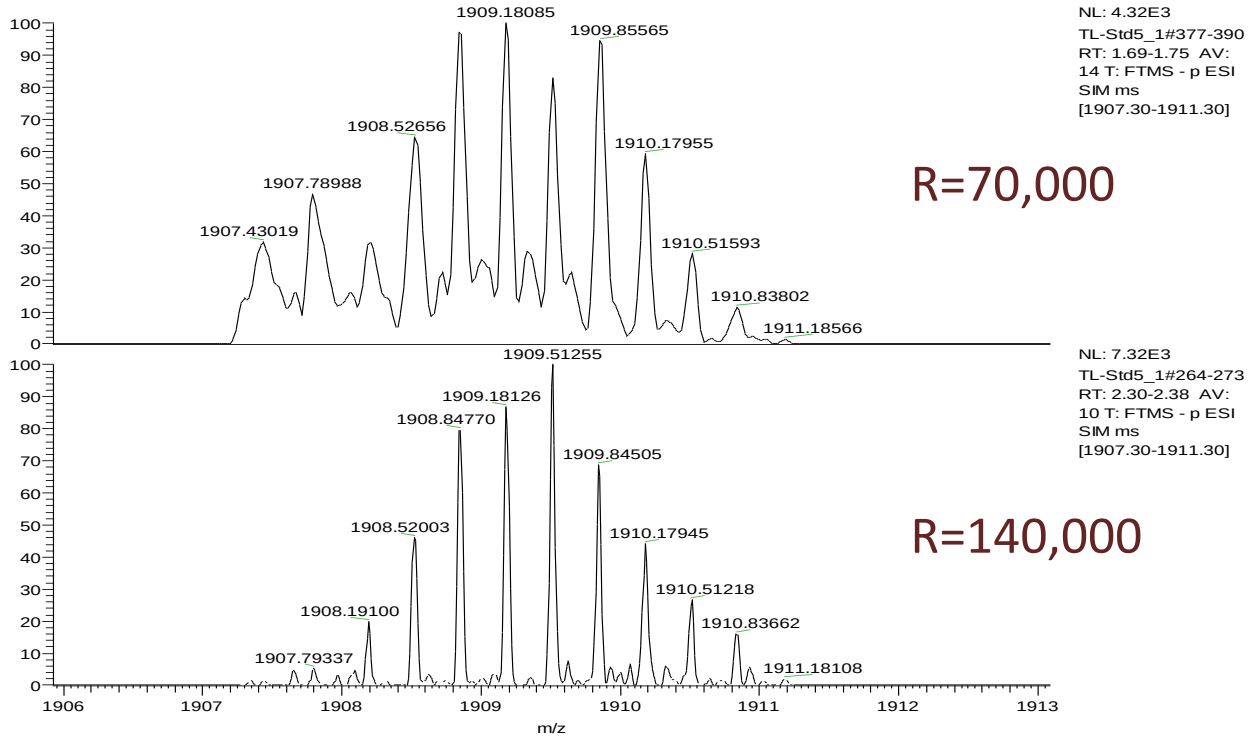


SRM Transitions evaluated for TL0901
715.5 → 319.0
715.5 → 304.2
818.0 → 319.0
818.0 → 304.2

LC Separation of a 18-mer PS-ODN and its Metabolites (Neat Mixture)



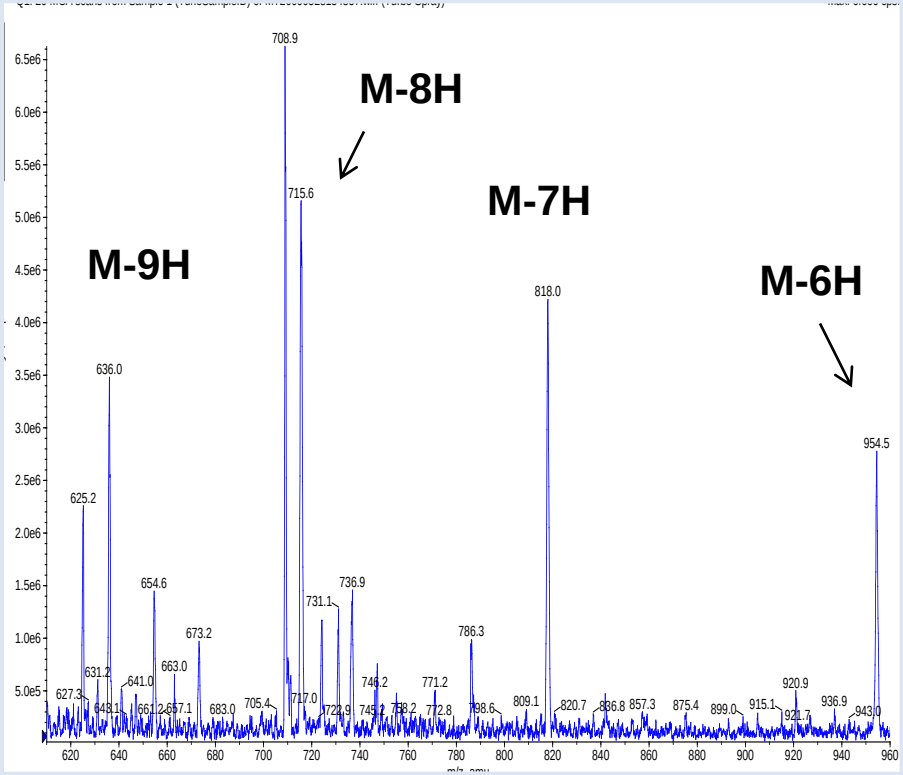
HRAM Improves Selectivity by Using Higher Resolution (Q Exactive)



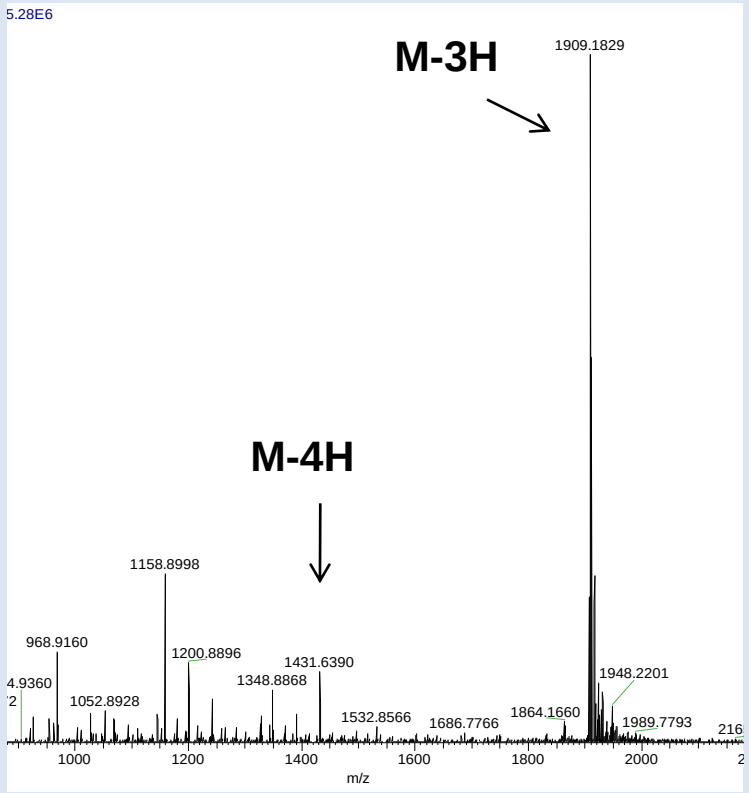
Isotope envelopes of the $[M-3H]^{3-}$ ion of an 18-mer PS-ODN on a high resolution Q-Exactive Orbitrap® (Thermo Finnigan, LLC)

Different Charge Distribution of a 18-mer PS-ODN on Different Platforms

API-5000

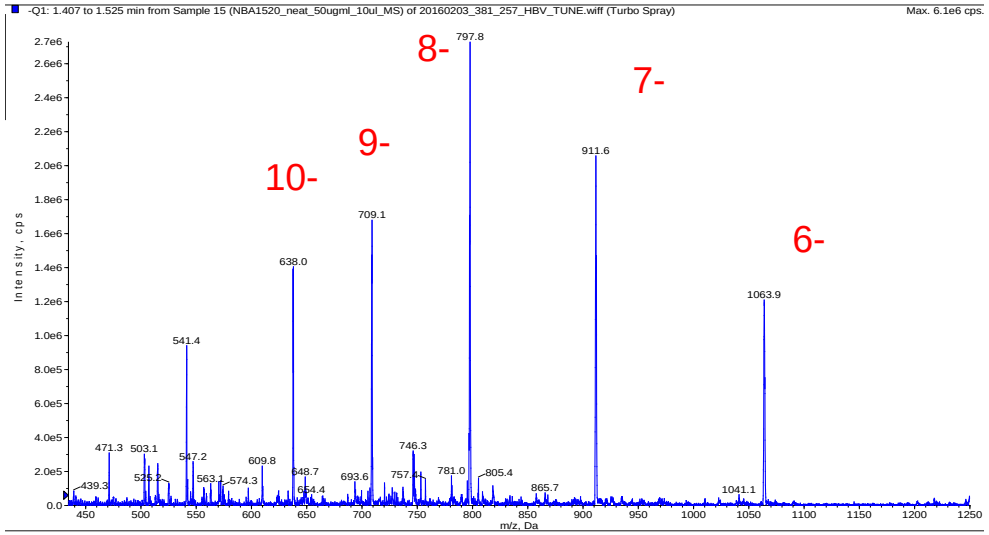


Q-Exactive

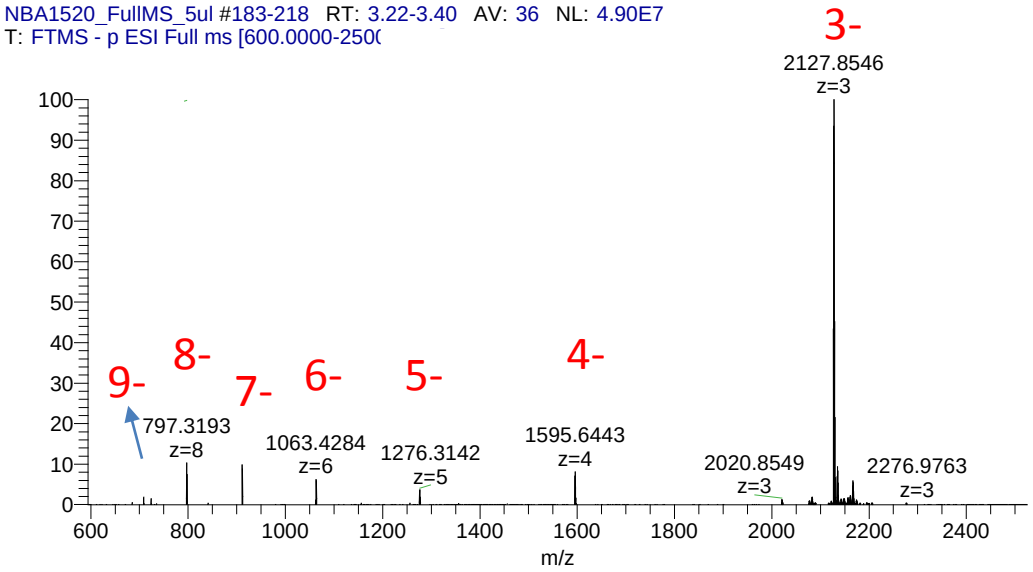


Weiwei Yuan, et. al. "Quantitative Determination of TL0901 Oligonucleotide in Human Plasma Using LC-HR/AM," *ASMS, 2012*

Different Charge Distribution of a 20-mer PS-ODN on Different Platforms

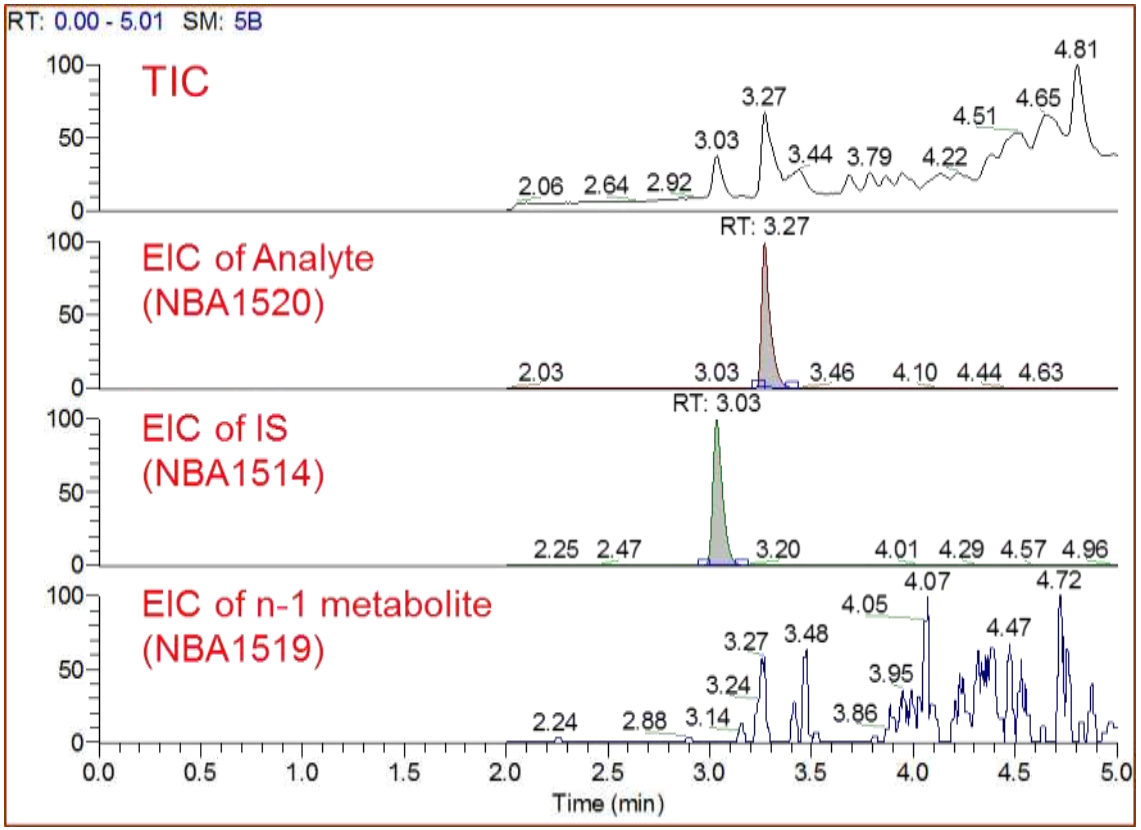


API5000 Triple Quads



Q Exactive Plus

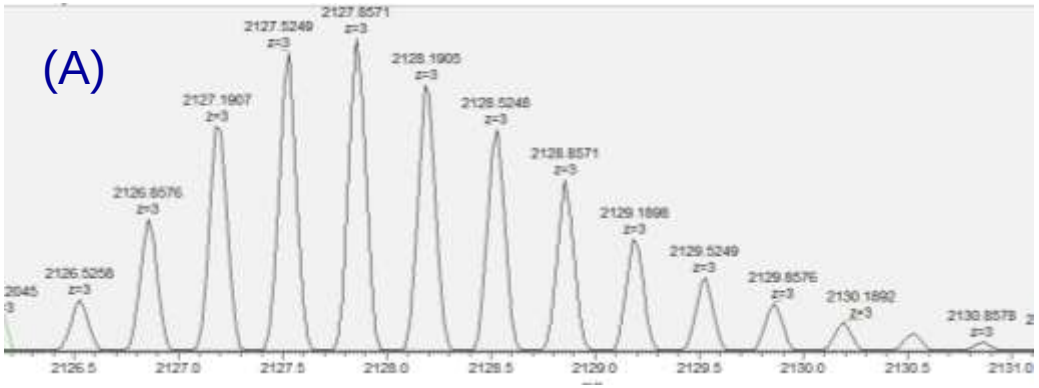
Full Scan Analysis of Extracted 20-mer PS-ODN (HuPL) on Q-Exactive Plus (1/3)



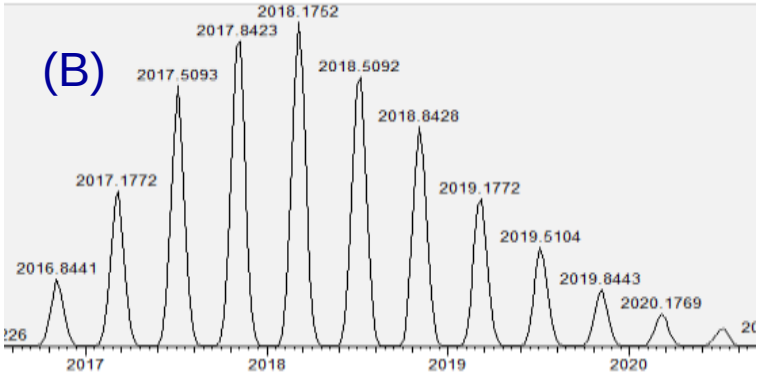
Extraction:
SPE from 200 µL HuPl
LC Column:
Thermo DNApac RP
4 µm, 2x50 mm
Column Temp: *60 °C*
Mobile Phase:
HFIP/TEA buffered water/ MeOH Gradient
Run Time: *8 minutes*
LC System:
Themo Vanquish UPLC
MS: *Q-Exactive Plus*
Ionization Mode: *Negative*
Resolution: *70K*
Scan Range *600-2500 m/z*

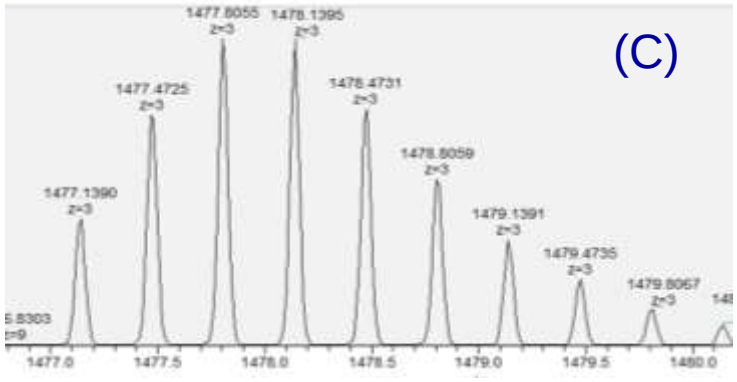
NBA1520:	5'--TAG TCA ATC TGC TTA TGT CA--3'	C196H249N68O102P19S19
NBA1519:	5'--TAG TCA ATC TGC TTA TGT C--3'	C186H237N63O98P18S18
NBA1514:	5'--TAG TCA ATC TGC TT--3'	C137H175N46O72P13S13

Full Scan Analysis of Extracted 20-mer PS-ODN (HuPL) on Q-Exactive Plus (2/3)



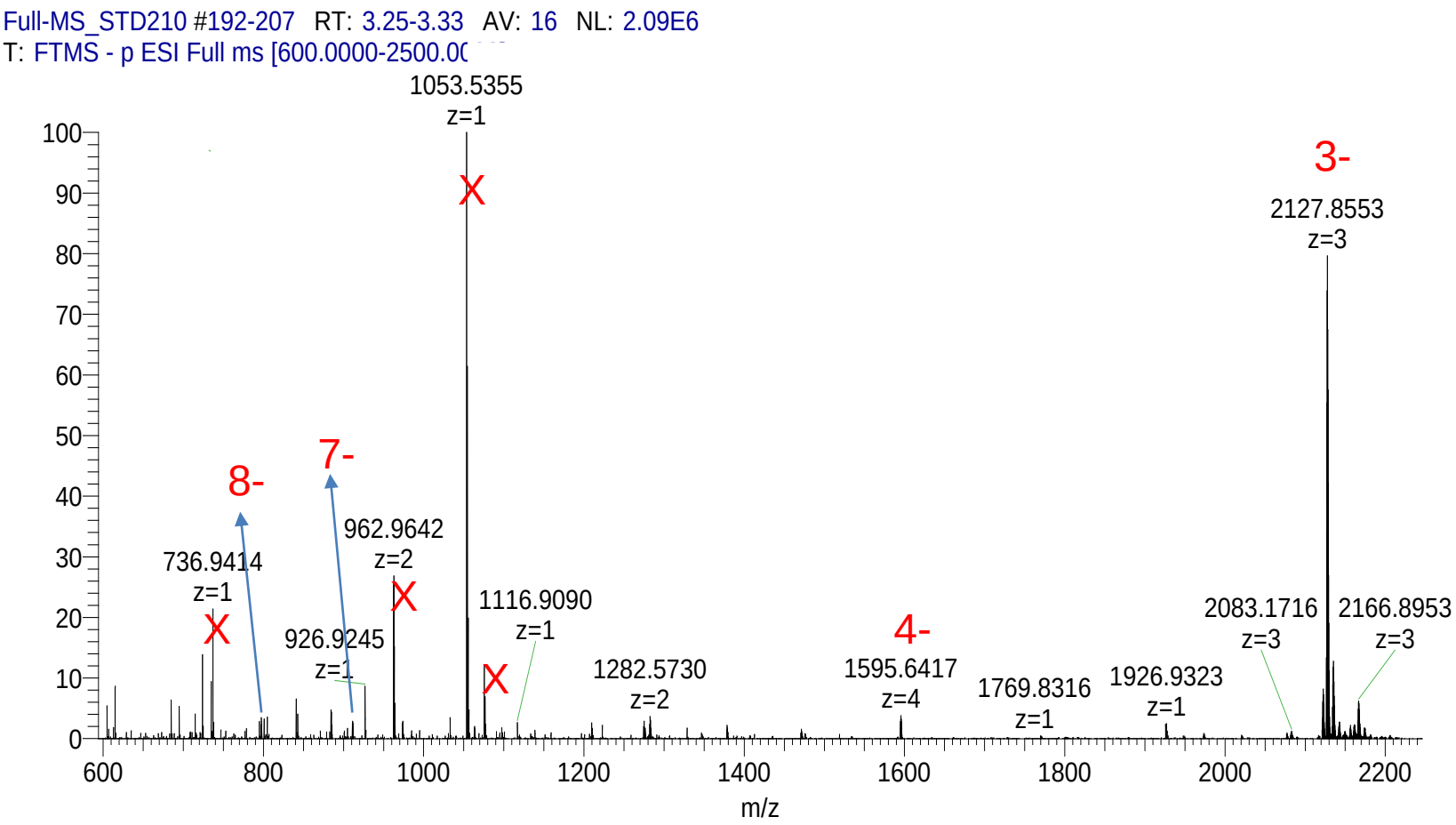
(A): NBA1520
(B): NBA1519
(C): NBA1514



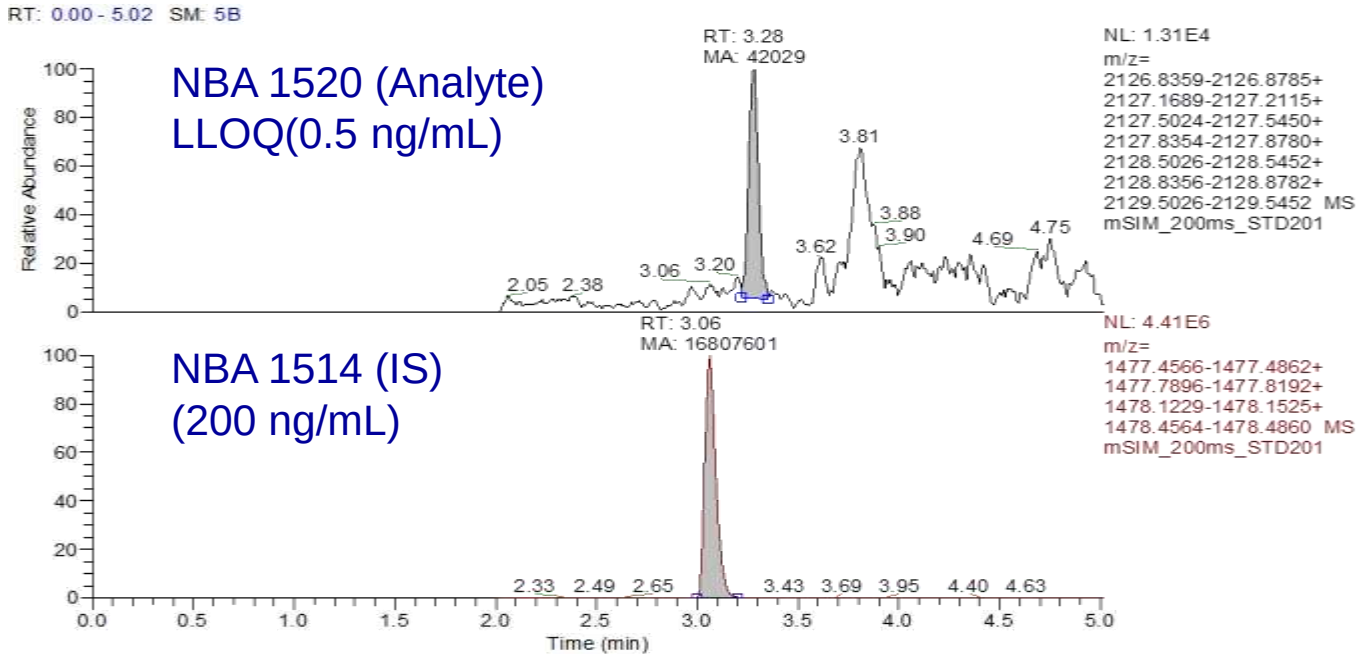


NBA1520:	5'--TAG TCA ATC TGC TTA TGT CA--3'	C196H249N68O102P19S19
NBA1519:	5'--TAG TCA ATC TGC TTA TGT C--3'	C186H237N63O98P18S18
NBA1514:	5'--TAG TCA ATC TGC TT--3'	C137H175N46O72P13S13

Full Scan Analysis of Extracted 20-mer PS-ODN (HuPL) on Q-Exactive Plus (3/3)



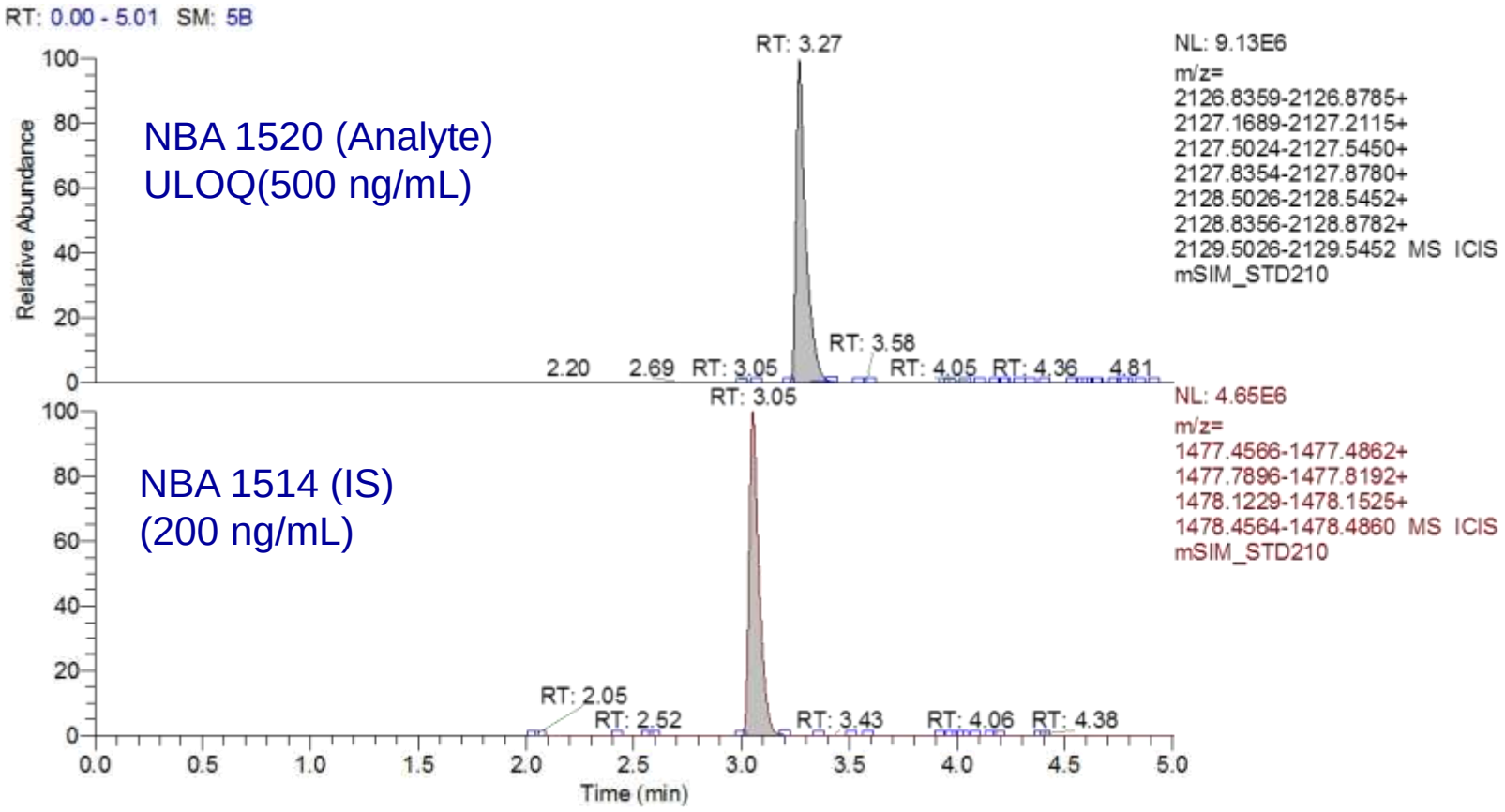
Target-SIM Analysis of Extracted 20-mer PS-ODN (HuPL) on Q-Exactive Plus (1/3)



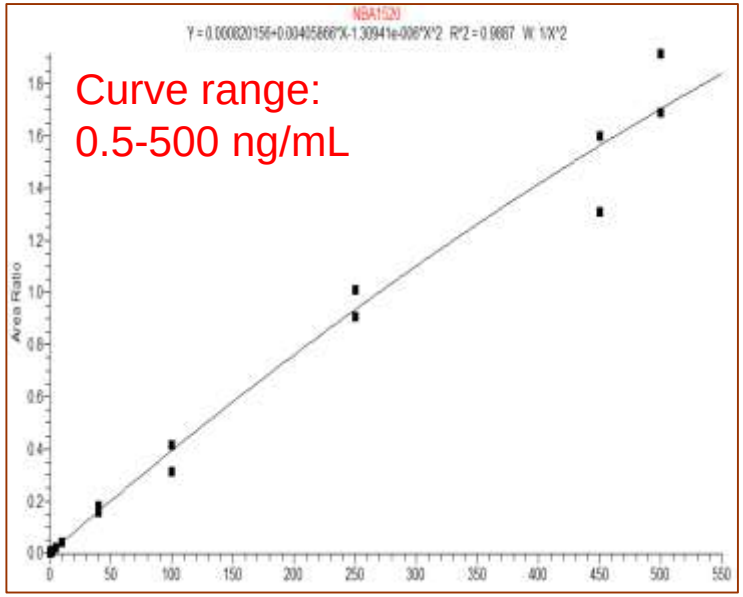
Extraction: ***SPE from 200 µL HuPl***
LC Column: ***Thermo DNApac RP***
4 µm, 2x50 mm
Mobile Phase:
HFIP/TEA buffered water/MeOH
LC System:
Themo Vanquish Flux UPLC

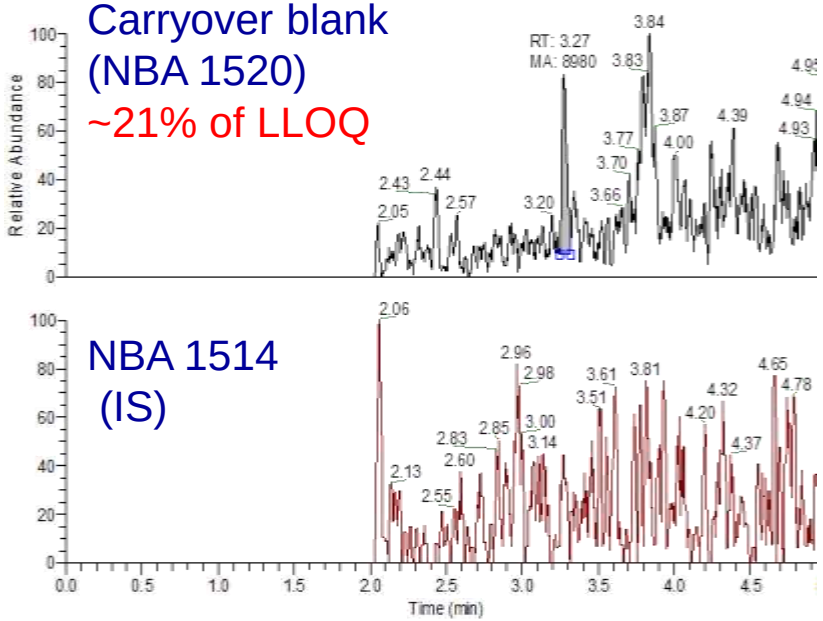
MS: ***Q-Exactive Plus***
Ionization Mode: ***Negative***
Resolution: ***70K***
t-SIM:
Analyte: 2123.8610-2131.8610
IS: 1473.8065-1481.8065

Target-SIM Analysis of Extracted 20-mer PS-ODN (HuPL) on Q-Exactive Plus (2/3)



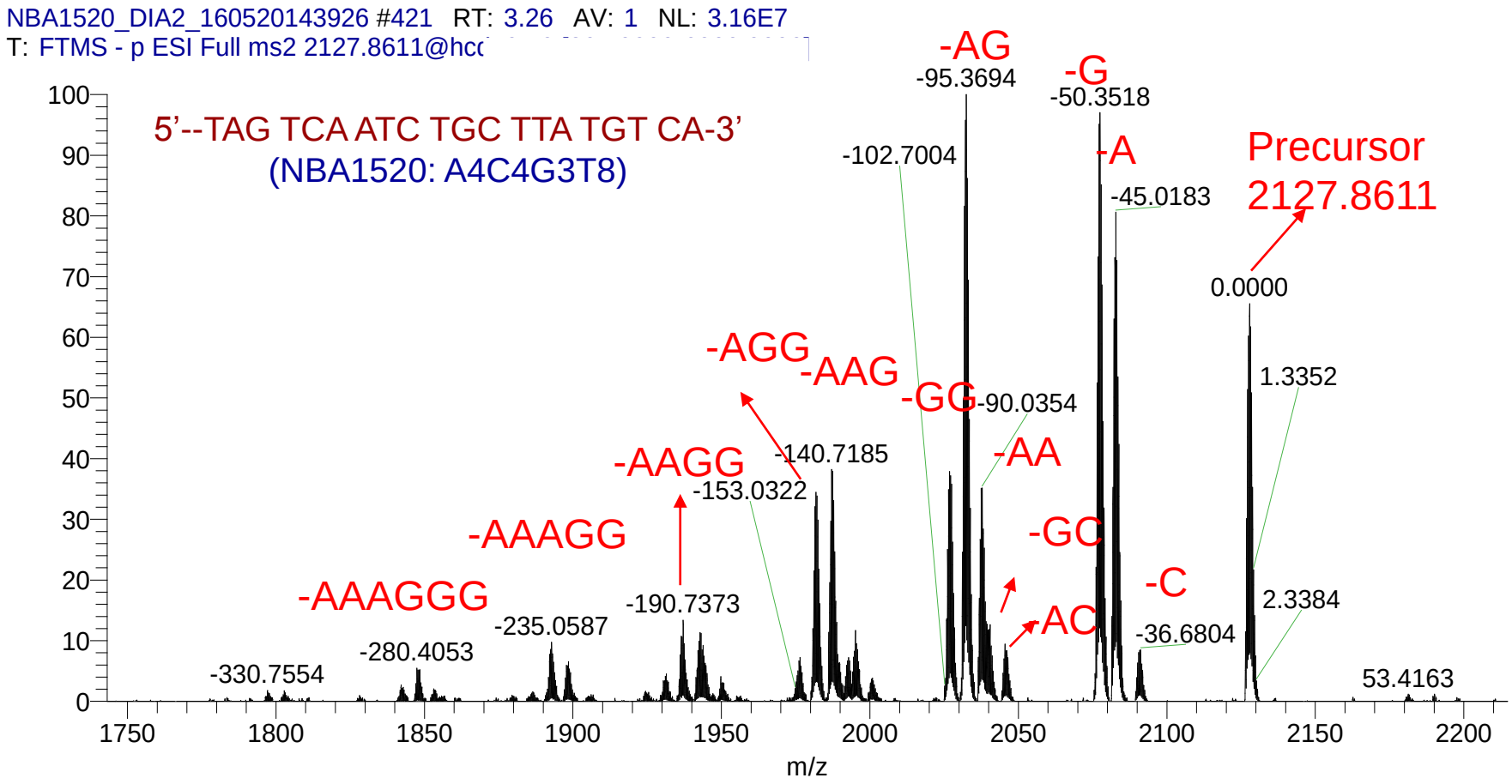
Target-SIM Analysis of Extracted 20-mer PS-ODN (HuPL) on Q-Exactive Plus (3/3)



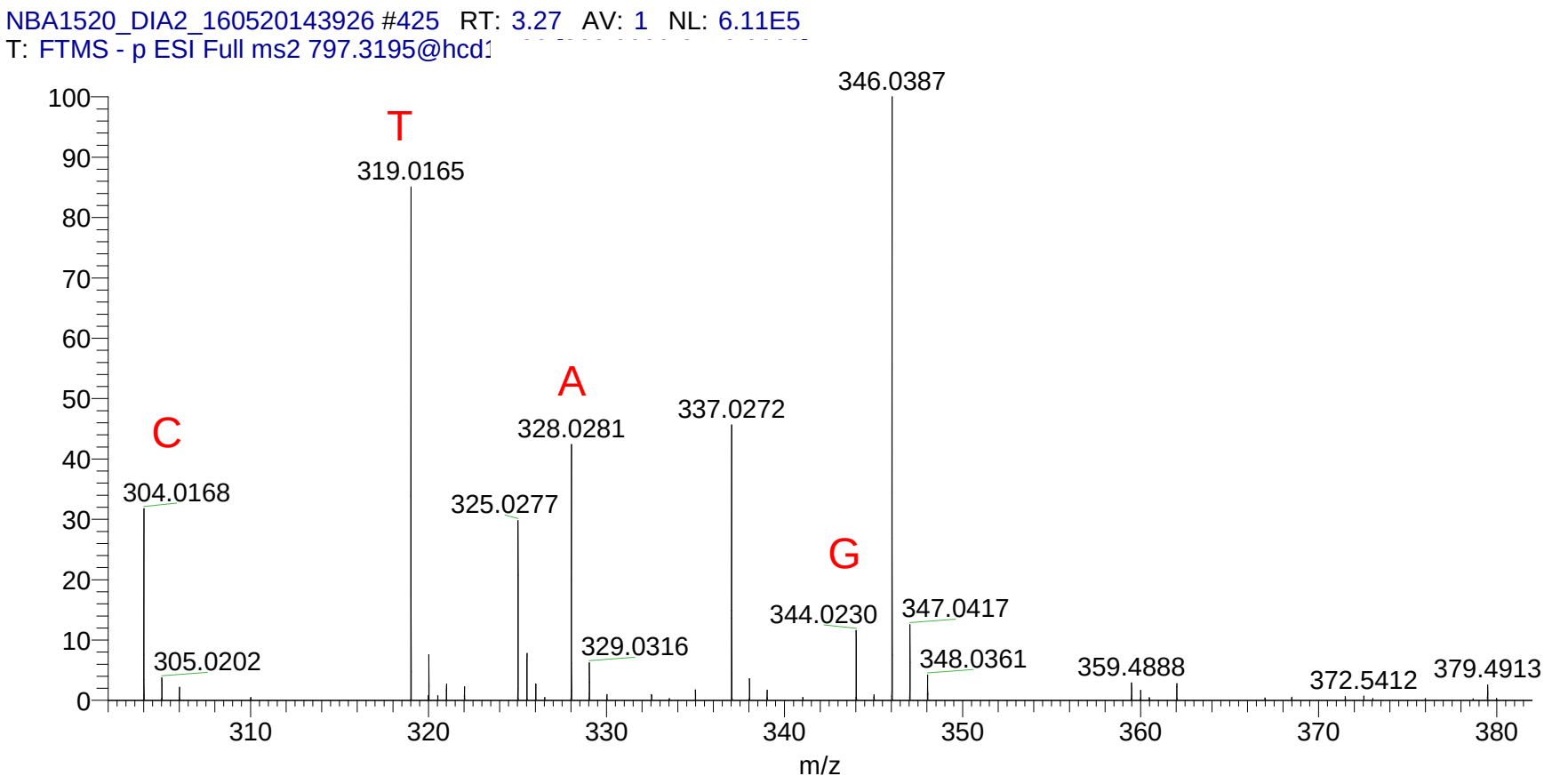


	Nominal C. (ng/mL)	Average C. (ng/mL)	% Bias	CV%
High QC	400.	395	-1.25%	4.51%
Medium QC	200.	193.	-3.50%	5.29%
Low-Medium	15.0	14.2	-5.33%	7.10%
Low QC	1.50	1.41	-6.00%	4.48%

Fragmentations of PS-ODN on Q-Exactive for PRM/DIA Analysis Plus (1/2)



Fragmentations of PS-ODN on Q-Exactive for PRM/DIA Analysis Plus (2/2)



SPE Extraction of the 20-mer PS-ODN From Human Plasma

Extraction SPE Plate: 96-Well WAX SPE plate, 30 mg,

Sample Aliquot: *200 µL Human Plasma*

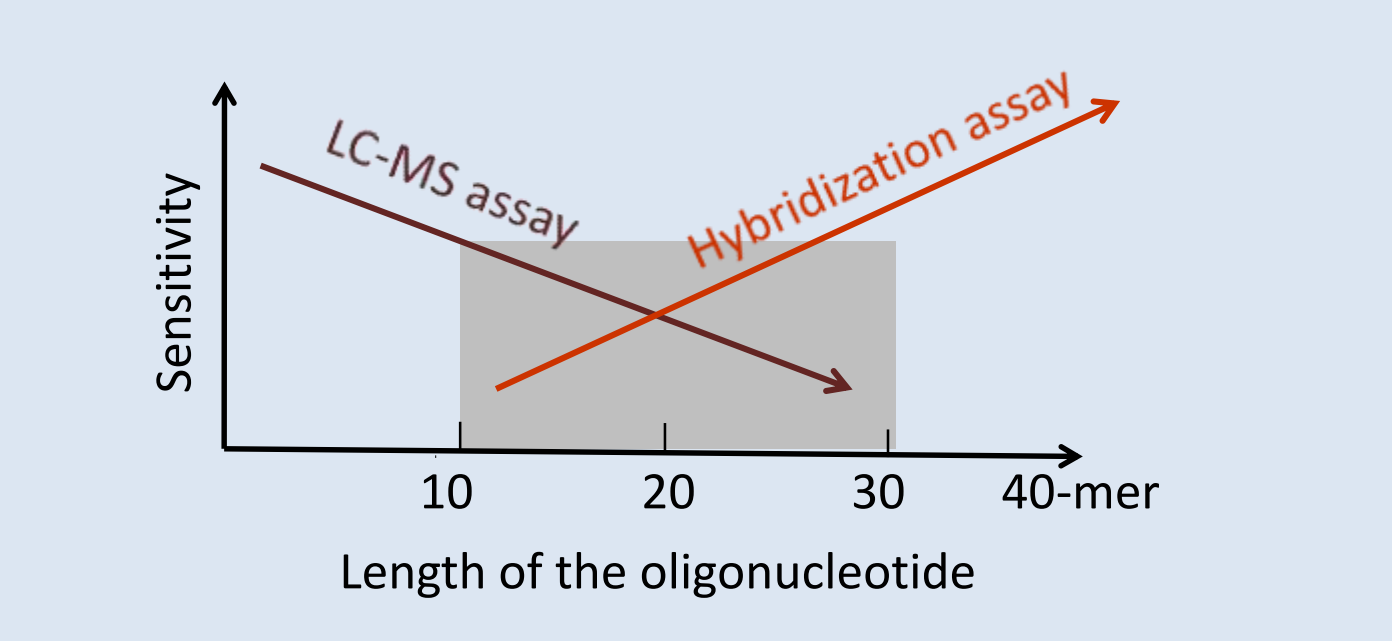
Extraction Procedure:

1) Aliquot 200 µl sample
   a) Add 50 µl IS to each well
   b) Add 500 µl loading buffer
   c) Vertex briefly and spin down
2) Load the samples onto the SPE plate
   (Pre-equilibrated with 1 mL MeOH and 1 mL loading buffer)
3) Wash the plate with 1 ml loading buffer and 1 mL of washing buffer
4) Heat sample plate for 1 hr incubation at 70°C
5) Elute the sample with 250-500 µl elution buffer (contains 10% methanol)
6) Inject 10 µl for LC-MS analysis

LC-MS/MS vs LC-HRAM

- Q1 mass spectra of oligos have different charge distributions on different instrument platforms
- LC-HRAM on Q-Exactive can achieve comparable sensitivity as LC-MS/MS on triple quads for some analytes.
- LC-HRAM can be performed without any prior knowledge of the compound, thus avoiding the need for compound-specific tuning (setting up the SRM transition). Therefore, it is very convenient for monitoring unknown metabolite information or estimate the known metabolite concentration(s) without standard reference material while quantifying the parent compounds.

Size Maters for The Assay Sensitivities



- LC-MS/MS (HRAM) assays are a good choice for small oligonucleotides;
- Hybridization-based assays are advantageous for large oligonucleotides.

THANK YOU