Mastering the art of Oligonucleotide Analysis via LCMS using the HALO 1000Å OLIGO C18 HPLC column

Joshua McBee, Ph.D, Barry Boyes, Ph.D., Peter Pellegrinelli, M.S.

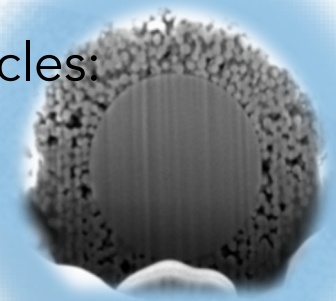
HALO®

innovated by

advancedmaterialstechnology

Background of AMT

- Founded in 2005 by Tim Langlois, Joe DeStefano
- The first company to commercially manufacture the <3 µm superficially porous particles:
  - *Fused-Core® Technology, "SPP" or HALO®*



Located: Wilmington, Delaware

Facility:

Fully equipped state of the art laboratories

All operations handled in Wilmington, DE

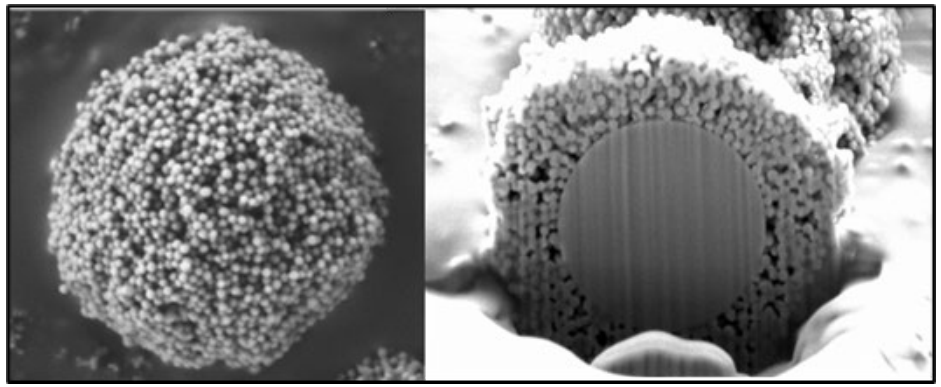
- Vertically integrated
- ISO 9001 QMS certified company

Sold & Distributed via:

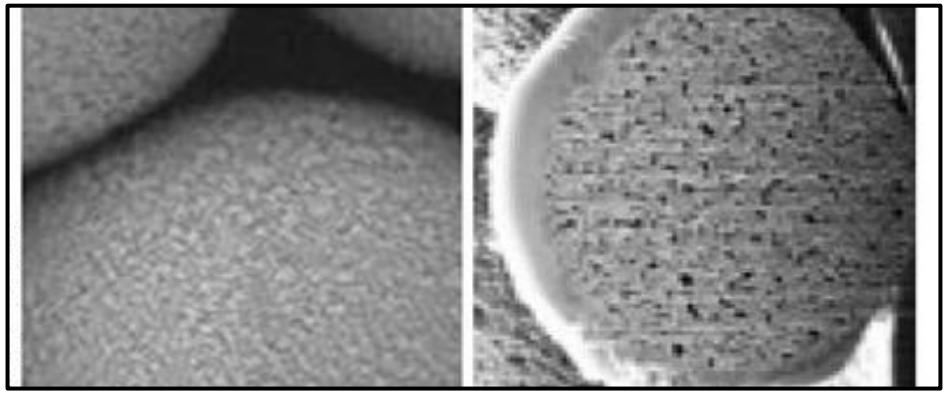
- US - Direct through AMT or 3rd party: Fisher/VWR
- Internationally- via our Channel Partners
  - individual by country

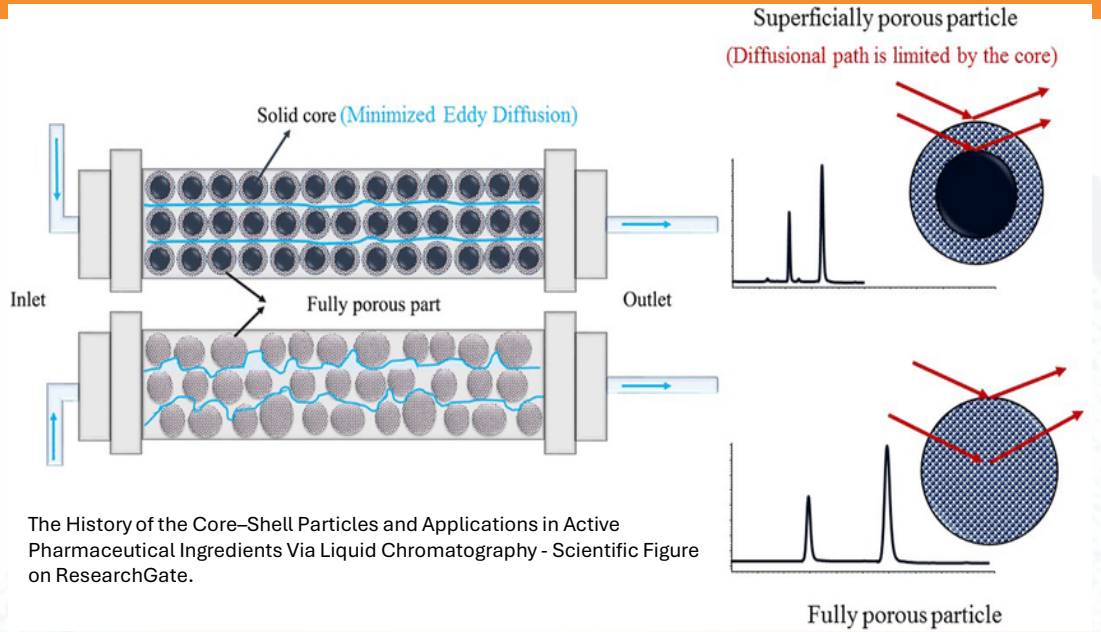
HALO® vs. FPP

Superficially Porous Particle (SPP)



Fully Porous Particle (FPP)



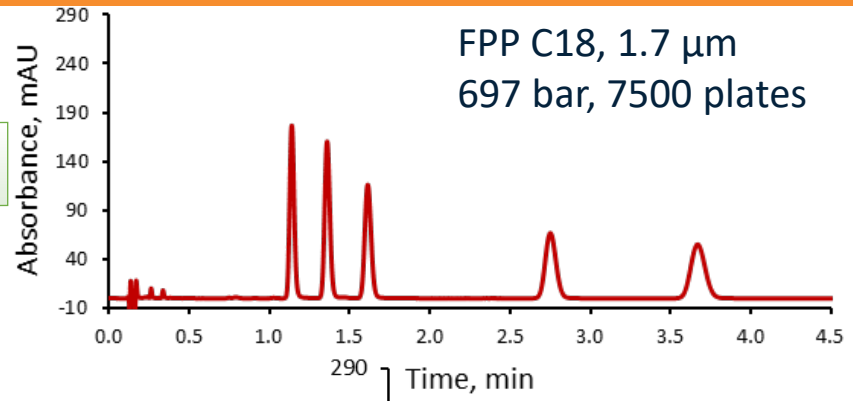


The History of the Core–Shell Particles and Applications in Active Pharmaceutical Ingredients Via Liquid Chromatography - Scientific Figure on ResearchGate.

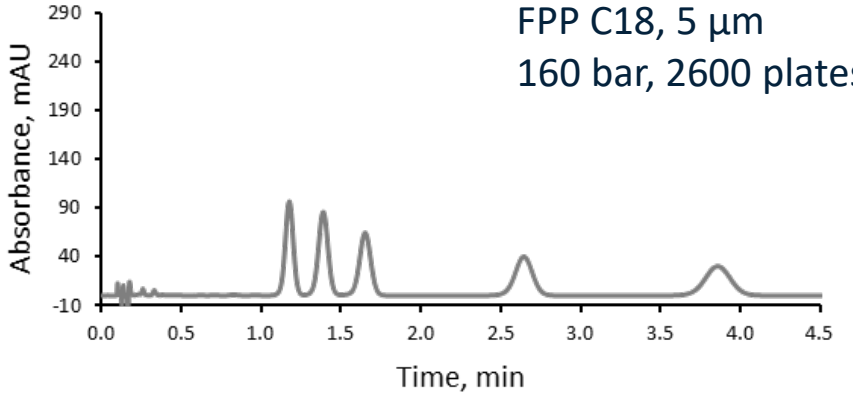
- Higher Efficiency/ Peak Capacities: HALO® provides a **30-50% increase in efficiency over FPPs** of the same particle size
- Faster Analysis Times: Due to enhanced mass transfer
- Great Performance at Lower Back Pressures: HALO® allows for performance similar to smaller FPPs but at significantly lower back pressures

ADVANTAGES of HALO® vs FPP

FPP C18, 1.7 µm
697 bar, 7500 plates



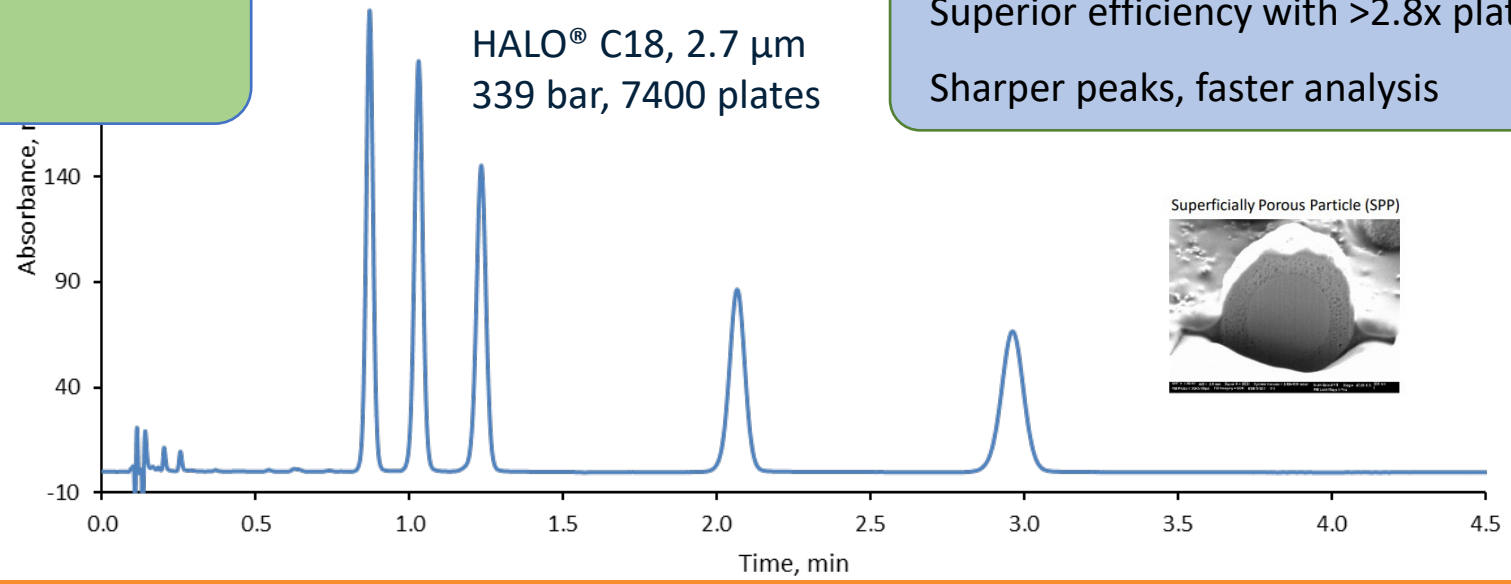
FPP C18, 5 µm
160 bar, 2600 plates



High performance with <1/2 the back pressure

Faster analysis

HALO® C18, 2.7 µm
339 bar, 7400 plates



Superior efficiency with >2.8x plates!

Sharper peaks, faster analysis

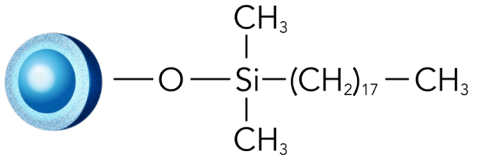
Superficially Porous Particle (SPP)

HALO®

BIOCLASS

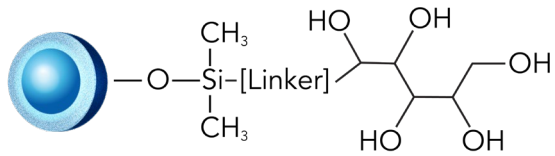
OLIGO

OLIGO C18



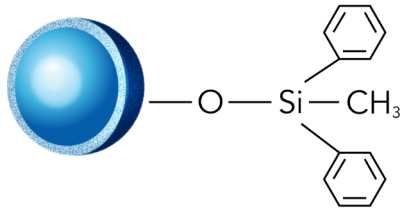
GLYCAN

GLYCAN

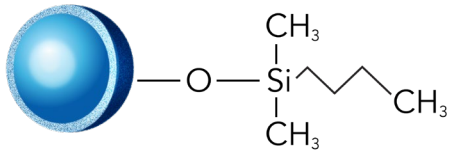


PROTEIN

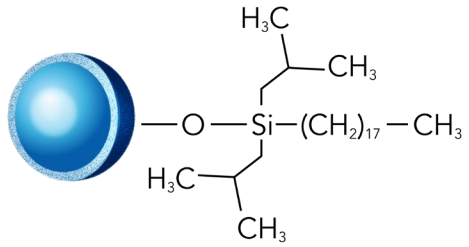
DIPHENYL



C4

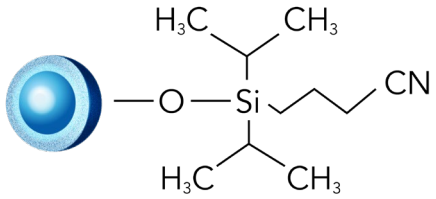


ES-C18

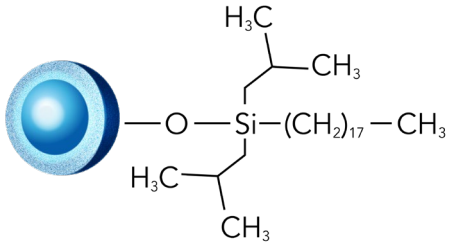


PEPTIDE

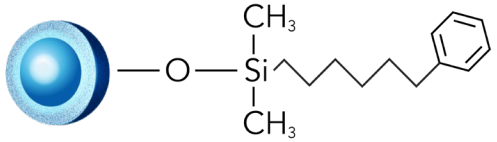
ES-CN



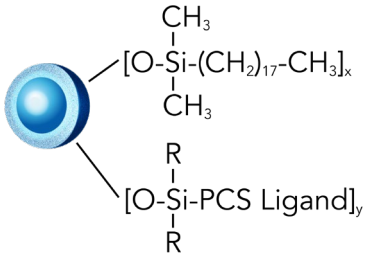
ES-C18



PHENYL-HEXYL

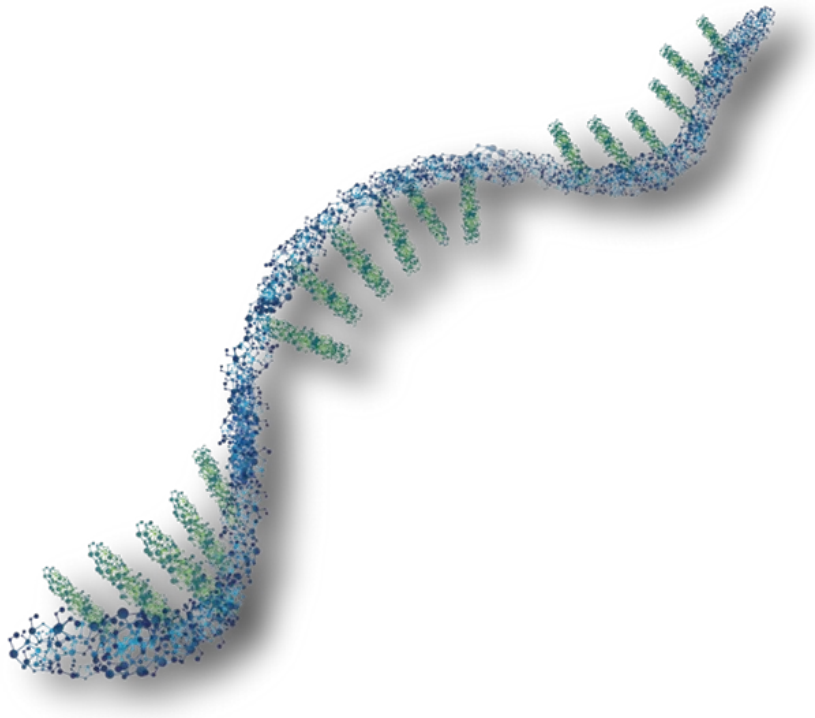


PCS C18



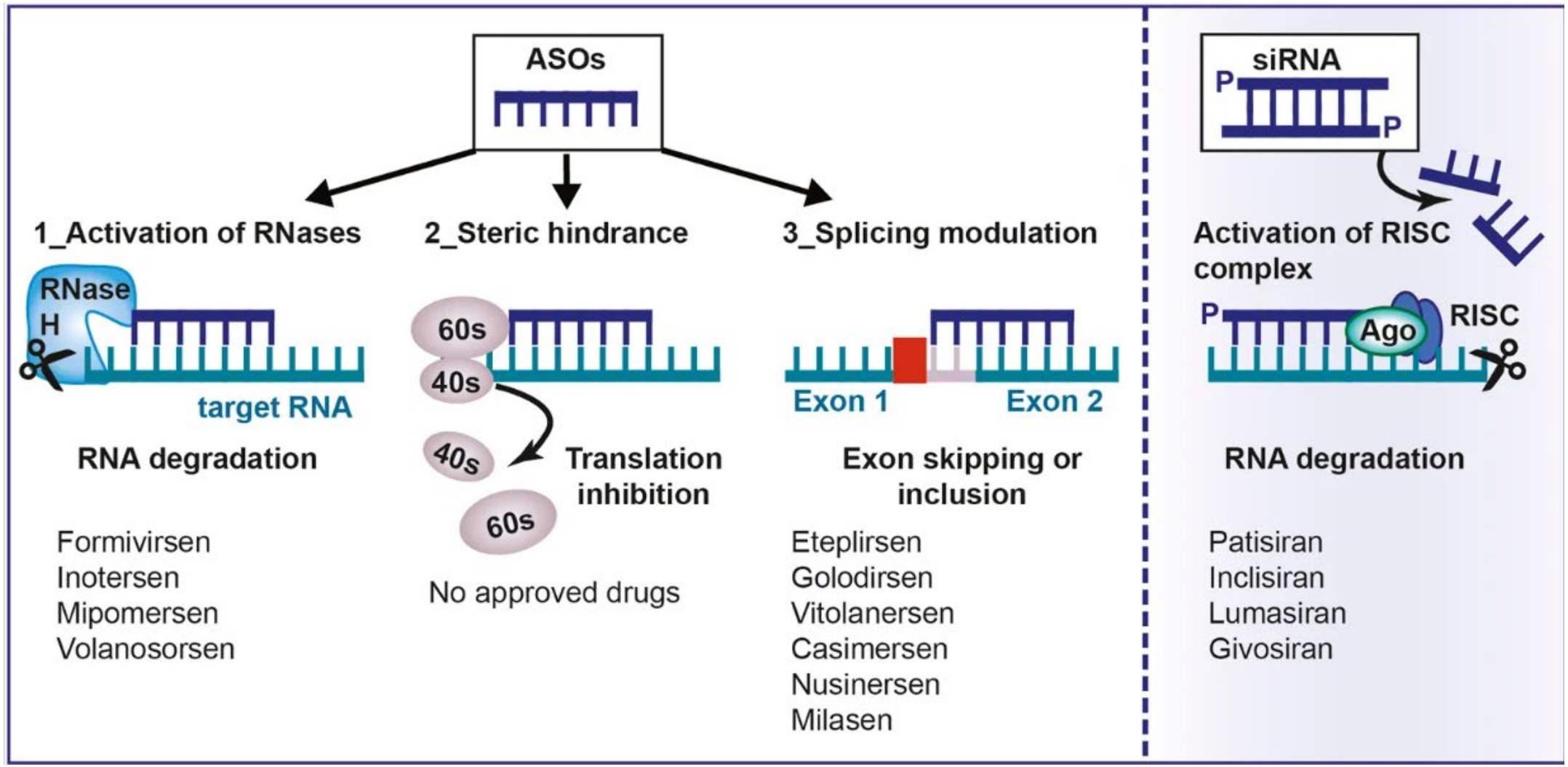
Overview

- How is Oligonucleotide RP LC different?
- The resurgence of oligonucleotides.
- Modifications currently in use in therapeutics.
- What makes Oligonucleotide RP LC so challenging.
- Methodological Approaches to Oligonucleotide HPLC.
- Considerations for MS analysis of Oligonucleotides.
  - Minimizing Adduction.
- Application Examples.



Oligonucleotide Therapeutics

"Short" Oligonucleotides (< 25 bases)



Collotta D, Bertocchi I, Chiapello E and Collino M (2023) Antisense oligonucleotides: a novel Frontier in pharmacological strategy. Front. Pharmacol. 14:1304342. doi: 10.3389/fphar.2023.1304342

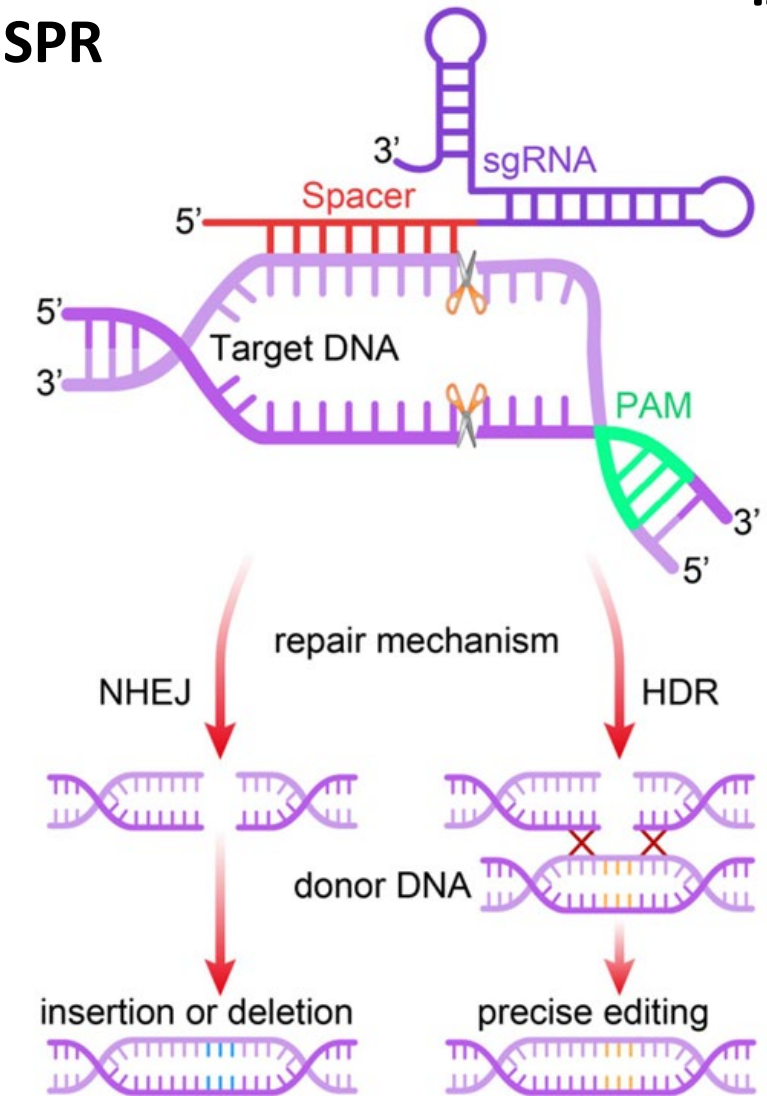
Oligonucleotide Therapeutics

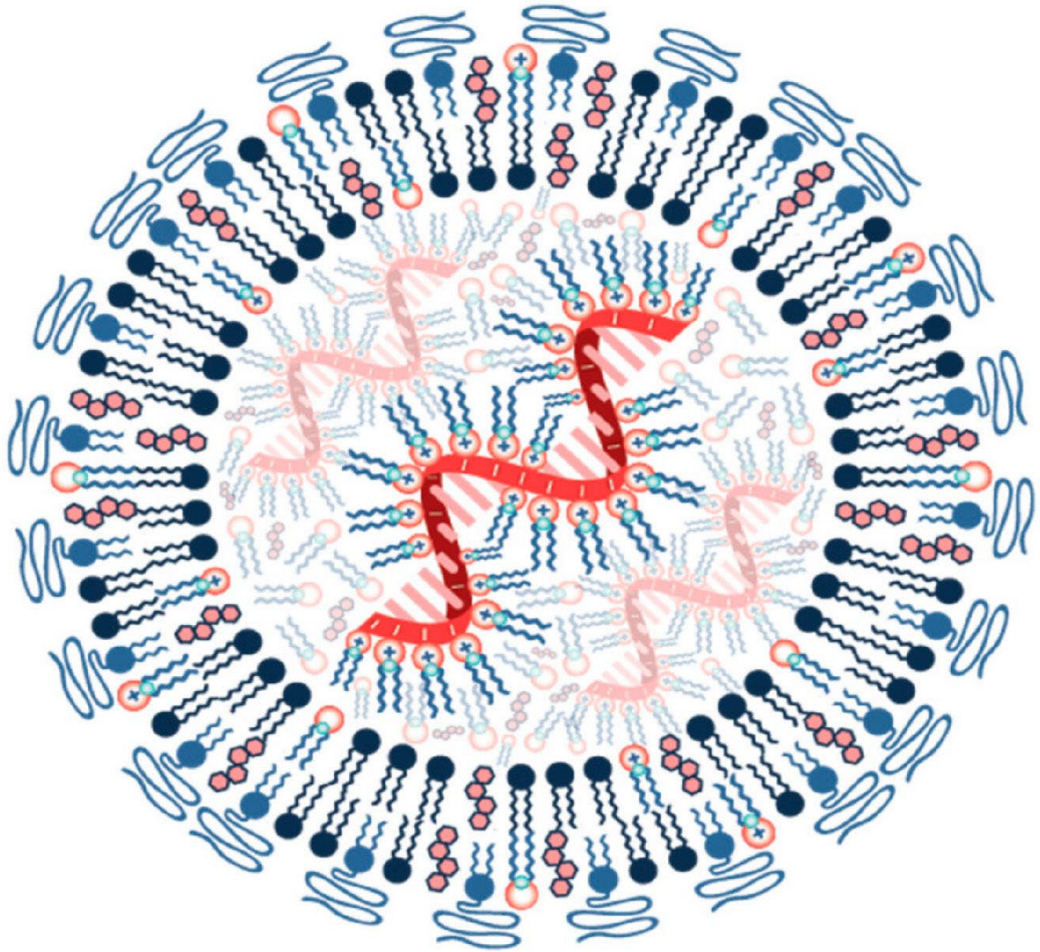
HALO

CRISPR

"long" Oligonucleotides

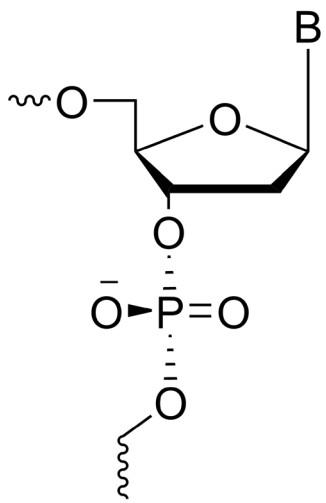
LNP mRNA



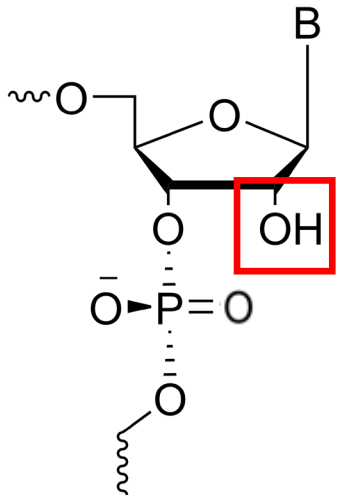


Zhang *et al,* **Mol. Cancer (2021)20:126**

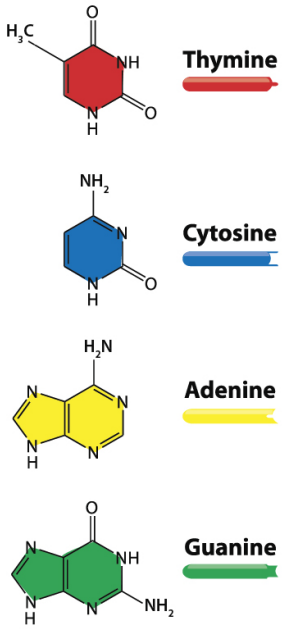
Oligonucleotide Fundamentals



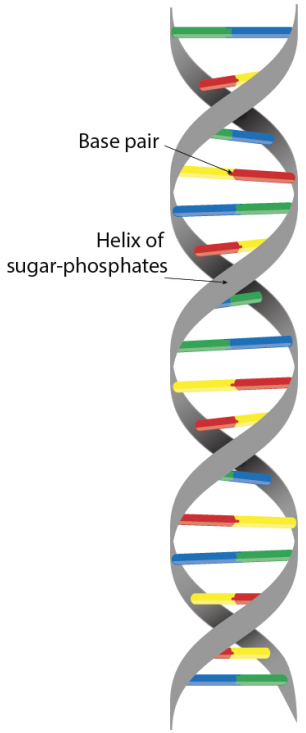
DNA



RNA

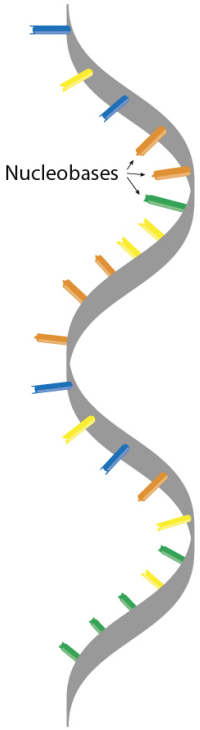


Nucleobases of DNA



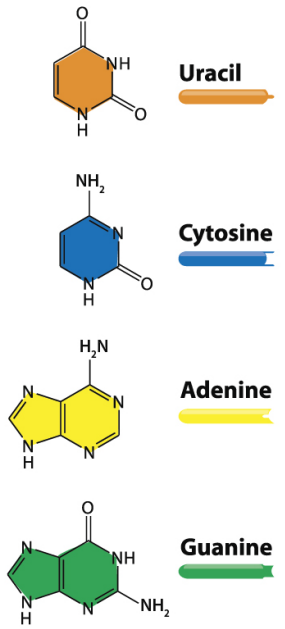
DNA
Deoxyribonucleic acid

A = T
G ≡ C



RNA
Ribonucleic Acid

A = U
G ≡ C



Nucleobases of RNA

Technical Considerations for Oligonucleotide Separations

- **Oligonucleotide Structural Forms**

  Oligonucleotides exist as single strands or duplexes with secondary structures affecting separation.

- **Chemical Modifications**

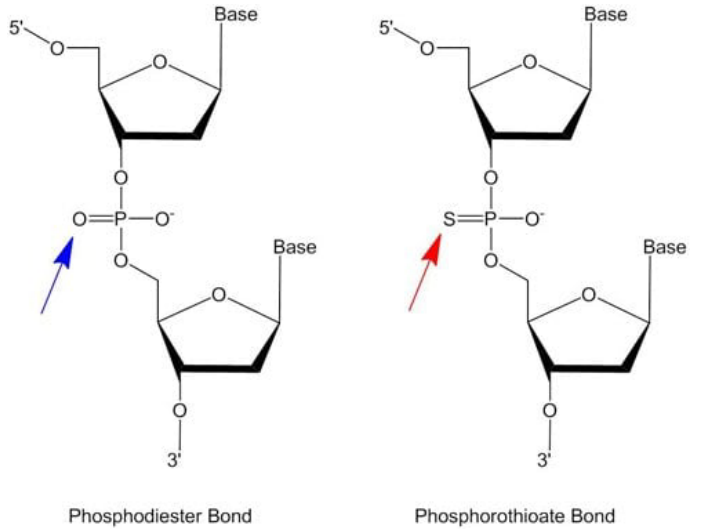
  Phosphorothioate and 2′-O-methyl modifications alter oligonucleotide stability and chromatographic properties.

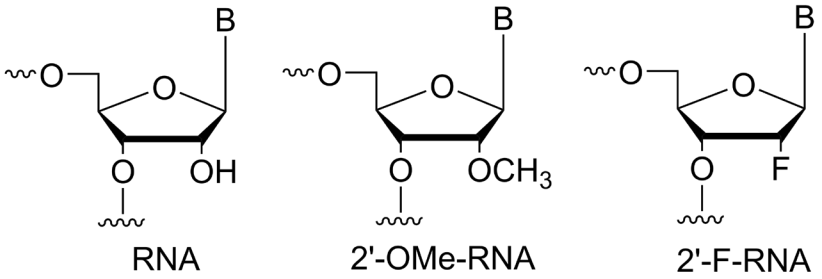
- **Conjugates in Oligonucleotides**

  Incorporating lipids or peptides enhances delivery but adds complexity to chromatographic analysis.

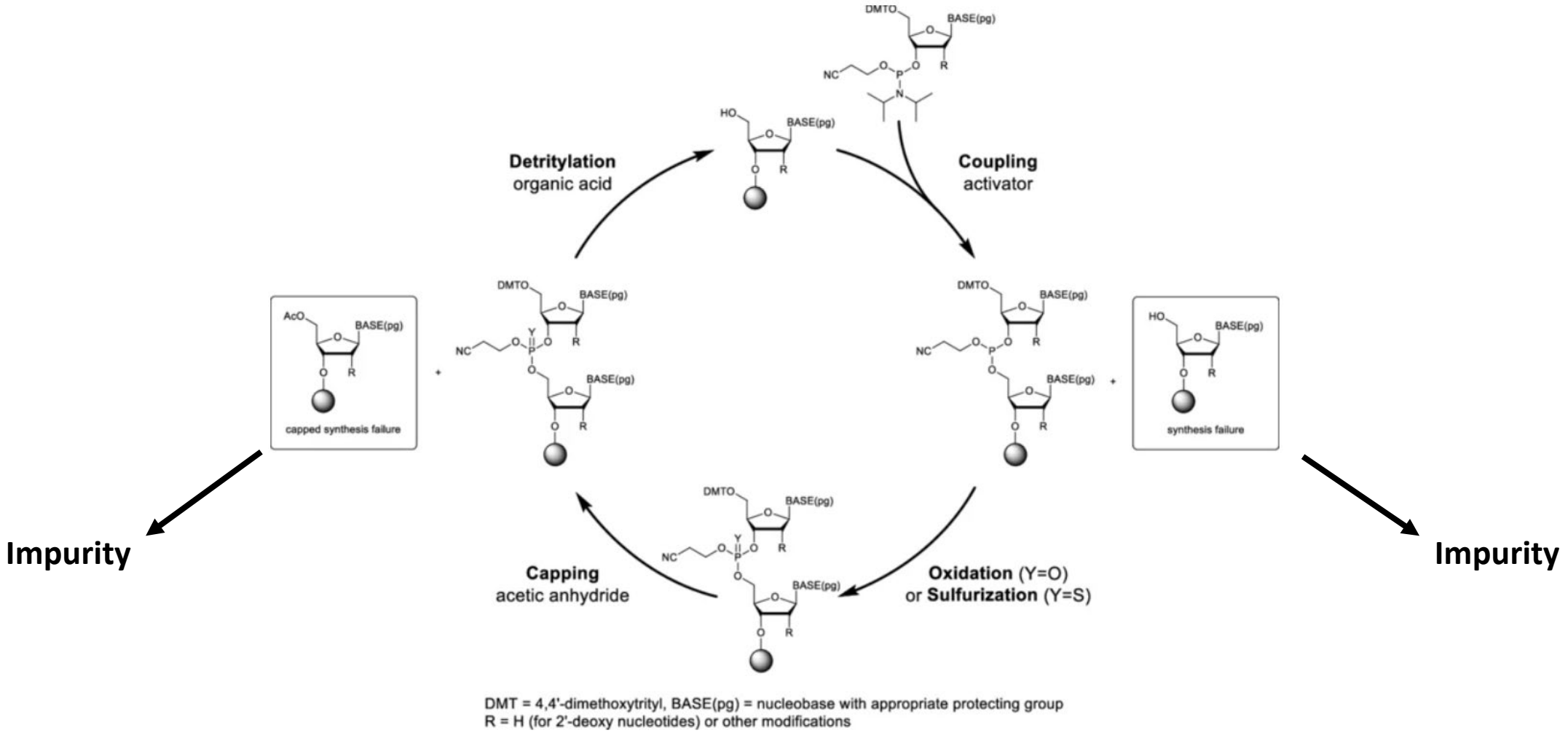
- **Chromatographic Challenges**

  Modifications create positional isomers and affect retention, requiring tailored chromatography strategies.





Solid Phase Oligo Synthesis



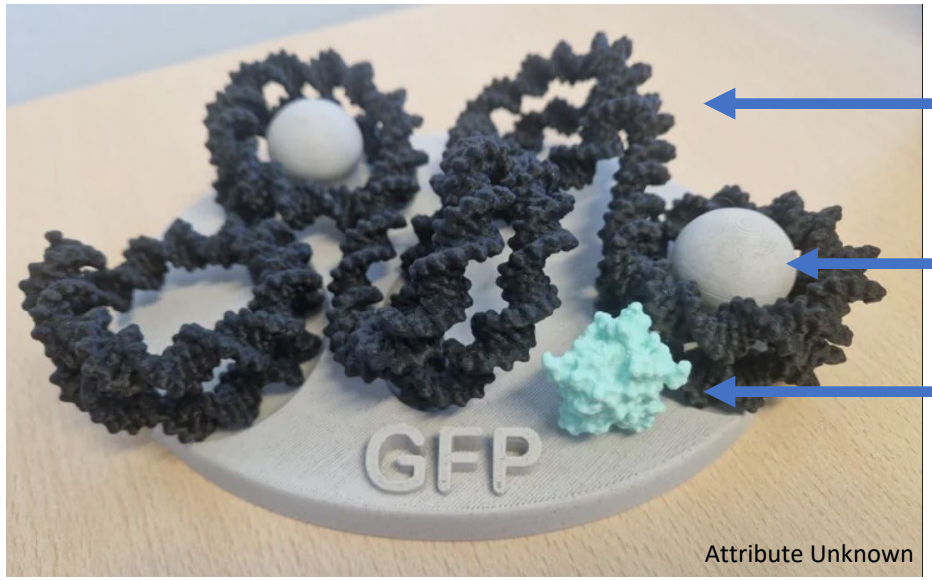
Andrews, Benjamin & Antia, Firoz & Brueggemeier, Shawn & Diorazio, Louis & Koenig, Stefan & Kopach, Michael & Lee, Heewon & Olbrich, Martin & Watson, Anna. (2020). Sustainability Challenges and Opportunities in Oligonucleotide Manufacturing. The Journal of Organic Chemistry. 86. 10.1021/acs.joc.0c02291.

Macromolecule Size Comparisons

Argonaute protein from *Aquifex aeolicus*

- **Argonaute protein 706AA, ~83kDa**
- **siRNA 22bp duplex, ~13.3kDa**

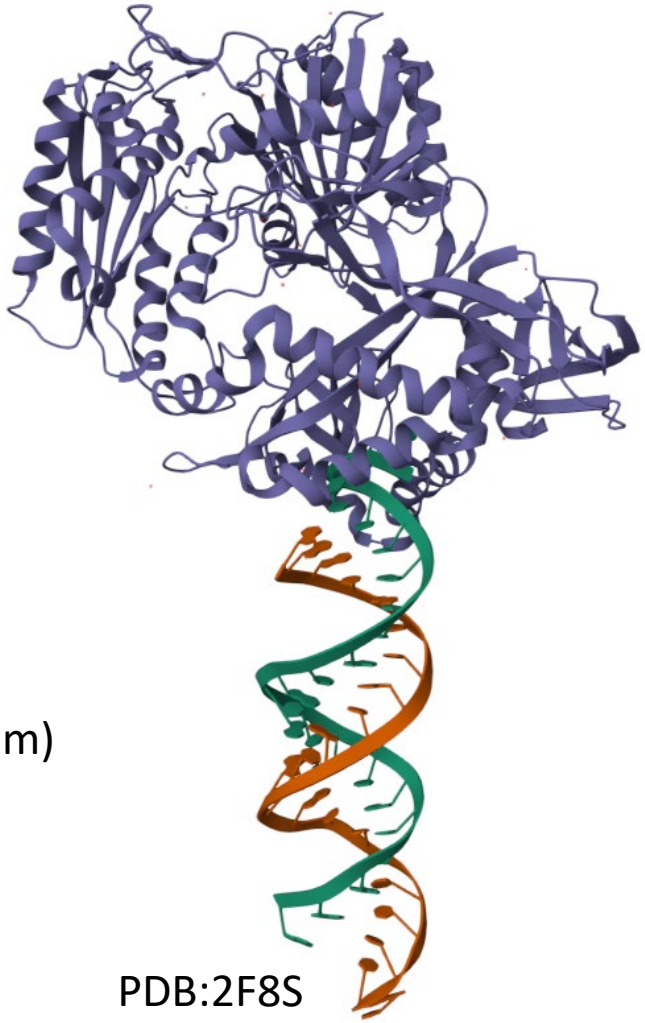
- **siRNA ~40% volume of Ago protein**



DNA

Histone/nucleosome (11nm)

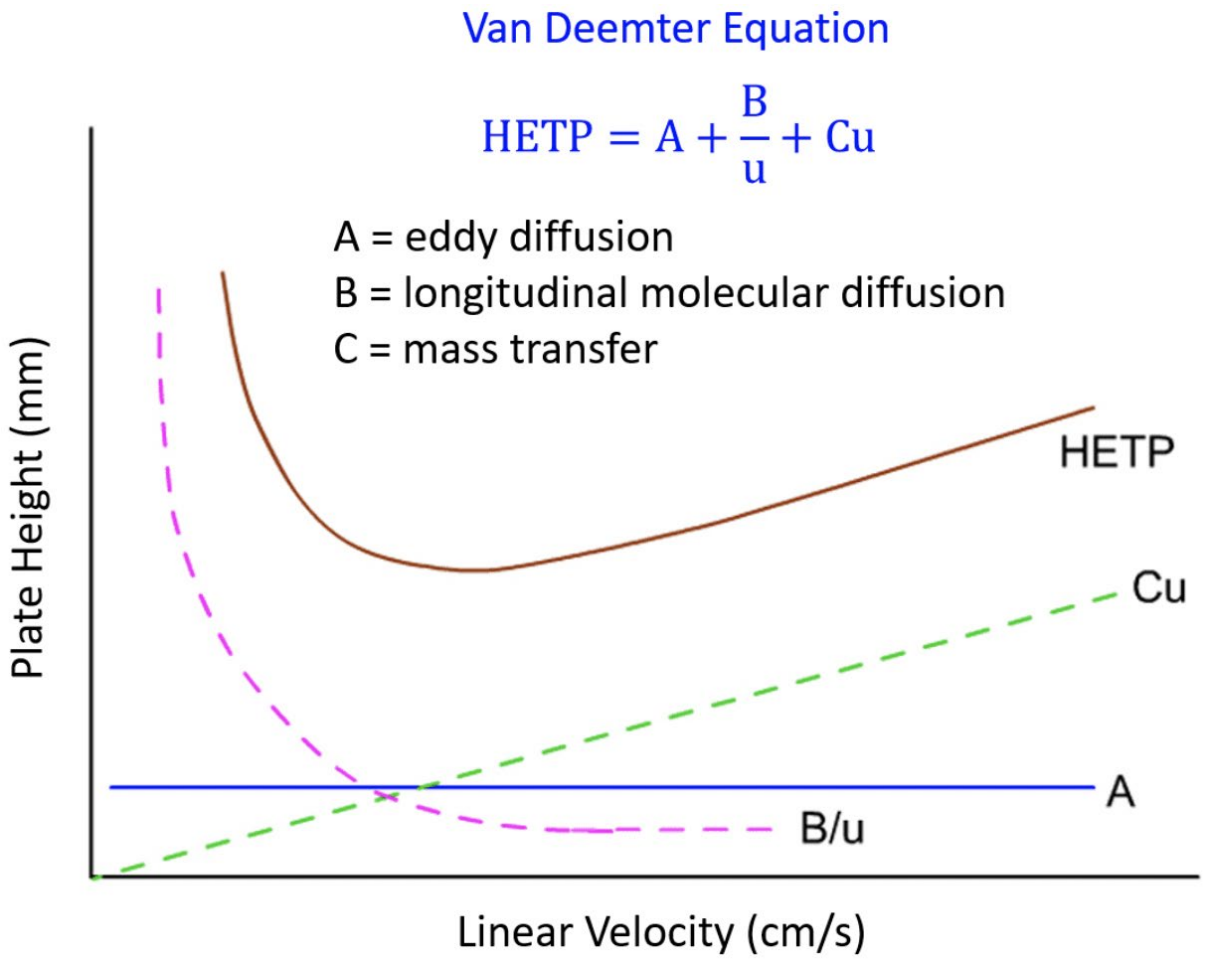
GFP (238AA; 27kDa)



PDB:2F8S

Chromatographic Fundamentals

Van Deemter Equation



Where:

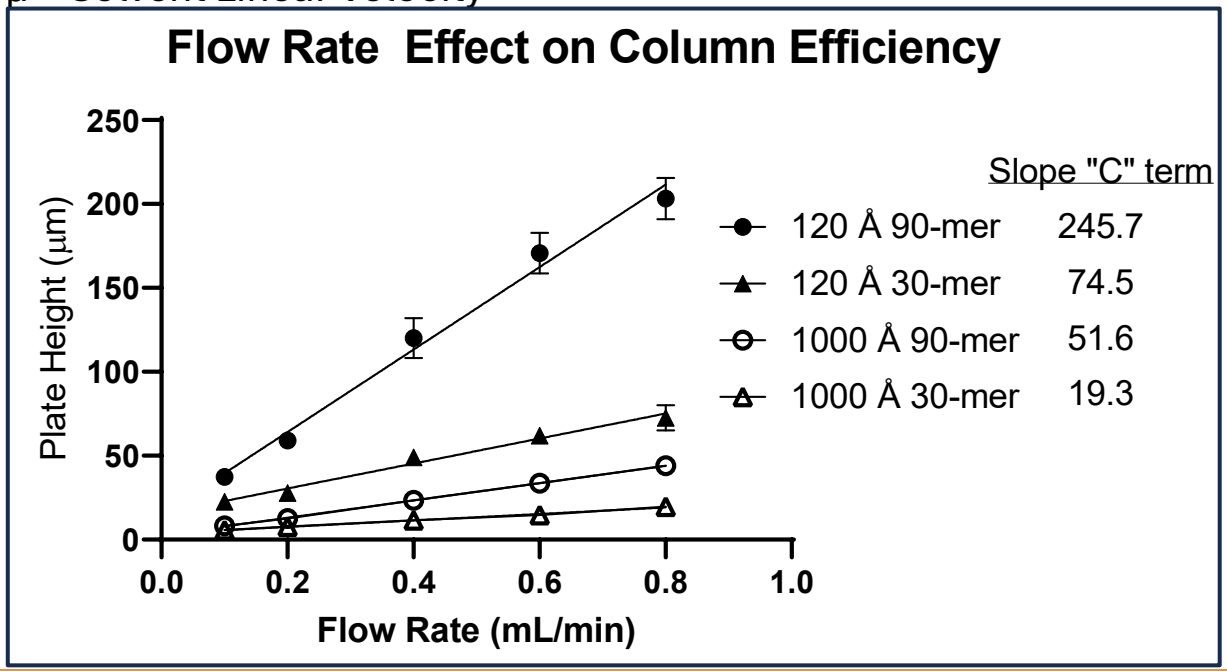
HETP = Height Equivalent to a Theoretical Plate

A = Eddy Diffusion Parameter (i.e. the solvent path around the silica particle packing)

B = Longitudinal Diffusion (Analyte dispersion along the axial path of the column.)

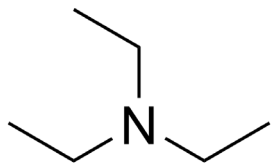
C = Mass Transfer Coefficient

μ = Solvent Linear Velocity

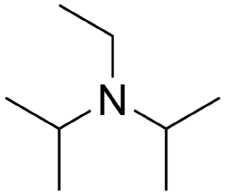


Ion Pair / Reversed Phase Chromatography

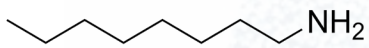
- **Oligonucleotides are very hydrophilic!**
- Ion Pair acts as a bridge
  - Neutral to high-pH range (pH 7-10)
- Alkylamines most common ion pair



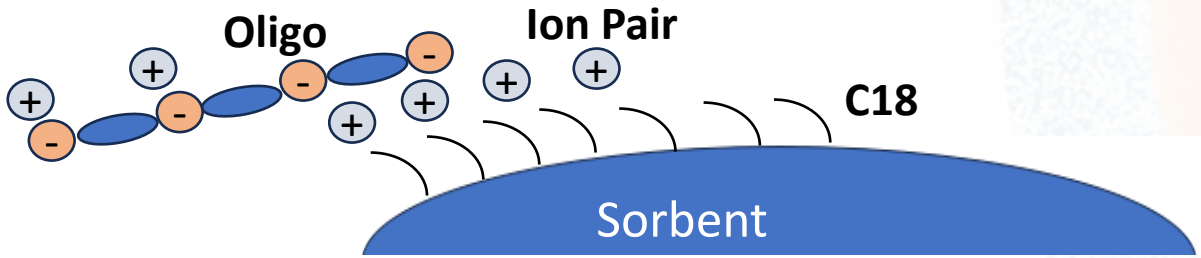
Triethylamine (TEA)
LogP = 1.65

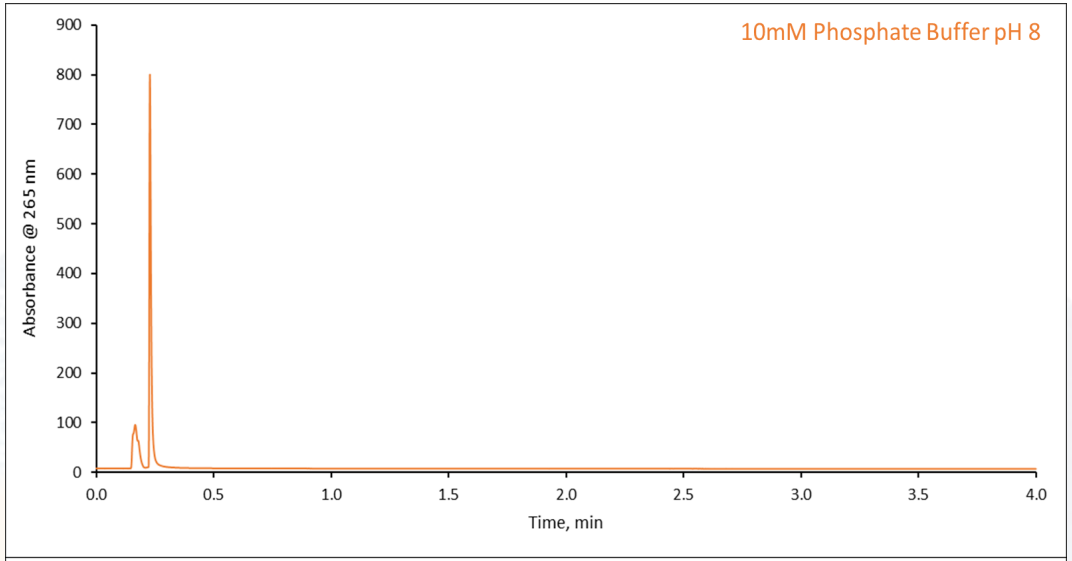


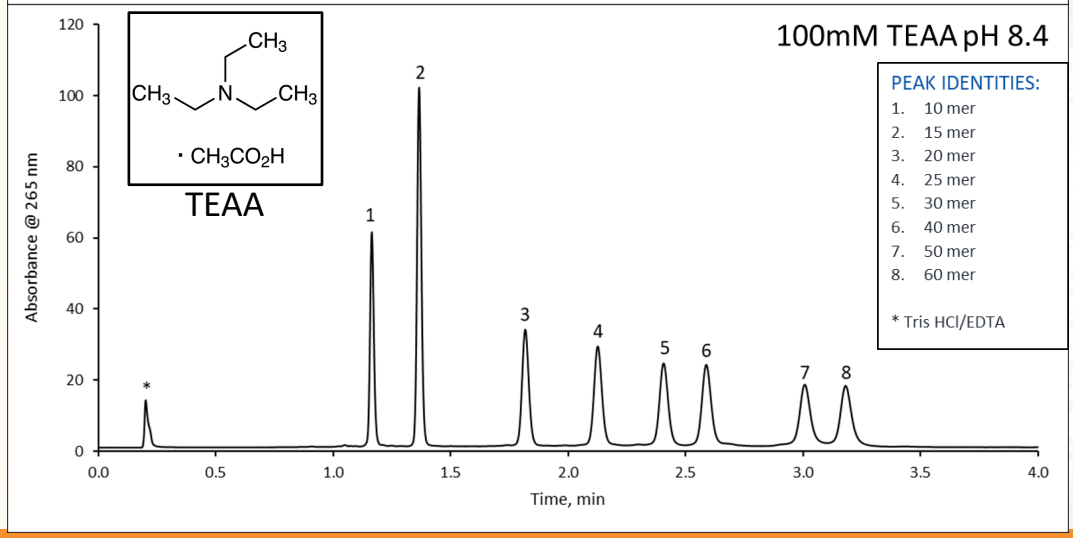
Diisopropylethylamine (DiPEA)
LogP = 2.2



OctylAmine
LogP = 3.1

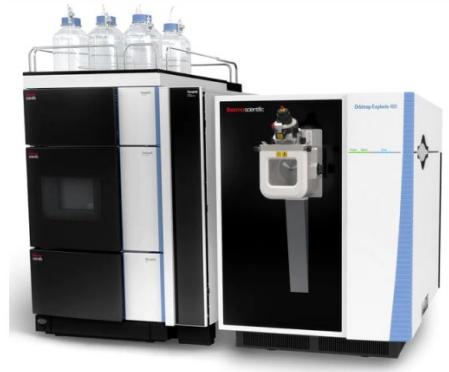




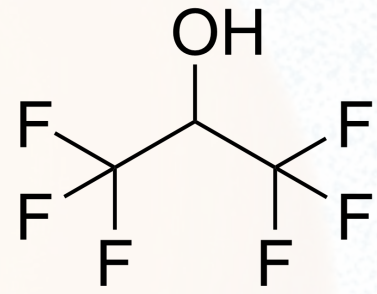


Important Considerations for IP/RP

Oligo Analysis by MS



- For IP/RP MS analysis is performed in negative polarity
- Typical anions used in MS
  - Acetate
  - Formate
  - Carboxylic acids



Hexafluoroisopropanol (HFIP)

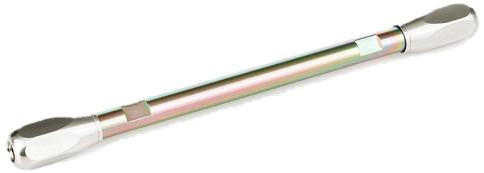
pKa ~9.3

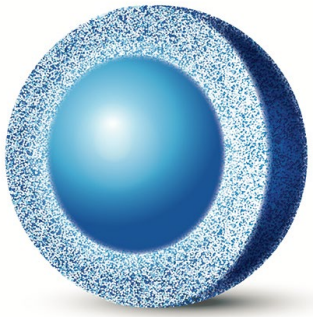
- Requires higher pKa which wont dominate cause ion suppression
- HFIP is most common counterion

HALO® ELEVATE Particle Technology

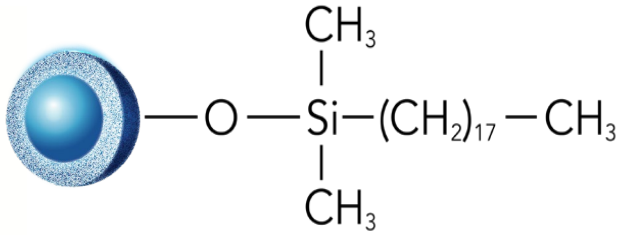
- ELEVATE C18 incorporates surface modified technology for alkaline resistance resulting in excellent stability in high pH/high temperature environments.
- With a wide operational use range of pH 2-12, HALO® ELEVATE allows improved separations for basic compounds that may present problems at lower pH, such as poor peak shapes, inadequate retention, or limited load tolerance.
- Ideal for use with high pH mobile phases, HALO® ELEVATE protects against degradation of the silica particle which is demonstrated by aggressive stability tests at elevated temperatures.
- Phases: C18 (C8 and Phenyl-Hexyl coming in 2026)

Introduction to HALO® OLIGO C18





120 Å



Fully Passivated Hardware

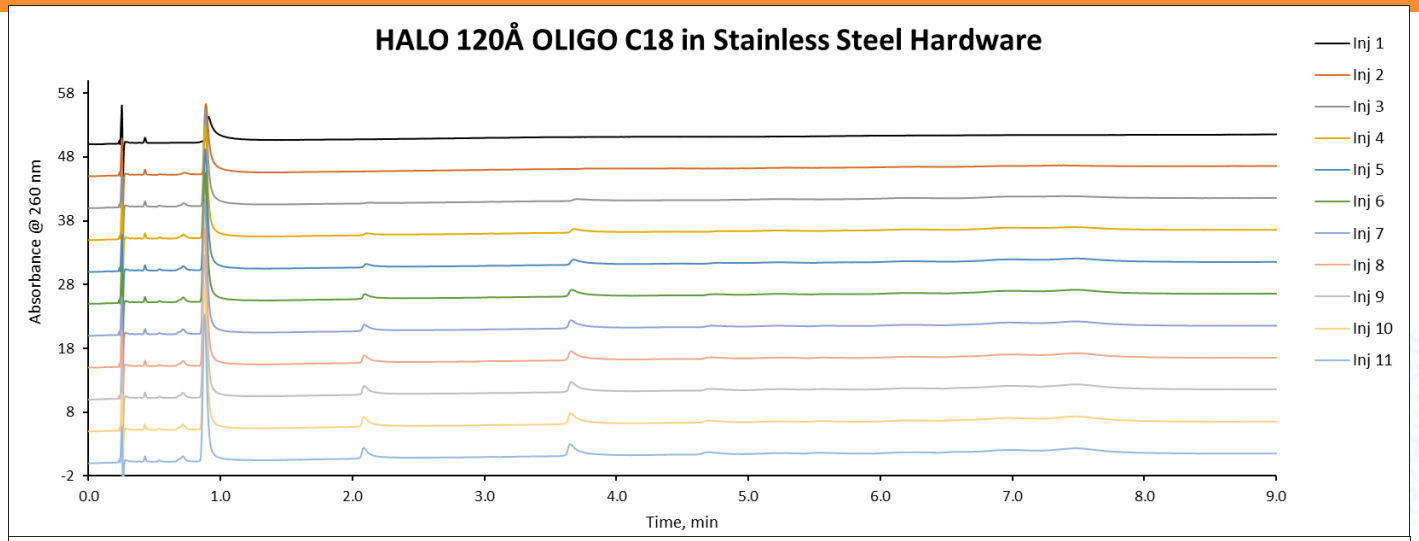
1000 Å

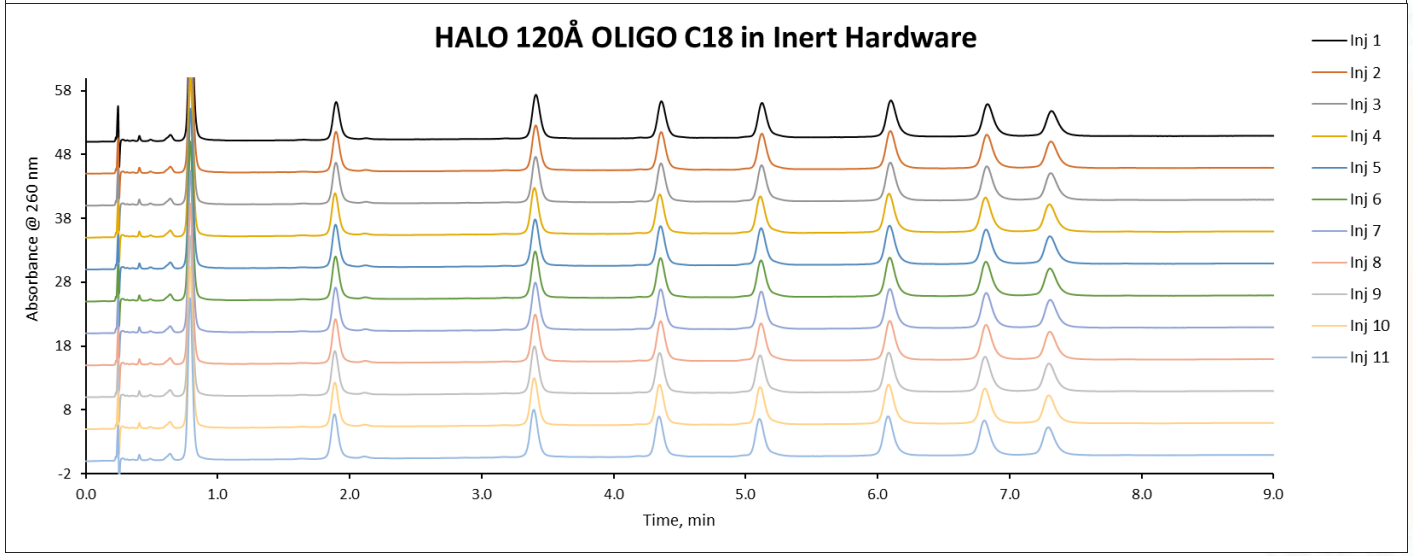
1000 Bar!

- Separations of Oligos up to 60 bases in length
- High pH and temperature stability, designed for conditions suited to oligo separations
- HPLC, UHPLC, MS compatible stationary phase
- Surface passivated hardware to reduce adsorption

- Separations of Oligos up to 100 bases in length and beyond
- Ideal for advanced applications like CRISPR and therapeutic development.
- Low Backpressure: Enables scalable workflows and faster analyses, even with longer columns.

Inert vs non-inert Hardware





Testing Conditions:

Mobile Phase: A: 25mM HAA @ pH 7.0
B: 50/50 25mM HAA/Acetonitrile

Gradient:

Time	%B
0.0	8
12.0	22
12.5	8
16.0	8

Flow Rate: 0.4 mL/min
Back Pressure: 130 bar
Temperature: 60 °C
Injection: 1 µL of 1:10 Dilution of 10/60 Ladder Standard (IDT)
Sample Solvent: RNAse free water
Wavelength: PDA, 260 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2

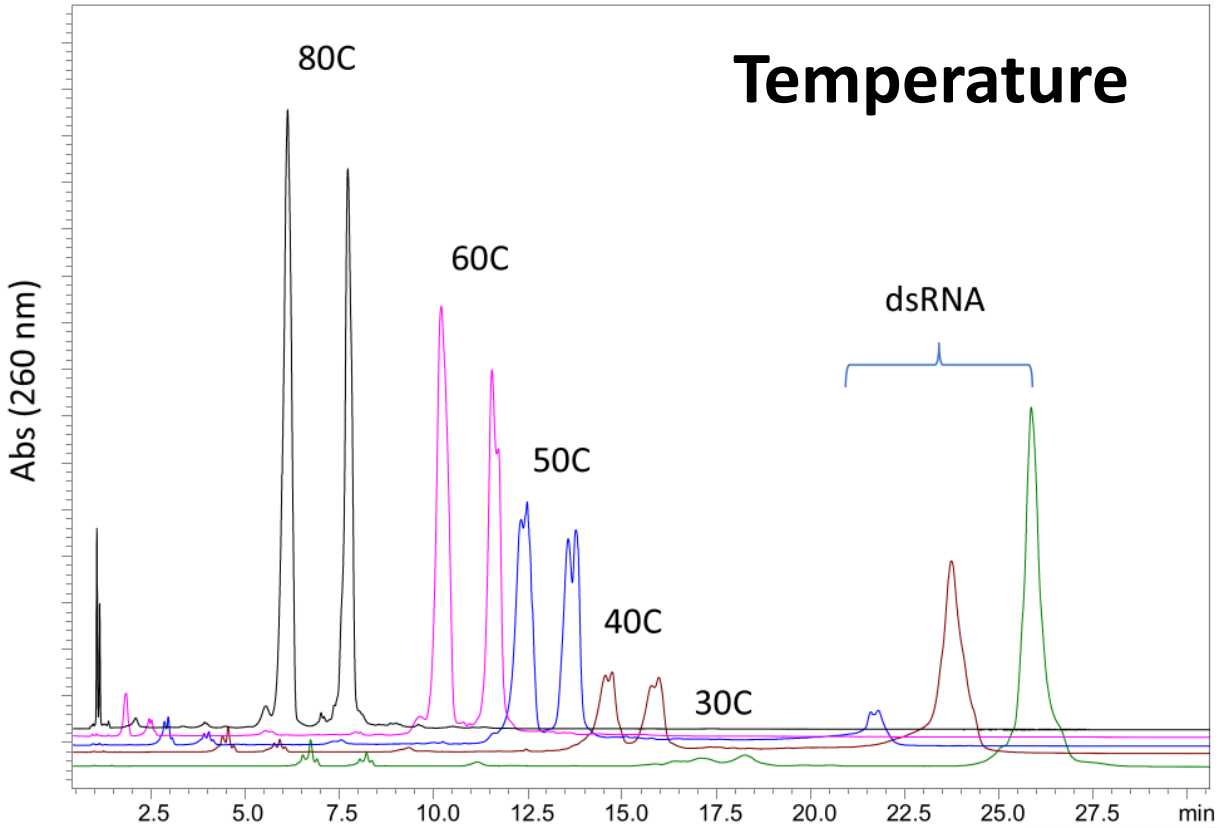
PEAK IDENTITIES:

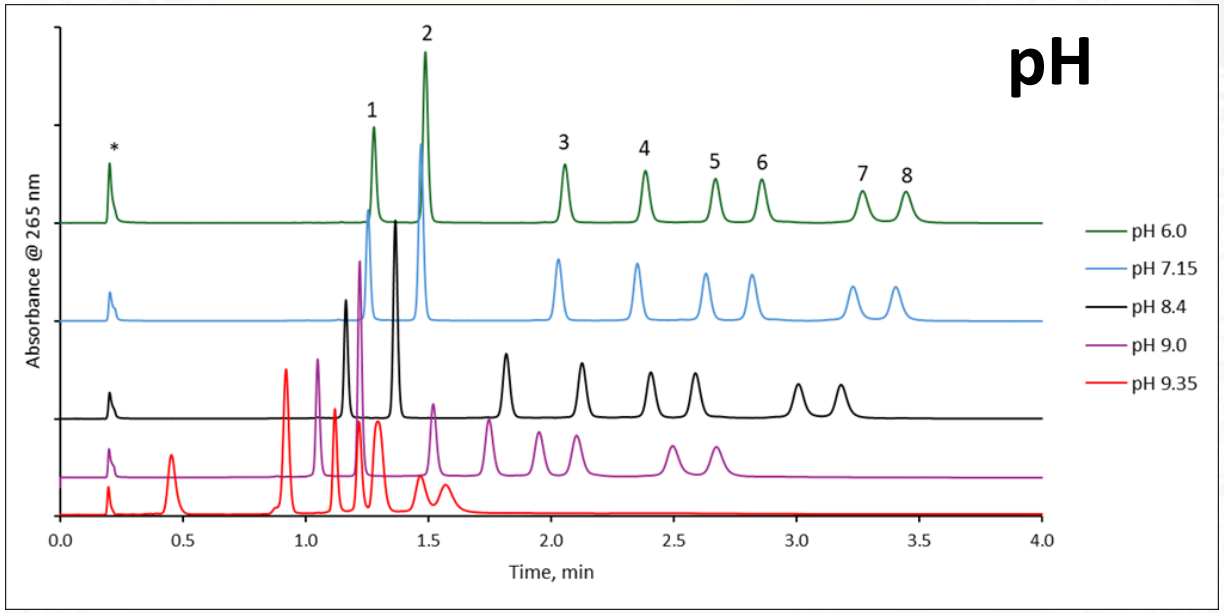
1. 10 mer
2. 15 mer
3. 20 mer
4. 25 mer
5. 30 mer
6. 40 mer
7. 50 mer
8. 60 mer

Building an Oligo LCMS Method

Building an Oligo LCMS Method

Parameters that can Impact your Separation via IP/RP





Building a Method

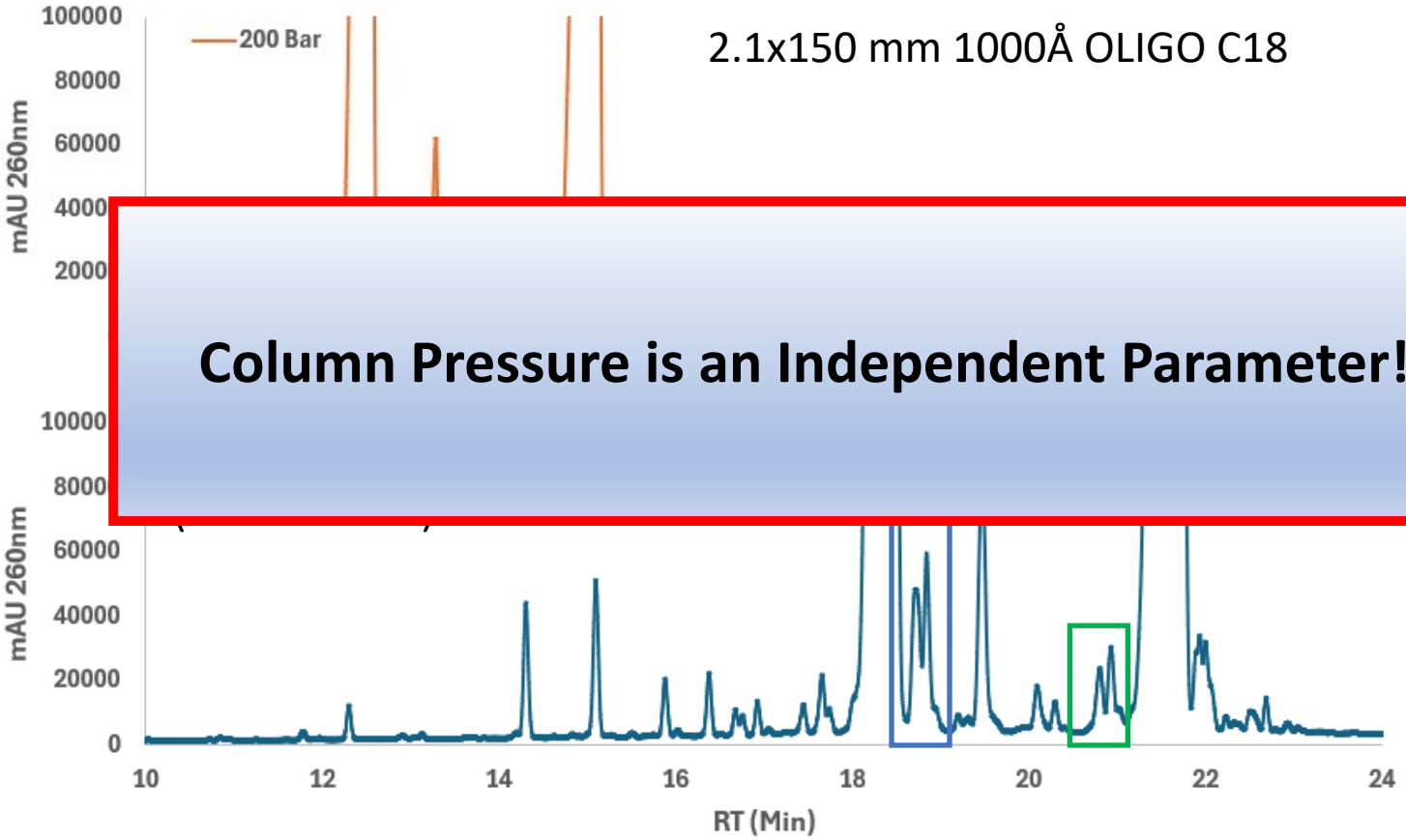
Ion Pair
(Increa

HFIP Cor
Decre

If Chromatographic Precision is Necessary,
IP/RP buffers should be made Gravimetrically

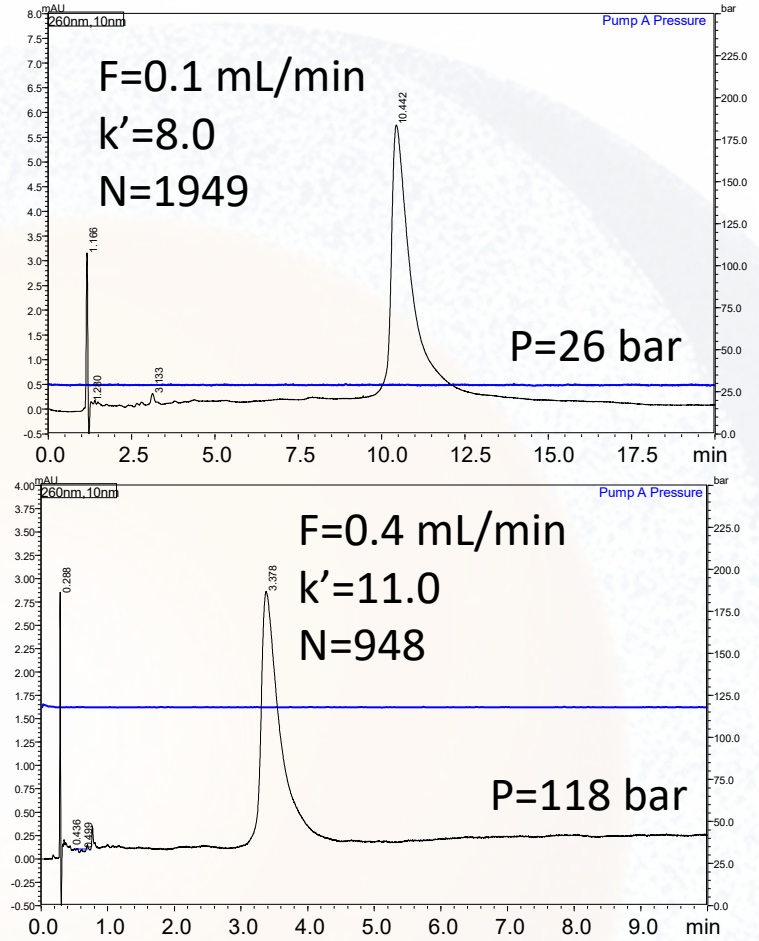
Building a Method

Column Length: Resolving power $\propto \sqrt{L}$



2.1x150 mm 1000Å OLIGO C18

Column Pressure is an Independent Parameter!!



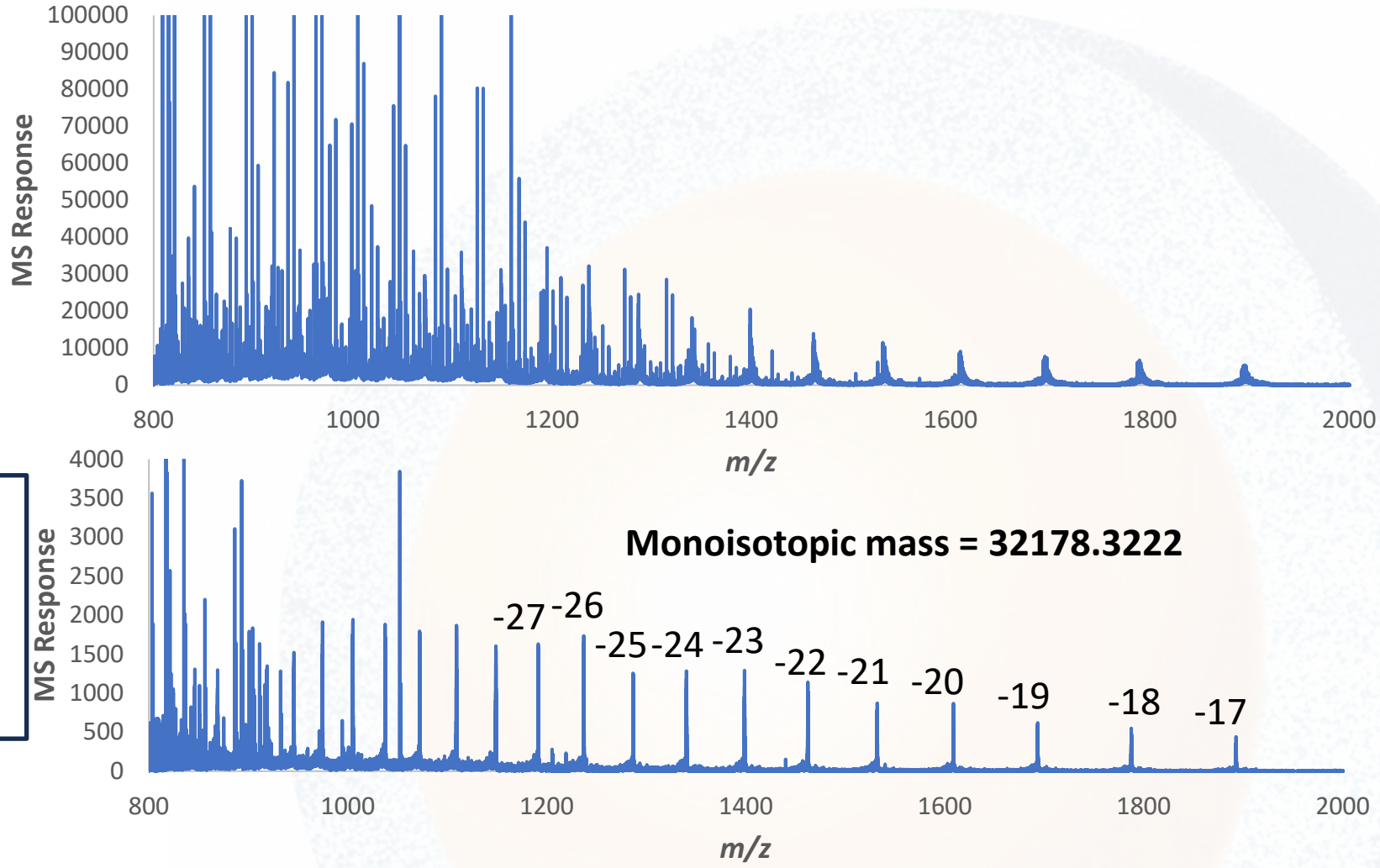
Minimizing Adduction

- Reduction of exposure to Metals
  - Na^+, K^+, etc
- Minimize glass exposure
- Use LCMS grade HFIP (Supelco)
- Use Type-1 18MOhm Water
  - Avoid bottled if possible

Guide RNA

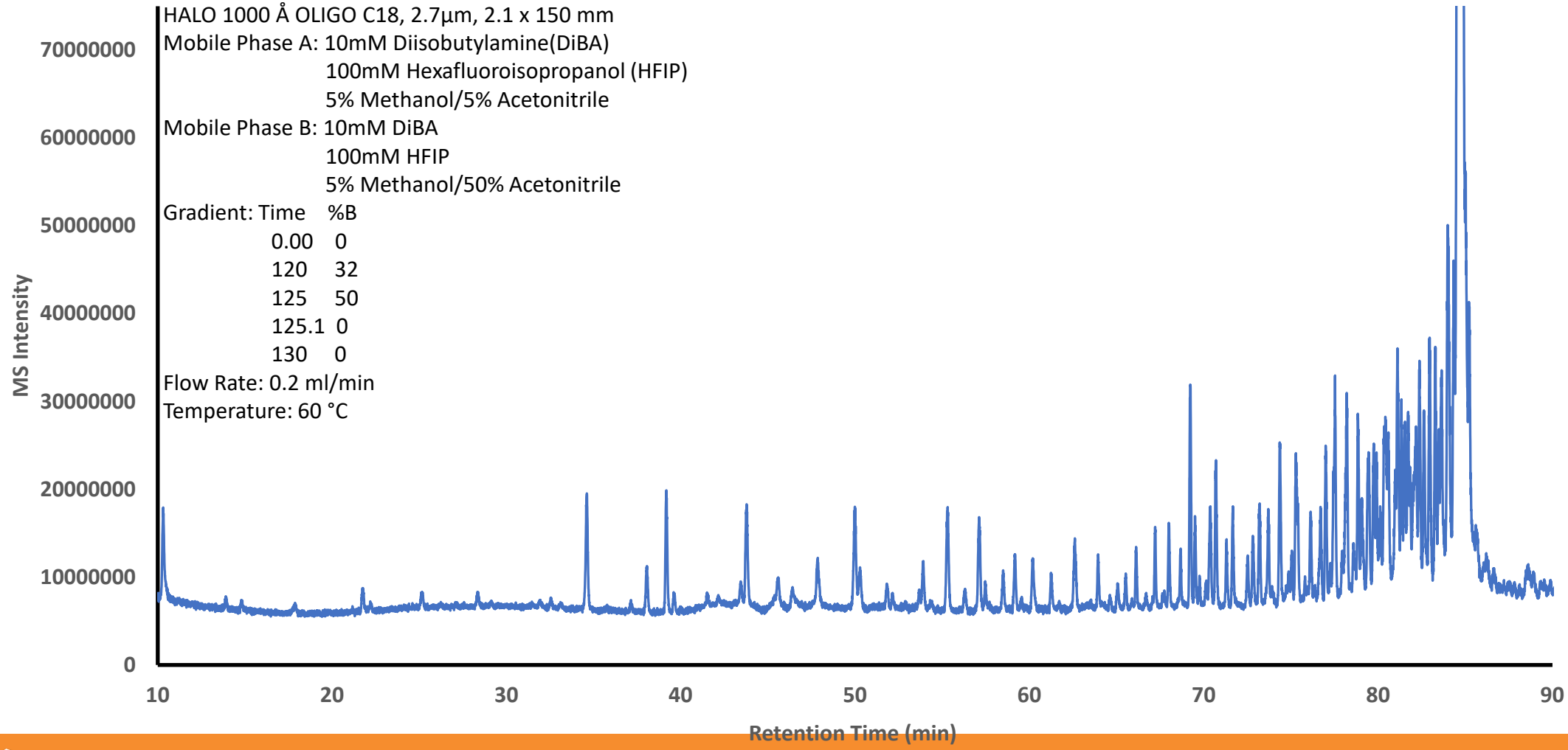
mU mA mG CAUAGACCUGCCACUCUGUUUUA GAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGU CCGUUAUCAACUUGAAAAAGUGGCACCGAGUCG GUGC mU mU mU rU

M = 2'-OMe modification

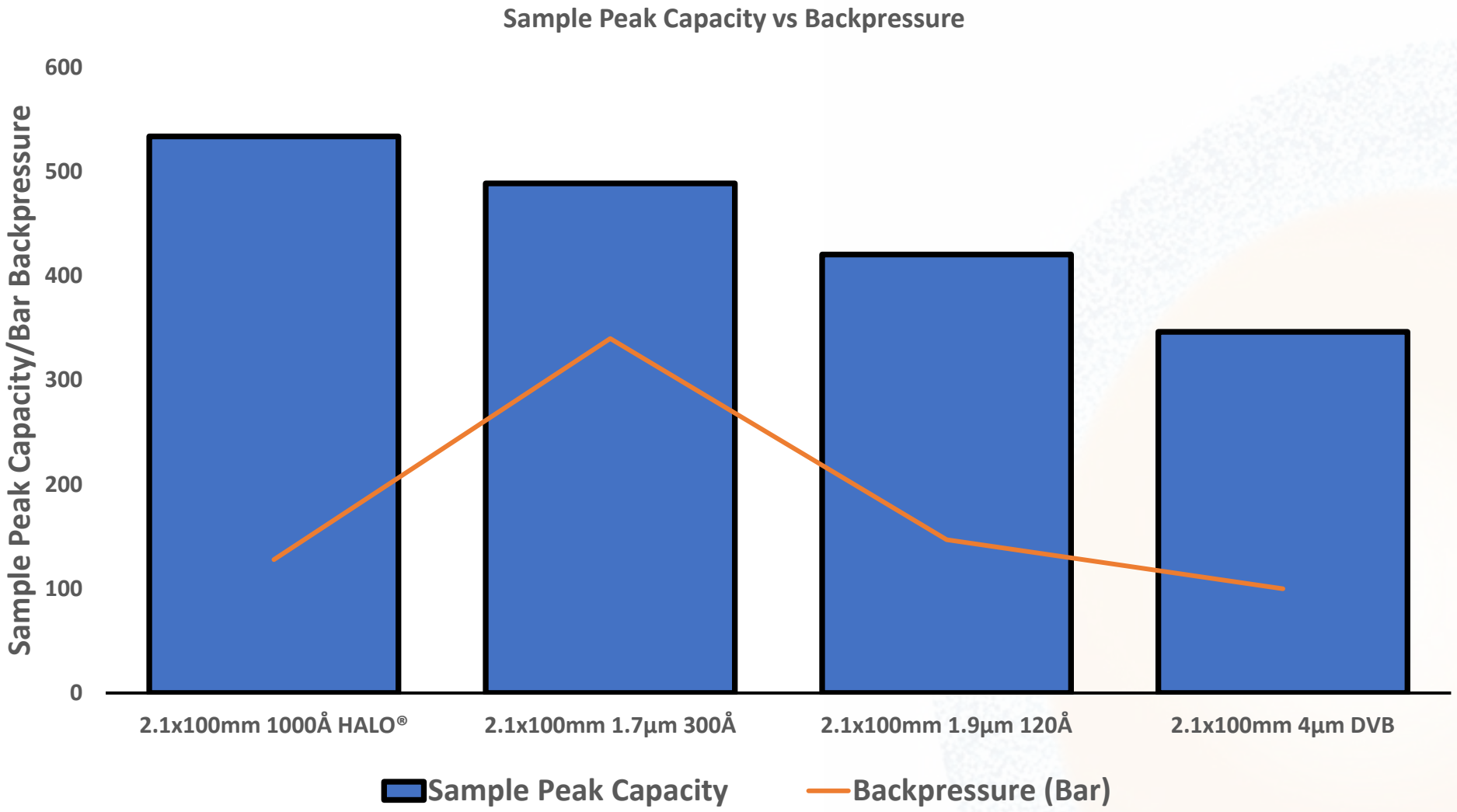


Separation of a 90-mer ssDNA

Impurity Analysis of crude 90-mer GDA EX



Sample Peak Capacity Comparison



Peak Capacity = $1+ (T_g / W_{1/2})$

T_g = Gradient Time
$W_{1/2}$ = Avg Peak Width @ HH

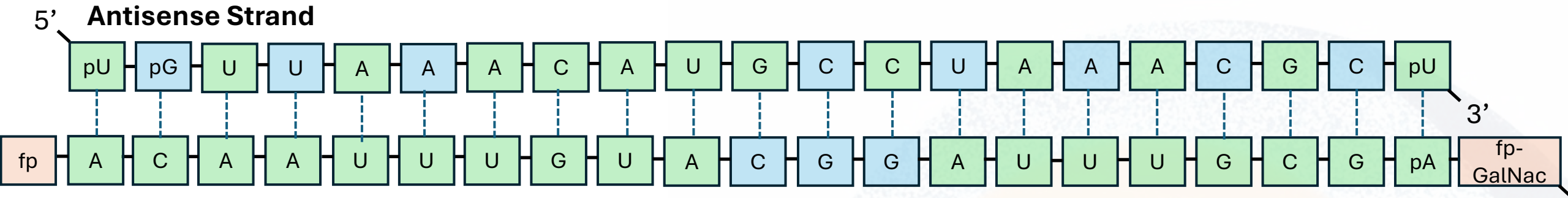
Sample Peak Capacity

$N_c = P_c \times (T_z - T_a)/T_g$

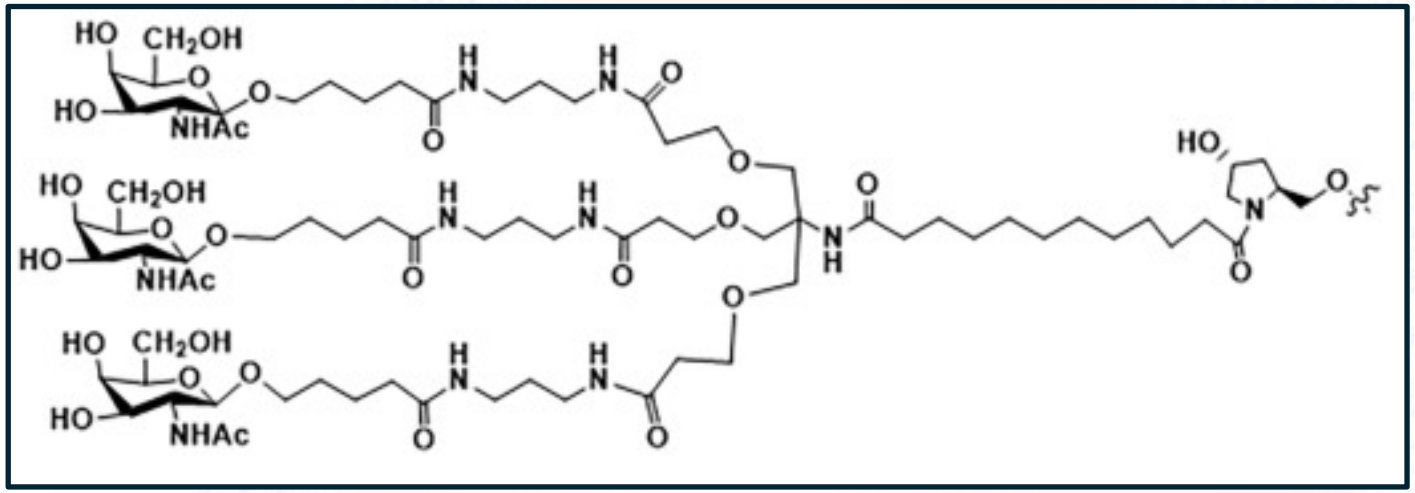
T_z = RT last eluting peak
T_a = RT first eluting peak

Applications

Fazirsiran

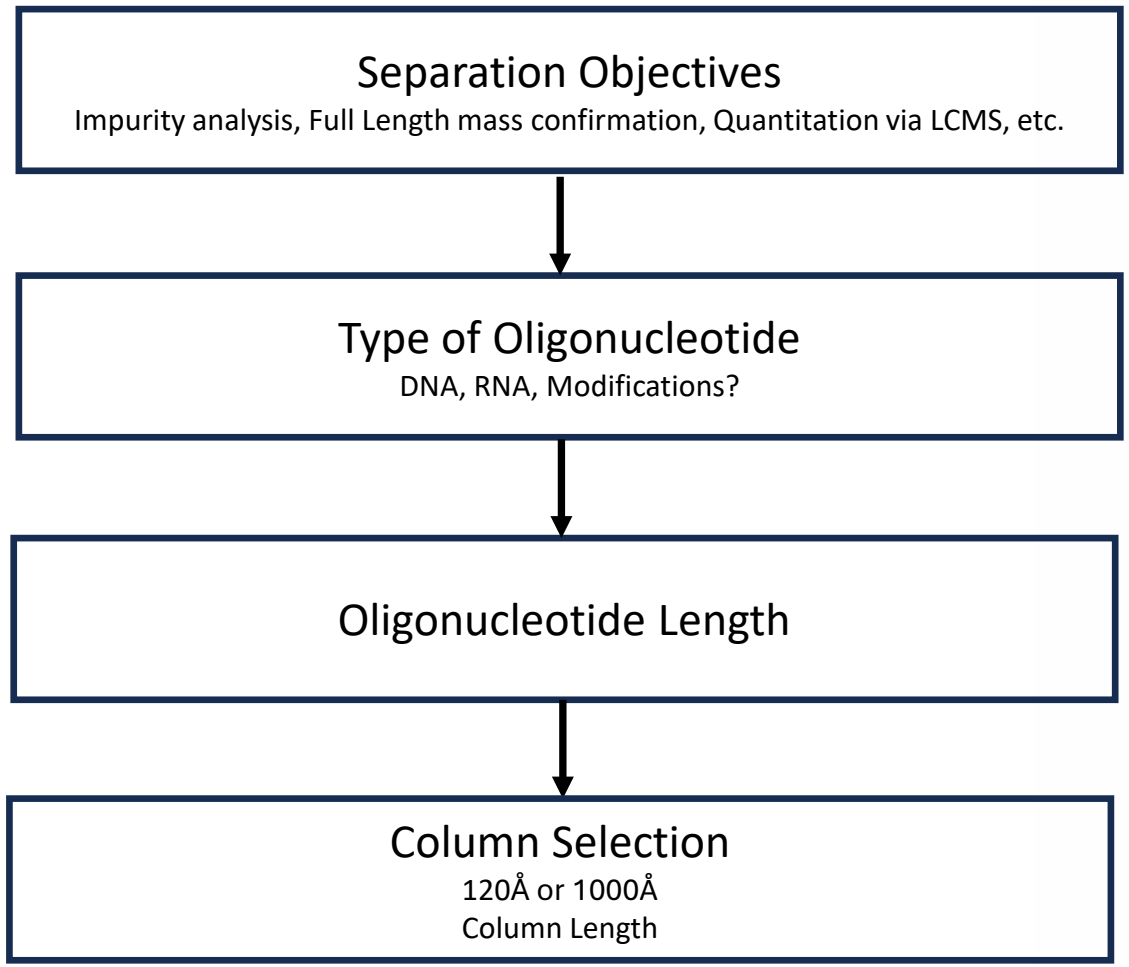


- 2’-OMe Modification
- 2’-F Modification
- p — phosphorothioation
- fp — Inverted abasic phosphorothioation
- GalNac — N-acetylgalactosamine conjugated linker



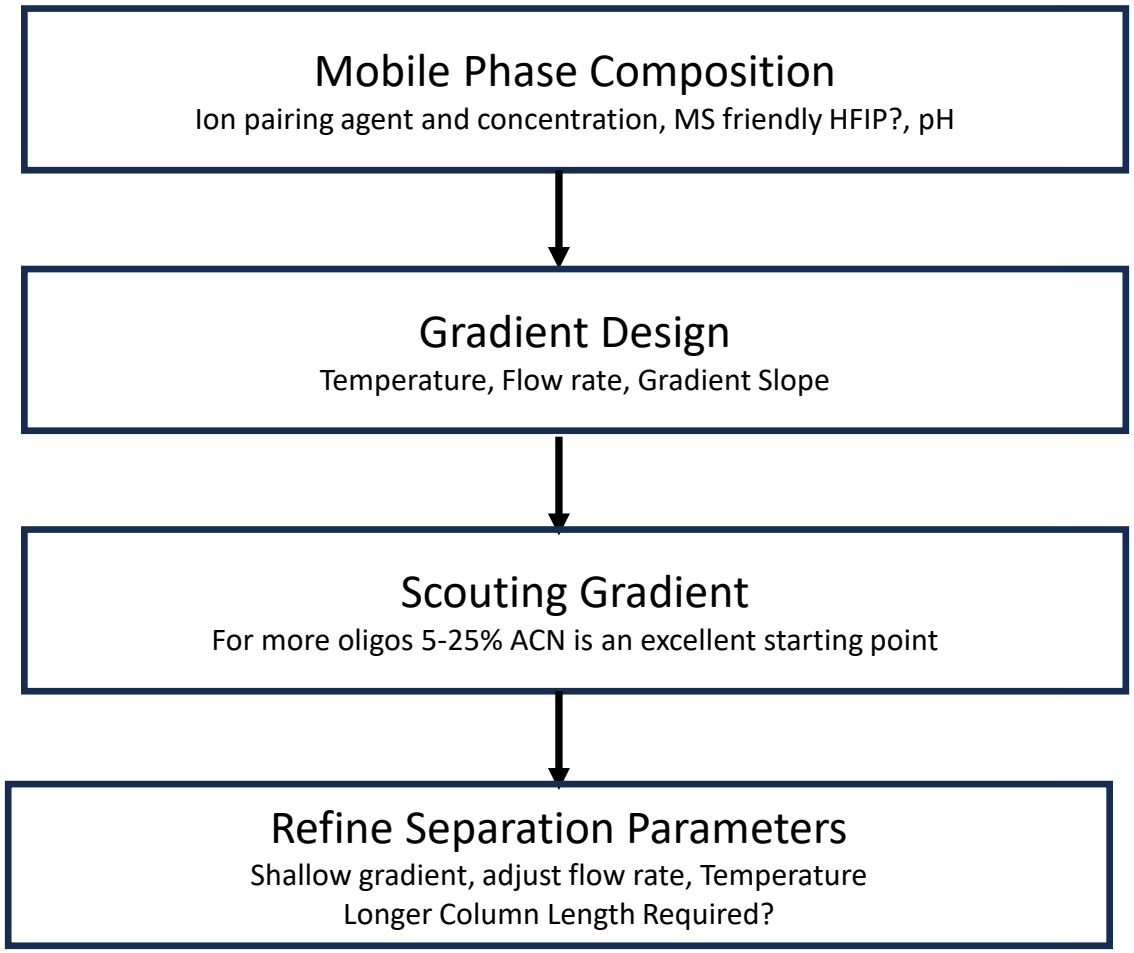
5’-Sense strand
Inverted abasic furan
With phosphorothioation

Method Development Preparation



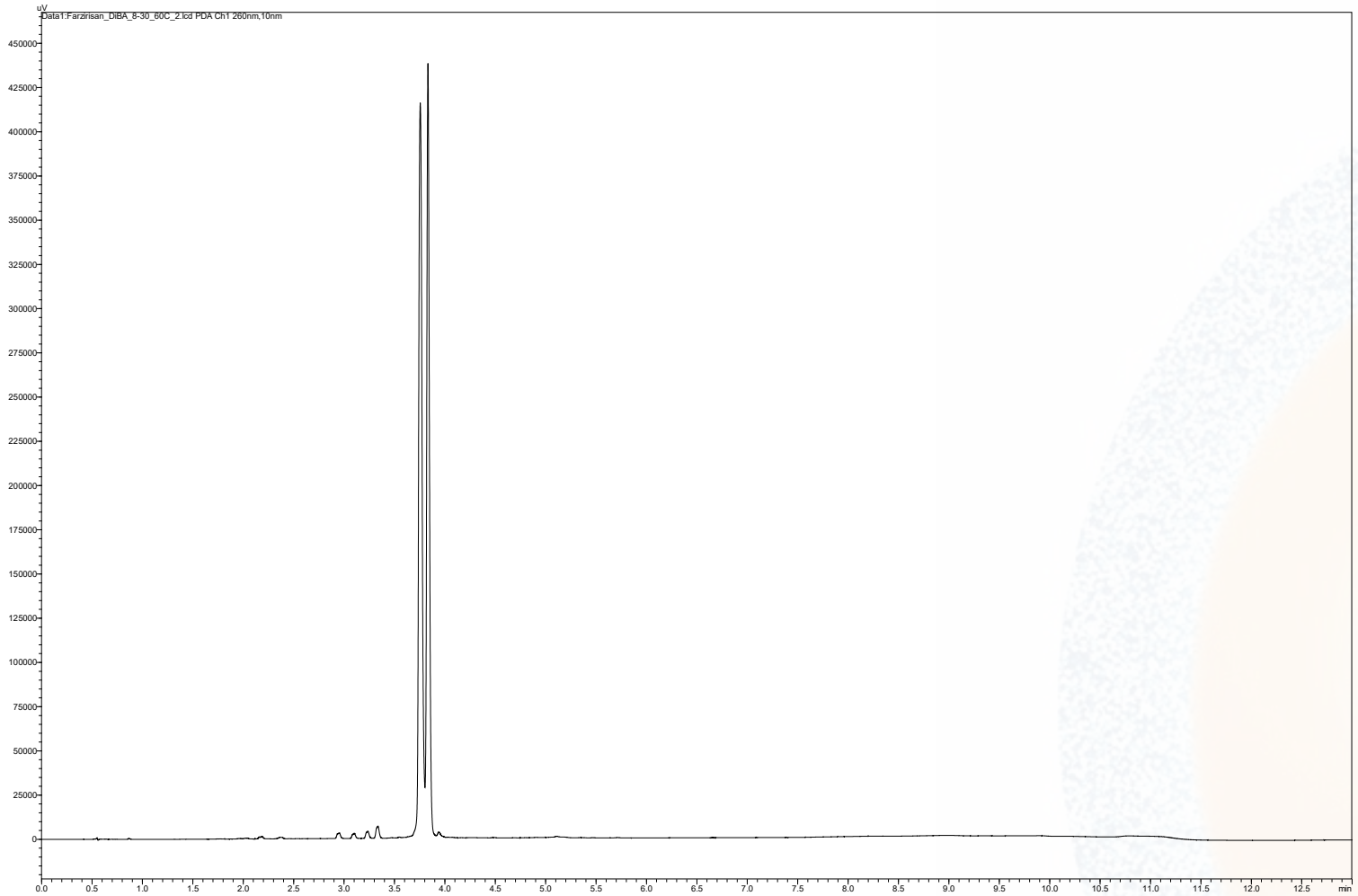
- For this example, we wish to perform an impurity analysis of Fazirsiran. Good separation of the two full length strands is required in order to see specific impurities of the later eluting strand.
- Our sample is a highly modified siRNA which adds hydrophobicity by addition of –OMe and –F to the furan. The conjugated linker adds additional complexity.
- The Anti-sense strand is 21 base pairs and the Sense strand has 23 base pairs including the inverted furan bases. The Sense strand also has a conjugated linker that terminates in 3 GalNac sugar residues.
- For this method, and given our objective is impurity characterization we will begin with the 1000Å HALO OLIGO C18 column in a 2.1x150mm format.

Method Development Continued



- For Fazirsiran, we have several 2'-F which increase hydrophobicity. Perhaps a more hydrophobic IP agent
- Resolve sense and anti-sense strands. ACN or MeOH/IPA?
  - Oligo gradients are very shallow (0.2%/min)
  - A: 0-5% organic ; B: 25-40% organic
- Optimize buffers, concentration, temperature, etc.

DiBA-HFIP Fazirsiran



Testing Conditions:

Column: 2.1x100mm 2.7µm 1000Å OLIGO C18

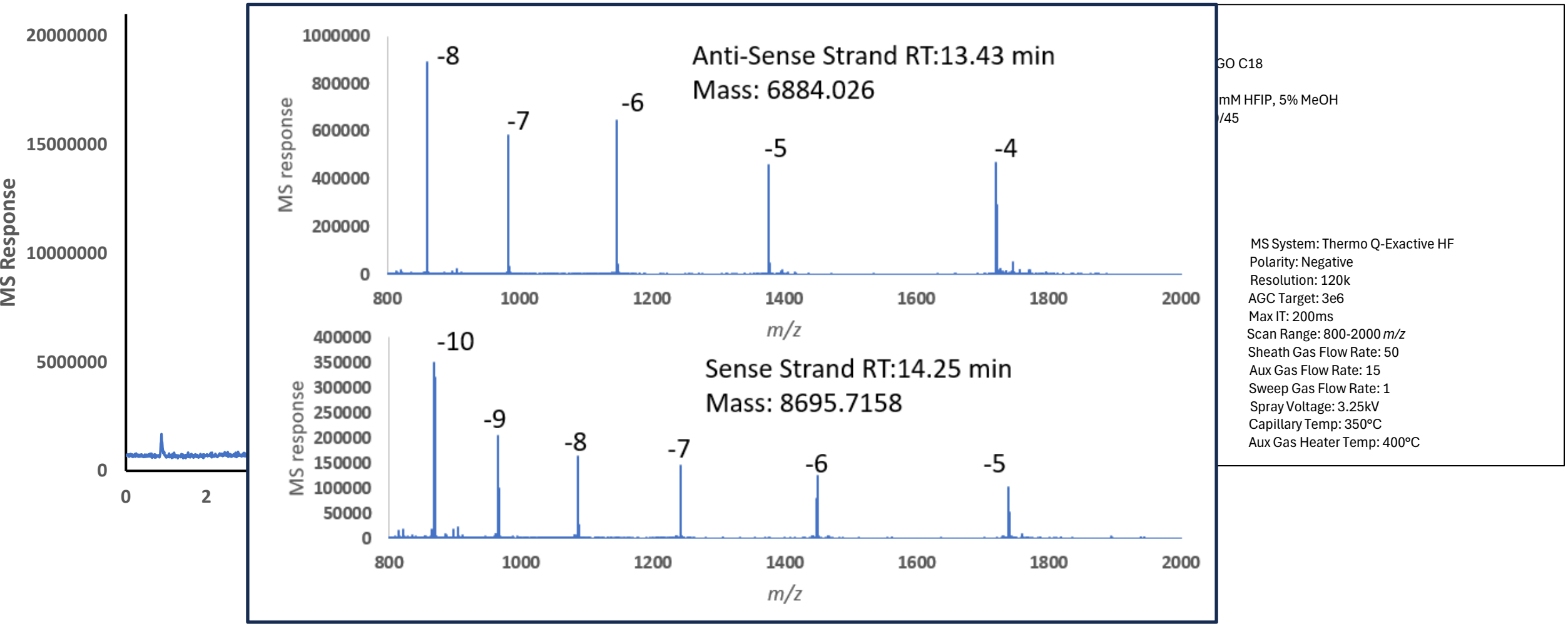
Mobile Phase: A: 10mM DiBA/100mM HFIP/5% MeOH
B: ACN

Gradient:

Time	%B
0.0	8
10.0	30

Flow Rate: 0.4 mL/min
Back Pressure: 231 bar
Temperature: 60 °C
Injection: 1 µL of 1 mg/mL Farzirisan
Sample Solvent: RNAse free water
Wavelength: PDA, 260 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.1 sec.
LC System: Shimadzu Nexera X2

Hexylamine-HFIP

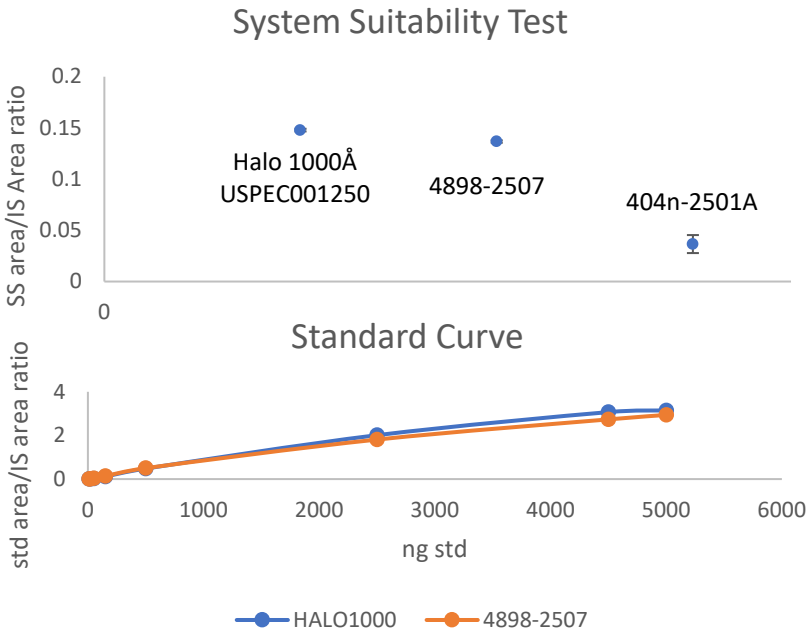


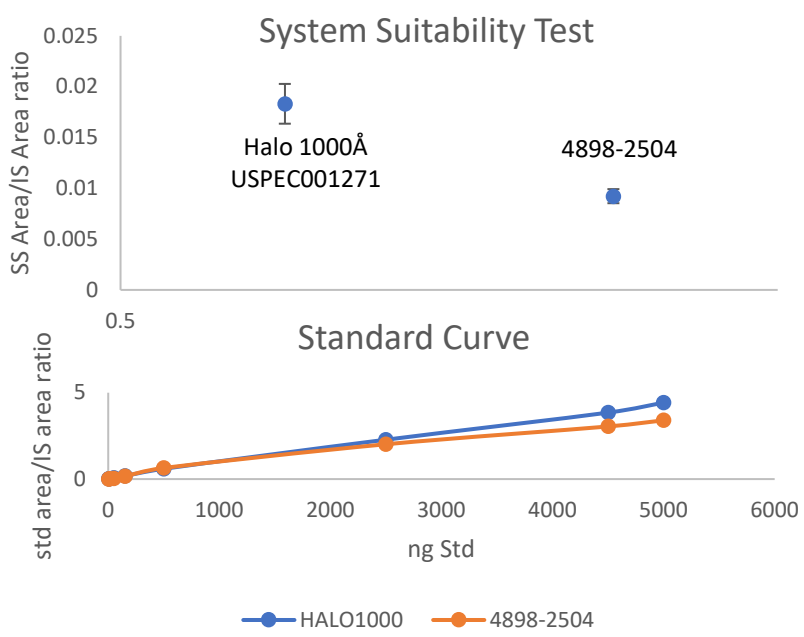
GO C18

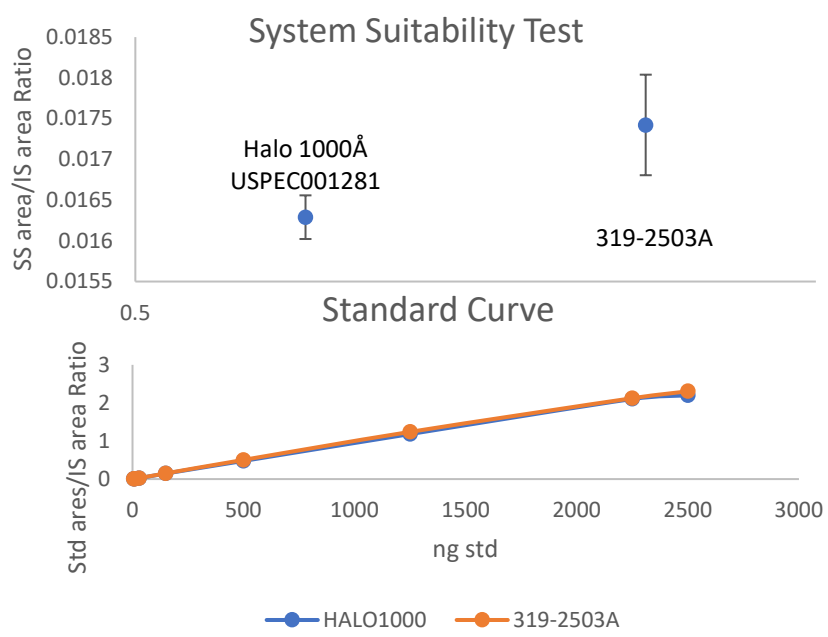
mM HFIP, 5% MeOH
/45

MS System: Thermo Q-Exactive HF
Polarity: Negative
Resolution: 120k
AGC Target: 3e6
Max IT: 200ms
Scan Range: 800-2000 *m/z*
Sheath Gas Flow Rate: 50
Aux Gas Flow Rate: 15
Sweep Gas Flow Rate: 1
Spray Voltage: 3.25kV
Capillary Temp: 350°C
Aux Gas Heater Temp: 400°C

Lot Testing of HALO OLIGO C18 1000Å In a Compliance Environment







- **The HALO OLIGO 1000Å C18 column passed the system suitability test for a validated oligo method**
- **Allowed for “plug and play” interchangibility in a real world assay for biological samples**

Summary

- Oligonucleotide IP/RP presents a challenging workflow methodologically
- It is LCMS compatible!
- Oligonucleotide Therapeutics are becoming increasingly complicated
- HALO OLIGO C18 in 120Å and 1000Å pore sizes
- Workflow for IP/RP method development approaches
- Method Development for Fazirsiran
- Plug and Play suitability for HALO OLIGO C18 1000Å in a compliance environment

ISPPP 2026

October 18th – 21st

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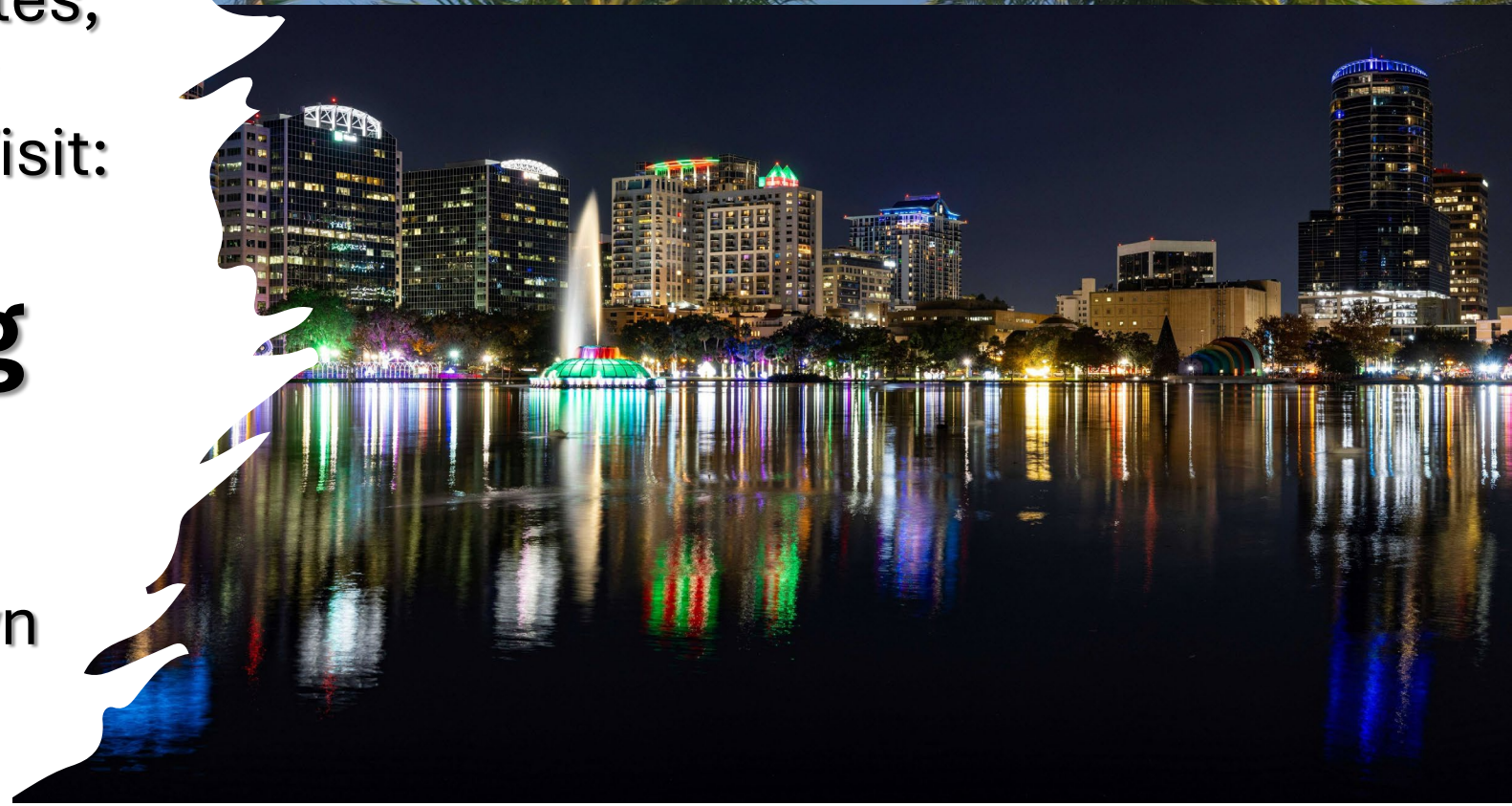

Marriott Orlando Downtown
Orlando, Florida

Thank You!

advancedmaterialstechnology

halocolumns.com

3521 Silverside Road, Suite 1-K
Quillen Building
Wilmington, DE 19810

(302) 992-8060