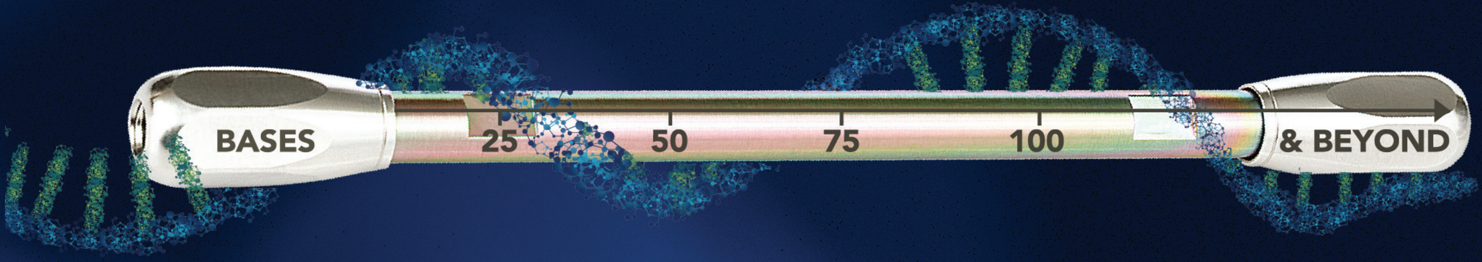
HALO®

OLIGO GUIDEBOOK



This guidebook provides a practical framework for the separation and analysis of oligonucleotides using ion-pair reversed-phase high-performance liquid chromatography (IP/RP-HPLC). It is intended to help scientists address the unique analytical challenges presented by oligonucleotide therapeutics, including high polarity, structural complexity, and the presence of closely related impurities.

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INTRODUCTION TO OLIGO

Pharmaceutical companies continue to make rapid advancements in developing new therapeutic modalities. Oligonucleotide-based therapeutics comprise one of these new modalities. The breadth of these therapeutics continue to push the limits of analytical separation technologies due to the complexity and chemical similarities of various impurities resulting from synthesis.

HPLC separations have become an indispensable component of the analytical testing pipeline for oligonucleotides. However, oligonucleotides present a number of unique challenges for chromatographic separation distinct from other biomolecules.

Traditional chromatographic columns utilize fully porous particle silica (FPP) and performance gains have come largely from reduction in particle size but at the cost of added backpressure. In contrast, Superficially Porous Particle (SPP) column technology presents a better solution for oligonucleotide separations. Unlike fully porous silica, SPP silica can achieve high resolution separations with reduced backpressures.

The HALO® OLIGO column line offers a unique combination of technologies that solve many of the challenges currently facing teams needing to characterize oligonucleotide formulations for impurities, modifications, and other critical quality attributes. Based on SPP Fused-Core® silica, HALO® OLIGO columns utilize a high pH resistance modification and are loaded in inert surface passivated stainless steel hardware. HALO® OLIGO columns utilize a C18 bonding phase and are available in 120 Å and 1000 Å pore sizes. This provides users with a wide range of options in regards to method development for their oligonucleotide separations.

Method development for oligonucleotide separations is just as critical for success as the choice of column and presents a much more complicated landscape compared to other biomolecules such as proteins and peptides. The choice of ion pair, temperature, backpressure, and more can have a significant effect of your separation.

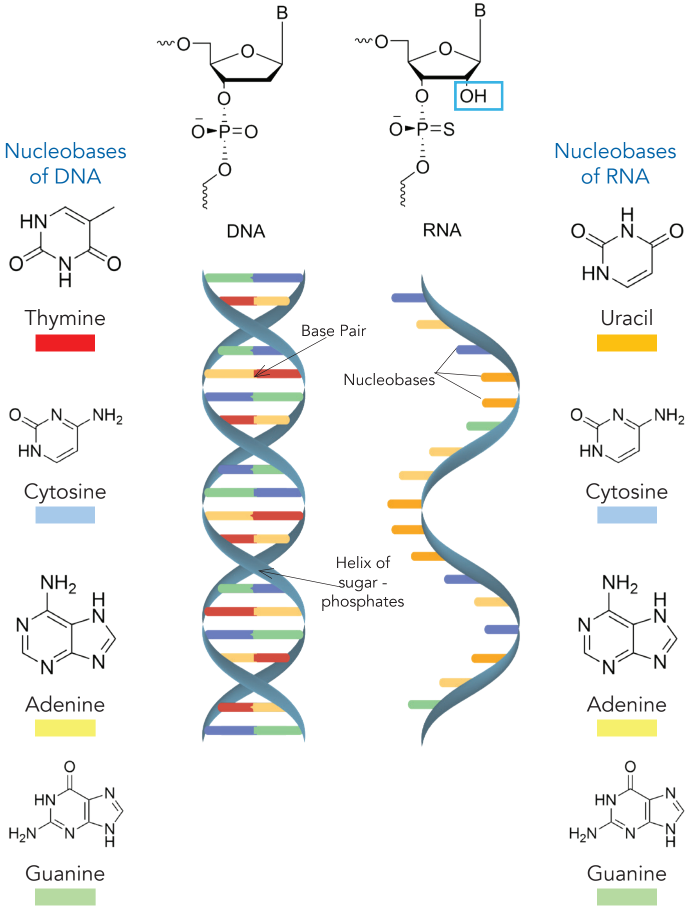
This guidebook will discuss many of the current approaches for oligonucleotide therapeutics, and a practical approach to using ion pair/reversed phase HPLC for the separation of these oligonucleotides for characterization, impurity analysis, and QA/QC purposes. This will provide a starting point for the development of a robust method for the analysis of your oligonucleotide via HPLC.

OLIGONUCLEOTIDE BASICS

Oligonucleotides make up the fundamental building blocks of our cells and serve countless functions. DNA serves as the blueprint for every cell providing instructions on reproduction, growth, and development. RNA, the transcription product, has been recognized in recent years to serve numerous functions in the cell, playing roles in protein polypeptide synthesis translation, ribosomal function, and gene regulation.

Both DNA and RNA have similar structures, having a sugar phosphate backbone, with RNA having a hydroxyl group at the 2′ position of the ribose sugar group. However, in RNA, uracil is substituted for thymine in base pairing.

DNA is typically found in a double stranded configuration and RNA is usually single stranded, but often forms hairpin loops, or longer complementary hybrids, base pairing with itself.

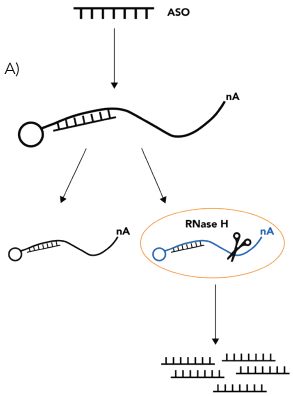


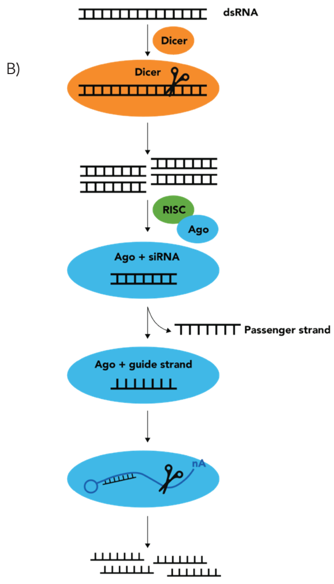
OLIGONUCLEOTIDE THERAPEUTICS

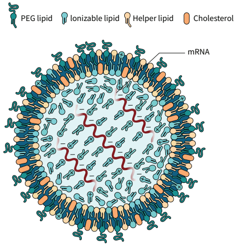
Molecular biology was has progressed over the last 50 years to incorporate the ability to splice DNA from one organism to another and express proteins of therapeutic value. This technology still plays a critical role in the development and manufacturing of biopharmaceuticals such as antibodies. However, many of the of the oligonucleotide-based therapeutics are RNA-focused due to its numerous functions within the cell.

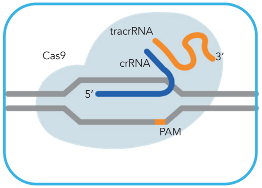
Current RNA based therapeutics can conveniently be broken into 3 groups. The first being small RNA's such as antisense and silencing RNA's which are often in the 15-25 base pair range. Second are the medium-sized emerging guide RNA's used for CRISPR applications. These often are 90-110 base pairs in length.

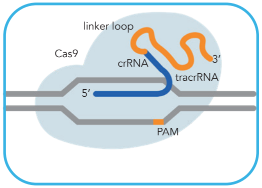
Finally, messenger RNA's make up the large RNA area which are a part of the lipid nanoparticle therapeutics such as COVID vaccines.









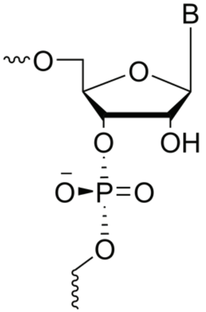


OLIGONUCLEOTIDE MODIFICATIONS

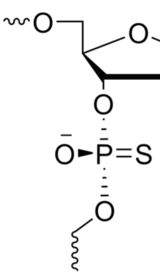
One challenge with RNA is that compared to DNA, it is considered quite fragile. Both must be handled with care, but RNA's are susceptible to ubiquitous nuclease degradation. This fragility has driven research into chemically modifying RNA in such a way that protects it from enzymatic degradation without sacrificing function. These modifications can significantly increase the half-life of the therapeutics in-vivo.

Many of these modifications are applied to the sugar-phosphate backbone. Phosphorothioation, where sulfur replaces an oxygen on a phosphate group, and modifications at the 2′ position of the ribose sugar where the –OH group is commonly replaced with a –OCH_3 or –F group. 5′ or 3′ linkers are commonplace as well allowing for specific cell or tissue targeting.

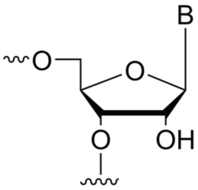
Similarly, uracil is commonly replaced with pseudouridine or N1-methyl pseudouridine which can reduce the risk of immunological response.



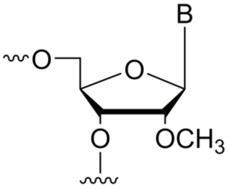
Phosphodiester



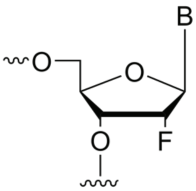
Phosphorothioate



RNA



2'-OMe-RNA



2'-F-RNA

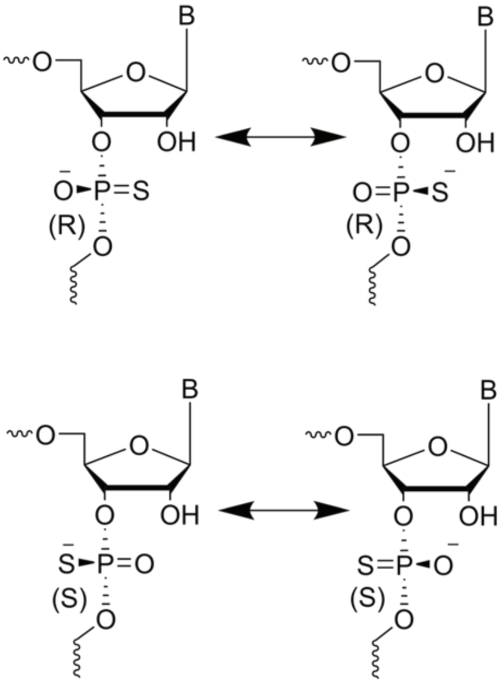
CHALLENGES WITH MODIFIED OLIGOS

While these modifications help prevent breakdown of oligo therapeutics in-vivo, they change the chemical nature of the oligos, frequently making them more hydrophobic.

Additionally, phosphorothioation creates a much stronger dipolar bond condition versus phosphate and creates an enantiomeric pair creating populations of variants depending on the number of phosphorothioate modifications the oligo contains.

For example, many siRNA’s have 5-6 phosphorothioations on the 5′ and 3′ ends for protection. An oligo with 5-6 phosphorothioations will create 32 or 64 individual populations of molecules depending on the enantiomeric position of each sulfur.

These changes create significant complexity in characterization and impurity analysis of these oligos representing an ongoing analytical challenge.

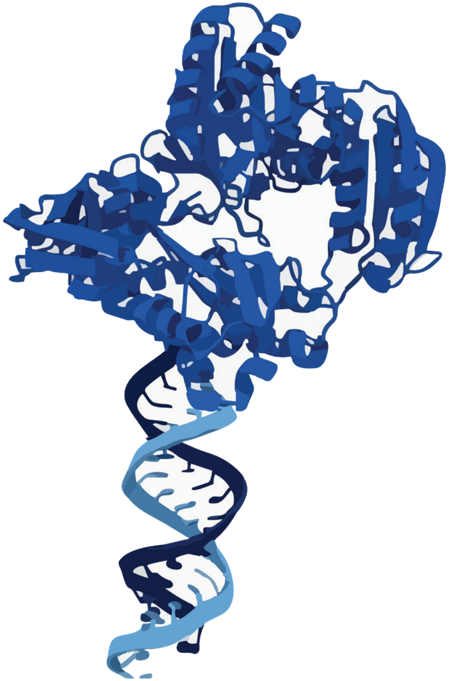


MACROMOLECULE SIZE COMPARISONS

On a per-residue basis, oligonucleotides tend to occupy more space per monomer than do proteins. Protein chains form secondary, tertiary, and even quartenary structures to form compact folded macromolecules. In contrast, oligo base pairs are larger and tend to form twisted, linear structures.

The image on the right shows the Argonaute protein from Aquifex aeolicus complexed with a 22-bp siRNA duplex (PDB:2F8S). The Argonaute protein contains 706 amino acids and has a molecular weight of 83 kDa. In contrast, the the 22 base pair siRNA duplex has a molecular weight of 13.3 kDa.

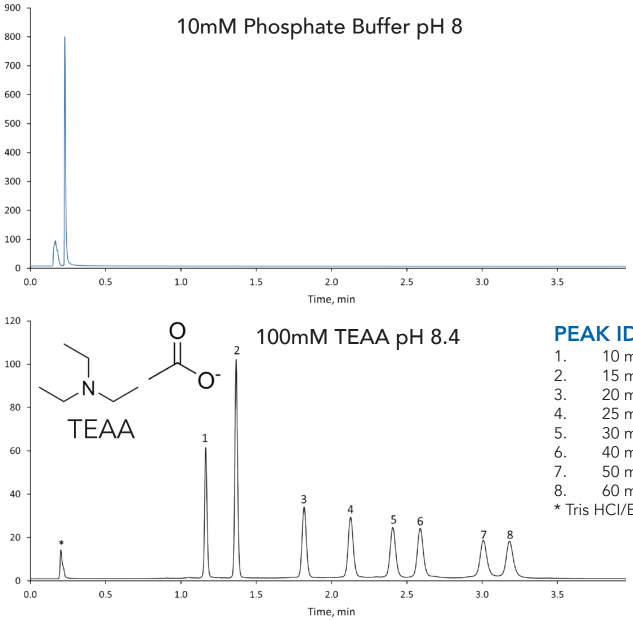
The comparative differences in volume to molecular weight ratios between oligonucleotides and proteins must be taken into consideration when thinking about chromatographic separation conditions as the size of oligonucleotides scale much faster than proteins as more residues are incorporated.



ION PAIR / REVERSED PHASE CHROMATOGRAPHY

Separation of oligonucleotides via reversed phase HPLC requires an approach unique from most small molecule or protein and peptide separations. Oligonucleotides are highly hydrophilic and thus suffer from poor retention on traditionally hydrophobic bonding phases. Oligonucleotides require an ion pairing agent, much like the use of trifluoroacetic acid or formic acid for more traditional chromatography. However, for oligonucleotides, the ion pairing agent requires a positive charge in addition to a hydrophobic moiety to create a non-covalent chemical bridge between the sorbent surface and the negatively charged backbone of the oligo.

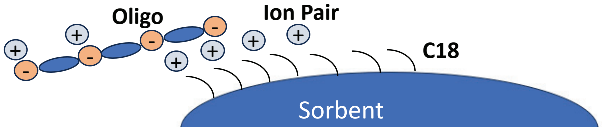
In the presence of phosphate buffer, a DNA ladder mix is poorly retained on C18. In contrast, the use of an ion-pairing system Triethylammonium Acetate (TEAA) facilitates interaction between the oligonucleotide and stationary phase, resulting in improved surface interaction and retention.



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

* Tris HCl/EDTA



ION PAIRING CHOICES

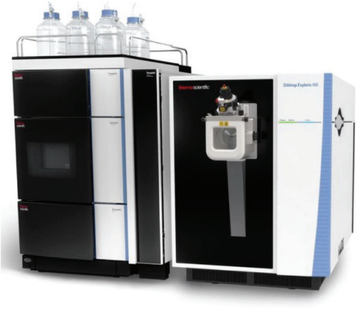
There are many choices for ion pairing reagents, some factors to take into consideration:

- Length and hydrophobicity of oligo
- Choice of Detection (UV/VIS or Mass Spectrometry)
- Solubility of the ion pairing system (amine and counterion)

The use of alkylamines with an acetate counterion are well suited for IP/RP. Triethylammonium acetate (TEAA) is a commonly used reagent for this purpose. However, depending on the length of your oligo, modifications, etc, other IP reagents may be more useful as well as higher IP reagent concentration within limits of solubility.

At present time, optimization of ion pairing conditions is complex and limited guidance is available from the literature. Frequently, finding the correct IP reagent and concentration may need to be empirically determined.

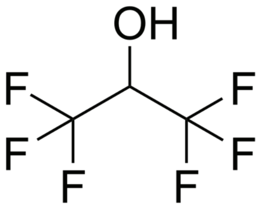
OLIGO ANALYSIS BY MS



Mass Spectrometry can be seen as a significant enhancement for the characterization of oligonucleotide separations. Some of the benefits include:

- Orthogonal separation and characterization
  - Accurate mass is a useful tool for confirmation of sugar, base, and modification identities composition
  - Tandem MS (MS/MS) can be used to confirm base pair sequence
- High sensitivity and specificity particularly important for PK and ADME
- Stability and modification monitoring

Unfortunately, some of the ion pair reagents used for UV/VIS cannot be used for MS purposes. Oligonucleotide MS is typically operated in negative polarity and acetic acid among other carboxylic acids are highly ion suppressing. For MS, hexafluoroisopropanol is commonly used as an alternative. It is highly volatile with a pKa of ~9.3, providing a suitable counterion that will readily volatilize during electrospray without competing for charge.



Hexafluoroisopropanol (HFIP)

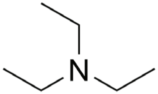
CHOOSING THE RIGHT IP AND COUNTERION

As seen in the graphic on the opposite page, there are numerous ion pairing agents one can choose from for the separation of oligonucleotides. Often, finding the correct combination of IP reagent and counterion and their concentrations must be empirically determined to be optimal for your needs, there are some broad guidelines to consider.

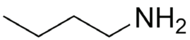
There are numerous alkylamines suitable for pairing with HFIP for LCMS analysis of oligonucleotides. The most common for small oligos is triethylamine (TEA). However, as your oligo gets longer and more complex due to modifications, a more hydrophobic IP agent may be beneficial.

In general, the longer the oligo the more likely a more hydrophobic IP agent may be useful as well as higher concentration of the IP agent. Solubility considerations must be taken into consideration and often the IP agent that provides the best chromatographic separation must be empirically determined. There is a complex relationship between solubility of IP, organic solvent choice, and temperature of operation.

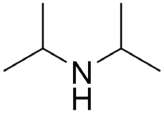
COMMON ION PAIR REAGENTS



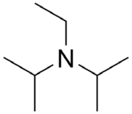
Triethylamine (TEA)
LogP = 1.65



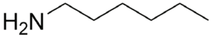
n-Butylamine
LogP = 1.06



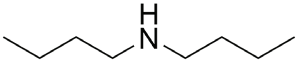
Diisopropylamine (DiPA)
LogP = 1.4



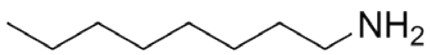
Diisopropylethylamine (DiPEA)
LogP = 2.2



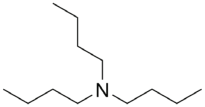
Hexylamine
LogP = 2.1



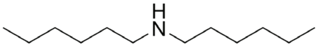
Dibutylamine (DBA)
LogP = 2.8



OctylAmine
LogP = 3.1

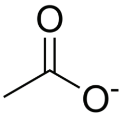


Tributylamine (TBA)
LogP = 4.0

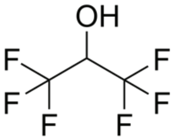


Dihexylamine (DHA)
LogP = 4.5

Anions Compatible with LC/UV

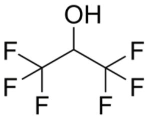


Acetate

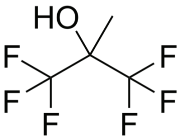


HFIP

Anions Compatible with LC/MS



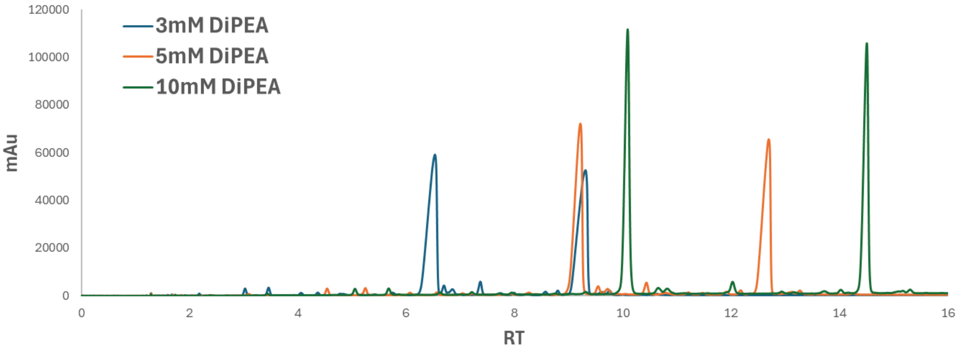
HFIP



Hexafluoro-2-methyl
Isopropanol (HFMIP)

EFFECTS OF ION PAIR STRENGTH

The figure demonstrates the impact of changing ion pair concentration on the retention of an siRNA, in this case, Patisiran. As the concentration of IP increases from 3mM to 10mM the strands are more strongly retained and peak shapes become much sharper. This is most likely due to greater coverage of both the oligo and the silica particle by the IP reagents, improving retention and peak shape.



TEST CONDITIONS:

Column: HALO 1000 Å OLIGO C18, 2.7µm, 2.1x150mm
Mobile Phase A: Diisopropylethylamine (DiPEA), 100mM HFIP
5% MeOH, 5% Acetonitrile
Mobile Phase B: DiPEA, 100mM HFIP
5% MeOH, 30% Acetonitrile

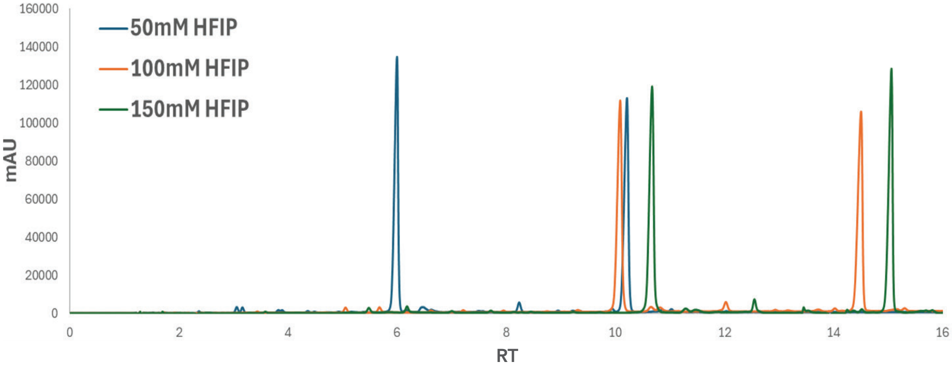
Gradient:

Time	%B
0	0
30	20
35	100

Flow Rate: 0.25 mL/min.
Back Pressure: 185 Bar
Temperature: 60 °C
Injection: 1µl of 200µg/ml of Patisiran (Research Grade)
Detection: 260nm
Sample Solvent: H_2O
LC System: Shimadzu Nexera X2

IMPACT OF HFIP

We see similar shifts with increasing HFIP concentration. However, we did not see any significant differences in MS response with increasing HFIP concentration. The IP and HFIP concentration effects are generally observed, but with significant variation between siRNAs, depending on length and modifications.



TEST CONDITIONS:

Column: HALO 1000 Å OLIGO C18, 2.7µm, 2.1x150mm

Mobile Phase A: 10mM Diisopropylethylamine (DiPEA), HFIP
5% MeOH, 5% Acetonitrile

Mobile Phase B: 10mM DiPEA, HFIP
5% MeOH, 30% Acetonitrile

Gradient:

Time	%B
0	0
30	20
35	100

Flow Rate: 0.25 mL/min.

Back Pressure: 185 Bar

Temperature: 60 °C

Injection: 1µl of 200µg/ml of Patisiran (Research Grade)

Detection: 260nm

Sample Solvent: H_2O

LC System: Shimadzu Nexera X2

COMMON ORGANIC MODIFIERS

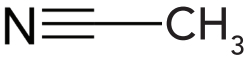
Much like other reversed phase separations, useful organic modifiers such as acetonitrile, methanol or 2-propanol are commonly used to elute oligonucleotides in an isocratic or gradient method.

Organic modifiers are also useful to assist in solubility of ion pairing agents and HFIP. If higher concentrations are needed, the addition of 5% MeOH to the A and B buffers often improves solubility. An example of this is shown on page 16.

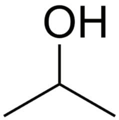
Organic Gradient Considerations

Oligonucleotide separations typically require a shallower gradient than most other analyte types due to their similar chemical properties and very high solvent retention parameter S.[1] It is not uncommon to operate a gradient of 0.2% B/min. As a result, unlike many preparations where water and acetonitrile comprise buffers A and B, it is often more practical to make an A buffer with 5-10% acetonitrile and B buffer with 30-50% ACN in order to better gain shallow gradient control.

Acetonitrile
BP: 82 °C
Polarity Index: 5.8



Methanol
BP: 65 °C
Polarity Index: 5.1



2-Propanol
BP: 82 °C
Polarity Index: 3.0

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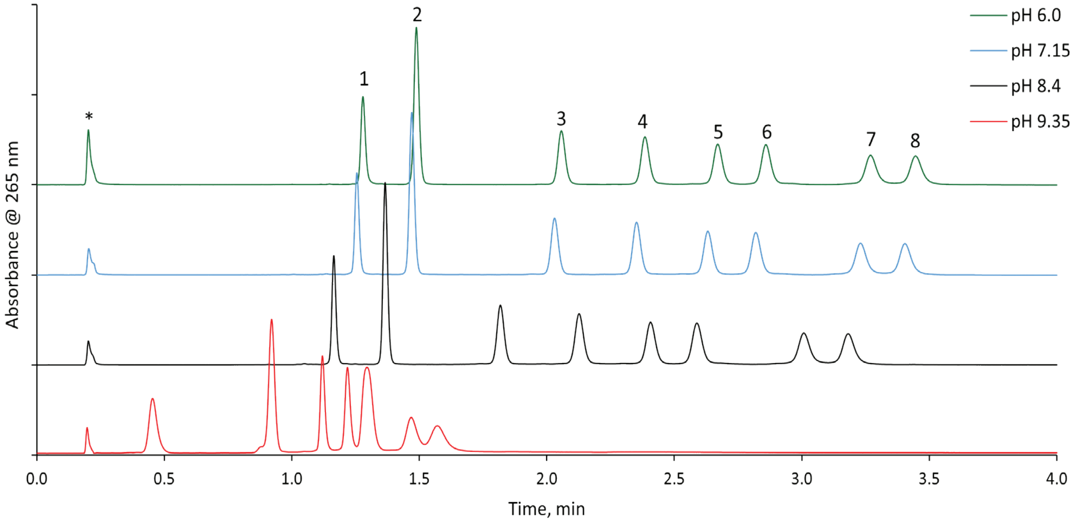
[1] Snyder LR, Dolan, JW. High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model. 2007. ISBN 0-471-70646-9

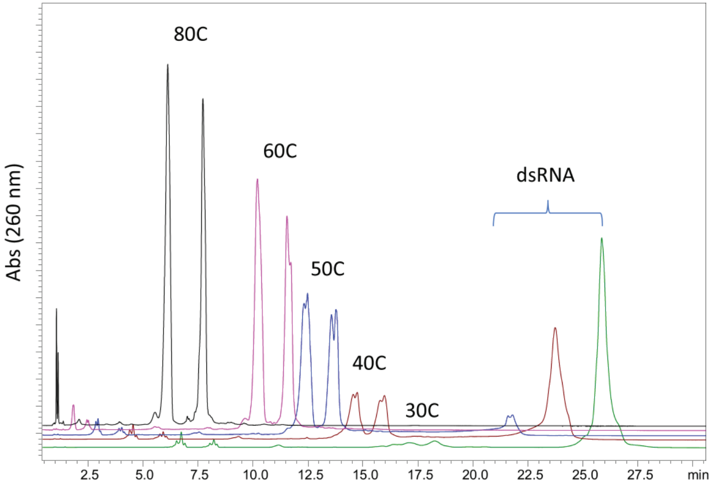
pH EFFECTS

The pH of your mobile phases may also have a significant impact on your separation. In this experiment, we evaluated the retention of a DNA ladder standard from a range of pH 6 to 9.5 using TEAA as the IP buffer and adjusting pH with acetic acid. The results show that as pH is increased, retention is reduced. This is likely due to the oligonucleotides increasingly adopting a folded, deprotonated state which will reduce retention. When preparing IP buffers, ensuring consistent pH will help to minimize shifts in retention time.



COLUMN TEMPERATURE

Column temperature can have a significant impact on a number of parameters related to retention and selectivity. As column temperature rises, mobile phase viscosity lowers which can impact the Van Deemter C-term of mass transfer. Additionally duplex oligos begin to dissociate and organic composition can also impact intramolecular bonding as well as intermolecular. As shown in the figure, the siRNA Inclisiran shows strand dissociation, improved peak shapes, and better recovery as column temperature increases. This reflects the faster on/off kinetics related to mass transfer. Choosing column temperature will depend on size of your oligo, its modifications, the GC content and sequence.



TEST CONDITIONS:

Mobile Phase A: 3 mM DiPEA/150 mM HFIP, 5% MeOH
Mobile Phase B: 40% H_2O, 15% IPA, 45% MeOH
Flow Rate: 0.35 mL/min.
Temperature: See Graph
Sample: Inclisiran, 1 µL, 1mg/mL in H_2O

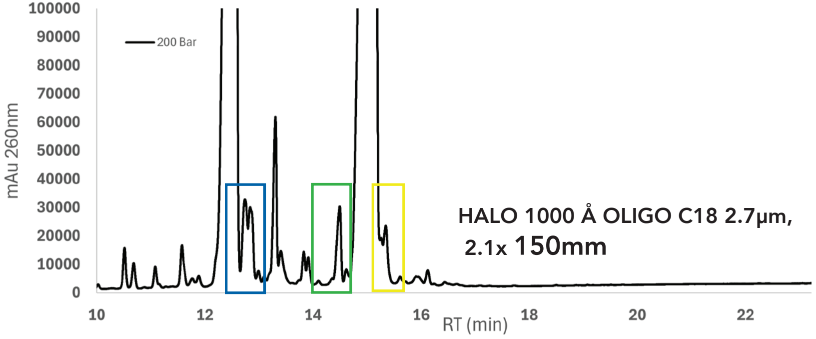
COLUMN LENGTH

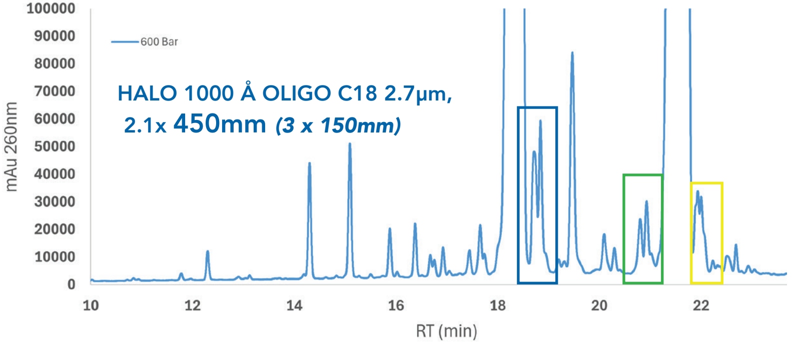
If high resolving power is desired for your separation, it is useful to remember that like many chemical separations, the following equation still holds true for oligonucleotide chromatography:

Resolving power $\propto \sqrt{L}$

Thus, one way to achieve additional resolution is to increase column length. The Fused-Core® silica of HALO® columns reduces backpressures relative to fully porous silica and can allow for longer column lengths that do not exceed the backpressure limits of your HPLC system.

Here we see an enlarged chromatogram of Patisiran. We still see the RT shift from increased backpressure, but we see features in the 450mm column that cannot be resolved on the 150mm column alone.

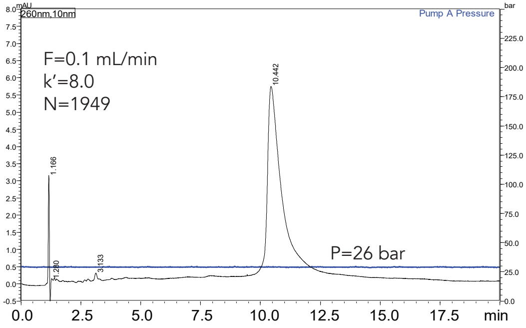


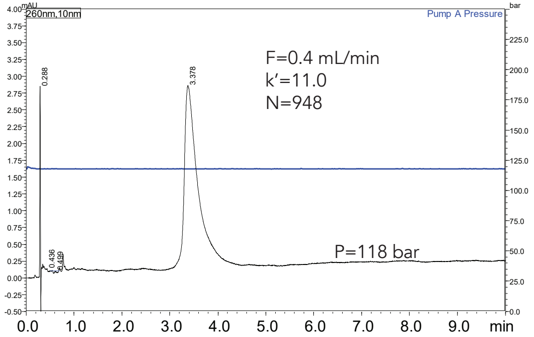


OPERATING PRESSURE AND RETENTION

It has been recently demonstrated that column backpressure can have a significant effect on retention time for oligonucleotide chromatography[1]. This has also been demonstrated for HILIC chromatography as well[2]. This parameter is independent of other factors that can influence retention time as described in the Van Deemter equation and should be taken into consideration when designing your method.

Here we demonstrate a retention time shift, both absolute RT and k’ of a 90-mer ssDNA by increasing column backpressure from 26 bar to 118 bar by increasing flow rate under isocratic conditions.





TEST CONDITIONS:

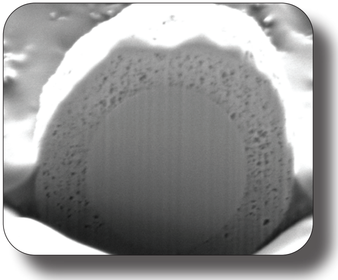
Columns: HALO 1000 Å OLIGO C18, 2.7 µm, 2.1x50mm
Mobile Phase A: 100 mM TEAA (pH 7.0)
Mobile Phase B: Acetonitrile –
Isocratic: 11.3% B (9.0% AcN)

Sample: 1 µL, 90-mer ssDNA, 10ng
Temperature: 60 °C
Detection: UV/PDA, 260 nm

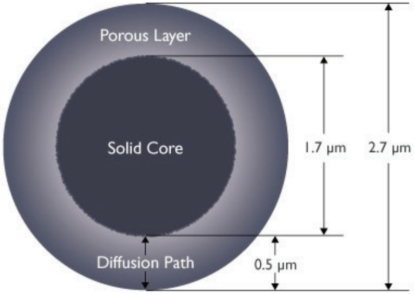
1. Stoll, D. et al. J. Chrom. A (2025) 1744, 465687
2. Meston, D et al. J Chrom. A (2025) 1742, 465643

SUPERFICIALLY POROUS PARTICLE TECHNOLOGY FROM AMT

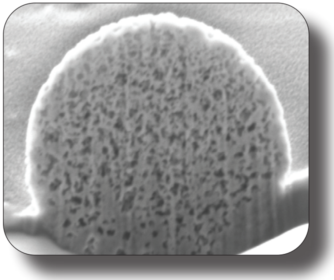
Most commercial columns are based on fully porous particle silica. In order to improve the efficiency of FPP silica, particle diameters are often reduced in size, often at the cost of significantly increased backpressures. HALO® superficially porous particle (Fused-Core®) technology creates a solid core shell with the outer 1/3 providing a porous layer for analyte interaction. The use of SPP technology improves all 3 terms of the Van Deemter equation, particularly the "C" term by creating shorter diffusion distances into the pores of the particles. SPP technology also significantly reduces backpressures without sacrificing performance.



Superficially Porous Particle (SPP)

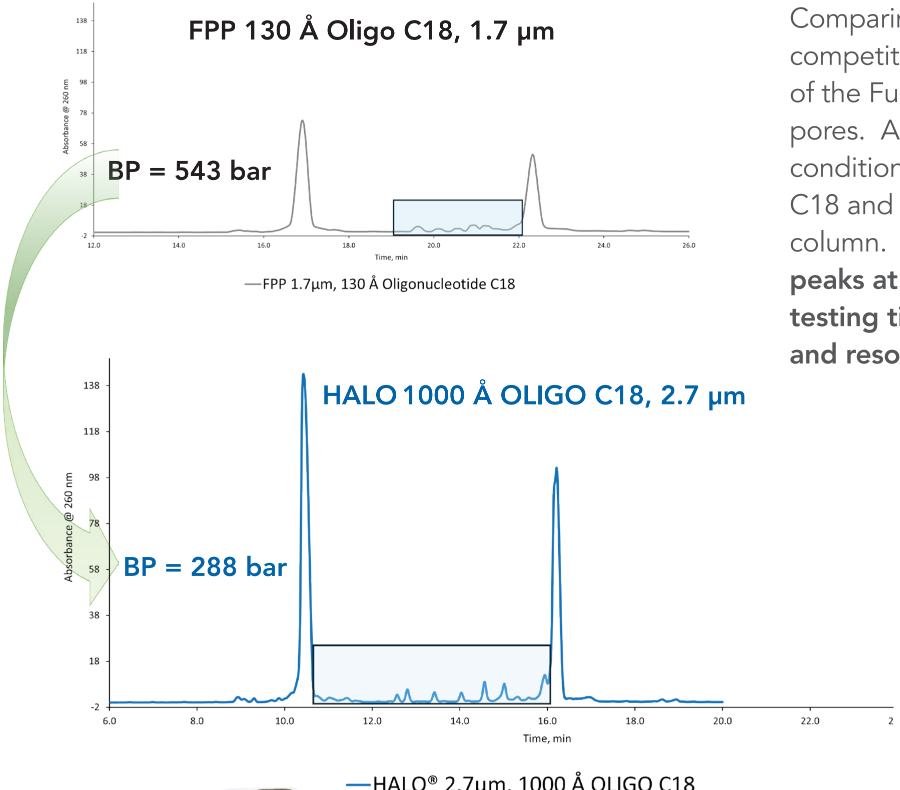


HALO 90 Å, 2.7 µm



Fully Porous Particle (FPP)

COMPETITIVE EXAMPLE: FPP VS SPP - LUMASIRAN (siRNA)



Comparing the HALO 1000 Å OLIGO C18 to a FPP competitor column demonstrates the advantages of the Fused-Core® SPP technology as well as wider pores. A sample of Lumisiran was run in identical conditions on A 2.1x150mm HALO 1000 Å OLIGO C18 and a competitor FPP 1.7µm 2.1x150mm 130 Å column. The HALO® column produces much **sharper peaks at half the backpressure and in shorter testing time.** This allows for **greater peak capacity and resolution.**

TEST CONDITIONS:

Mobile Phase A: 3 mM DiPEA/150 mM HFIP/5% MeOH

Mobile Phase B: 40/15/45 Water/IPA/MeOH

Gradient:

Time	% B
0.0	14
25	24
26	50
28	50
29	14

Flow Rate: 0.4 mL/min.

Pressure: HALO® - 288 bar
FPP - 543 bar

Temperature: 70 °C

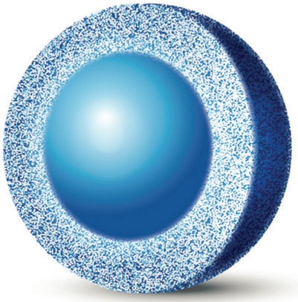
Injection Volume: 1.0 µL (1 mg/mL of Lumasiran)

HALO® OLIGO C18

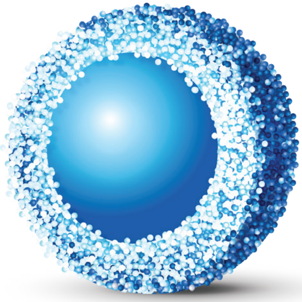
HALO® OLIGO C18 columns, part of the BIOCLASS offerings from Advanced Materials Technology, provide an excellent solution for your challenging oligonucleotide separations. Whether your need is to analyze a short DNA primer, or perform an impurity analysis of a guide RNA for CRISPR, we have unique column solutions that meet the most demanding requirements. The 120 Å offers excellent separation for oligonucleotides up to 60 bases or bp and high surface area for increased loading capacity. The 1000 Å has larger pores for excellent mass transfer performance of longer oligonucleotides.

HALO® OLIGO C18 columns have several features that offer best-in-class performance for your chromatographic needs:

- Fused-Core® silica technology for low backpressures
- Our proprietary ELEVATE high-pH treatment for operation in pH 2-12
- INERT column hardware
- 120 Å and 1000 Å pore sizes available
- 1,000 bar operating pressures

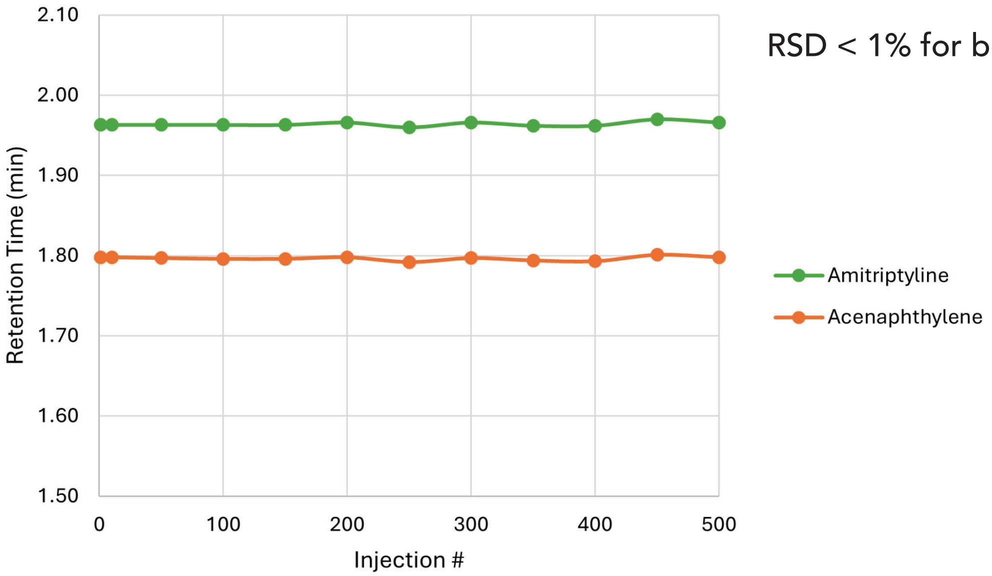


120 Å



1000 Å

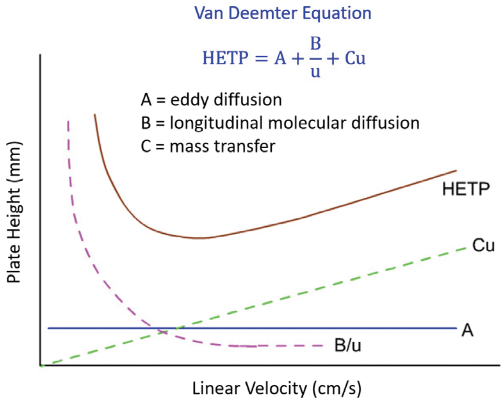
STABILITY DATA



RSD < 1% for both analytes

Testing the packing material stability that is used in the HALO® OLIGO C18 column, a less than 1% change in retention is achieved over 20,000 column volumes.
This stability run was performed at both **high pH (10) and high T (60 °C).**

VAN DEEMTER EQUATION



The Van Deemter Equation determines chromatographic theoretical plate height based on flow and kinetic parameters that can cause peak broadening:

$$HETP = A + \frac{B}{\mu} + C \cdot \mu$$

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

Each term above has an impact on the efficiency of a column. For example, the A-term is improved via more efficient packing. Similarly, while longitudinal diffusion is reduced with increased linear velocity, mass Transfer, the ability for the analyte to have time enter the silica pores and interact with the surface bonding is reduced.

By combining all terms, an optimal linear velocity to minimize plate height can be determined. This is typically done using isocratic conditions.

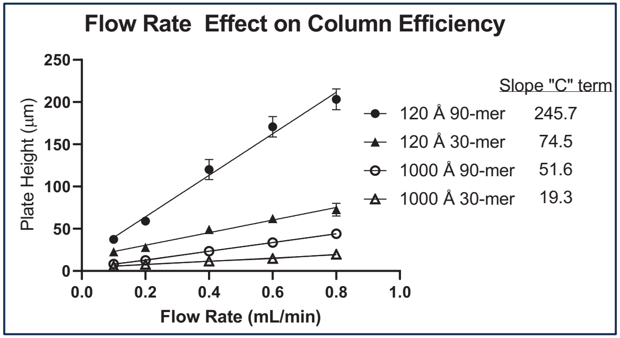
PORE SIZE AND MASS TRANSFER

Impact of Pore Size on Diffusion: Small pores restrict diffusion of large biomolecules, reducing column efficiency in chromatography.

Optimal Pore Sizes for Oligonucleotides: Optimal pore size generally scales with oligonucleotide length, with shorter oligos favoring smaller pores and longer oligos requiring larger pores to minimize steric hindrance and maintain efficient mass transfer.

Trade-offs in Pore Size Selection: Increasing pore size reduces surface area, affecting loading capacity and retention.

Mass Transfer Optimization: Understanding mass transfer dynamics aids in selecting columns for efficient and robust oligonucleotide separation.

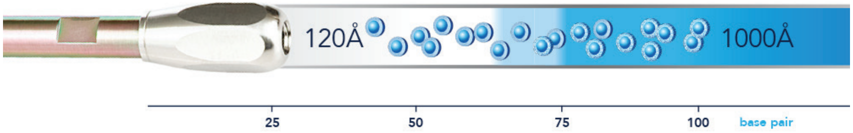


The figure above demonstrates the importance of pore size for separation. As oligonucleotides get longer, diffusion into pores becomes more challenging and column performance suffers. Larger pores allow easier diffusion, particularly as flow rates aka linear velocity increases.

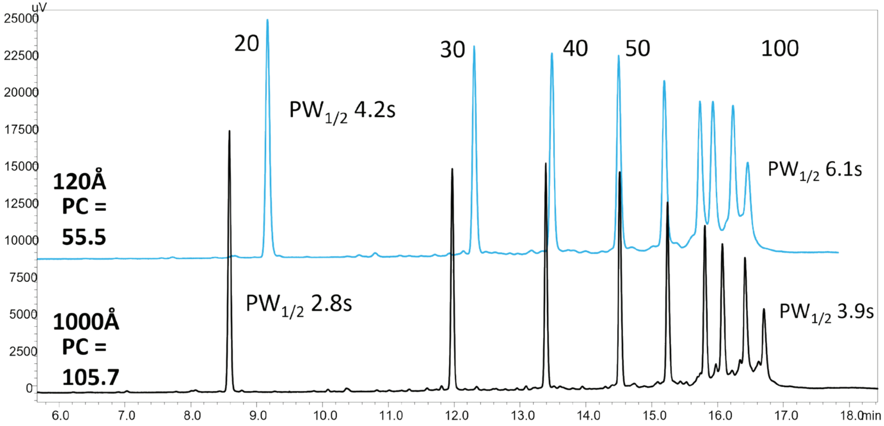
PEAK CAPACITY COMPARISON FOR ssDNA LADDER

Selection of the proper pore and column size for your oligonucleotide application can depend on several considerations:

- Oligo Size
- Loading Capacity Required
- Desired Run Time



For small oligos up to 10-60 bases or bp, the 120 Å is an excellent solution with good loading capacity. As oligo size enters the range of 30-60 bases or bp, both the 120 Å and 1000 Å may be good choices. As oligos increase above 60 basesor bp. The 1000 Å will be the best choice for your separation requirements.



TEST CONDITIONS:

Columns: HALO 120 Å OLIGO C18, 2.1x100mm, INERT
HALO 1000 Å OLIGO C18, 2.1x100mm, INERT
Mobile Phase A: 15 mM TEA/50 mM HFIP, pH 8.9
Mobile Phase B: Acetonitrile
Gradient:

Time	%B
0.0	1.5
20	6.5
21	15.0
22	15.0
22.5	1.5
30	Stop

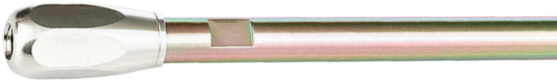
Flow Rate: 0.5 mL/min.
Temperature: 60 °C
Detection: 260nm, 10nm
Sample: 1 µL, 20/100 IDT @ 10ng

PREVENTING METAL ADDUCTION

In IP/RP buffers, oligonucleotides typically have a net negative charge on the phosphate backbone. These negatively charged sites can attract a variety of adduction ions including sodium, potassium, and metal cations from interaction and absorption onto metal and glass surfaces from the HPLC tubing, the column hardware, and even from glass bottle storage. The longer the oligonucleotide to be analyzed, the greater the risk of attracting positively charged metal ions. This can negatively impact your sample recovery and needlessly complicate your LCMS analysis. Some common ways to minimize adduction include:

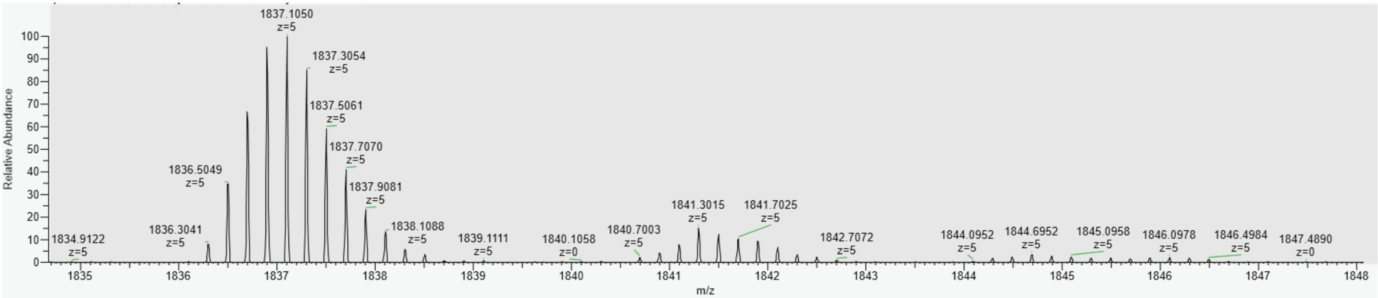
- Using glassware that is acid/EDTA treated during washing or using polypropylene bottles which are less likely to leach alkali metals
- Passivation of HPLC systems using phosphoric acid (Note: please use care when passivating an HPLC system. Follow directions carefully and use proper safety.) 50/50 MeOH/Type-1 H_2O + 0.1% LCMS grade formic acid is also useful as a system flush
- Use of high grade solvents (typically LCMS grade, including HFIP) 18 MΩ type-1 water is preferred over bottled LCMS grade water due to risk of alkali metals leaching from borosilicate storage bottles

HALO® INERT COLUMNS



To help minimize metal adduction to analytes such as oligonucleotides, HALO® offers stainless steel hardware that has a special inert coating to minimize adduction and adsorption of negatively charged analytes.

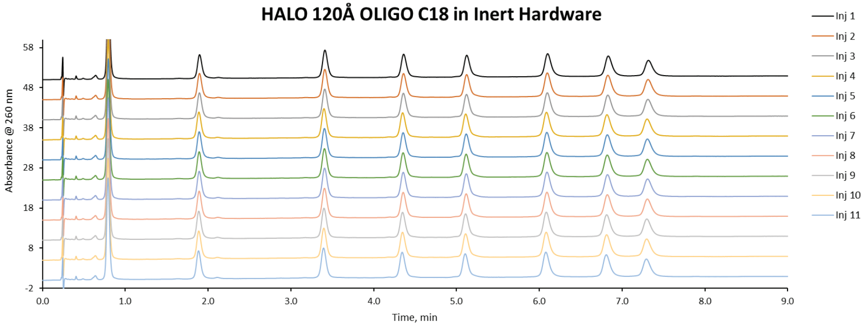
INERT hardware dramatically improves peak shape and analyte retention by minimizing secondary interactions with metal surfaces.



HRMS scan of 30-mer ssDNA showing Na+ and K+ adducts of the [-5] charge state

INERT VS NON-INERT HARDWARE

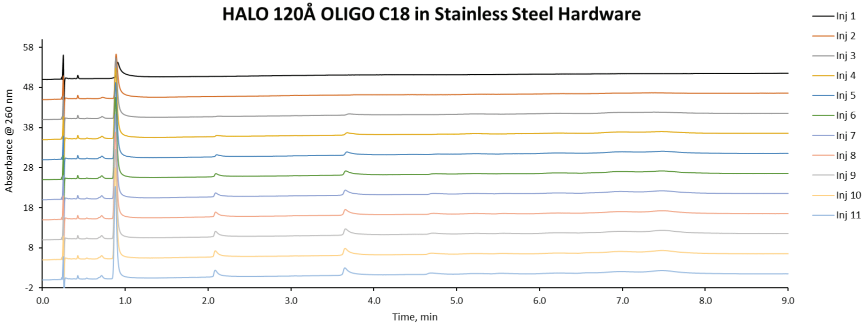
ssDNA Ladder run on non-INERT vs INERT Hardware 11 Consecutive Injections


HALO 120Å OLIGO C18 in Inert Hardware

TEST CONDITIONS:

Mobile Phase A: 25mM HAA @ pH 7.0

Mobile Phase B: 50/50 25mM HAA/ Acetonitrile


HALO 120Å OLIGO C18 in Stainless Steel Hardware

SAMPLE CARE AND HANDLING OF OLIGONUCLEOTIDES

- Oligonucleotides are typically shipped lyophilized and should be stored at -20° C or -80 °C
- Resuspension of oligos should be done in nuclease-free water or in nuclease-free Tris-EDTA (TE) buffer
- After resuspension, aliquot and freeze oligos to minimize multiple freeze-thaw cycles that can degrade the sample
- Resuspended DNA can be stored at -20 °C
- Resuspended RNA is considerably more fragile and susceptible to nucleases and should be frozen at -80 °C for storage
- Both DNA and RNA samples should be handled using nitrile gloves and nuclease-free pipette tips and plasticware
- Unmodified RNA samples may require additional considerations, including adding ribonuclease inhibitors to the sample or spraying gloves with Thermo RnaseZap^{TM} in conjunction with operating in sterile conditions in a culture hood

STRATEGIES FOR METHOD DEVELOPMENT

Separation Objectives
Impurity analysis, full length mass confirmation, quantitation via LCMS, etc.

Type of Oligonucleotide
DNA, RNA, Modifications?

Oligonucleotide Length

Column Selection
120Å or 1000Å Column Length

Mobile Phase Composition
Ion pairing agent and concentration, MS friendly HFIP?, pH

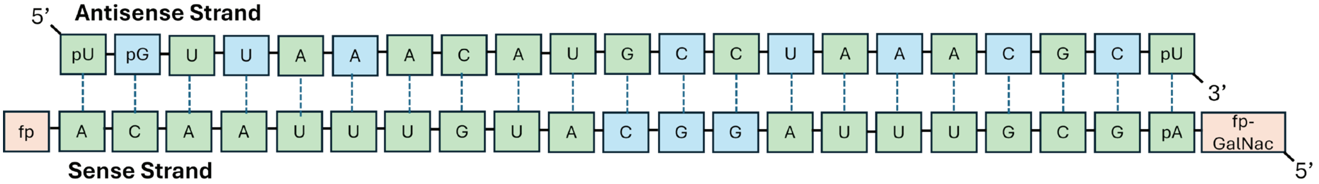
Gradient Design
Temperature, flow rate, gradient slope

Scouting Gradient
For most oligos 5-25% ACN is an excellent starting point

Refine Separation Parameters
Shallow gradient, adjust flow rate, temperature, longer column length required?

METHOD DEVELOPMENT EXAMPLE: **Fazirsiran siRNA**

As an example of method development, we have chosen a recently developed siRNA, Fazirsiran. Fazirsiran, like many siRNA's has a GalNac conjugation linked on the sense strand for targeting to hepatocytes. Both strands are heavily modified with 2'-F, 2'-OMe, and phosphorothioates on the ends to slow degradation. In addition, the sense strand contains two inverted bases, where the furan sugar ring is inverted. This combination of modifications help with tissue targeting, increasing half-life, and reducing the risk of inducing an unwanted immune response.



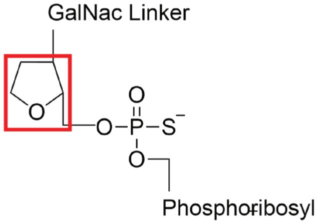
🟩 2'-OMe Modification

🟦 2'-F Modification

p phosphorothioation

fp Inverted abasic phosphorothioation

GalNac N-acetylgalactosamine conjugated linker



5'-Sense strand
Inverted abasic furan
With phosphorothioation

SAMPLE CONSIDERATIONS: SPECIFIC APPROACH

Separation Objectives
Impurity analysis, full length mass confirmation, quantitation via LCMS, etc.

Type of Oligonucleotide
DNA, RNA, Modifications?

Oligonucleotide Length

Column Selection
120Å or 1000Å Column Length

- 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 HALO 1000 Å OLIGO C18 column in a 2.1x150mm format.

METHOD DEVELOPMENT OPTIONS

Mobile Phase Composition
Ion pairing agent and concentration, MS friendly HFIP?, pH

Gradient Design
Temperature, flow rate, gradient slope

Scouting Gradient
For most oligos 5-25% ACN is an excellent starting point

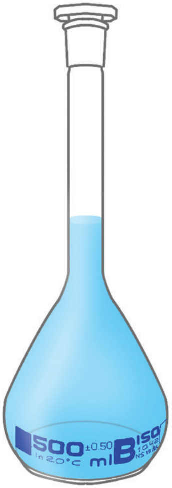
Refine Separation Parameters
Shallow gradient, adjust flow rate, temperature, longer column length required?

- We wanted to begin with a simple buffer condition that was UV friendly but not MS friendly, 100mM TEAA to determine how challenging our separation would be, then move to a more MS friendly buffer containing HFIP.
- We want to begin at a temperature where we know that strand melting is likely to occur. Thus we began at 60 °C and a flow rate that is optimal for our column, 0.3ml/min. The HALO 1000 Å OLIGO C18 column is much more tolerant of changes in solvent linear velocity.
- This is where we began our scouting, at 5-25%, then we will begin to refine parameters.
- Finally, we come to a method condition of IP reagent, concentration, temperature, pH, flow rate, etc. that maximizes separation in a reasonable time.

GRAVIMETRIC PRECISION FOR BUFFERS

Making mobile phases for IP/RP oligonucleotide separations can be a complex operation. Many of the components have solubility issues and small changes in the concentration of each can have a significant impact on retention time and separation quality. Because precision is critical for reproducibility, we recommend making buffers gravimetrically (via weight) rather than volumetrically (via volume).

Ideally, this should be done using a scale that can weigh reagents out to +/- 1 milligram. We also recommend having an ultrasonication bath and a stirring plate which also help with solubilization. An example recipe is listed on the next page. Densities and other chemical properties of each component are readily available at the NIST Chemistry WebBook.



WARNING!!

Many of the ion pairing reagents as well as HFIP are potentially toxic and proper care should be used in handling. This includes using proper PPE and handling chemicals in a suitable fume hood.

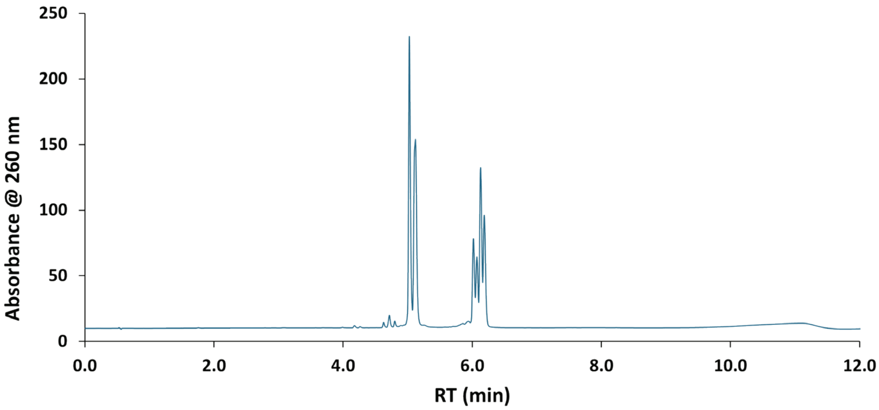
EXAMPLE PROCEDURE FOR MOBILE PHASE PREPARATION: TRIAL 2

The following is an example procedure for making a 1 liter buffer containing 10mM Diisobutylamine (DiBA), 100mM HFIP, 5% Methanol and 5% ACN for Mobile Phase A and B. The 5% Methanol is added to improve solubility of higher concentrations of HFIP:

1. Thoroughly clean and rinse volumetric flask before use
2. Place volumetric flask on scale and Tare to 0
3. Add Diisobutylamine (DiBA)
    a. DiBA: 129.34 g/mol ; Density = 0.74 g/ml
    b. .01 mol x 129.243 g/mol = **Add 1.292g DiBA to Volumetric flask**
4. Add 150-200ml LC/MS grade H_2O. Mix Well
5. Tare scale to 0
6. Add 5% by weight LC/MS grade methanol **(50ml * 0.792g/ml = 39.6g)**
7. Tare scale to 0
8. Add Hexafluoroisopropanol (HFIP) by weight
    a. HFIP: 168.038 g/mol; density: 1.596 g/ml
    b. 0.1mol * 168.038g/mol = **Add 16.804 g HFIP to Volumetric Flask**
9. Tare scale to 0
10. Add LC/MS grade acetonitrile (ACN) by weight
    a. **For buffer A: 5% (50ml * 0.786g/ml = 39.3g)**
    b. **For buffer B: 20% (200ml * 0.786g/ml = 157.2g)**
11. Add LC/MS grade H_2O to 900-950ml in volumetric flask
12. Sonicate volumetric flask for 5 minutes
13. Close volumetric flask and invert several times
14. Repeat steps 12 and 13 until HFIP is completely dissolved
15. Add stir bar and stir for 10 minutes
16. Repeat 5 minute sonication.
17. Let sit for air bubbles to settle out of solution
18. Add LCMS grade H_2O to 1L line in volumetric flask
19. Rinse HPLC bottle with LCMS grade H_2O
20. Transfer Oligo buffer to HPLC bottle

TRIAL 1: FAZIRSIRAN INITIAL TESTING CONDITIONS

Initial testing on a 2.1x100mm 1000 Å column in 100mM TEAA showed good separation of strands at 60 °C. The peak splitting is believed to be individual populations of phosphorothioates. However, we need to find HFIP buffer conditions to confirm via LC/MS.



TEST CONDITIONS:

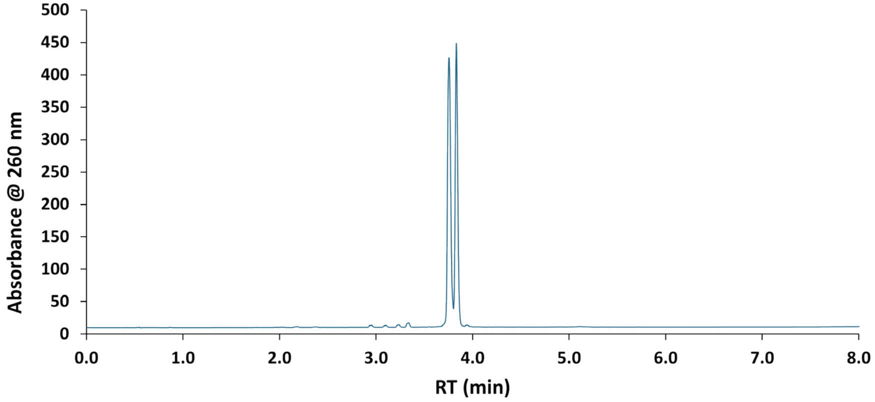
Column: HALO 1000 Å OLIGO C18, 2.7 µm, 2.1x100mm
Mobile Phase A: 100mM TEAA, pH 7.0
Mobile Phase B: ACN
Gradient:

Time	%B
0.0	8
10.0	18

Flow Rate: 0.4 mL/min.
Backpressure: 222 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

TRIAL 2: DiBA-HFIP FAZIRSIRAN

Changing to a alkylamine with medium hydrophobicity with 100mM HFIP demonstrated poor results.



TEST CONDITIONS:

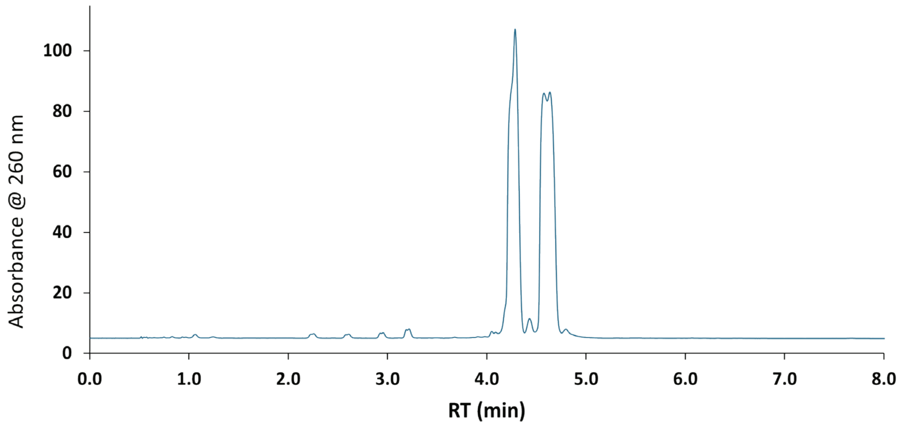
Column: HALO 1000 Å OLIGO C18, 2.7 µm, 2.1x100mm
Mobile Phase A: 10mM DiBA/100mM HFIP/5% MeOH
Mobile Phase B: ACN
Gradient:

Time	%B
0.0	8
10.0	30

Flow Rate: 0.4 mL/min.
Backpressure: 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

TRIAL 3: DiPEA-HFIP

Changing to a slightly more hydrophobic IP reagent but at a lower concentration, allows some separation of the strands. However, we are not yet seeing the same phosphorothioate separation of the TEAA conditions.



TEST CONDITIONS:

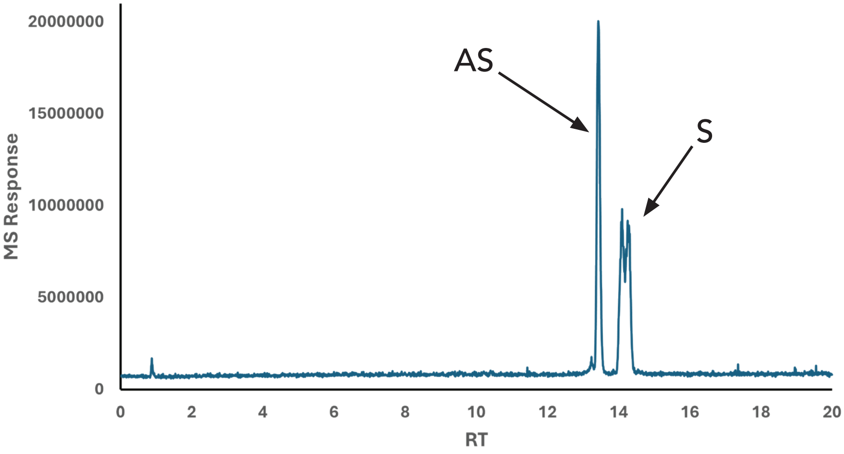
Column: HALO 1000 Å OLIGO C18, 2.7 µm, 2.1x100mm
Mobile Phase A: 3mM DiPEA/150mM HFIP/5% MeOH
Mobile Phase B: IPA/H_2O/MeOH 15/40/45
Gradient:

Time	%B
0.0	13
10.0	23

Flow Rate: 0.4 mL/min.
Backpressure: 223 bar
Temperature: 70 °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

FINAL RESULT: HEXYLAMINE-HFIP

Further optimization allowed for sharper peak shape and better separation of the strands. The Sense strand still shows separation of phosphorothioate populations. The identity of the strands was confirmed via HRMS. With these conditions, we achieve baseline separation of the strands, and good signal to noise.



TEST CONDITIONS:

Column: HALO 1000 Å OLIGO C18, 2.7 µm, 2.1x150mm
Mobile Phase A: 15mM Hexylamine, 10mM HFIP, 5% MeOH
Mobile Phase B: IPA/H_2O/MeOH 15/40/45

Gradient:	Time	%B
	0.0	35
	30.0	45

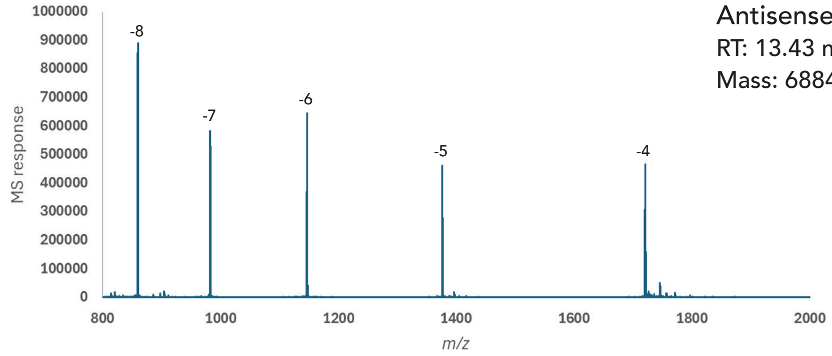
Flow Rate: 0.3 mL/min.
Backpressure: 250 bar
Temperature: 85 °C
Injection: 200 ng of 1 mg/mL Farzirisan
Sample Solvent: RNAse free water
Wavelength: PDA, 260 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.1 sec.
LC System: Shimadzu Nexera X2

MS CONDITIONS:

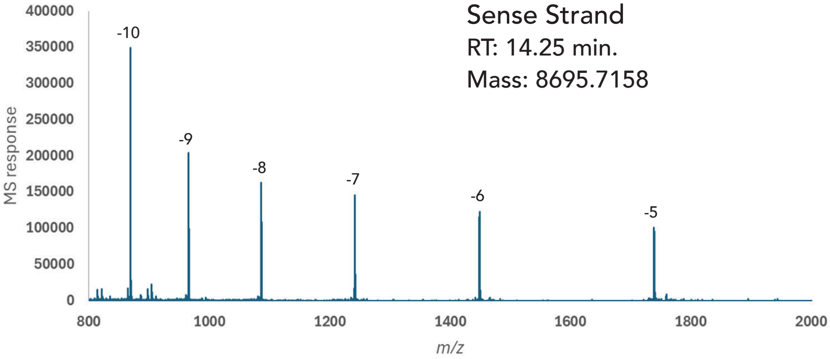
MS System: Thermo Q-Exactive HF
Polarity: Negative
Resolution: 120k
AGC Target: 3e6
Max IT: 200ms
Scan Range: 800-2000 *m/z*
Sheath Gas Flow Rate: 50
Aux Gas Flow Rate: 15
Sweep Gas Flow Rate: 1
Spray Voltage: 3.25kV
Capillary Temp: 350 °C
Aux Gas Heater Temp: 400 °C

FINAL RESULT: MS DATA OF FAZIRSIRAN

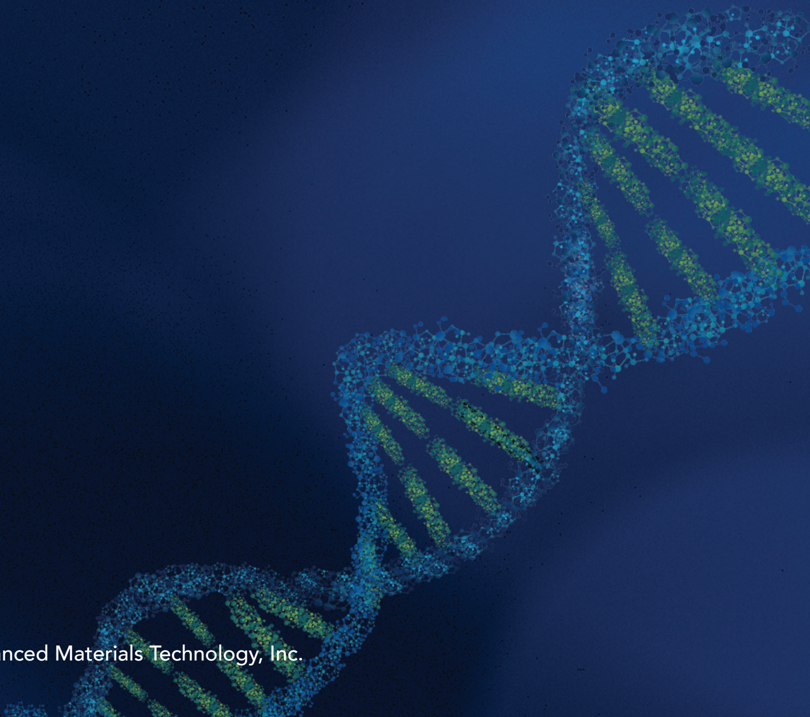
The MS1 spectra assist to identify the peaks. The peak eluting at 13.4 min is identified as the antisense strand with a MW of 6884.026. In contrast, the more complex eluting peak at 14.2 minutes is identified as the sense strand with a MW of 8695.716. The larger molecular weight representing the conjugated linker. Additionally, the two closely spaced peaks of the sense strand have the same mass, confirming the partial separation of the phosphorothioate species.



Antisense Strand
RT: 13.43 min.
Mass: 6884.026



Sense Strand
RT: 14.25 min.
Mass: 8695.7158



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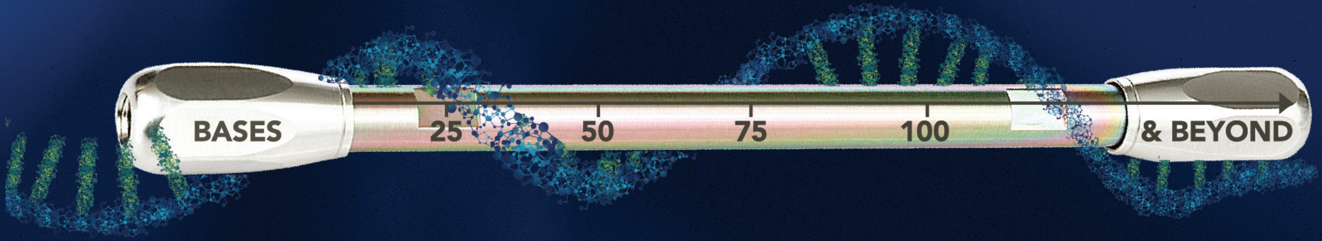
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