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Analysis & Purification of Therapeutic Oligonucleotides: Method Development Optimization from the Analytical Scale Through Semi-prep and Preparative Purification

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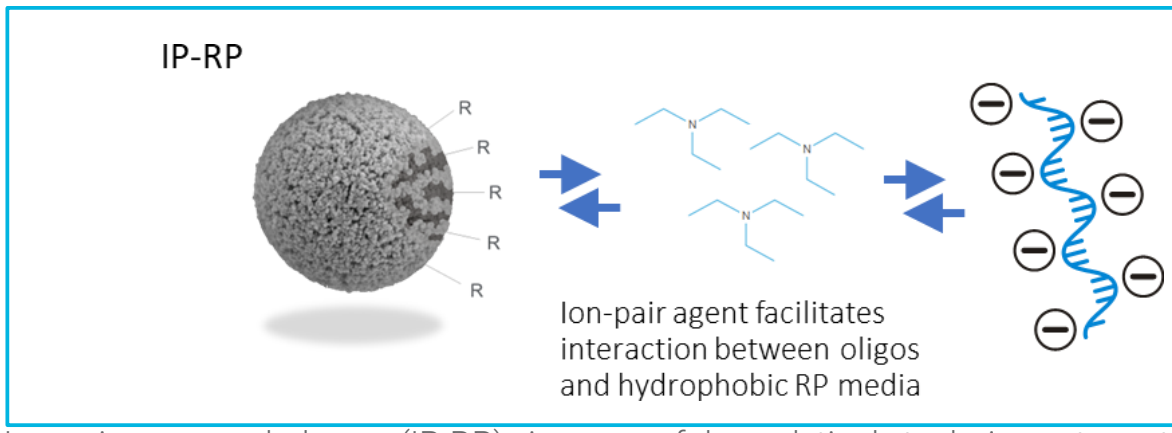
Introduction

Oligonucleotides are short nucleic acid sequences that regulate gene expression through the suppression or enhancement of target gene sequences. Investment in nucleic acid research in this market has led to great advancements in the potential to target different biological pathways and disease states, creating a fast growing and dynamic market.

Oligos can range in size, sequence complexity, and overall backbone modifications, creating significant analytical challenges. The step-wise process by which oligos are manufactured or synthesized leads to complex crude samples with closely related impurities or sequence failures. As a result, the analysis and purification of oligonucleotides by liquid chromatography plays a critical role throughout the entire drug development pipeline from discovery through production purification.

Experimental

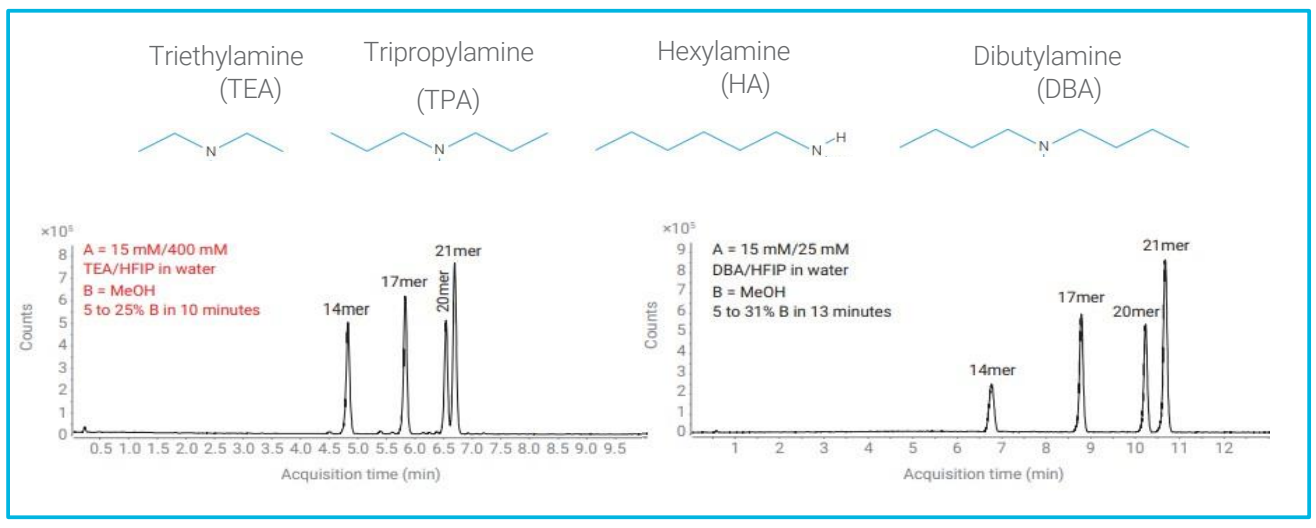
Understanding Ion-Pair Reversed-Phase (IP-RP)



Ion-pair reversed-phase (IP-RP) is a useful analytical technique to retain and separate charged molecules that would not normally be retained by a reversed-phase column. In the case of oligonucleotides, which are polar with an anionic backbone, the introduction of an alkylamine ion-pair agent facilitates an interaction with a C18 stationary phase. The positively charged nitrogen group creates an "ion pair" with the oligos' anionic backbone. The alkyl chain of the amine is retained by the hydrophobic C18 stationary phase, creating retention and separation on column.

When trying to optimize your IP-RP separations there are several factors or variables that you can work with; column temperature and the choice of your ion-pairing agent can impact the overall chromatography.

Ion-Pairing Effect on Oligo Resolution and Retention

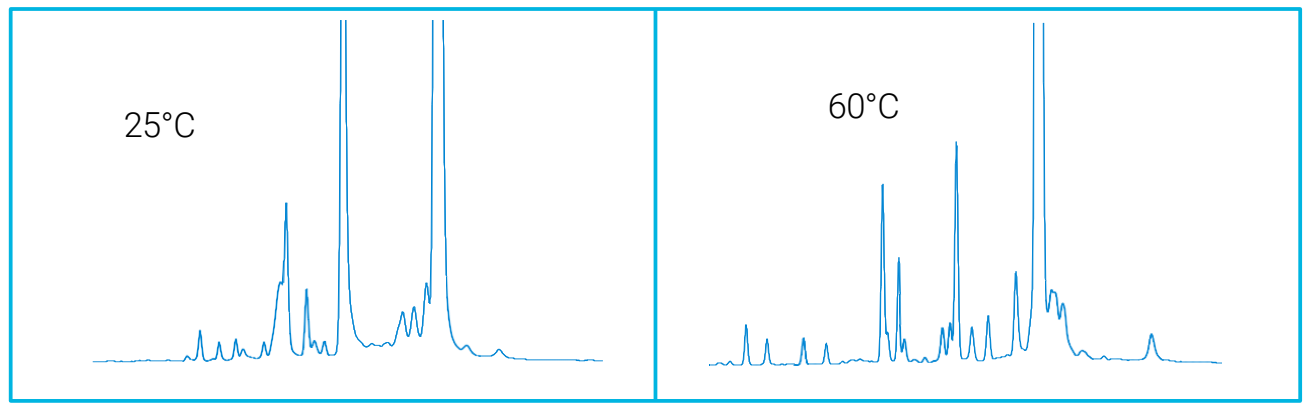


Significant improvement of resolution and sensitivity can be obtained by careful development and optimization of your method. Depending on the ion pairing reagent that you choose and the concentration, it will influence the retention and separation on column. More hydrophobic ion-pairing agents will increase retention and can be used to separate the target product from close eluting impurities.

Elevated temperature is often used for oligonucleotide analysis because it enables inter- and intramolecular secondary interactions to be overcome, leading to sharper peaks. This is illustrated in the following figure, comparing the separation at 25 °C and 60 °C. At elevated temperatures, the crude sample favors the full length product confirmation, and resolution from closely eluting impurities is improved. At higher temperatures, mobile phase viscosity is also reduced, which can further benefit resolution while lowering operating pressures.

Experimental

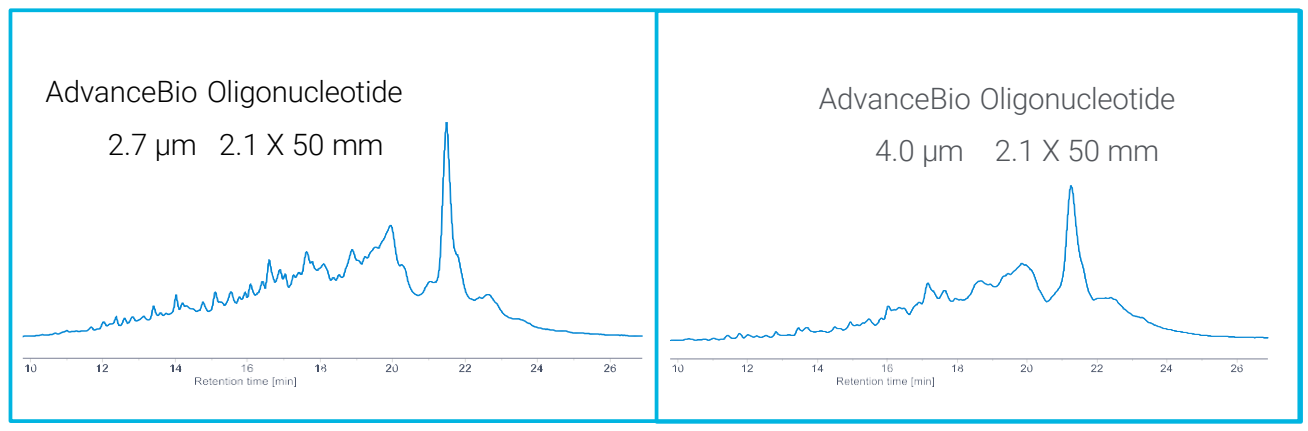
Effect of Temperature on Oligo Resolution



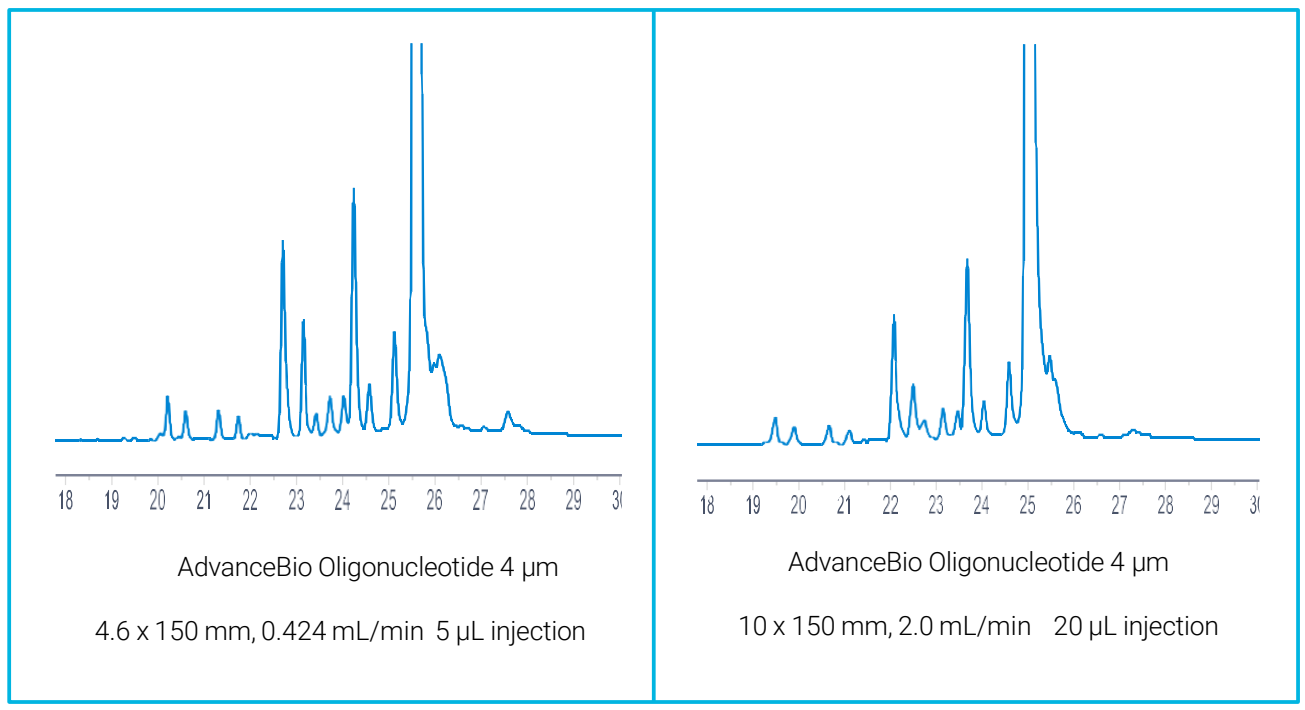
Results and Discussion

Scaling Up to Semi-prep & Prep Purifications

The ion pairing agents and elevated temperatures, require a particle support that is stable under high pH and high temperature. A traditional silica particle is not stable at high pH. A particle support is needed that can handle these harsh mobile phase conditions to be able to achieve the separation benefits these mobile phase optimizations offer. The AdvanceBio Oligonucleotide particle support leverages the resolution benefits that the Poroshell particle offers while including a novel surface chemistry that is stable under these harsh mobile phase conditions of high pH and temperature. When thinking about scaling up from the analytical scale to preparative purification, the ability to maintain column chemistry and particle size greatly simplifies the process of scaling across column internal dimensions.



As demonstrated in the figure above, the AdvanceBio Oligonucleotide column chemistry is easily scalable across the 2.7 and 4 µm particle sizes for a crude sgRNA sample allowing for easy method transfer and scale up.



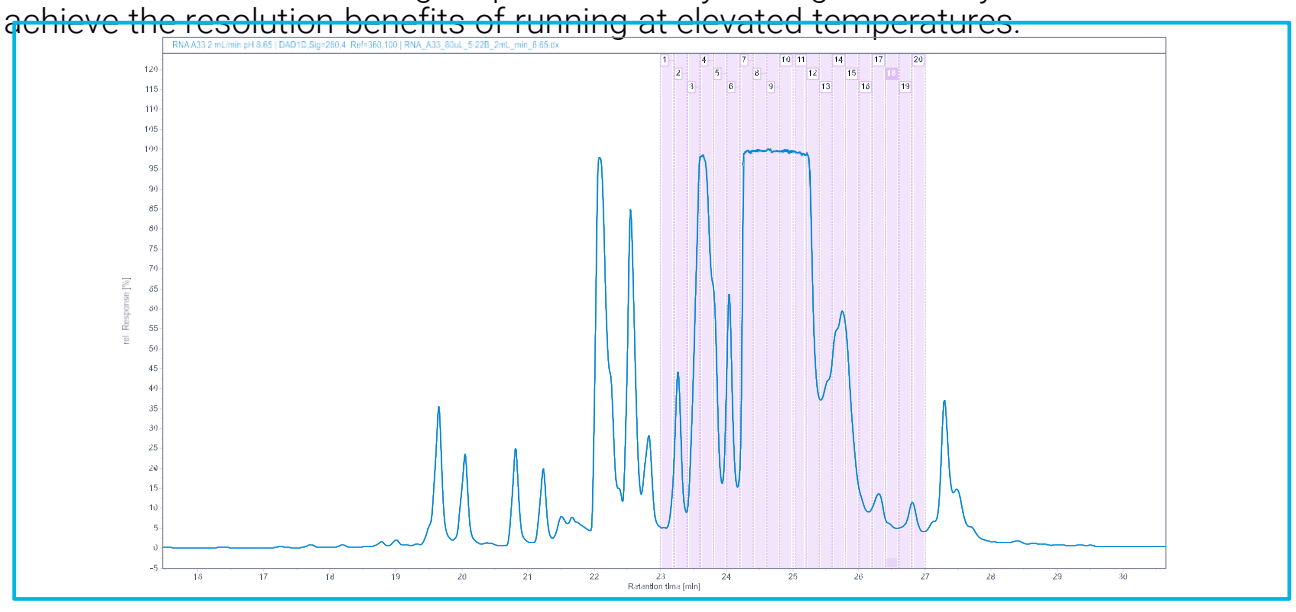
After optimizing the analytical method, it can be easily scaled to larger column formats for larger sample injections. The amount of sample that can be injected on column is directly related to the column volume, determined by column inner diameter (ID) and length, and surface area of the media itself. When maintaining the same column chemistry and column length, the method can be easily transferred within different column IDs. As is shown in the example above, the separation of a crude antisense oligo (ASO) on a 4.6 x 150mm column, is easily transferred to a semi-prep 10 x 150mm column dimension, for benchtop purification.

Results and Discussion

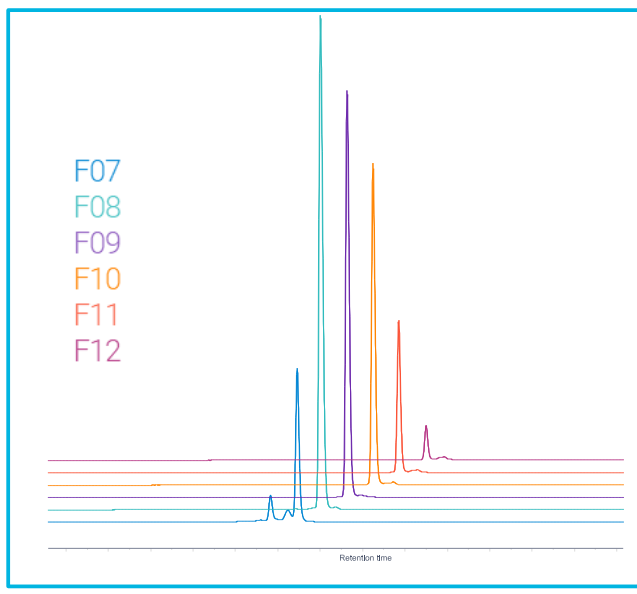
Maximize Your Resources: Semi-prep Benchtop Purification

Leverage the flexibility of the new AdvanceBio Oligonucleotide 10mm ID semi-prep columns paired with your Agilent analytical instrument for high resolution ion-pair reversed-phase separations for high yield and high purity benchtop purification.

Utilize the column heating capabilities of your Agilent analytical instrument to achieve the resolution benefits of running at elevated temperatures.



The figure above shows an optimized semi-prep separation of a crude oligo sample run at 60°C on an AdvanceBio Oligo 10x150mm column. Utilizing the gradient to focus the sample at the head of the column, a quad injection, of 160 µL (3.2 mg) total is loaded onto the column. The fractions were collected and analyzed on an analytical column to demonstrate purity and yield in the figure below.



Fraction	Product Purity	Peak Yield
F07	65.2%	9.4%
F08	99.2%	33.4%
F09	98.9%	25.9%
F10	97.9%	20.2%
F11	76.8%	9.0%
F12	46.9%	2.0%

Conclusions

AdvanceBio Oligonucleotide Scalar, Semi-prep, & Preparative Columns

New Agilent AdvanceBio Oligonucleotide columns deliver high resolution separations critical for accurate sequence confirmation of target product and closely related impurities at the analytical scale. Agilent's portfolio of AdvanceBio Oligonucleotide columns enable characterization and purification workflows of small and large scale oligonucleotides from siRNAs to larger CRIPSR guide RNAs.

- Simplify method development when scaling up from analytical to purification with scalar, semi-prep and preparative dimensions to achieve high yield purification.

- Whether you want to purify small quantities for bench-top research, or are needing ultimate purity for translational applications, Agilent's broad portfolio of AdvanceBio Oligonucleotide columns cover you from analytical to purification.

References

1. Superficially Porous Columns for Semi-Preparative Purification of Synthetic Oligonucleotides. Agilent Technologies application note, publication number 5994-7478EN, 2024.

2. Fast and Selective Purification of Oligonucleotides Using Preparative HPLC/MS and Software Support. Agilent Technologies application note, publication number 5994-4877EN, 2022.

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