White Paper

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# Maximize Your Resources and Simplify Scale-up with AdvanceBio Oligonucleotide Columns

## Competitive comparison from analytical to semipreparative purification


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## Introduction

The Agilent AdvanceBio Oligonucleotide product family offers a high-efficiency, high-resolution, superficially porous particle chemistry that is available in 2.7 and 4 µm particle sizes with 120 Å pore size. It is a fully scalable ion pair reversed-phase column chemistry platform with dimensional offerings from analytical through 21.2 mm id preparative. Ion pair reversed-phase (IP-RP) chromatography stands out as a prevalent method for analyzing and purifying oligonucleotides on a small scale. Oligonucleotides are polar substances containing numerous anionic groups, usually one phosphate group per nucleotide. If you need increased resolution and separation of your full-length synthetic oligonucleotide from closely related impurities for characterization, AdvanceBio Oligonucleotide is the ideal choice.

This work evaluated the performance of the Agilent AdvanceBio Oligonucleotide 4 µm column chemistry versus the Waters XBridge BEH C18 OBD 5µm Prep Column. The Waters BEH C18 is a fully porous particle with a higher internal surface area than the superficially porous AdvanceBio particle, which may affect the binding capacity, or the amount of sample that can be loaded onto the column. The increased surface area of the fully porous particles comes with the trade-off of lower column efficiency compared to a superficially porous particle of the same size. The data demonstrate that, when purifying the same crude oligo sample, the Agilent AdvanceBio Oligonucleotide column can achieve higher resolution and, as a direct result, better yield and purity compared to the Waters XBridge BEH C18 OBD. This is without significant loss of sample capacity.

## Experimental

### Analytical columns

- Agilent AdvanceBio Oligonucleotide, 4.6 × 150 mm, 2.7 µm (part number 653950-702)
- Agilent AdvanceBio Oligonucleotide, 4.6 × 150 mm, 4 µm (part number 693971-702)

### Semipreparative columns

- Agilent AdvanceBio Oligonucleotide, 10 × 50 mm, 4 µm (part number 639750-702)
- Agilent AdvanceBio Oligonucleotide, 10 × 150 mm, 4 µm (part number 633750-702)
- Waters XBridge BEH C18 OBD, 10 × 150 mm, 5 µm

### Instrument

All experiments were carried out on an Agilent 1290 Infinity II analytical system comprising the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler with sample thermostat (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7117C) with a 10 mm InfinityLab Max-Light cartridge cell (G7117-60020)
- Agilent 1260 Infinity bio-inert analytical-scale fraction collector (G5664A)

| Mobile Phase | |
|---|---|
| **Method 1 – Agilent Suggested Conditions** | |
| Eluent A1 | 100 mL 1M TEAA + 900 mL water, pH 8.65 |
| Eluent B1 | 100 mL 1M TEAA + 900 mL acetonitrile (ACN) |
| **Method 2 – Waters Suggested Conditions** | |
| Eluent A2 | 100 mL 1M TEAA + 900 mL water, pH 7.00 |
| Eluent B2 | 75% Eluent A + 25% ACN |
| Gradient | As described in Tables 1, 2, and 3 |
| Flow Rate | Analytical: 1 mL/min<br>Semipreparative: 2 mL/min |
| Column Temperature | 60 °C, Agilent InfinityLab Quick Connect heat exchanger, 0.17 mm id (G7116-60500) |
| Injection Volume | 4 × 40 µL of 20 mg/mL oligonucleotide was injected (3.2 mg of sample loaded) |
| Sample Loop | Sample loop assembly, stainless steel (G4267-60500) |
| Sample | Crude all-2'-O-methylated 22-mer |

**Table 1.** Gradient profile for semipreparative runs using method 1.

| Time | %A | %B | %ACN | TEAA, M |
|---|---|---|---|---|
| 0 | 94.5% | 5.5% | 5.0% | 0.10 |
| 5 | 94.5% | 5.5% | 5.0% | 0.10 |
| 35 | 77.8% | 22.2% | 20.0% | 0.10 |
| 40 | 16.7% | 83.3% | 75.0% | 0.10 |
| 45 | 16.7% | 83.3% | 75.0% | 0.10 |
| 50 | 94.5% | 5.5% | 5.0% | 0.10 |
| 60 | 94.5% | 5.5% | 5.0% | 0.10 |

**Table 2.** Gradient profile for semipreparative runs using method 2.

| Time | %A | %B | %ACN | TEAA, M |
|---|---|---|---|---|
| 0 | 75.0% | 25.0% | 5.0% | 0.094 |
| 5 | 75.0% | 25.0% | 5.0% | 0.094 |
| 35 | 0.0% | 100.0% | 20.0% | 0.075 |
| 40 | 0.0% | 100.0% | 20.0% | 0.075 |
| 45 | 0.0% | 100.0% | 20.0% | 0.075 |
| 50 | 75.0% | 25.0% | 5.0% | 0.094 |
| 60 | 75.0% | 25.0% | 5.0% | 0.094 |

**Table 3.** Gradient profile for analysis of fractions using method 1.

| Time | %A | %B | %ACN | TEAA, M |
|---|---|---|---|---|
| 0 | 94.5% | 5.5% | 5.0% | 0.10 |
| 20 | 77.8% | 22.2% | 20.0% | 0.10 |
| 21 | 16.7% | 83.3% | 75.0% | 0.10 |
| 25 | 16.7% | 83.3% | 75.0% | 0.10 |
| 26 | 94.5% | 5.5% | 5.0% | 0.10 |
| 30 | 94.5% | 5.5% | 5.0% | 0.10 |

## Results and discussion

When preparing for oligonucleotide purification, taking the ultimate scale into consideration is crucial. The amount of oligonucleotide requiring purification dictates the column size and instrument setup needed. When scaling up from analytical to semipreparative or preparative columns, determining the optimum conditions required to maximize sample yield and purity is essential.

It is important to have a full view of the compound-related impurities present in the crude sample, so that there are no surprises when going from analytical to a larger scale of purification. A comparison between analytical columns packed with the same stationary phase and particle size as the larger semipreparative columns was performed. The optimized method conditions found in a previous study

(Agilent publication number 5994-7478EN) were used. Figures 1A and 1B demonstrate the excellent analytical separation of the crude all-2'-O-methylated 22-mer RNA molecule on both columns (Figure 1A, full length run time). Figure 1B shows the expanded view with the increased sensitivity and performance when using the AdvanceBio Oligonucleotide column. The superficially porous nature of this column means it can achieve a better separation for the most difficult impurities, especially those closest to the target molecule.

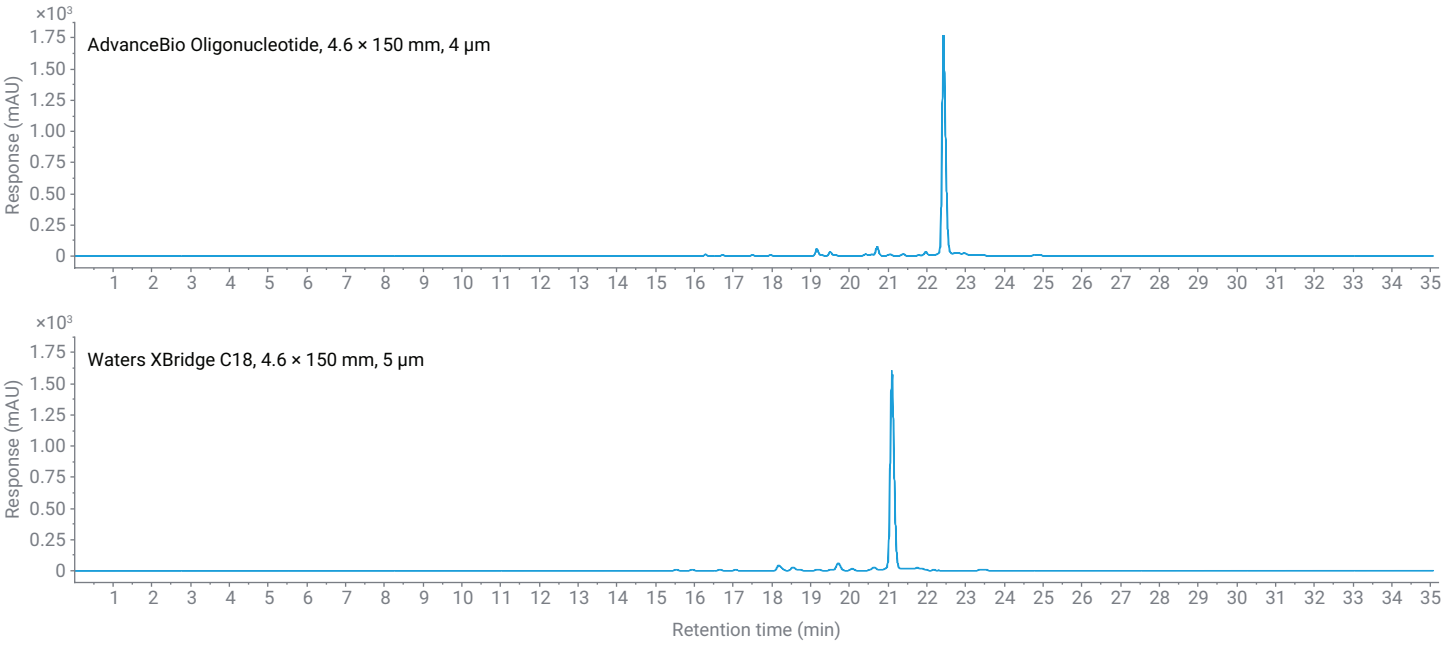


**Figure 1A.** Analytical LC/UV chromatograms (260 nm) using method 1 at 1.0 mL/min for the RNA oligonucleotide sample on a 4.6 × 150 mm, 4 µm Agilent AdvanceBio Oligonucleotide column, and a Waters XBridge BEH C18 OBD, 4.6 × 150 mm, 5 µm column.

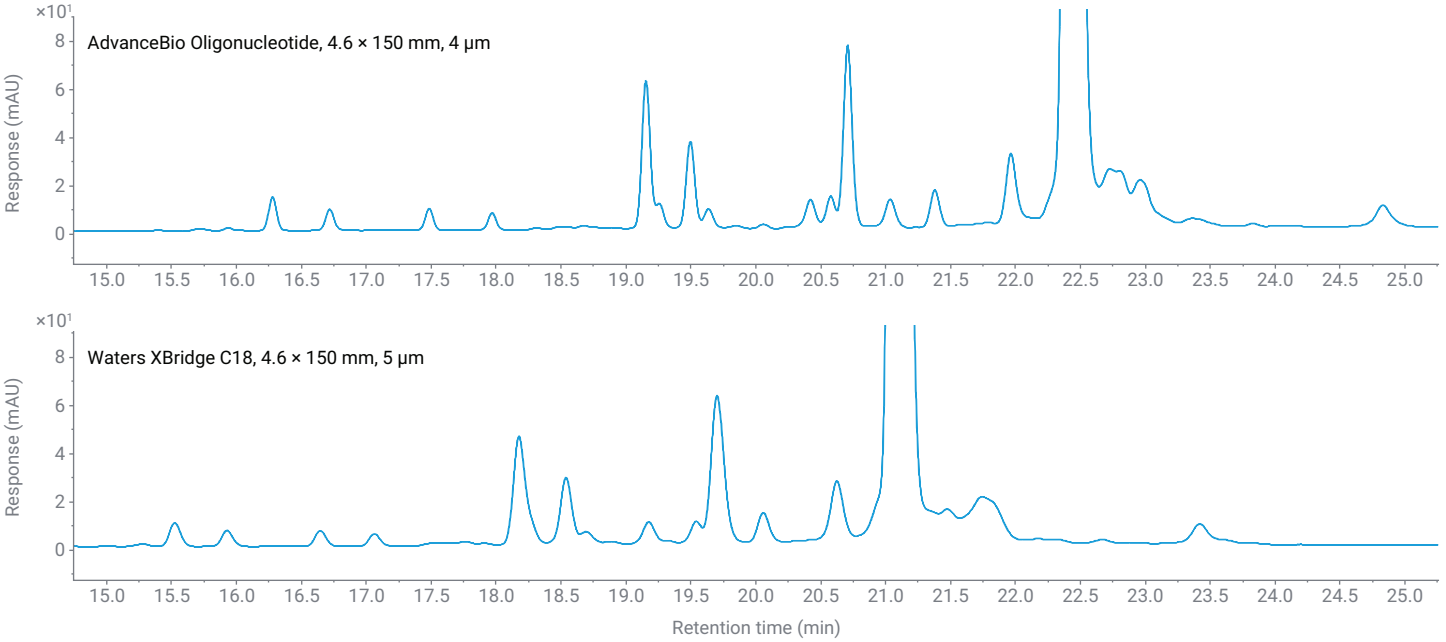


**Figure 1B.** A closer view of the analytical chromatograms (260 nm) showing the difference between the two columns. This highlights the increased performance when using Agilent AdvanceBio Oligonucleotide for the oligonucleotide under investigation.

## Adjust your gradient for best performance

For the purification runs, two methods were compared. Method 1 uses two mobile phase solutions that contain the same amount of ion-pair reagent in each, ensuring the concentration of ion-pair reagent remains constant throughout the gradient. In addition, eluent B contains 90% v/v ACN, which means the gradient used for each run can include a more effective "clean up" to higher organic solvent concentrations. This ensures that any strongly retained impurities potentially present are flushed from the column, and any carryover from run-to-run is minimized.

Method 2 is based on conditions often used in oligonucleotide separations where the amount of ACN is significantly reduced. However, the composition of eluent B (75% eluent A + 25% ACN) also means that the ion-pair reagent concentration changes during the gradient, and that the amount of ACN is restricted to just 25%. This means that the column may not be as rigorously cleaned during each run, potentially allowing a buildup of strongly retained impurities.

Following the investigation at analytical scale, and after reviewing the chromatographic profile of the crude oligonucleotide, the experiment then moved into the semipreparative scale purification. The same molecule was dissolved at a concentration of 20 mg/mL in eluent A (method 1). Figures 3A and 3B show two chromatographic comparisons for both columns. Chromatogram 3A highlights the higher resolution power obtained when using the AdvanceBio Oligonucleotide column under the same conditions, with better sensitivity and product recovery.

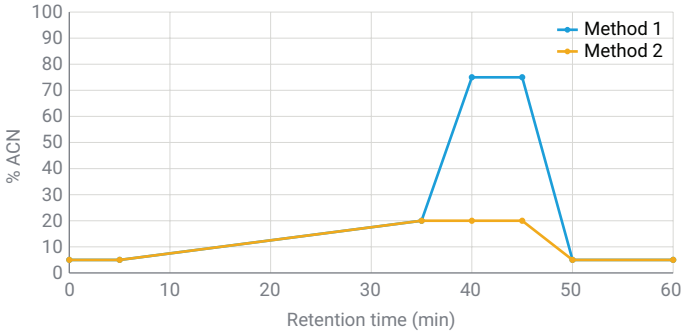


**Figure 2A.** Gradient profiles for the purification runs, showing ACN volume (%).

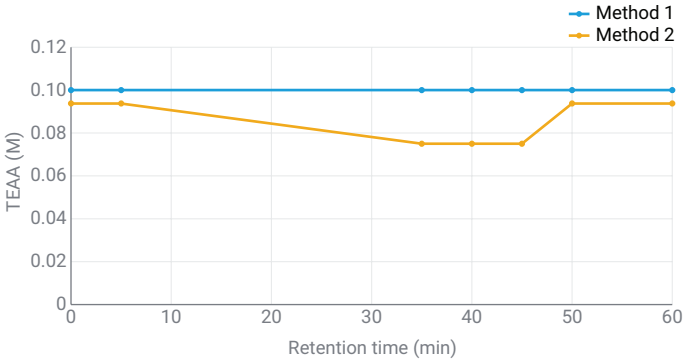


**Figure 2B.** Gradient profiles for the purification runs, showing TEAA concentration.

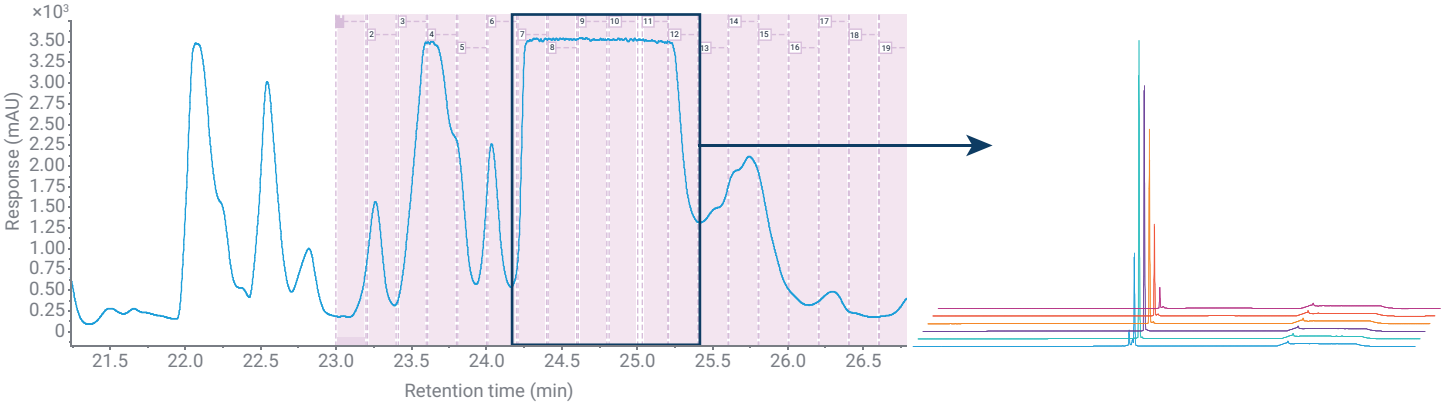


**Figure 3A.** Chromatogram (UV 260 nm) of a 160 µL (3.2 mg on column) injection on the semipreparative Agilent AdvanceBio Oligonucleotide column. The separation was performed using method 1 (Table 1) at 2.0 mL/min. The analysis of individual fractions was performed under method 1 conditions with a faster gradient (Table 3).

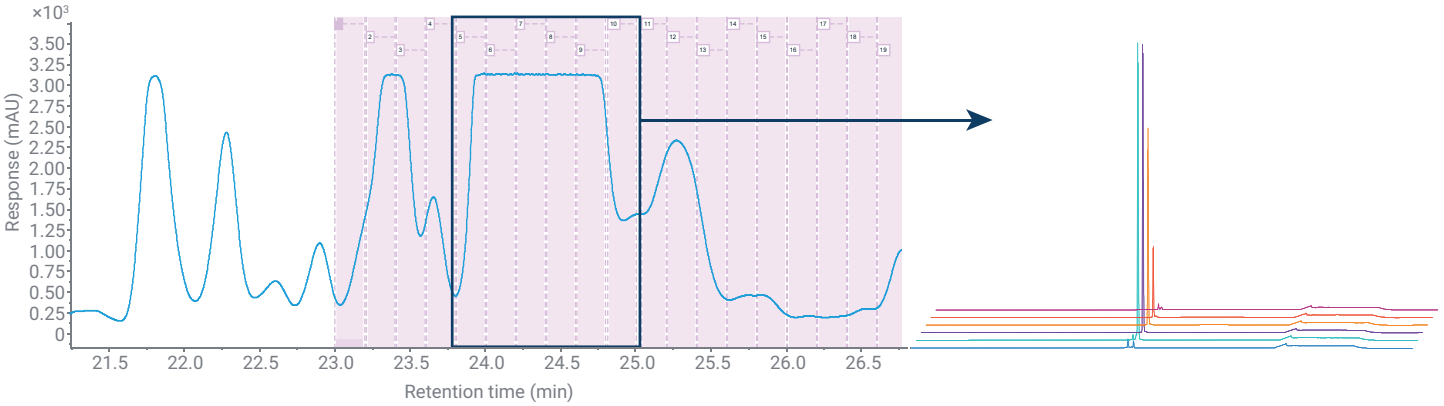


**Figure 3B.** Chromatogram (UV 260 nm) of a 160 µL (3.2 mg on column) injection on the semipreparative Waters XBridge BEH C18 OBD column. The separation was performed using method 1 (Table 1) at 2.0 mL/min. The analysis of individual fractions was performed under method 1 conditions with a faster gradient (Table 3).

Following the investigation on both semipreparative columns using method 1, the purification analysis was extended using the same oligonucleotide sample and amount to be purified with method 2 conditions (see Table 2 for gradient profile). Figures 4A and 4B illustrate the separation comparison on the two columns. With closer inspection, the separation using the AdvanceBio Oligonucleotide column demonstrates better resolution power for several impurity peaks (Figure 4A), although both columns show slight differences in selectivity compared to the method 1 approach. This is due to the changing ion-pair reagent concentration during the gradient.

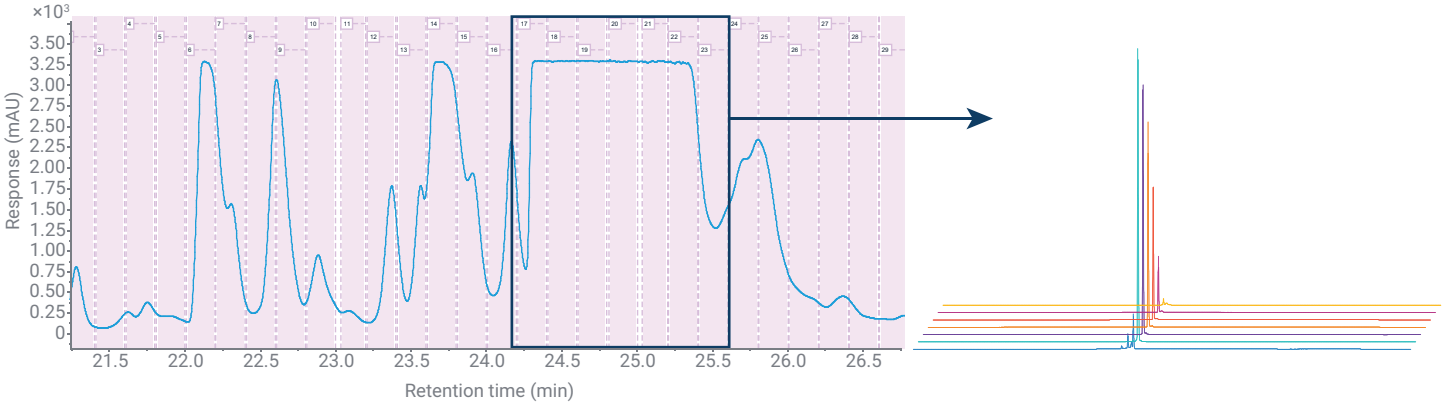


**Figure 4A.** Chromatogram (UV 260 nm) of a 160 µL (3.2 mg on column) injection on the semipreparative Agilent AdvanceBio Oligonucleotide column. The separation was performed using method 2 (Table 2) at 2.0 mL/min. The analysis of individual fractions was performed under method 1 conditions with a faster gradient (Table 3).

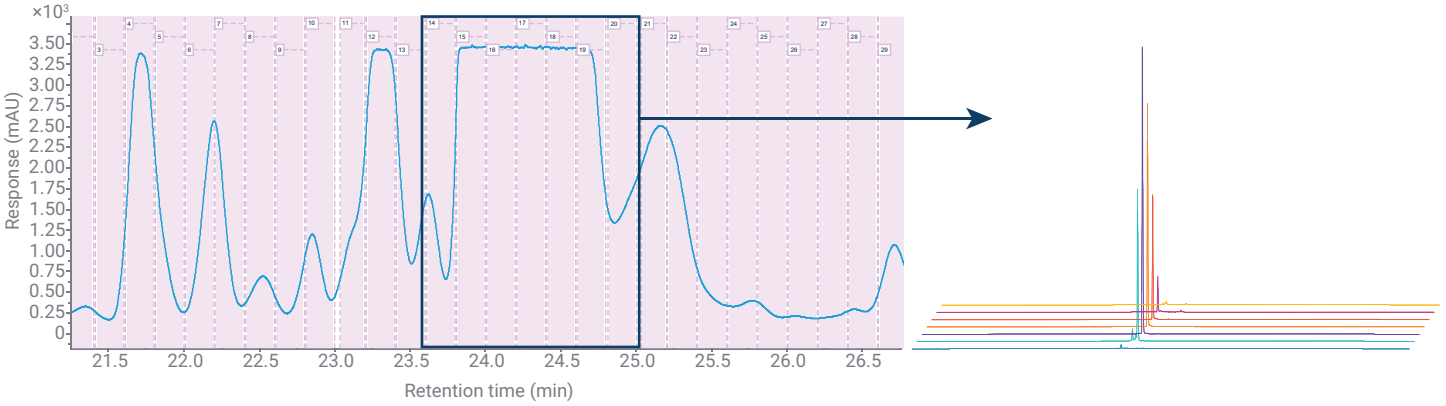


**Figure 4B.** Chromatogram (UV 260 nm) of a 160 µL (3.2 mg on column) injection on the semipreparative Waters XBridge BEH C18 OBD column. The separation was performed using method 2 (Table 2) at 2.0 mL/min. The analysis of individual fractions was performed under method 1 conditions with a faster gradient (Table 3).

The analysis results for individual fractions show that there are a larger number of fractions with higher purity using the AdvanceBio Oligonucleotide column than there are for the Waters XBridge BEH C18 OBD column (Figure 5), providing a greater yield of purified product.

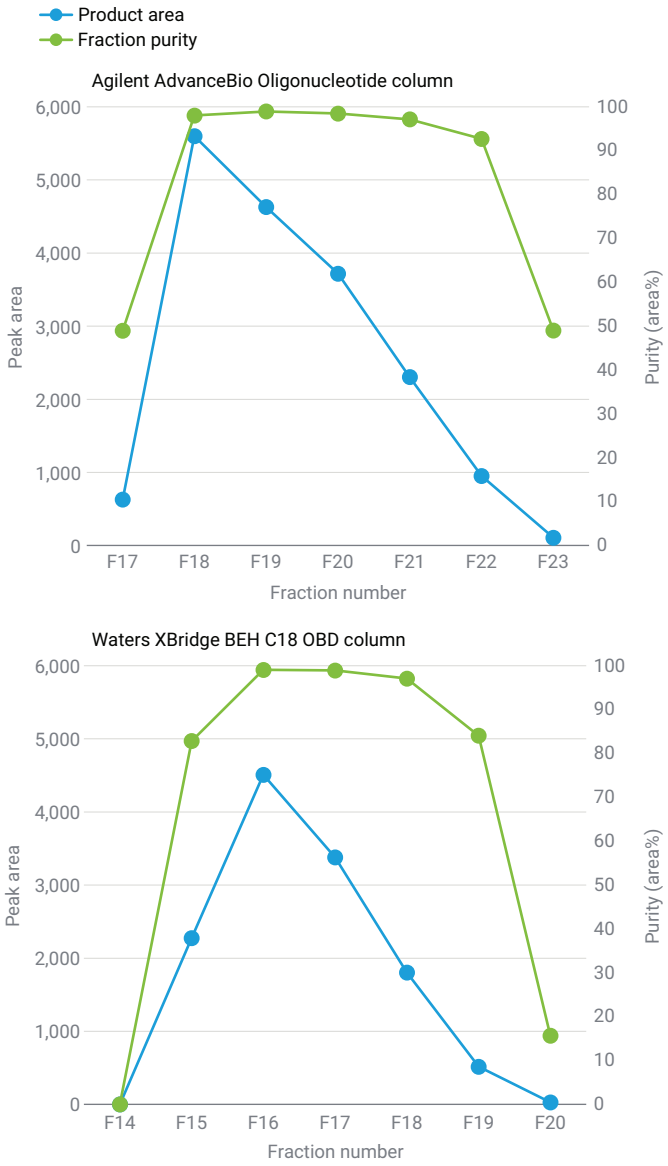


**Figure 5.** Fraction analysis details using method 2, showing peak area and %peak area for individual fractions.

## Conclusion

The Agilent 1290 Infinity II analytical-scale LC purification system, with the addition of a fraction collector, proved to be the ideal tool for larger-scale, semipreparative purification of a synthetic oligonucleotide. With only minor modifications to the autosampler, allowing larger injections, and the inclusion of flexible fraction collection (saving fractions into 1.5 mL glass vials), purification of 3.2 mg of the crude oligonucleotide was achieved. This investigation also highlighted that the superficially porous AdvanceBio Oligonucleotide 4 µm column provides the increased resolution, higher sensitivity, and greater selectivity needed to purify your target molecule in comparison to the Waters XBridge BEH C18 OBD 5 µm column. Finally, use of the same HPLC analytical system and the benefits of using the same particle size, from analytical to semipreparative and preparative scale, pave the way for a more accurate approach, with minimal risks when heading to higher-scale purification processes with your crude oligonucleotide sample.

## Ordering information

| Agilent AdvanceBio Oligonucleotide Columns | |
|---|---|
| **Description** | **Part Number** |
| **Analytical** | |
| AdvanceBio Oligonucleotide, 2.1 × 50 mm, 2.7 µm | 659750-702 |
| AdvanceBio Oligonucleotide, 2.1 × 100 mm, 2.7 µm | 655750-702 |
| AdvanceBio Oligonucleotide, 2.1 × 150 mm, 2.7 µm | 653750-702 |
| AdvanceBio Oligonucleotide, 4.6 × 50 mm, 2.7 µm | 659950-702 |
| AdvanceBio Oligonucleotide, 4.6 × 100 mm, 2.7 µm | 655950-702 |
| AdvanceBio Oligonucleotide, 4.6 × 150 mm, 2.7 µm | 653950-702 |
| **Scalar** | |
| AdvanceBio Oligonucleotide, 4.6 mm, guard, 4 µm | 820750-941 |
| AdvanceBio Oligonucleotide, 4.6 × 50 mm, 4 µm | 699971-702 |
| AdvanceBio Oligonucleotide, 4.6 × 100 mm, 4 µm | 695971-702 |
| AdvanceBio Oligonucleotide, 4.6 × 150 mm, 4 µm | 693971-702 |
| **Semipreparative** | |
| AdvanceBio Oligonucleotide, 10 × 50 mm, 2.7 µm | 639950-702 |
| AdvanceBio Oligonucleotide, 10 × 100 mm, 2.7 µm | 635950-702 |
| AdvanceBio Oligonucleotide, 10 × 150 mm, 2.7 µm | 633950-702 |
| AdvanceBio Oligonucleotide, 10 × 50 mm, 4 µm | 639750-702 |
| AdvanceBio Oligonucleotide, 10 × 100 mm, 4 µm | 635750-702 |
| AdvanceBio Oligonucleotide, 10 × 150 mm, 4 µm | 633750-702 |
| **Preparative** | |
| AdvanceBio Oligonucleotide, 21.2 × 50 mm, 4 µm | 671050-702 |
| AdvanceBio Oligonucleotide, 21.2 × 150 mm, 4 µm | 671150-702 |
| **Fast Guard** | |
| AdvanceBio Oligonucleotide, 2.1 mm, fast guard | 821725-921 |
| AdvanceBio Oligonucleotide, 4.6 mm, fast guard | 820750-921 |

**Want to learn more about superficially porous particles and how to scale up?**

Download these resources and discover the power of AdvanceBio Oligonucleotide:

- Superficially Porous Columns for Semi-Preparative Purification of Synthetic Oligonucleotides (Agilent publication number 5994-7478EN)
- Purification of Single-Stranded RNA Oligonucleotides Using High-Performance Liquid Chromatography (Agilent publication number 5994-3514EN)

**Supporting information:** Agilent Preparative LC Scaling Calculator

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