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アプリケーションノート

# HPLC and UPLC Columns for the Analysis of Oligonucleotides

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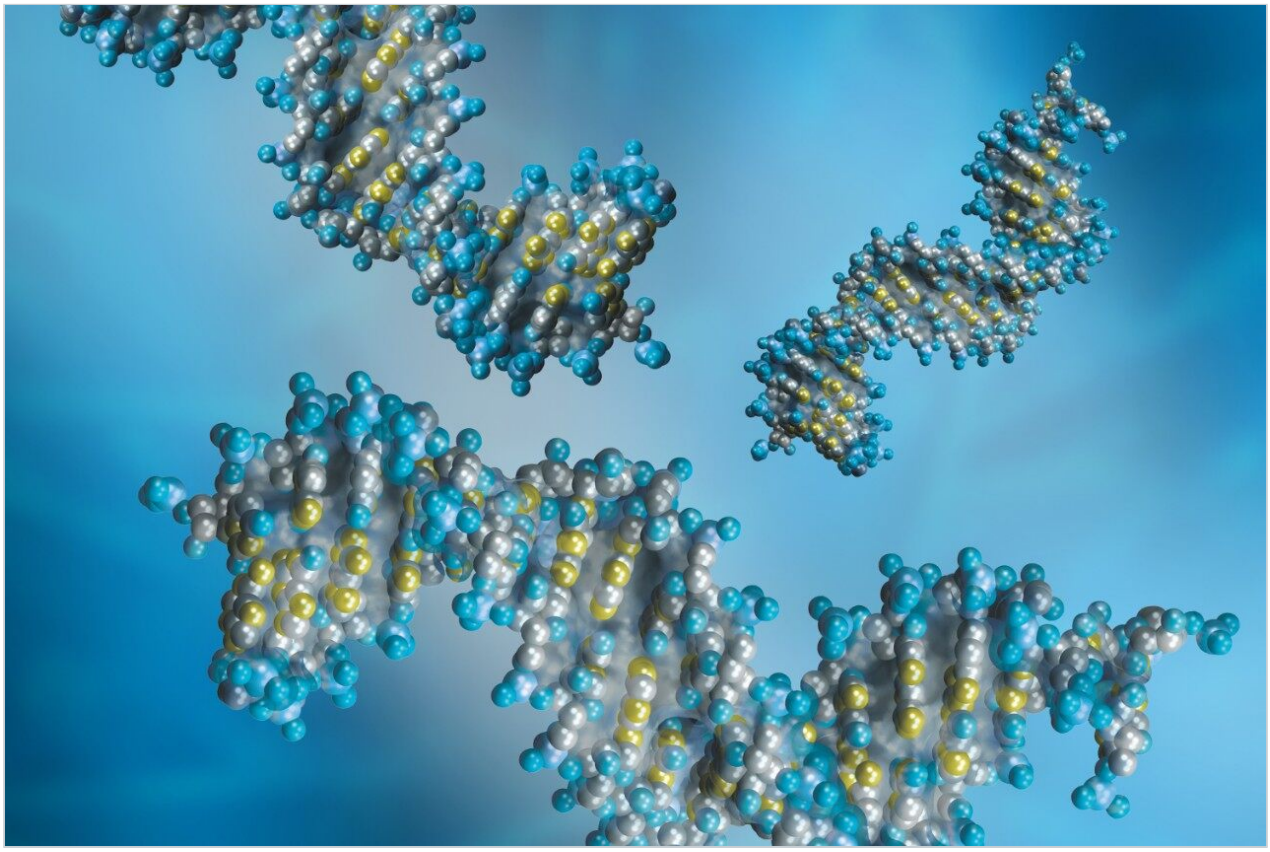

## Abstract

This application note details about the analysis of oligonucleotides using HPLC and UPLC columns.

## Introduction

Typical oligonucleotides used as therapeutic or potential therapeutic compounds are 15 to 30 nucleotides (nt) long, with the exception of aptamers, which are often 40 to 60 nucleotides in length. While shorter oligonucleotides (<15 nt) can be readily resolved by liquid chromatography (LC), the separation of longer sequences becomes progressively more challenging. Ion-pair reversed-phase (IP-RP) LC has been traditionally used for oligonucleotide analysis.

Recently, the Waters ACQUITY UltraPerformance LC (UPLC) System has been introduced offering separation benefits for a variety of compounds including oligonucleotides.

The traditional IP-RP LC eluent system typically employs 100 mM triethylammonium acetate (TEAA) at pH ~ 7. An alternative IP-RP eluent consists of 8.6–15 mM triethylamine (TEA) and 100 to 400 mM hexafluoroisopropanol (HFIP). The ion-pairing agent in both IP-RP LC eluent systems is the triethylammonium ion.[1, 2, 3]

The separation is most often carried out using $C_{18}$ columns at 60 °C. Using an elevated temperature is important to prevent the potential contribution of oligonucleotide secondary structure from impacting retention. Under such conditions, the column’ s hydrolytic stability becomes crucial.

Waters Oligonucleotide Separation Technology (OST) Columns have been developed to ensure excellent oligonucleotide resolution and column life time.

## Experimental

### LC conditions (Figure 1)

| | |
|---|---|
| LC system: | Waters Alliance HPLC 2695 HPLC System with 2996 PDA Detector |
| Column: | Waters XBridge BEH OST $C_{18}$ 4.6 x 50 mm, 2.5 µm |
| Column temp.: | 60 °C |
| Flow rate: | 1 mL/min |

| | |
|---|---|
| Mobile Phase A: | 10% methanol, 90% aqueous,<br>14.3 mM Triethylamine (TEA),<br>385 mM Hexafluoroisopropanol (HFIP), pH 7.9 |
| Mobile Phase B: | 25% methanol, 75% aqueous,<br>14.3 mM Triethylamine (TEA),<br>385 mM Hexafluoroisopropanol (HFIP), pH 7.9 |
| Gradient: | 0 to 100% B in 30 min |
| Detection: | UV 260 nm |

## LC conditions (Figure 3)

| | |
|---|---|
| LC system: | Waters Alliance HPLC 2796 Bioseparations System with 2996 PDA detector |
| Column: | Waters XBridge OST $C_{18}$ 2.1 x 50 mm (sorbent size is indicated in labeled chromatograms) |
| Column temp.: | 60 °C |
| Flow rate: | 0.2 mL/min |
| Mobile phase A: | 100 mM TEAA, pH 7 |
| Mobile phase B: | 80% A, 20% acetonitrile |
| Gradient: | 40 to 62.5% B in 30 min |
| Detection: | UV 260 nm |

## LC conditions (Figure 4)

| | |
|---|---|
| LC system: | Waters ACQUITY UPLC System for oligonucleotide analysis, with ACQUITY UPLC |

| | |
|---|---|
| | PDA detector |
| Column: | Waters ACQUITY UPLC OST $C_{18}$ 1.7 µm, 2.1 x 50 mm |
| Column temp.: | 60 °C |
| Flow rate: | 0.2 mL/min. |
| Mobile phase A: | 15 mM TEA, 400 mM HFIP in water, pH 7.9 |
| Mobile phase B: | 50% m.p. A, 50% methanol |
| Gradient: | 45 to 49.5% B in 15 min |
| Detection: | UV 260 nm |

## Results and Discussion

### Column Lifetime

Figure 1 illustrates Waters XBridge OST Column lifetime for ~1000 injections, demonstrating no loss of retention or resolution. Traditional silica-based columns operated at similar separation conditions frequently fail after only tens of injections.

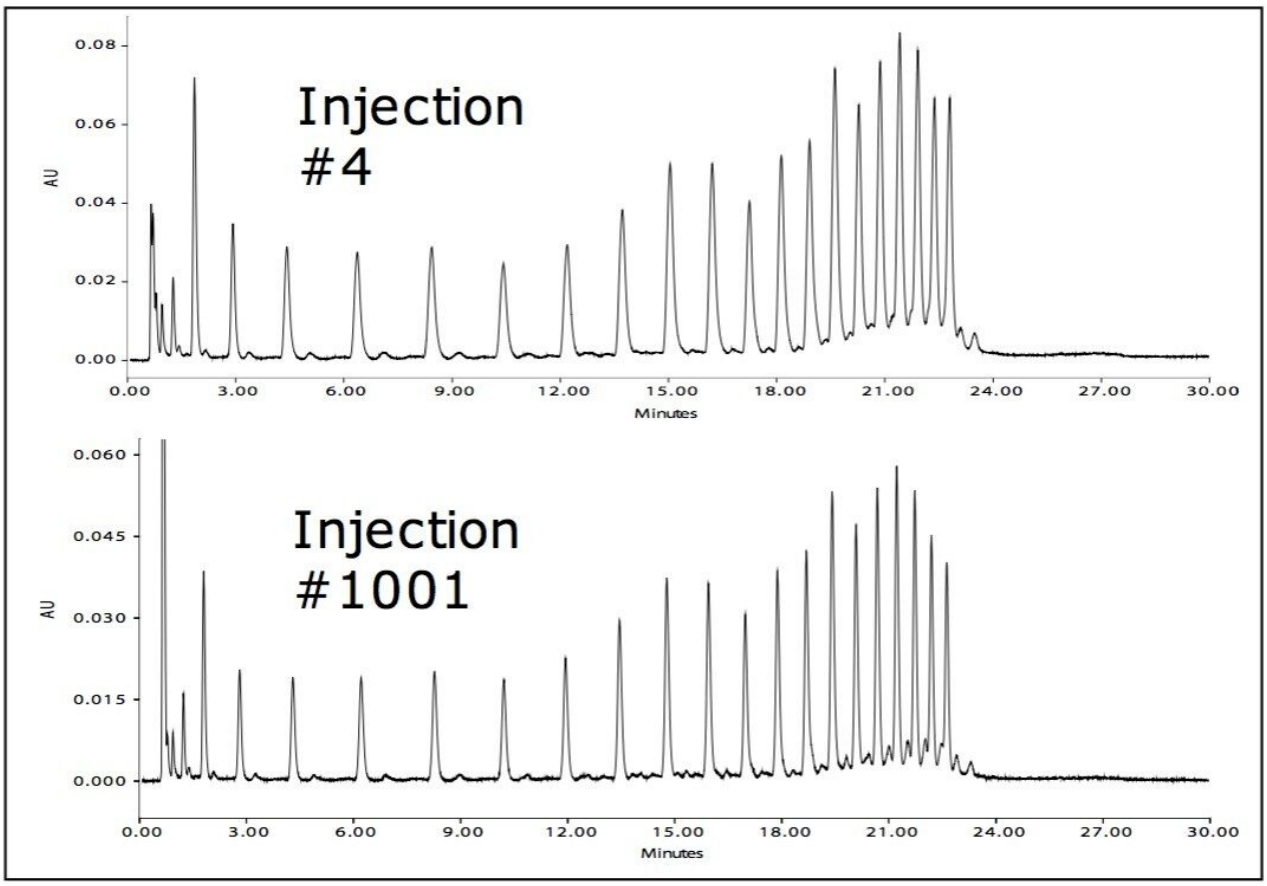


*Figure 1. BEH OST Column longevity exceeds 1000 injections. Separation of 5 to 25 nt oligodeoxythymidine ladder.*

The hydrolytic stability of OST Columns is achieved by a patented second-generation bridged ethyl hybrid (BEH) organic-inorganic sorbent. This BEH Technology is used in Waters ACQUITY UPLC Columns and XBridge HPLC Columns (Figure 2).

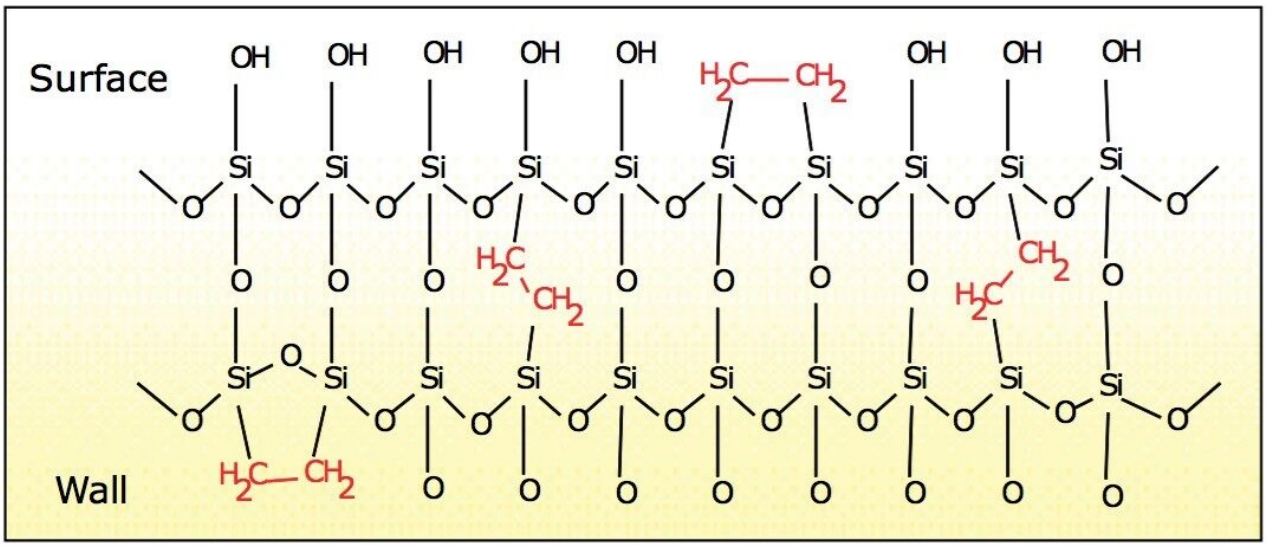


*Figure 2. Schematic structure of BEH sorbent. Hydrolytic stability is achieved by bridging ethyl groups. For oligonucleotide analysis, the surface of sorbent is alkylated by $C_{18}$ functional groups.*

BEH Technology columns demonstrate outstanding performance over a wide pH range (pH 1 to 12) typical of polymer packings, yet attain peak shape, efficiency, retention properties, and high temperature stability equal to or better than silica-based reversed-phase columns.

## BEH OST Column Separation Performance

Column separation performance in gradient elution mode is frequently measured as peak capacity. The peak capacity represents the maximum theoretical number of peaks that can be resolved within the gradient time. For oligonucleotides, where the target compound (N) elutes in close proximity to shorter species (N-1, N-2, etc.), and the separation selectivity cannot be easily altered, column peak capacity is critical.

In order to maximize peak capacity, OST Columns are packed with small sorbent particles. XBridge HPLC OST Columns are packed with 2.5 µm $C_{18}$ sorbent, while ACQUITY UPLC OST Columns are packed with 1.7 µm $C_{18}$ sorbent. The impact of sorbent particle size on the resolution of oligonucleotides is illustrated in Figure 3. An oligodeoxythymidine ladder (15 to 60 nt) is analyzed using 2.1 x 50 mm XBridge OST $C_{18}$ Columns packed with 2.5, 3.5, and 5 µm sorbent. All separation conditions are identical for each tested column. Since the selectivity of separation does not change, the improvements in resolution are achieved by the higher peak capacity of columns packed with increasingly smaller sorbent.

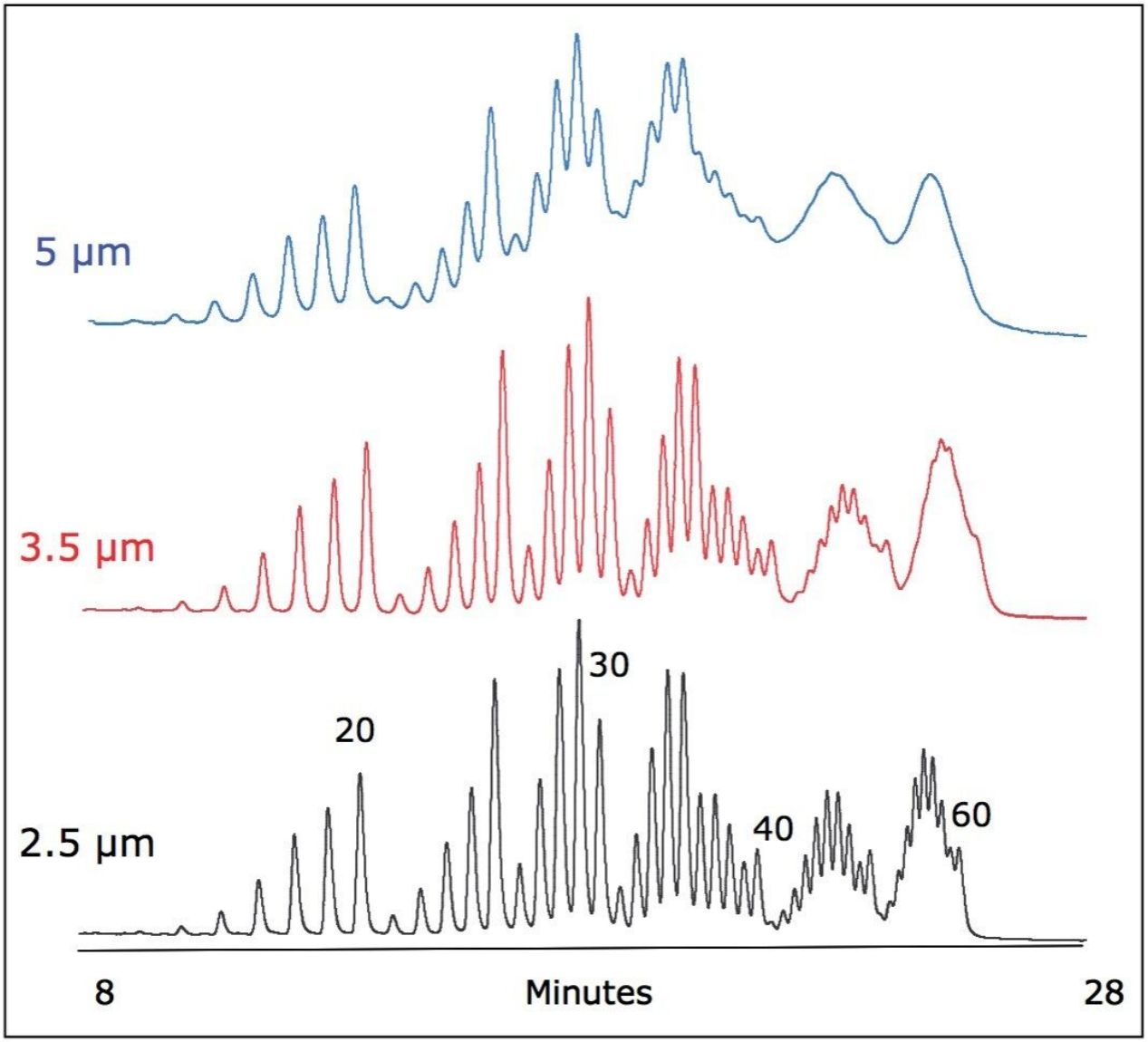


*Figure 3. Impact of sorbent particle size on oligonucleotide ladder separation. Improved resolution of 15 to 60 nt oligodeoxythymidine ladder is observed for columns packed with smaller particles.*

Understandably, using 2.5 µm sorbent for XBridge OST Columns leads to elevated backpressure. For relatively short OST Columns operated at elevated temperatures, the pressure is well within the range of conventional HPLC pumps.

## UPLC Separation of Oligonucleotides

The ACQUITY UPLC System enables the use of columns packed with sub-2 µm sorbent. Enhanced diffusion of macromolecules in such columns leads to greater peak capacity and faster analyses than those attained with HPLC technology. Figure 4 illustrates the improved resolution of 30 to 60 nt oligodeoxythymidines;

rapid, high resolution separation conditions were optimized for UPLC.

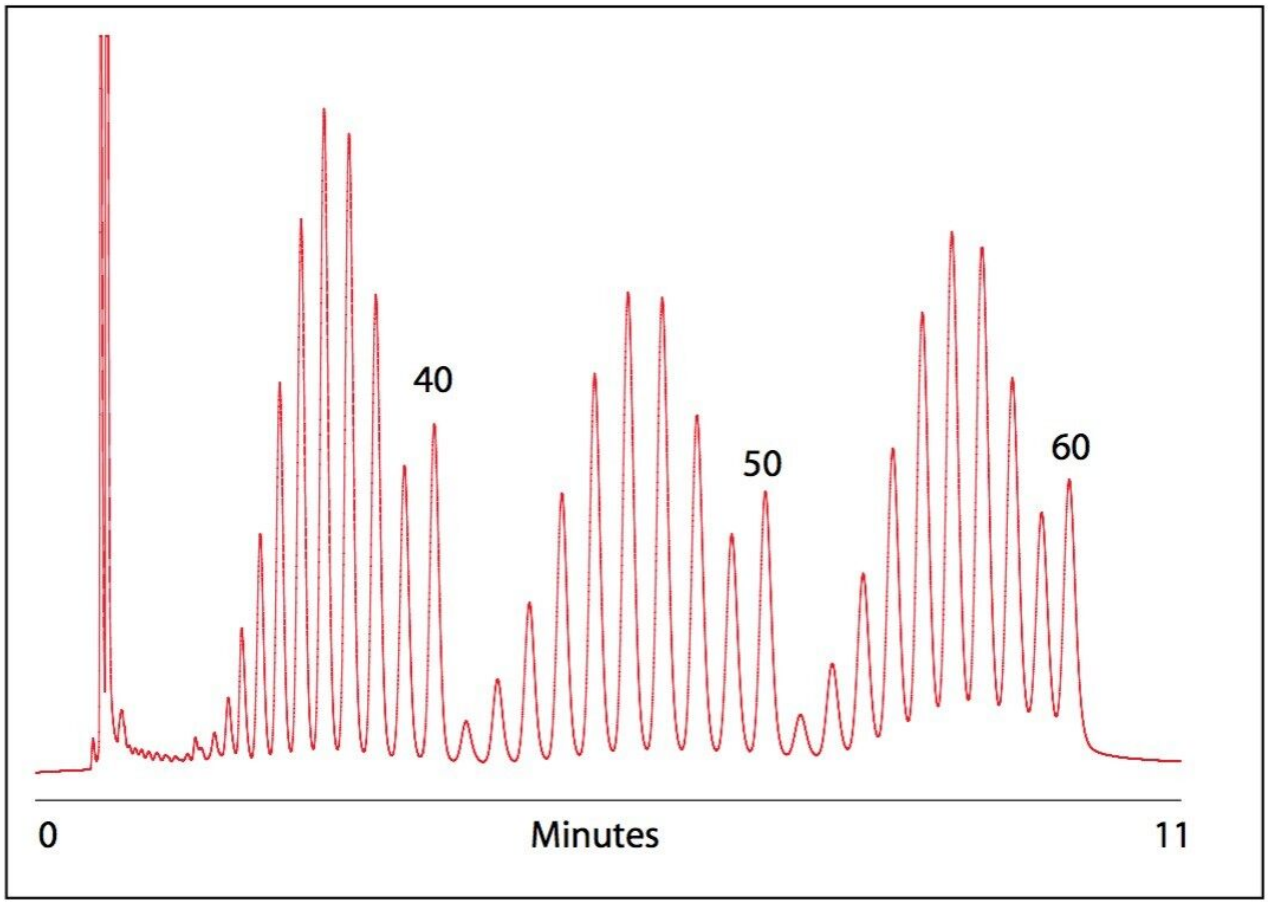


*Figure 4. Resolution of 30 to 60 nt oligodeoxythymidine ladder in UPLC mode.*

ACQUITY UPLC BEH OST Column dimensions are listed in Table 1. The sorbent is identical in HPLC and UPLC OST columns, enabling simple method transfer from HPLC to the ACQUITY UPLC System.

| Description | Particle size | Pore size | Dimension | Part number |
|---|---|---|---|---|
| XBridge OST $C_{18}$ | 2.5 µm | 135Å | 2.1 x 50 mm | 186003952 |
| XBridge OST $C_{18}$ | 2.5 µm | 135Å | 4.6 x 50 mm | 186003953 |
| XBridge OST $C_{18}$ | 2.5 µm | 135Å | 10 x 50 mm | 186003954 |
| Custom XBridge OST $C_{18}$ | -- | -- | -- | On request |
| ACQUITY UPLC OST $C_{18}$* | 1.7 µm | 135Å | 2.1 x 50 mm | 186003949 |
| ACQUITY UPLC OST $C_{18}$* | 1.7 µm | 135Å | 2.1 x 100 mm | 186003950 |
| Custom ACQUITY UPLC OST $C_{18}$* | -- | -- | -- | On request |

*Table 1. BEH OST Columns information.*

**For use on Waters ACQUITY UPLC Systems.*

*Figure 5. ACQUITY UPLC System and Oligonucleotide Separation Technology (OST) Columns.*

## Conclusion

The second generation of hybrid sorbents, utilizing bridged ethyl hybrid (BEH) chemistry, are extremely

stable, providing for a robust platform for separation of oligonucleotides at neutral-basic pH and at elevated temperature. Waters OST Columns packed with 2.5 µm particle size sorbent exhibit a superior performance for oligonucleotide separations. Routinely, 15 to 25 nt oligonucleotides can be baseline-resolved within 10 minutes with UPLC, compared to traditional LC and capillary electrophosresis analyses that usually take 30 to 60 minutes to accomplish. Longer oligonucleotides can be separated at moderately longer retention times.

OST Columns for UPLC analyses are suitable for efficient and fast separations of oligonucleotides in lengths up to 60 nt or longer. Smaller oligonucleotides can be analyzed within minutes using the ACQUITY UPLC System and columns. The faster analysis will result in savings in both time and resources, allowing for faster sample throughput and higher laboratory productivity.

## References


1. Gilar, M. *et. al.* Ion-Pair Reversed-Phase High-Performance Liquid Chromatography Analysis of Oligonucleotides: Retention Prediction. *J of Chrom.* 2002; 958: 167-182. 2.
2. Gilar, M. *et. al.* Characterization of Therapeutic Oligonucleotides Using Chromatography with On-Line Mass Spectrometry Detection. *Oligonucleotides.* 2003; 13: 229-243.
3. Fountain, F. *et. al.* (2003) Analysis of Native and Chemically Modified Oligonucleotides by Tandem Ion-Pair Reversed-Phase High-Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry. *Rapid Commun. Mass Spetrom.* 2003; 17: 646-653.


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720002376, September 2020