# Evaluation of new high pH stable, superficially-porous particle columns for the reversed-phase separation of oligonucleotides

Brian A. Bidlingmeyer, *Xiaoli Wang*, Stephen Luke* and Phu T Duong
Agilent Technologies, Inc., 2850 Centerville Road, Wilmington, DE 19808, US and *Essex Road, Church Stretton, Shropshire, SY6 6AX, UK

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## Introduction

Synthetic oligonucleotides have emerged as promising therapeutic agents. They are synthesized using a multi-step process. Although coupling efficiencies are high, the overall yield decreases as the cycles increase with failure coupling with single (N-1) and double (N-2) deletions as the major impurities. Therefore, fast, high resolution analyses are needed. HPLC is usually done in basic pH mobile phases at high temperature; thus, requiring chemically stable columns. Totally porous hybrid particles are commonly used but the mass transfer for the larger size oligos is not ideal. In this work, we evaluate the uses of new high pH stable, superficially-porous particles for oligo separations for fast and high resolution analysis, and compare them with totally porous particles.

## Experimental

### Materials

| Description | Part Number |
|---|---|
| AdvanceBio Oligonucleotide 2.1 x 50 mm, 2.7 um | 659750-702 |
| AdvanceBio Oligonucleotide 2.1 x 100 mm, 2.7 um | 655750-702 |
| AdvanceBio Oligonucleotide 2.1 x 150 mm, 2.7 um | 653750-702 |
| AdvanceBio Oligonucleotide 2.1 mm Fast Guard | 821725-921 |
| AdvanceBio Oligonucleotide 4.6 x 50 mm, 2.7 um | 659950-702 |
| AdvanceBio Oligonucleotide 4.6 x 100 mm, 2.7 um | 655950-702 |
| AdvanceBio Oligonucleotide 4.6 x 150 mm, 2.7 um | 653950-702 |
| AdvanceBio Oligonucleotide 4.6 mm Fast Guard | 820750-921 |
| | |
| Oligonucleotides Resolution Standard | 5190-9028 |
| Oligonucleotides Ladder Standard | 5190-9029 |

**Column characteristics**

| Bonded Phase | Pore Size | Temp. Limits | pH Range | End Capped |
|---|---|---|---|---|
| C18 | 100Å | 65°C | 3.0 - 11.0 | Double |

Acetonitrile, methanol, TEAA, HFIP, TEA (Sigma-Aldrich)

### Method

Column: AdvanceBio Oligonucleotide, 2.1 x 50 mm (p/n 659750-702)

**Option 1**

Mobile phase A: 100 mM TEAA in water
Mobile phase B: 100 mM TEAA in acetonitrile

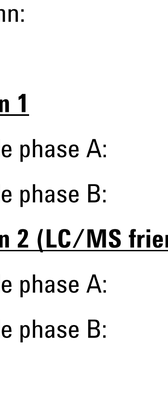
**Option 2 (LC/MS friendly)**

Mobile phase A: HFIP:TEA (400 mM:15 mM) in water
Mobile phase B: Methanol : mobile phase A (50:50)

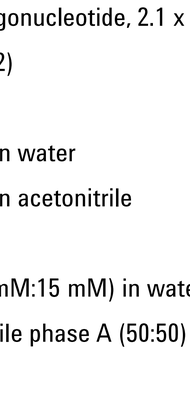
Gradient: See chromatogram
Stop time: See chromatogram
Post run: 5 min
Flow rate: 0.6 mL/min (or other flow rates)
Col. temp: 65 °C
Sample: See figures
Injection: See figures
Detection: UV at 260 nm

### LC instruments

Compatible with



1260 Infinity Quaternary



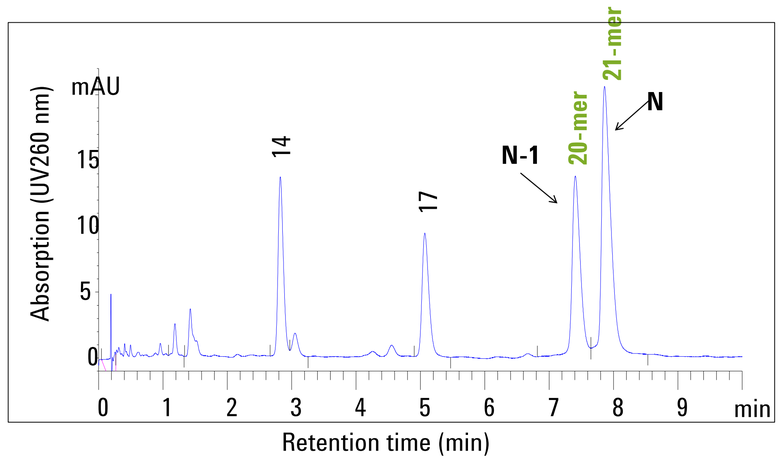
1290 Infinity Binary

Note: Conventional (green) capillaries are 0.17 mm ID can be used. However, red capillaries, 0.12 mm ID, will reduce connector volumes by 50%. Black capillaries (0.075 mm ID) can also be used but be careful of increased back pressure.

## Results and Discussion

### Resolution of N and N-1 Oligonucleotides

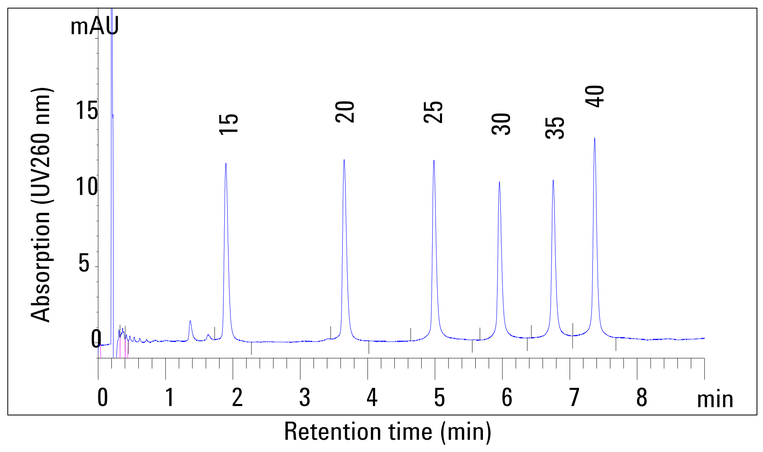
**Separation of RNA-oligonucleotides**




Column: AdvanceBio Oligonucleotide, 2.1 x 50 mm (p/n 659750-702)
Mobile phase A: 100 mM TEAA in water
Mobile phase B: 100 mM TEAA in acetonitrile
Gradient: 10 to 14%B in 10 min
Sample: 10 µL (Agilent Oligonucleotide Resolution Standard (p/n 5190-9028)
14, 17, 20 21-mer
21-mer= rGrUrCrArUrCrArCrArCrUrGrArArUrArCrCrArArU
LC: Agilent 1290 Infinity Binary

The AdvanceBio Oligonucleotide column generates sharp peaks and high resolution for RNA-oligos. The N and N-1 RNA-oligonucleotides (21 and 20-mers) were separated close baseline resolution. This data suggested that column is very capable of resolving a main oligo from impurities.

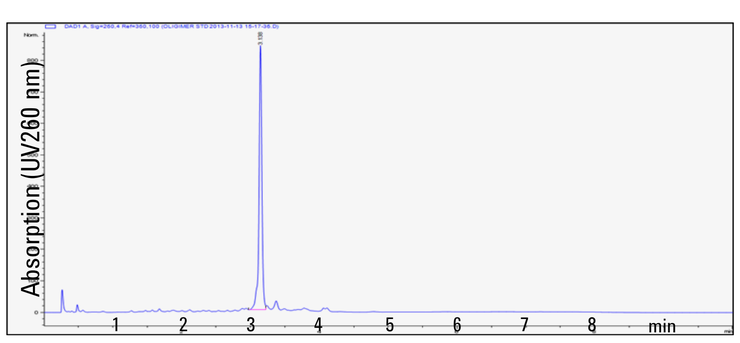
**Separation of DNA-oligonucleotides**




Column: AdvanceBio Oligonucleotide, 2.1 x 50 mm
Mobile phase A: 100 mM TEAA in water
Mobile phase B: 100 mM TEAA in acetonitrile
Gradient: 6 to 8% B in 12 min
Sample: 0.5 µL (Agilent Oligonucleotide Ladder Standard (p/n 5190-9029)
15, 20, 25, 30, 35, 40-mer
LC: Agilent 1290 Infinity Binary

All 6 DNA-oligos were separated with baseline resolution. The separation was completed in less than 10 min. The 15-mer DNA oligo was eluted as soon as 2 min and the average 20 and 25-mers were eluted at 4 and 5 minute, respectively. The data indicated that AdvanceBio Oligonucleotide columns are suitable for high through-put separation of a wide range of oligonucleotide sequence length.

### MS compatibility- Separation of oligonucleotide



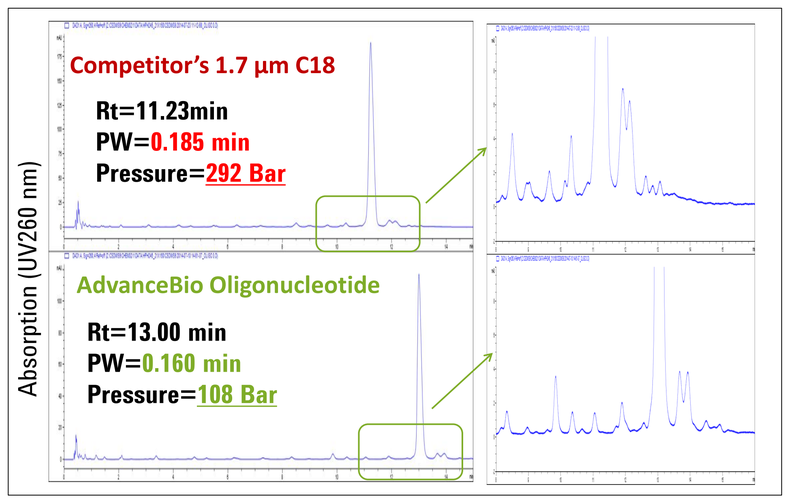

Column: AdvanceBio Oligonucleotide, 2.1 x 50 mm
Mobile phase A: HFIP:TEA (400 mM:15 mM) in water
Mobile phase B: Methanol : mobile phase A (50:50)
Gradient: 30-40% B in 0.5 min; 40-70% B in 5 min
Sample: 1.0 µL (0.5mg/mL 25-mer *DNA-oligo)*
LC: Agilent 1290 Infinity Binary

With MS compatible mobile phase, the AdvanceBio Oligonucleotide column gives high chromatographic resolution and the 25-mer DNA oligo peak and its impurities were eluted in about 3 min.

## Results and Discussion

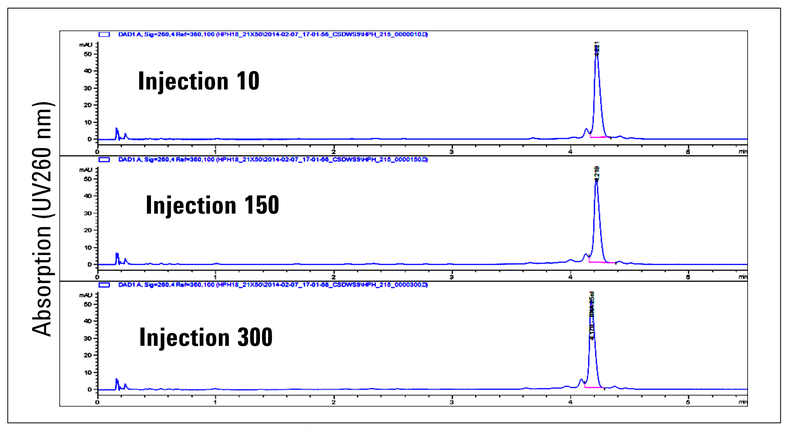
### Comparison

**Separation of 23-mer RNA-oligonucleotide (2.1 x 50 mm)**




The peak width of the superficially-porous column was slightly narrower than the totally porous material 1.7um, this supports the fact that there is shorter distances required for diffusion into/out of the superficially-porous stationary phase. This faster mass transfer results in higher resolution capability . With 2.7 um particle size, AdvanceBio Oligonucleotide column operates at low backpressure compare to 1.7 um particle size column, it is compatible with 600 bar HPLC system as well as 1200 bar HPLC system.

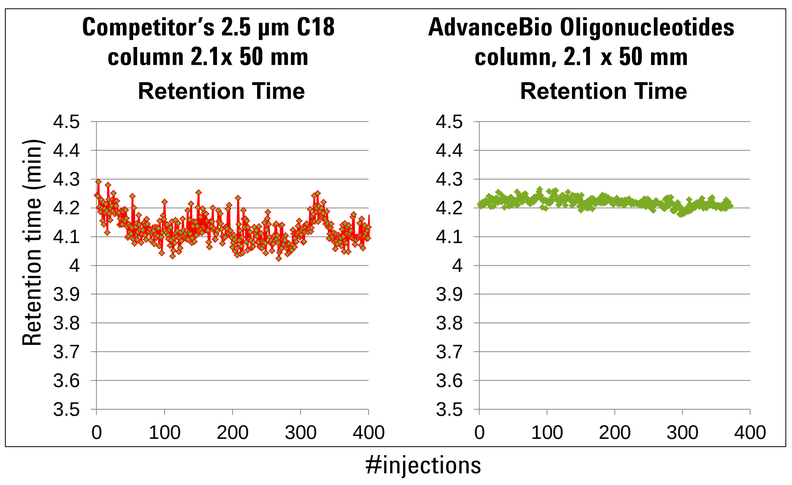
**Column stability (2.1 x 50 mm)- 300 consecutive injections of 25-mer DNA-oligo**




Gradient: 7% B to 11% B in 5 min, 11% B to 80% B in 5.01 min
Hold at 80% B for 5.50 mim, 80% B to 7% B in 5.56 min
Sample: 1uL (0.5mg/mL 25-mer DNA-oligo)
Total run time: 8.5 min

Column stability of 300-consecutive injections of 25-mer (DNA-oligo) showed that though out 300 injections, the retention time of main peak and its impurities were highly reproducible. Column still maintains its performance after these 300 injections.

**Comparison of column stability (2.1 x 50 mm)**




The peak retention time from ~400 injections of 25-mer which was generated by AdvanceBio Oligonucleotide column (high pH stable superficially-porous particles) showed to be as stable as the totally porous hybrid particles. This data also indicated that the AdvanceBio Oligonucleotide column has a long lifetime .

## Conclusions

- AdvanceBio Oligonucleotide columns are designed for separating RNA and DNA-oligonucleotides of different sizes with fast and high resolution.
- RNA and DNA-oligonucleotides can be successfully analyzed by AdvanceBio Oligonucleotides column using both LC/UV and LC/MS mobile phases.
- High numbers of injections - column stability data generated with TEAA (>pH 8.0 )- indicated that columns packed with high pH stable superficially porous particles have long life time.
- AdvanceBio Oligonucleotide columns are packed with 2.7 um superficially-porous particles- low backpressure columns- can be compatible with 600 bar and 1200 bar HPLC systems.