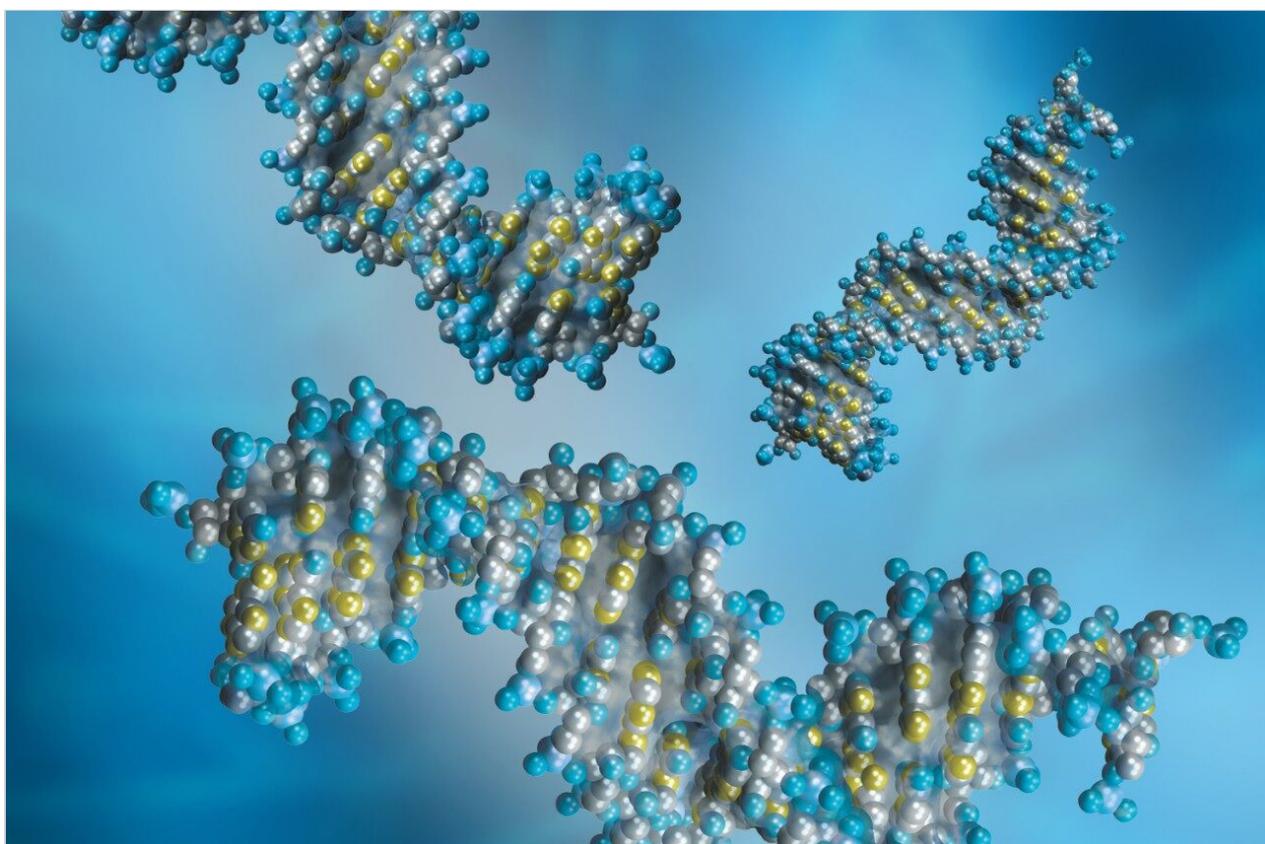


# RP-HPLC Method For Quality Control Of Synthetic Oligonucleotides Using XTerra MS C<sub>18</sub> Columns

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Waters Corporation



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## Abstract

This application note demonstrates RP-HPLC method for quality control of synthetic oligonucleotides using

## Introduction

### Oligonucleotide synthesis yield

Although state-of-the-art oligonucleotide synthesis is a fast and reliable process, the yield of the target product is limited. The full-length oligonucleotide (N) is typically contaminated with shorter failure products called N-1, N-2, N-3... Oligonucleotide purity decreases with the complexity of synthesis. The typical purity of crude 25mer product is ~75% while the purity of a 60mer product is ~20%. Occasionally the synthesis or post synthesis deprotection result in a product of inferior purity, which may compromise quality of the polymerase chain reaction (PCR) or other molecular biology assays.

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## Experimental

### Preparation of 0.1 M TEAA buffer

Dissolve 5.6 mm glacial acetic acid in ~950 mL of water. While mixing add 13.86 mL of TEA. Adjust pH with diluted acetic acid to ~7 and adjust volume to 1L with water.

### Preparation of 16.3 mM TEA - 400 mM HFIP buffer

Dissolve 42.1 mL of HFIP in ~950 mL of water. While mixing vigorously add 2.28 mL of TEA. Adjust volume to 1L with water. The pH of solution should be close to 7.9.

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## Results and Discussion

### XTerra Columns for efficient oligonucleotide separations

XTerra MS C<sub>18</sub> Columns are packed with porous 2.5 μm, hybrid particles. The sorbent has extended stability at temperatures and pH's typically used for oligonucleotide separations (50–60 °C; pH 7–9). The column yields high resolution efficiency required for successful analysis of oligonucleotides (Figure 2). Volatile ion-pairing mobile phases are utilized to separate oligonucleotides according to their length. The resolution performance rivals that seen with capillary gel electrophoresis (Figure 1). With XTerra MS C<sub>18</sub> Columns,

baseline resolution of N from N-1 oligonucleotides is typically achieved for <30mer. Good resolution can be achieved for larger oligonucleotides (e.g. 60mer or larger: see (Figures 2 & 3)).

Triethylammonium acetate (TEAA) is an inexpensive volatile ion-pairing agent used for routine oligonucleotide analysis (Figure 2) and purification. Alternatively, triethylammonium-hexafluoroisopropanol buffer (TEA-HFIP) can be used for more efficient separation of long oligonucleotides (>45mer, Figure 3), and for applications using mass spectrometry detection.

<b>HPLC system:</b>	Alliance® 2690 Separations Module	<b>CGE system:</b>	Waters Quanta® 4000 CE
<b>Column:</b>	XTerra®MS C <sub>18</sub> , 2.5 μm, 4.6 x 50 mm	<b>Column:</b>	BioRad BioCAP™ 75 μm, 34.5 cm
<b>Part Number:</b>	18600602	<b>Separation:</b>	15 kV
<b>Flow rate:</b>	0.5 ml/min	<b>Sieving matrix:</b>	PEG 35 kDa, 2 g/10 ml 0.1mM Tris-Boric acid, pH 8.3
<b>Solvent A:</b>	10 % MeOH in 16.3 mM TEA, 400 mM HFIP buffer, pH 7.9	<b>Injection:</b>	4 s @ 5kV
<b>Solvent B:</b>	40 % MeOH in 16.3 mM TEA, 400 mM HFIP buffer, pH 7.9	<b>Column temp.:</b>	30 °C
<b>Gradient:</b>	0-10 min from 16.7 to 30 % B, 10-30 min from 30 % to 47.2 % B	<b>Detection:</b>	UV @ 254 nm
<b>Injection volume:</b>	5 μl		
<b>Column temp.:</b>	60 °C		
<b>Detection:</b>	UV @ 260 nm		

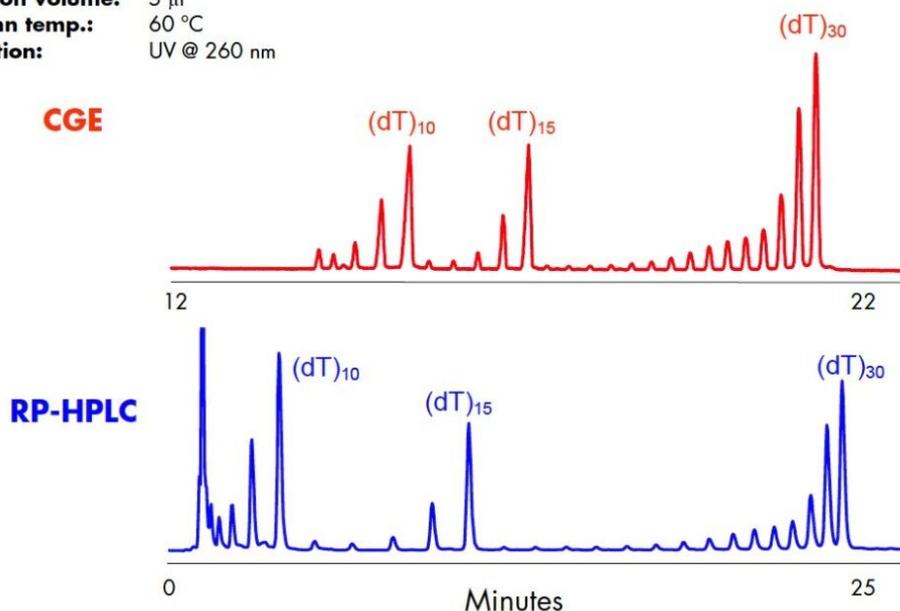


Figure 1. Separation of 6-30mer oligodeoxythymidines by CGE and RP-HPLC using XTerra MS C<sub>18</sub>.

**HPLC system:** Alliance® 2690 Separations Module  
**Column:** XTerra®MS C<sub>18</sub>, 2.5 µm, 4.6 x 50 mm  
**Part Number:** 186000602  
**Flow rate:** 1 ml/min  
**Solvent A:** 5 % ACN in 0.1 M TEAA, pH 7  
**Solvent B:** 20 % ACN in 0.1 M TEAA, pH 7

**Gradient:** From 9.3 % B to 44 % B in 20.8 min  
**Injection volume:** 5 µl (1 nmole of oligonucleotide)  
**Column temp.:** 60 °C  
**Detection:** UV @ 260 nm

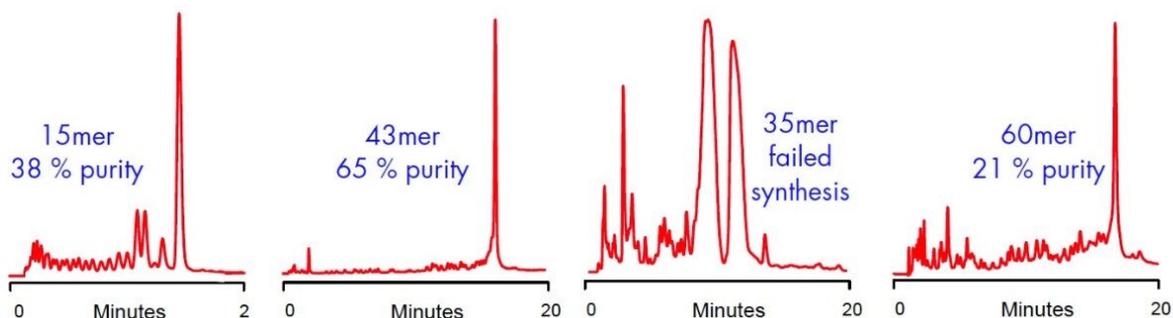
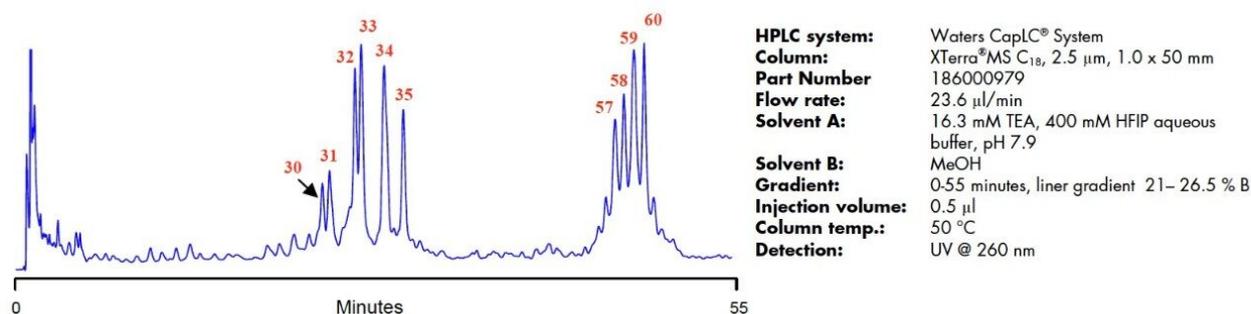


Figure 2. RP-HPLC quality control of synthetic oligonucleotides. Purity was measured as the % of peak area at UV<sub>260</sub> nm.



**HPLC system:** Waters CapLC® System  
**Column:** XTerra®MS C<sub>18</sub>, 2.5 µm, 1.0 x 50 mm  
**Part Number:** 186000979  
**Flow rate:** 23.6 µl/min  
**Solvent A:** 16.3 mM TEA, 400 mM HFIP aqueous buffer, pH 7.9  
**Solvent B:** MeOH  
**Gradient:** 0-55 minutes, liner gradient 21–26.5 % B  
**Injection volume:** 0.5 µl  
**Column temp.:** 50 °C  
**Detection:** UV @ 260 nm

Figure 3. RP-HPLC separation of 30–35mer and 57–60mer heterooligonucleotides.

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Alliance HPLC System <<https://www.waters.com/534293>>

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