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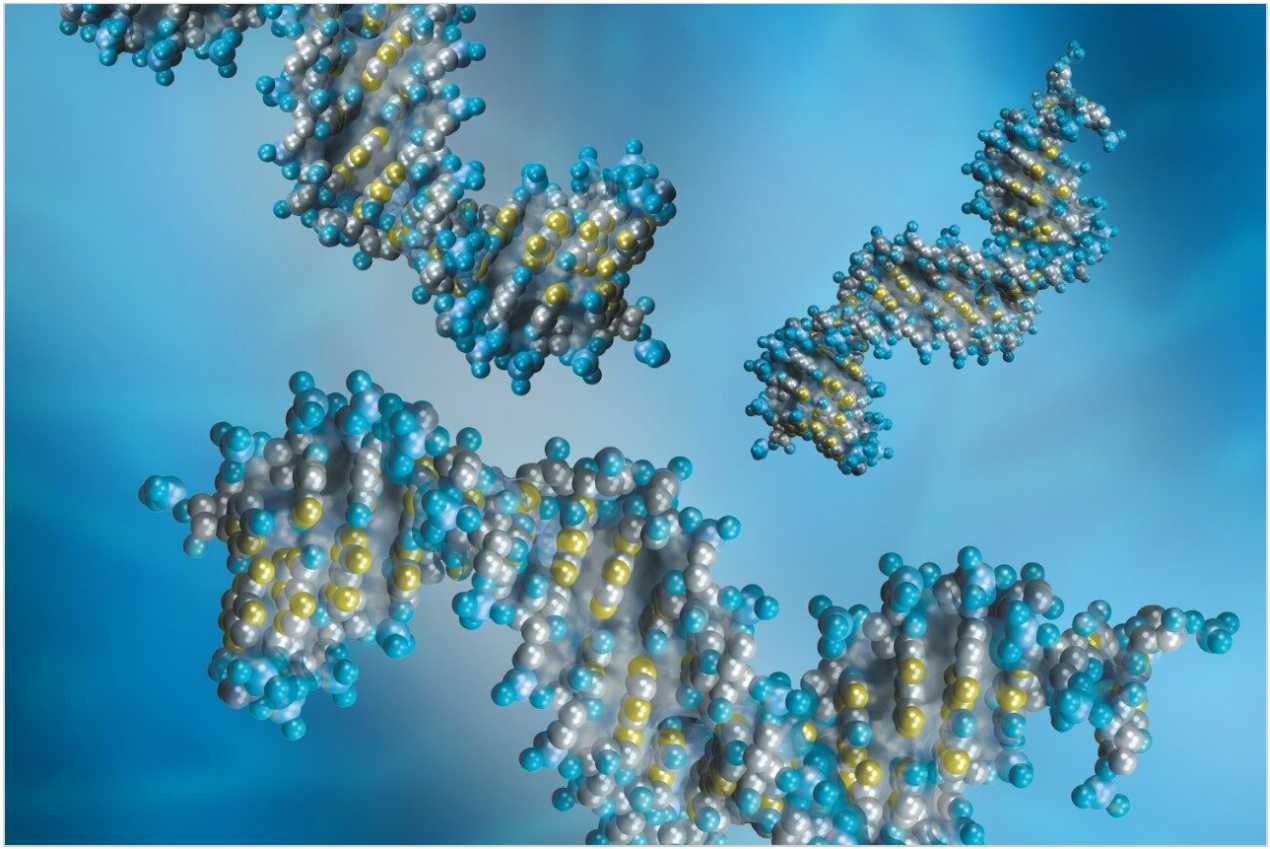
アプリケーションノート

RP-HPLC Method For The Purification Of Labeled Synthetic Oligonucleotides Using XTerra MS C_{18} Columns

Waters Corporation



Abstract

This application note demonstrates RP-HPLC method for the purification of labeled synthetic

oligonucleotides using XTerra MS C_{18} Columns.

Introduction

Labeled oligonucleotide probes

Labeled oligonucleotides are utilized in quantitative polymerase chain reactions (qPCR), genotyping, DNA sequencing, and diagnostics. Among the most common labels are fluorescent dyes, various linkers, and biotin. Synthesis of labeled oligonucleotides is challenging, especially for multiple-labeled probes. The target product is typically contaminated with labeled and non-labeled failure products; both types of impurities, as well as the excess of uncoupled label, may compromise quality of the molecular biology assays.

Results and Discussion

XTerra Columns for enhanced oligonucleotide purification

Non-labeled oligonucleotides, labeled failure sequences, and excess of free label (dye) can be easily removed from the target product using XTerra HPLC Columns (Figure 1). XTerra MS C_{18} Columns are packed with porous 2.5 µm, hybrid particles. XTerra hybrid particle technology has extended stability at temperatures and pH’ s typically used for oligonucleotide separations (50–60 °C; pH 7-9). The column yields the high resolution efficiency required for successful oligonucleotide purification (See Lib. Ref #WA21955). The mobile phases are volatile and can be removed from the collected fractions by evaporation. The 4.6 x 50 mm column is suitable for purification of 50–200 nmole scale syntheses in a single injection. Larger amounts of oligonucleotides (200 nmole–1 µmole) can be purified using a 10 x 50 mm column or greater.

HPLC system:	Alliance® 2695 Separation Module	**Gradient:**	0-100 % B in 15 minutes followed with column cleanup with 100 % ACN for 1-2 minutes and equilibration with mobile phase A for 8 minutes
Column:	XTerra®MS C_{18}, 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	**Injection volume:**	50 µl (sample dissolved in 0.1 M TEAA)
Solvent B:	30 % ACN in 0.1 M TEAA, pH 7	**Column temp.:**	60 °C
		Detection:	UV @ 260 nm,

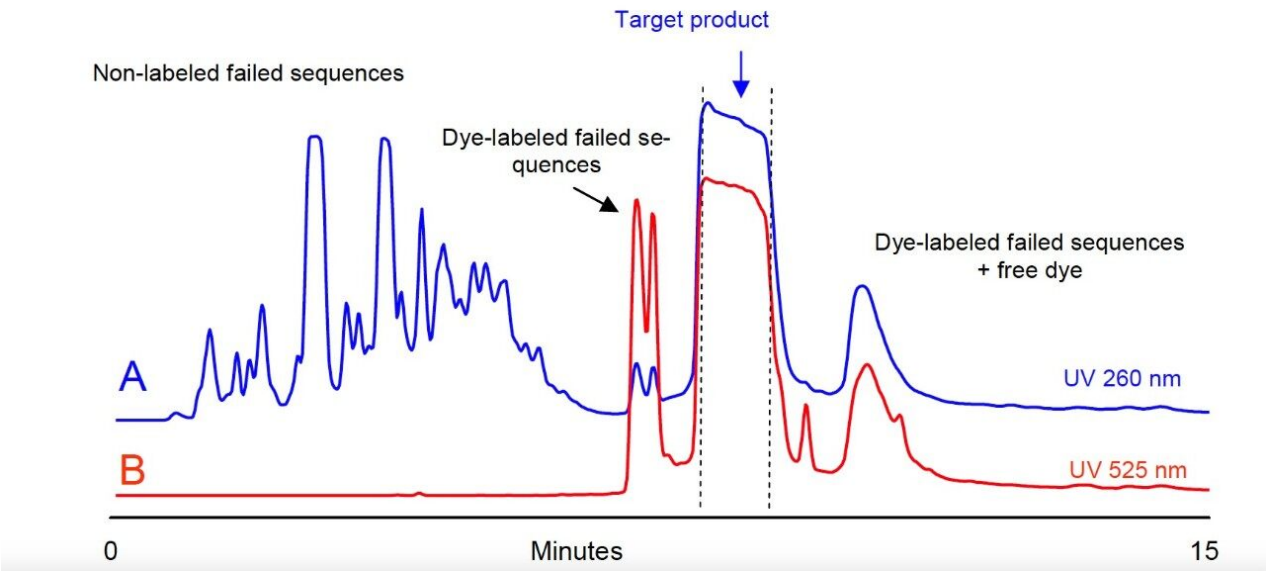


Figure 1. Purification of 100nmole of 5′ TET labeled oligonucleotide.

High purification yield and oligonucleotide purity

XTerra’ s high performance allows for collection of the target fraction without sacrificing large amounts of the product (Table 1). When purifying 100 nmole with the 4.6 x 50 mm column, 75–80% of the target compound is typically recovered at a purity greater than 90%. Since RP-HPLC separates isomers of dye labels, we recommend this method for purification of single isomer dye-labeled oligonucleotides. The mobile phase components triethylammonium acetate (TEAA) and acetonitrile are volatile. Collected fractions are simply dried down and ready for use. Purification was monitored at two wavelengths: 260 nm (DNA detection, alternatively at 290 nm) and λmax of the label (dye labeled species only are detected). Real-time monitoring of two traces (chromatograms A & B, Figures 1 & 2) helps to identify a proper collection time window. Impurities eluting early during chromatography are the non-labeled failed products. The impurities eluting after the main peak are mostly dyelabeled failed sequences (Fountain *et al* ., *J. Chromatogr. B*, 783 (2003) 61–72) (See Lib. Ref # WA20742).

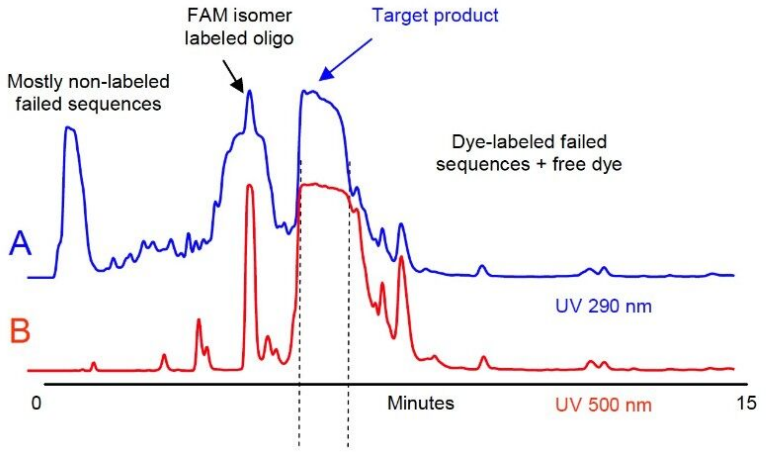
Oligonucleotide	Yield @ 260 nm [a] (%)	Yield @ dye l max absorbance (%)	crude purity [b] by CGE (%)	purified purity by CGE (%)
25mer (no label)	91.7	-	85.4	91.5
25mer 5′ TET	55.2	73.8	58.1	91.1
25mer 5′ 6FAM	31.1	45.8	52.3	97.7
25mer 5′ Cy3	59.2	71.8	87.4	96.9
25mer 5′ HEX	63.0	85.4	77.3	93.5
25mer 3′TAMRA	80.0	81.5	65.5	80.1 [c]

[a] Yields were determined as an area percent of the target fraction versus total peaks area at 260 nm (and at the maximum absorbance wavelength of the dye). Yield was calculated from the analytical scale injection.

[b] The purity of crude and the HPLC purified oligonucleotides were monitored by CGE at 260 nm.

[c] Main CGE peak. CGE resolved several peaks (dye isomers); the actual purity by LC-MS is greater than 90 %.

Table 1. Purity and yield of XTerra-purified oligonucleotides.



HPLC system: Alliance® 2695 Separations Module
Column: XTerra®MS C_{18}, 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: 30 % ACN in 0.1 M TEAA, pH 7
Gradient: 0-100 % B in 15 minutes followed with column cleanup with 100 % ACN for 1-2 minutes and equilibration with mobile phase A for 8 minutes
Injection volume: 50 µl (sample dissolved in 0.1 M TEAA buffer)
Column temp.: 60 °C
Detection: UV @ 260 nm,

Figure 2. Purification of 100 nmole of 5′ 6FAM labeled 25mer oligonucleotide.

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WA20771, June 2003