Quality Control of DNA Synthesis using Fast Ion-pair Reversed-phase High Performance Liquid Chromatography

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Overview

- A high performance liquid chromatography (HPLC) method has been developed for the evaluation of DNA synthesizer performance and routine quality control of DNA/RNA synthesis.
- Baseline separation of oligonucleotides up to 30mer in length from synthesis impurities was consistently achieved.
- The 5-minute per sample duty cycle allows for fast diagnosis of the performance of DNA synthesizers.
- The method is capable of routine detection of 0.5 % of failed products in the target oligonucleotide synthesis.

Introduction

Current methods of DNA synthesis can routinely produce high quality oligonucleotides, provided that automated synthesizers are in optimal operating condition. While suboptimal synthesis can still yield acceptable and usable oligonucleotides, diagnostic and therapeutic applications require high purity probes.

A method utilizing ion-pair reversed-phase high performance liquid chromatography (IP-RP-HPLC) with ultraviolet (UV) detection has been developed for the routine quality control (QC) of synthetic oligonucleotides up to 30mer in length. Separation was performed on a 20 × 4.6-mm MS C_{18}, 2.5 µm column. Mobile phases were comprised of 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and triethylamine (TEA) with a concave methanol (MeOH) gradient. Resolution of the target product from N – 1 impurities was routinely achieved for all DNA fragments analyzed, including oligonucleotides with mixed base sequences (up to 30mer). The 5-minute per sample duty cycle allows for fast diagnosis of the performance of DNA synthesizers.

Two sets of four homooligonucleotides ($(dG)_{20}$, $(dC)_{20}$, $(dA)_{20}$, and $(dT)_{20}$) and a 30mer heterooligonucleotide were purchased from five different vendors. The quality of each homooligonucleotide is indicative of a particular problem of the synthesis process; coupling efficiency, incomplete deprotection, side reactions, depurination, and poor solvent and reagent quality. Since detection is performed by UV, the absolute amount of impurities in each synthesis was quantitated. The HPLC method is capable of routine detection of 0.5 % of failed products in the target oligonucleotide synthesis, and can be utilized for monitoring DNA/RNA production quality.

Experimental

HPLC System:	Waters Alliance® HT Separations Module (Milford, MA)
Column:	XTerra® Intelligent Speed (IS™) MS C_{18}, 20 × 4.6 mm, 2.5 µm
Flow Rate:	1.0 mL/minute
Mobile phase A:	5 % MeOH in TEA/HFIP (16.3 mM/400 mM), pH 7.9
Mobile phase B:	30 % MeOH in TEA/HFIP (16.3 mM/400 mM), pH 7.9
Gradient:	0—60 % B (for 20mers) or 0—65.8 % B (for 30mers) in 4.25 minutes; gradient profile # 4 (concave)
Injection volume:	2 µL was routinely injected onto the system, which equals 200 pmole total mass load
Column temp.:	60 °C
Detection:	Waters® 2487 Dual λ Absorbance Detector; 254 nm
Buffer preparation:	Dissolve 41.5 mL of HFIP in ~ 950 mL of water. While mixing vigorously, add 2.3 mL of TEA. Adjust final volume to 1 L with water. The pH of the solution should be about 7.9.
Samples:	Oligonucleotides were provided by Midland Certified Reagents (Midland, TX), One Trick Pony (Ramona, CA), IDT (Coralville, IA), Qiagen (Valencia, CA), and Sigma-Genosys (The Woodlands, TX).

Results and Discussion

Figure 1: Separation performance of the IP-RP-HPLC method. The N—1 impurity is separated from the target 20mer oligodeoxycytidine (A). Further proof is provided when the sample is spiked with a purified N—5 impurity, $(dC)_{15}$ (B).

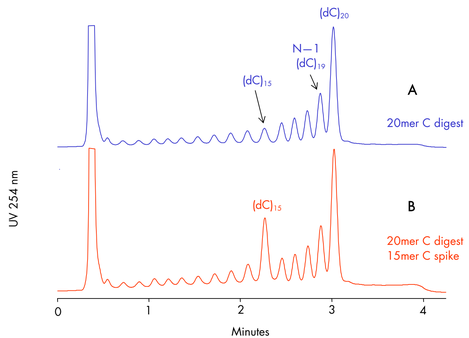


Figure 2: Separation of the four different 20mer homooligonucleotides by IP-RP-HPLC. The short column (20 mm) allows for this separation in under 5 minutes for each oligonucleotide. Separation of closely eluting impurities (i.e. N—1, 2, 3, etc.) from the target oligonucleotide was routinely achieved.

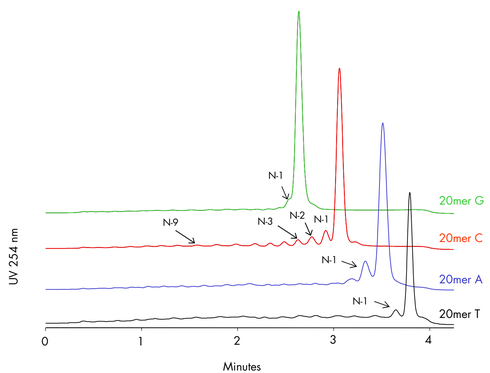


Figure 3: HPLC analysis of an oligodeoxyguanosine ($(dG)_{20}$) from two different vendors. Notice that synthesis impurities and incomplete deprotection of the target product are distinguishable (Vendor E). A wide range of synthesis quality was observed between vendors.

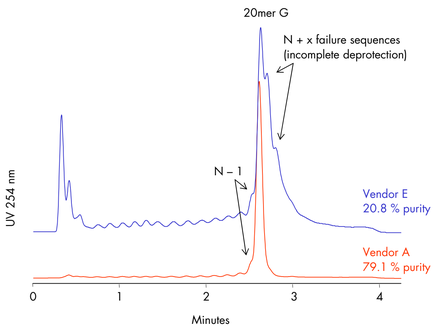
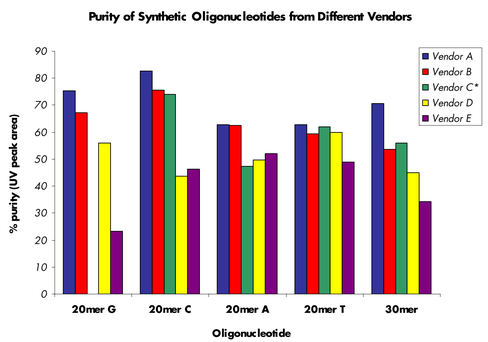


Figure 4: DNA product quality was evaluated for 5 oligonucleotide vendors. The % purity of two sets of four homooligonucleotides and a 30mer heterooligonucleotide was monitored using the fast IP-RP-HPLC method. The purity values listed in the plot below represent the average purity of the two oligonucleotides from each vendor.



*Vendor C does not synthesize oligonucleotides containing more than 6 consecutive guanine (G) residues.

Figure 5: Quantification of impurities in a 20mer oligodeoxycytidine ($(dC)_{20}$) synthesis. Since detection is performed by UV, the amount of impurities in each synthesis is calculated as an area % at 254 nm. Routine detection of 0.5 % (~ 5 pmoles) of failed products in the target synthesis was achieved.

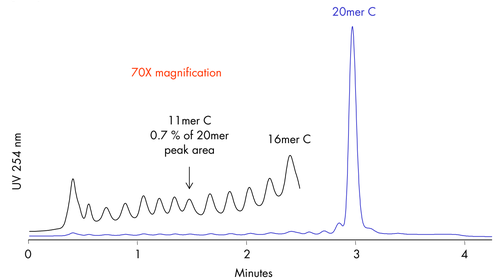
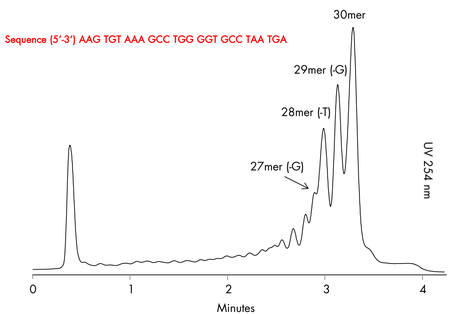


Figure 6: Separation of a 30mer mixed sequence oligonucleotide that was digested with 3′ exonuclease to generate "failure sequences". Adequate resolution of N—x impurities is routinely achieved.



Conclusions

- An IP-RP-HPLC method was developed for the routine quality control of synthetic oligonucleotides up to 30mer in length.
- The 5-minute per sample duty cycle allows for fast diagnosis of DNA synthesizer performance.
- Absolute amounts of oligonucleotides as low as 5 pmoles can be quantified by UV detection.
- The HPLC method can be coupled on-line with electrospray ionization mass spectrometry (ESI-MS) due to the compatibility of the mobile phases with MS.