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Ion-pair Reversed-phase High Performance Liquid Chromatography of Dye-labeled Oligonucleotides

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Introduction

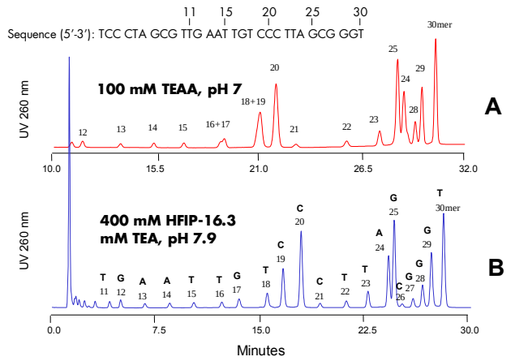
Synthetic oligonucleotides are used in many biological applications such as DNA sequencing, genotyping, quantitative polymerase chain reaction (PCR), and molecular diagnostic applications. Fluorescent oligonucleotides are often used to improve the sensitivity and throughput of these assays.

Oligonucleotide synthesis yields a target product that is contaminated with truncated failure sequences. Synthesis is especially challenging when producing dye-labeled oligonucleotides. Since the performance of bioassays is sensitive to the quality of fluorescent probes, it is crucial to purify these oligonucleotides prior to use .

We developed a simple but high performance purification method based on ion-pair reversed-phase high performance liquid chromatography (IP-RP-HPLC) [1]. Singly- and dually-labeled synthetic oligonucleotides were purified using a 4.6 x 50 mm XTerra® MS C_{18} column packed with porous, 2.5 µm sorbent. We purified oligonucleotides labeled with 6-carboxyfluorescein (6FAM), hexachlorofluorescein (HEX), tetrachlorofluorescein (TET), carboxytetramethylrhodamine (TAMRA) and indodicarboxycyanine (Cy™3) dyes, as well as dually-labeled TaqMan™ probes. Purification of a 0.1 µmole oligonucleotide synthesis in a single injection was demonstrated.

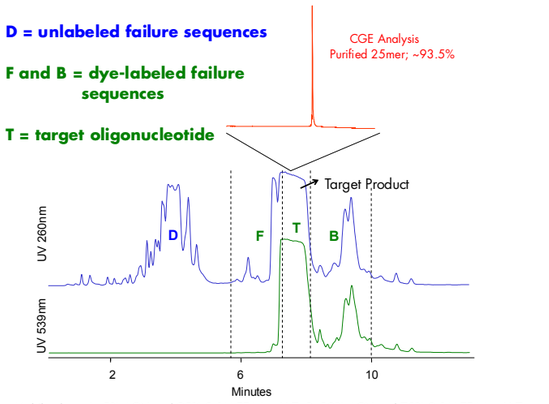
The retention of native oligonucleotides in IP-RP-HPLC is dictated by both length (charge) and sequence (Figure 1) [2]. However, retention of dye-labeled oligonucleotides is determined by the hydrophobicity of the fluorescent label that is attached rather than the sequence or length [3]. This is evident from Figure 2, which shows six 25mers with the same sequence but a different dye. Also notice that the dye-labeled oligonucleotides are retained much longer than the native one. We adjusted our HPLC purification conditions accordingly to account for this longer retention, and an example of purification (0.1 µmole scale) of a dye-labeled oligonucleotide can be seen in Figure 3.

Figure 1: Separation of a 10-30mer heterooligonucleotide ladder using different ion-pairing buffers. Retention of native heterooligonucleotides is dictated by both length and sequence. Ladder was generated by digestion with 3′ exonuclease. Oligonucleotide length and 3′ terminal base indicated at peak apex.



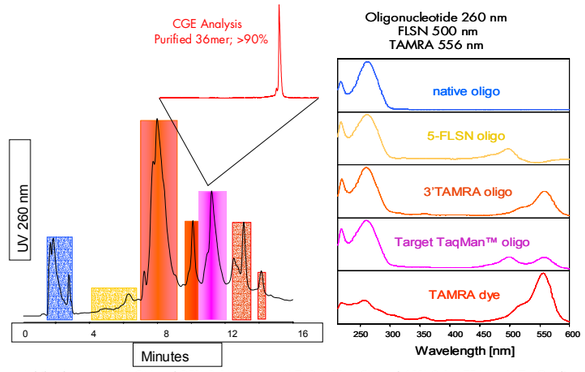
A: Mobile phase A: 5 % acetonitrile and 95 % 0.1M TEAA, pH 7 (v:v). Mobile phase B: 15 % acetonitrile and 85 % 0.1M TEAA, pH 7 (v:v). Gradient starts from 5 % acetonitrile; gradient slope was 0.25 % of acetonitrile per minute. Flow rate 1 ml/min.
B: Mobile phase A: 10 % methanol, 90 % 16.3mM TEA-400mM HFIP buffer, pH 7.9. Mobile phase B: 40 % methanol, 60 % 16.3mM TEA-400mM HFIP buffer, pH 7.9. Gradient starts at 16 % methanol, gradient slope was 0.23 % per minute. Flow rate 1 ml/min.

Figure 3: RP-HPLC purification of a 0.1 µmole crude synthesis of a 25mer mixed sequence oligonucleotide labeled with 5′ HEX fluorescent dye. The analysis was monitored at two wavelengths; 260 nm (DNA absorbance) and 539 nm (absorbance of HEX dye). Comparison of 260 nm and 539 nm chromatograms reveals which oligonucleotide failure sequences are dye-labeled



Mobile phase A: 5% ACN and 95% 0.1M TEAA, pH 7; B: 30% ACN and 70% 0.1M TEAA, pH 7. Gradient from 0% B (5% ACN) to 100% B (30% ACN) in 15 minutes, 1 ml/min.

Figure 4: RP-HPLC separation of a crude synthetic 36mer Taqman™ oligonucleotide using real-time UV-VIS spectrum monitoring. Taqman™ probes are synthesized in a "one-pot" approach. This type of synthesis saves time and cost, however it yields a complicated mixture of failure products. The analysis is monitored at 260 nm (chromatogram on left); real-time UV-VIS spectra monitoring (spectra on right) was used to determine the time of fraction collection. Product was collected when the dually-labeled spectrum (pink trace) was detected.

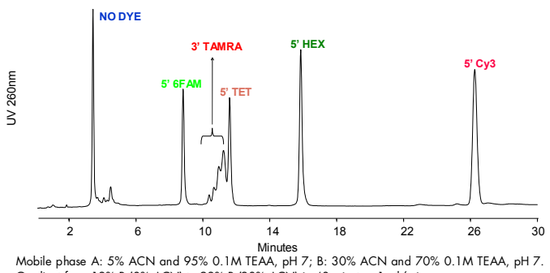


Mobile phase A: 5% ACN and 95% 0.1M TEAA, pH 7; B: 40% ACN and 60% 0.1M TEAA, pH 7. Gradient from 17.1% B (11% ACN) to 60% B (26% ACN) in 30 minutes, 0.5 ml/min.

Experimental

HPLC System: Alliance® 2690 (Waters) equipped with a 996 photodiode array detector
Column: XTerra® MS C_{18}, 4.6 x 50 mm, 2.5 µm
Column temp.: 60 °C
Flow rate: 0.5 or 1.0 ml/min.
HPLC Conditions: See figure captions
Ion-pairing buffers: 100 mM triethylammonium acetate (TEAA) buffer, pH 7, was prepared by titrating the triethylamine (TEA) ion-pairing agent with glacial acetic acid. Oligonucleotides were eluted from the column with an acetonitrile gradient. Alternatively, hexafluoroisopropanol-triethylamine (HFIP-TEA) buffers with a methanol gradient were used for HPLC separations. The TEA ion-pairing reagent (16.3 mM) was buffered with 400 mM HFIP (weak acid); pH was 7.9 [2,4].

Figure 2: Impact of fluorescent dye on oligonucleotide retention. 25mer heterooligonucleotides with identical sequence but different fluorescent labels exhibit different retention behavior. The TAMRA-labeled oligonucleotide elutes in several peaks. These peaks are all 25mer target products labeled with different dye isomers, as determined by LC-MS.



Mobile phase A: 5% ACN and 95% 0.1M TEAA, pH 7; B: 30% ACN and 70% 0.1M TEAA, pH 7. Gradient from 12% B (8% ACN) to 92% B (28% ACN) in 40 minutes, 1 ml/min.

Table 1: Purity and Yield of HPLC-purified Oligonucleotides [a]

Oligonucleotide	Yield @ Dye Absorbance (%)	CGE Crude Purity (%)	CGE Purified Purity (%)
25mer no dye	91.7	85.4	91.5
25mer 5' TET	73.8	58.1	91.1
25mer 5' 6FAM	45.8	52.3	97.7
25mer 5' CY3	71.8	87.4	96.9
25mer 5' HEX	85.4	77.3	93.5
25mer 3' TAMRA	81.5	65.5	80.1[b]

[a]Purity was determined as an area percent of target product from CGE. Yield was calculated as an area percent at the maximum UV absorbance of each dye. This takes into account labeled oligonucleotide sequences only.

[b]This value represents the main peak found by CGE. Some impurities resolved by CGE are also full-length products (Figure 2), resulting in a higher purity than is shown. This separation is caused by dye isomers.

Conclusions

- An IP-RP-HPLC method was developed for the purification of dye-labeled oligonucleotides.
- Purification of a 0.1 µmole synthesis can be routinely performed in a single injection.
- Method was used for isolation of dually-labeled oligonucleotides synthesized in the "one-pot" approach.
- IP-RP-HPLC method was successfully utilized for the quality control of synthetic oligonucleotides.
- Quality of synthetic oligonucleotides varies dramatically from synthesis to synthesis.

References

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