Purification of DNA oligonucleotides Using Oasis® HLB 96-well Extraction Plates

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ABSTRACT

Purification of target oligodeoxyribonucleotides from DNA synthesis was developed based on a solid phase extraction ("trityl on" purification) using a 96-well Oasis® HLB extraction plate. The Oasis® HLB sorbent combines excellent pH stability and a high loading capacity allowing for single step purification of 0.2 µmol scale synthesis. After washing failure sequences off, the oligonucleotide trityl group is cleaved in situ with 2% TFA, and target oligonucleotide is eluted with acetonitrile/0.36 mM triethylamine acetate, pH 11.3 (10/90, v/v). Typical yield of purified product is 75-95 %. Final purity, measured by capillary gel electrophoresis, was found to be 90 % or greater. Alternatively, >99 % purity oligonucleotides can be obtained using RP-HPLC "Trityl off" method with an XTerra™ MS C18 column.

Approaches to synthetic DNA purification:

- Slab Gel Electrophoresis (trityl off)
- Ion-exchange HPLC (trityl off)
- Reversed-phase HPLC (trityl on, trityl off)
- Reversed-phase solid phase extraction (trityl on)

"Trityl on" method separates target oligonucleotide containing hydrophobic trityl group from "trityl off" failure sequences. We used 96-well SPE plate for high throughput purification. "Trityl on" SPE purification is capable of providing oligonucleotide purity up to ~ 95 %.

The source of oligonucleotide impurities:

1) Incomplete coupling - although the reaction is very efficient, a small portion of CPG anchored chain fails to react. These failure sequences, "capped" with acetic anhydride, are major source of impurities (Figure 1).

2) Inefficient detritylation or capping - these fragments are further elongated in the synthesis cycle. Because of missing mononucleotide(s) somewhere inside of the oligonucleotide sequence, these products are called mismatch failure sequences. Because these fragments contain the terminal trityl group, it is difficult to separate them using "trityl on" purification method.

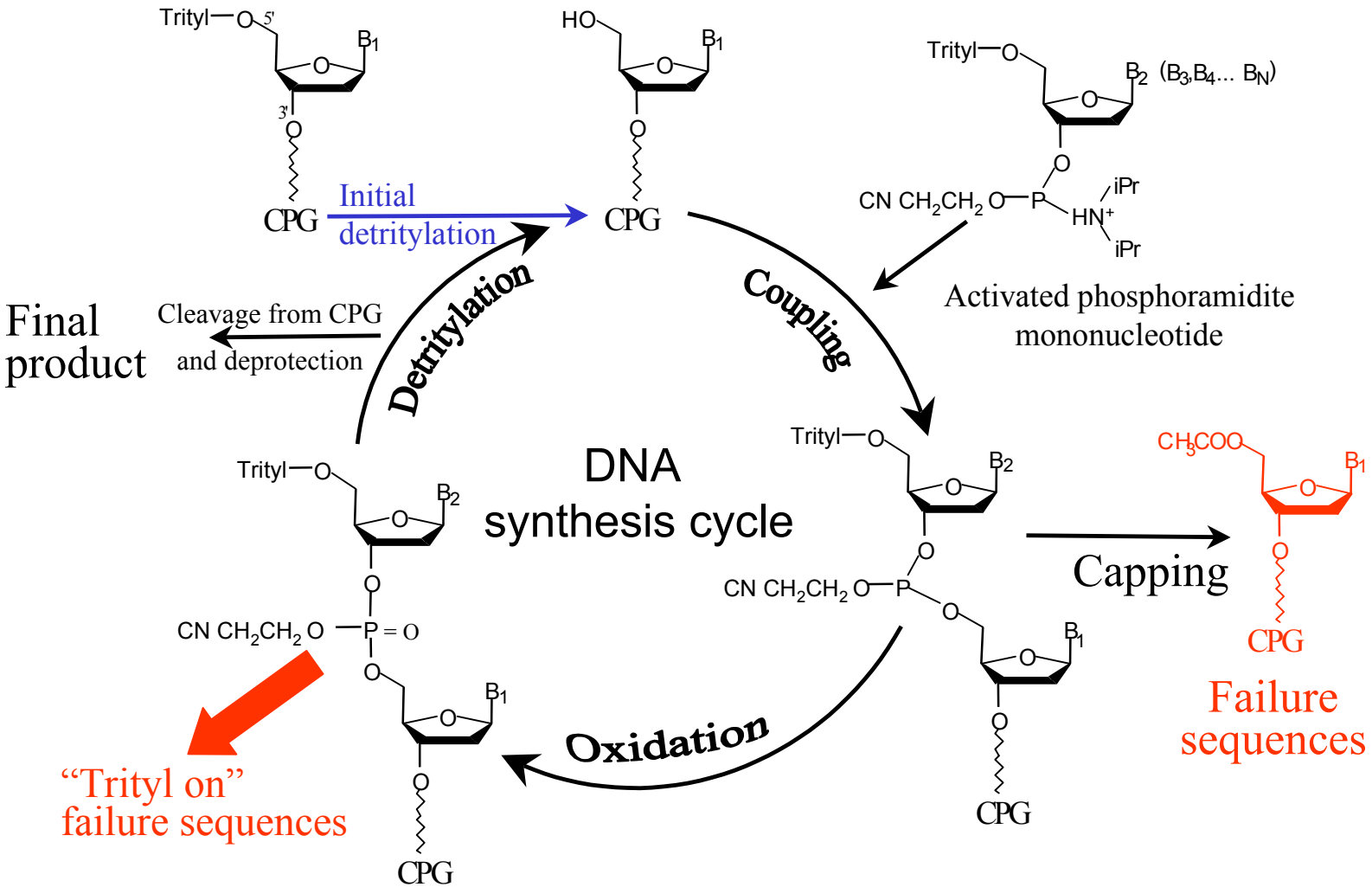
Instrumentation and separation conditions:

Alliance 2690 HPLC separation module with a 996 PDA detector (Waters Corp., Milford, USA) were used. Columns were thermostated in a water bath. Waters Quanta 4000E capillary electrophoresis system was used for Capillary Gel Electrophoretic separation of oligonucleotides.

For quantitation and separation of "trityl off" and "trityl on" oligonucleotides we used SymmetryShield™ RP8, 50 x 3.9 mm, 5 µm. A fast gradient from 10 to 100 % of acetonitrile in 0.1 M TEAAc, pH 7, in 6 minutes was used for phosphorodiester DNA, and a gradient from 30 to 90 % acetonitrile in 0.1 M TEAAc, pH 7, in 4 minutes was used for "trityl on" and "trityl off" separation of phosphorothioate DNA. For SPE method development we used a 20 x 2.1 mm HPLC column packed with 5 µm Oasis HLB sorbent. SPE purification was performed using standard 30 mg Oasis® HLB 96-well plate (30 µm sorbent) and prototype 96-well plate packed with 30 mg Oasis® HLB, 9 µm sorbent. CGE capillaries (13 T, 7M urea, 15 % formamide, 0.1 M Tris-TAPS buffer, pH 8.3) were prepared in house.

RP-HPLC "trityl off" separations were obtained with 50 x 4.6 and 75 x 4.6 mm XTerra™ MS C18, 2.5 µm. For separation conditions see figure captions.

Figure 1: Phosphoramidite DNA synthesis cycle



Oligonucleotide SPE purification protocol

Standard 30 µm, 30 mg Oasis® HLB 96-well extraction plate.

Cartridge conditioning: Apply 1 ml of acetonitrile to each well in the plate by gravity, followed by 1 ml of 0.1M triethylamine acetate (TEAAc) pH 8.

Sample loading: Dissolve lyophilized 0.2 µM oligonucleotide crude synthesis in 1 ml of 0.1M TEAAc pH 8 and apply solution to plate. Flow solution by gravity through the sorbent bed (3-4 minutes). See Figure 2.

Washing out failure sequences: Wash failure sequences from the cartridge using a weak mobile phase. For phosphorodiester (natural) oligonucleotides, use 1 ml of 14 % acetonitrile in 0.1M TEAAc, pH 8. Phosphorothioate oligonucleotides require a stronger eluent: use 20 % acetonitrile in 0.1M TEAAc, pH 8. To increase purity of oligonucleotide product further, a second wash (1ml) of washing solvent can be applied.

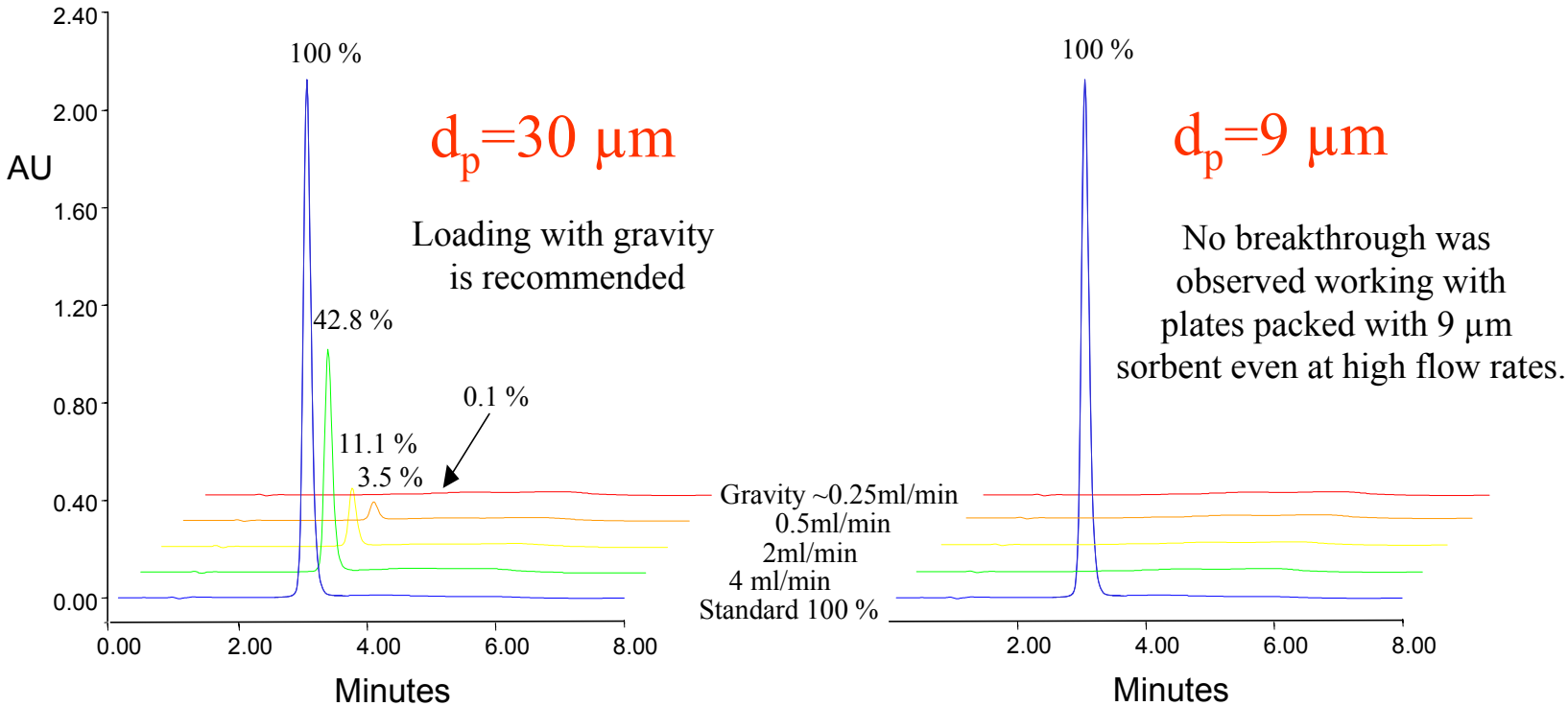
Oligonucleotide SPE purification protocol (Cont.)

Standard 30 µm, 30 mg Oasis® HLB 96-well extraction plate.

Detritylation on sorbent: Detritylate target product with 1 ml of 2 % trifluoracetic acid. It takes 3-4 minutes to pass through sorbent by gravity flow resulting in 95-98 % detritylation. If more efficient detritylation is desired, pass another 1 ml of 2 % TFA through sorbent.

Elution: Use vacuum to elute the detritylated sample with 1 ml of 10 % acetonitrile in 0.36 M TEAAc pH 11.3 at a flow rate 1-2 ml/min. Lyophylize sample to remove traces of TFA and TEAAc from the DNA sample. See also Figure 3.

Figure 2: Effect of loading flow rate on sample breakthrough.

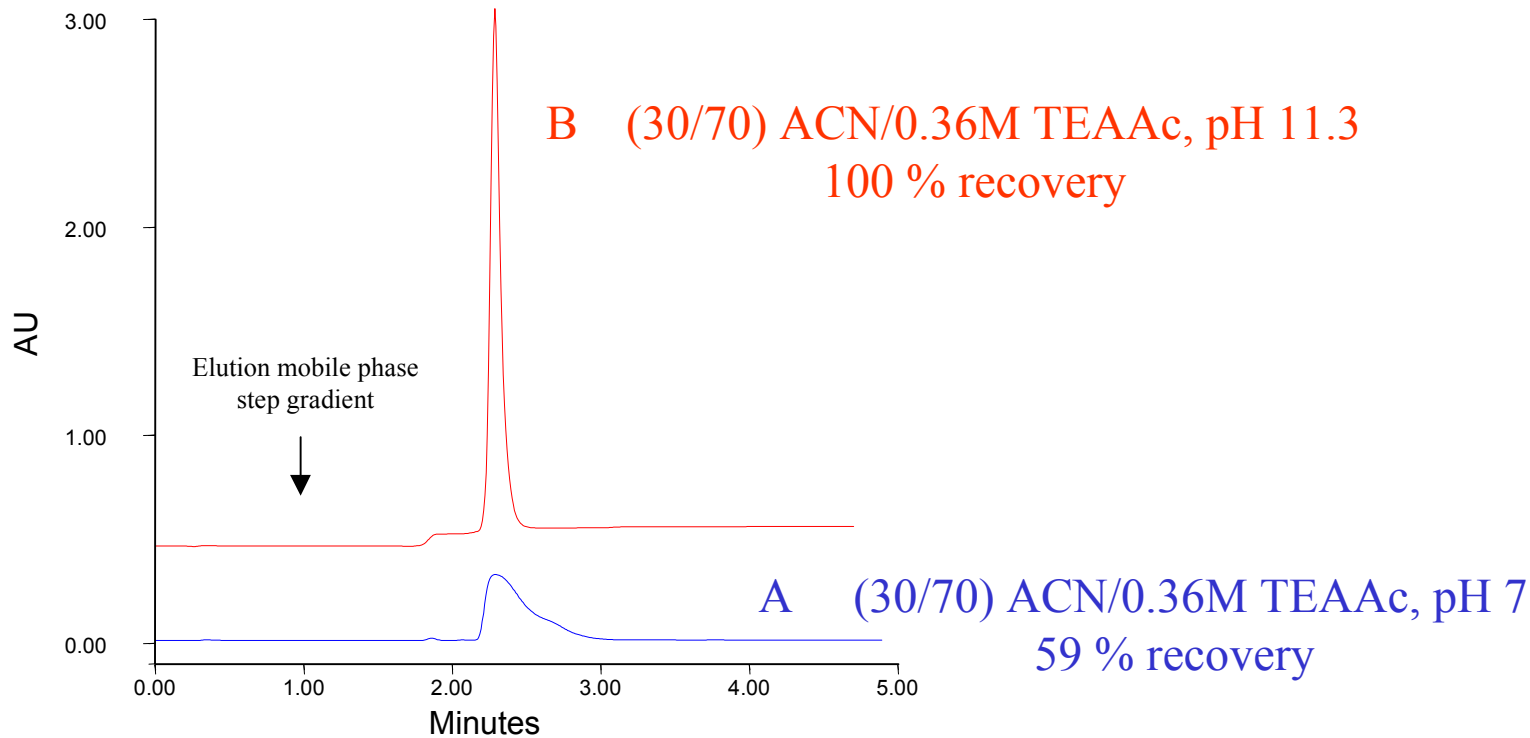


Standard 96-well extraction plate
30 mg/30 µm Oasis® sorbent

Prototype 96-well extraction plate
30 mg/9 µm Oasis® sorbent

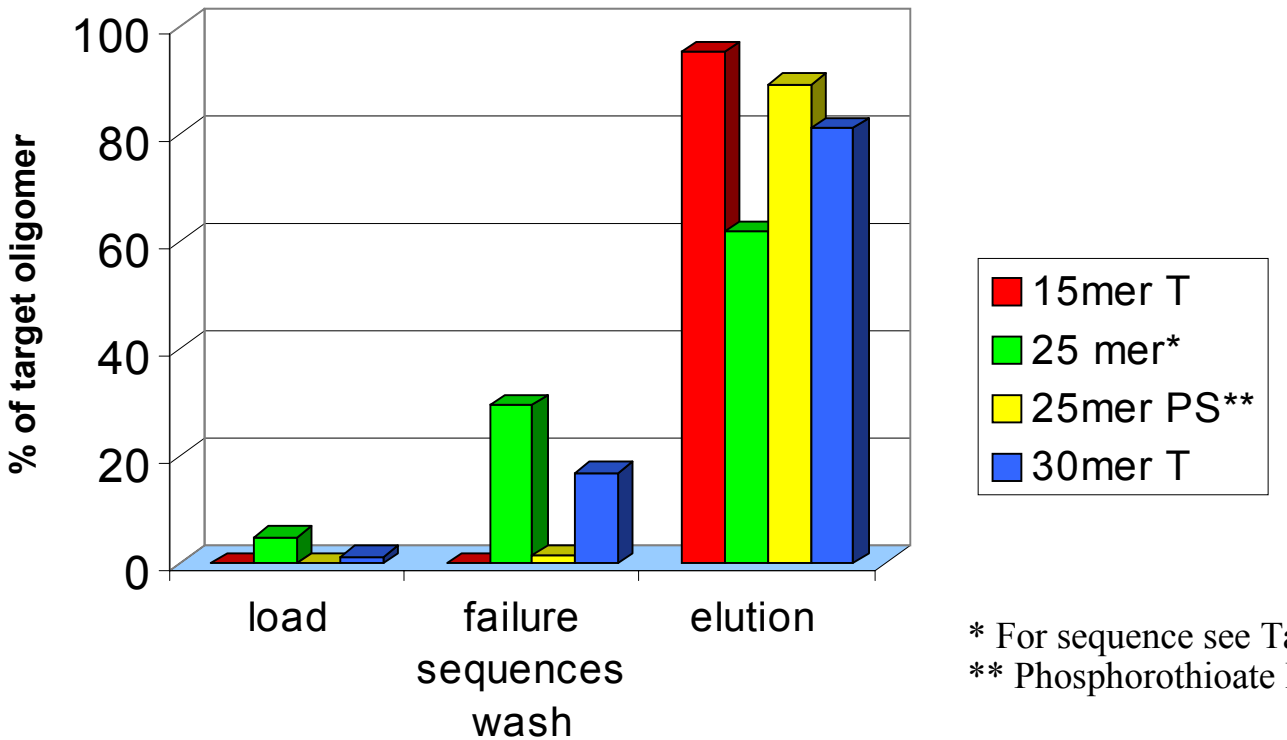
1 mg of 25-mer oligonucleotide was loaded in 1 ml of 0.1 M triethylamine acetate, breakthrough was measured by HPLC

Figure 3: Influence of mobile phase pH on oligonucleotide elution recovery. Studied on 20 x 2.1 mm, 5 µm Oasis® HLB HPLC column.



After oligonucleotide injection (25-mer PS), the column was equilibrated with 2 % TFA, and then eluted with 30 % acetonitrile in triethylamine acetate (TEAAc) buffer. High pH helps to dissolve the oligomer precipitated on column by TFA wash. High pH elution buffer is recommended.

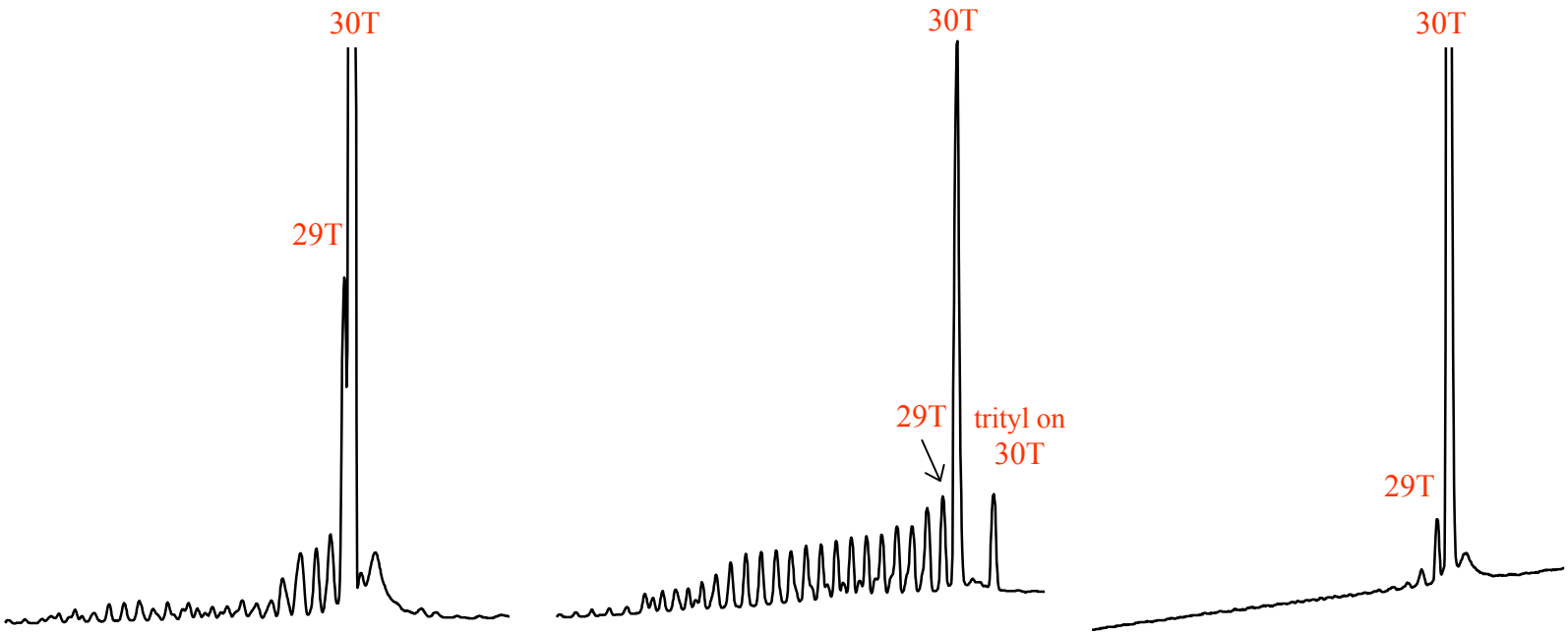
Figure 4: Mass balance of oligomers in purification process using Oasis® HLB 96-well extraction plate (30 mg/30µm).



* For sequence see Table 1.
** Phosphorothioate DNA

Load: gravity. Wash: 1 ml of 14% acetonitrile in 0.1M TEAAc buffer, pH 8 (20% acetonitrile for PS - phosphorothioate DNA) . Elution: 1 ml of 10% acetonitrile in 0.36 TEAAc buffer, pH 11.3 (20% acetonitrile for PS oligonucleotide). Less than 1 % of oligonucleotide was lost in detritylation step (data not shown in the plot).

Figure 5: Capillary gel electrophoresis analysis of fractions from oligodeoxythymine (30-mer) SPE purification



Crude 30T
79.0 % purity

Wash fraction
contains failure sequences, detritylated 30T, and small amount of "trityl on" target 30T

Purified 30T
93.2 % purity

Figure 6: CGE analysis of SPE purified (A) and crude (B) phosphorothioate 25-mer.

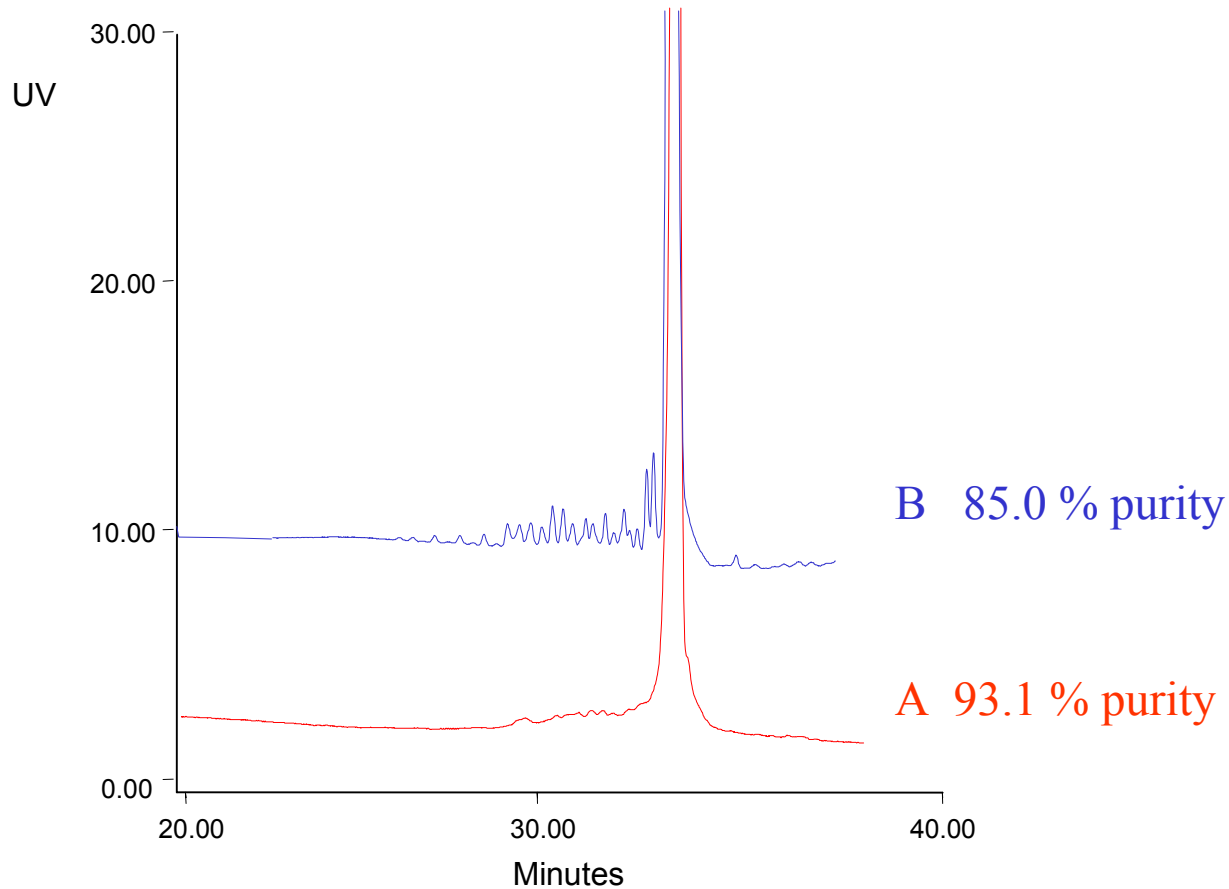


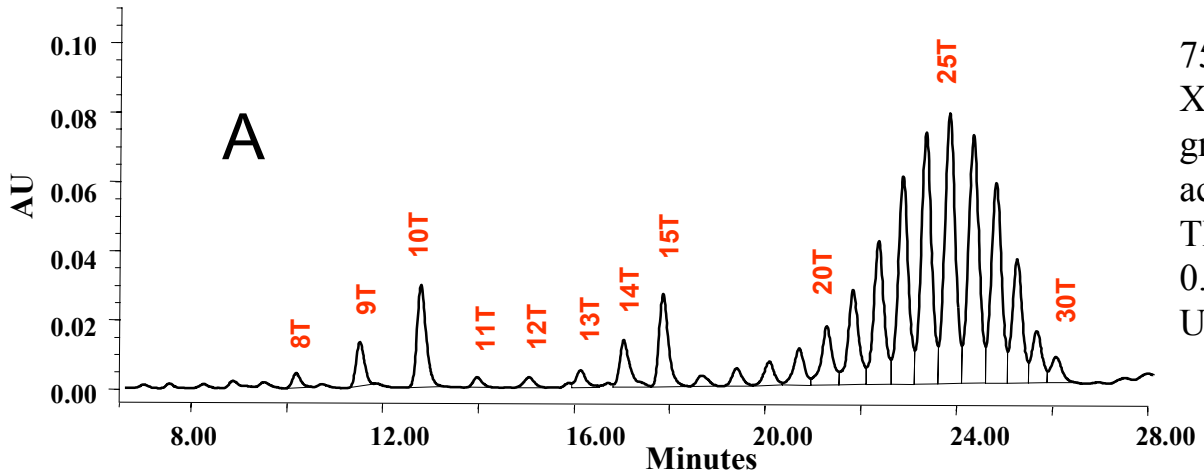
Table 1: Recovery and purity of oligonucleotides obtained from "trityl on" purification using Oasis® HLB 96-well extraction plates

Oligonucleotide sequence (5'-3')	length	crude purity (%)	30 um extraction plate final purity (%)	30 um extraction plate yield (%)	9 um extraction plate final purity (%)	9 um extraction plate yield (%)
TTT TTT TTT TTT TTT	15-mer	89.1	96.0	95.3		
AAA AAA AAA AAA AAA	15-mer	72.6	89.8	83.8		
CCC CCC CCC CCC CCC	15-mer	77.2	89.9	89.3		
CTC TCG CAC CCA TCT CTC TCC TTC T	25-mer	80.7	91.6	61.9	95.1	62.2
CTC TCG CAC CCA TCT CTC TCC TTC T	25-mer PS	85.0	93.1	89.3	94.4	91.1
TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT	30-mer	79.0	93.2	81.1	93.9	96.0

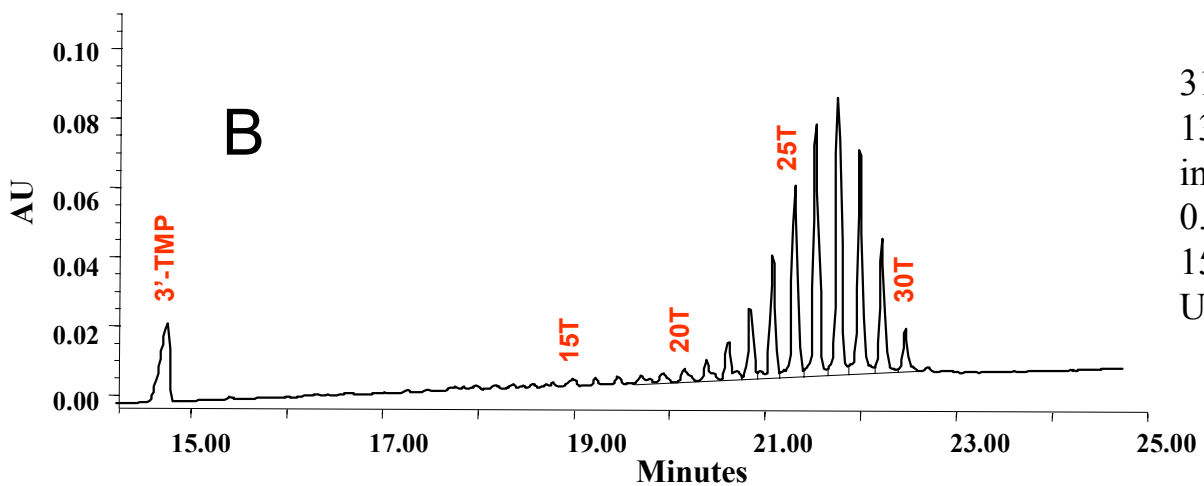
PS - phosphorothioate DNA

Standard 30 µm plate: sample processed by gravity ~ 45 min turnover/96 wells
Prototype 9 µm plate: vacuum processed sample ~ 10 min turnover/96 wells

Figure 7: Monitoring of "trityl off" oligonucleotides purity. (A) - RP-HPLC of $p(dT)_{8-30}$, (B) - CGE separation of $p(dT)_{15-30}$

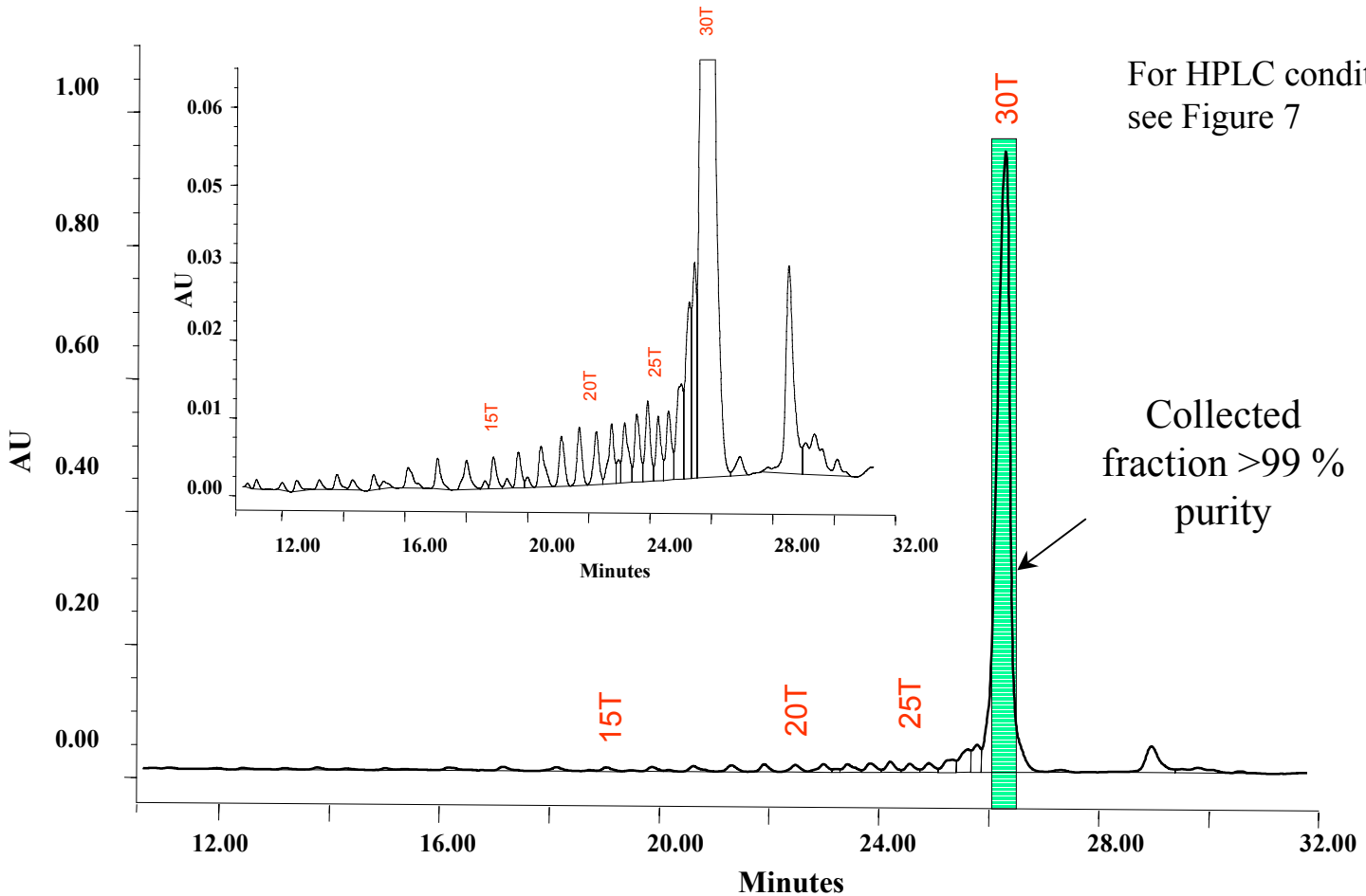


75 x 4.6 mm, 2.5 µm XTerra™ MS C18, 36 min gradient from 10 to 14.5 % acetonitrile in 0.1 M TEAAc, pH 7, 0.5 ml/min, 51.5°C, UV 255 nm

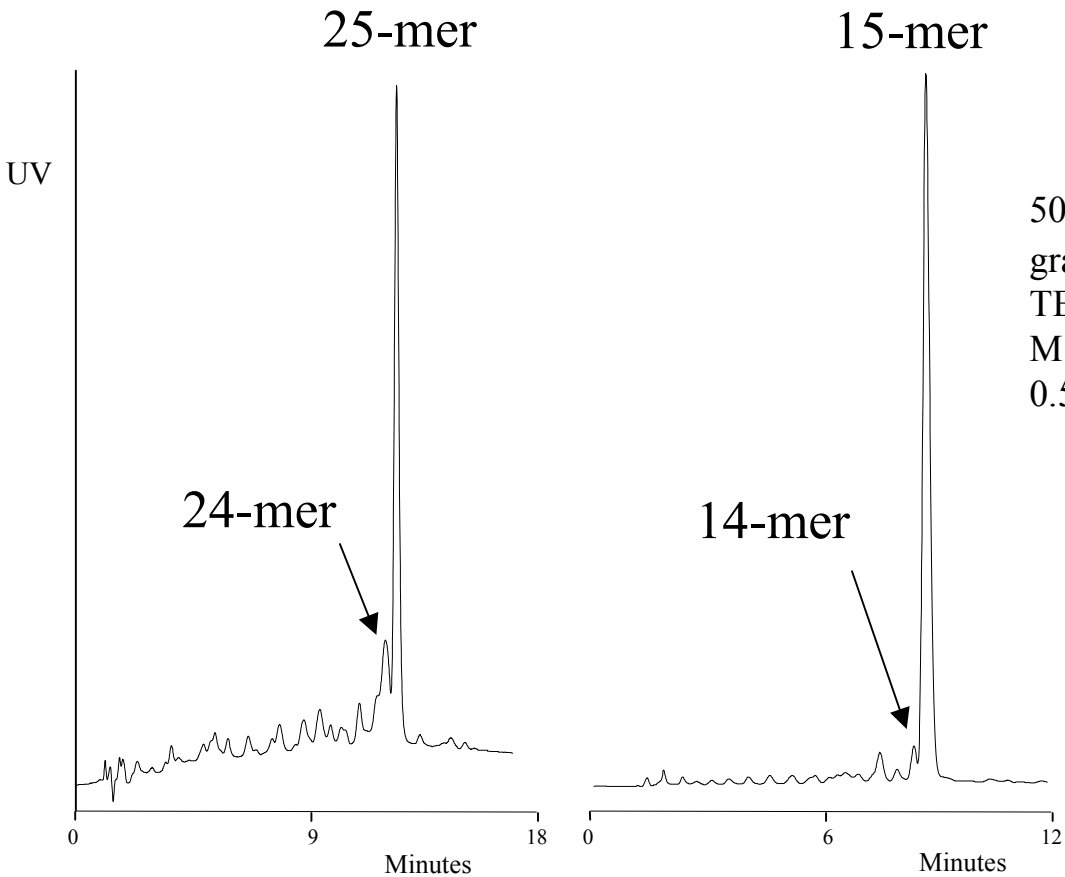


31/24 cm, 75 µm capillary 13 % linear polyacrylamide in 7M urea, 15% formamide 0.1 M Tris-TAPS, pH 8.3, 15 kV run, 30°C UV 270 nm

Figure 8: HPLC purification of crude 30-mer oligodeoxythymidine



Fast DNA purification by HPLC using 50x4.6mm, 2.5 µm XTerra™ MS C18 column



50 x 4.6 mm, 2.5 µm XTerra™ MS C18, gradient from 7.5 % acetonitrile in 0.1 M TEAAc, pH 7, to 10 % acetonitrile in 0.1 M TEAAc, pH 7, in 20 minutes
0.5 ml/min, 51.5 °C, UV 255 nm

CONCLUSION

Unique properties of Oasis® HLB sorbent, including its pH resistance, high capacity, and water wettability, make Oasis® 96-well plates an ideal tool for automated high throughput SPE purification of crude oligonucleotides. High recoveries and purity were demonstrated.

In addition to CGE, we used RP-HPLC for oligonucleotide purity monitoring and high performance "trityl off" purification. Excellent separations of detritylated oligonucleotides were obtained for 8 - 30-mers using an XTerra™ MS C18 column.