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Purification of crude DNA oligonucleotides by solid-phase extraction and reversed-phase high-performance liquid chromatography

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Abstract

Purification of target oligodeoxyribonucleotides from failure sequence by-products of synthesis is often required for polymerase chain reaction primers, DNA sequencing and other oligonucleotide applications. We have developed purification protocols based on a reversed-phase mechanism (''trityl on'' purification) using a 96-well Oasis HLB extraction plate. The Oasis HLB sorbent combines excellent pH stability with a high loading capacity allowing for single-step purification of 0.2 μM scale synthesis. After sample loading and washing, the oligonucleotide trityl group is cleaved on the plate with 2% trifluoroacetic acid. Target DNA is eluted with acetonitrile–0.36 mM triethylamine acetate, pH 11.3 (10:90, v/v). Typical yield of purified product is 60–95%. Final purity, measured by capillary gel electrophoresis, was found to be 90% or greater. Alternatively, highly pure oligonucleotides can be obtained by a RP-HPLC ''trityl off'' method using an XTerra C_{18} column. The use of volatile triethylamine acetate buffer as an ion-pair for RP-HPLC eliminates the need for further desalting.

Keywords: DNA; Oligonucleotides

1. Introduction

Synthetic oligonucleotides are routinely used as a primers for DNA sequencing, as well as for polymerase chain reaction (PCR). Short oligonucleotide sequences (8–30mers) are being investigated as drug candidates for regulation of viral reproduction cycle and modulation of gene expression [1]. These applications require high-throughput chemical synthesis of natural and modified oligonucleotides. State-of-the-art DNA synthesis is largely based on phosphoroamidite chemistry synthesizers capable of parallel production of tens to hundreds of oligonucleotide sequences a day. Although phosphoroamidate synthesis chemistry gives yields greater than 99% for each step in the reaction cycle, the multi-step nature of synthesis results in the target oligonucleotide of limited purity [2,3]. Typical crude synthesis of 25mer oligonucleotides yields product of 80–85% purity; impurities consist of shorter ''failure'' oligonucleotide sequences. Some applications do not require pure DNA. However, even a small contamination of mismatch sequences in PCR primer may cause amplification of unwanted DNA sequences, and therefore extensive PCR background noise.

While oligonucleotide synthesis is relatively fast and inexpensive, the purification is generally time consuming and requires complex procedures resulting in low sample throughput. Two main techniques, electrophoresis and chromatography, are used for target oligonucleotide separation from failure

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sequences [3]. Highly cross-linked polyacrylamide slab gel electrophoresis allows for excellent separation of oligonucleotide ladders from 2 to 100mers. This technique is used when high product purity (>98%) is required. Although electrophoresis is a superior method for DNA separation, it suffers from several problems. UV shadowing and laborious excising of product bands from a gel poses risk of human error, which ultimately compromises purity and recovery of target oligomer. DNA isolated from a gel needs to be desalted prior to further use. Gel loading capacity is low which limits purification to a <1 μM scale. Also, slab gel electrophoresis is difficult to automate.

Two modes of high-performance liquid chromatography (HPLC), anion-exchange and reversed-phase, are frequently used for oligonucleotide purification. Anion-exchange chromatography is suitable for separation of 2–30mer oligonucleotides based on the number of charged phosphate groups in oligonucleotide backbone [4–6]. Selectivity of separation decreases with the length of the oligonucleotide; poor separation is obtained for longer than 30mer DNA fragments. Purity of target DNA therefore depends on its length; yield of the sample is usually sacrificed to obtain better purity by heart-cutting the component of interest. Typical purity varies between 95 and 98%. Collected fractions then require desalting, usually by reversed-phase (RP) HPLC [3].

RP-HPLC for purification of DNA employs volatile ion-pairing buffers, most often triethylamine acetate (TEAAc) buffer [3,7,8]. Similar to anion-exchange HPLC, the separation capability of RP-HPLC decreases with increasing oligonucleotide length. Good separations are obtained for up to 10–12mers. For fragments longer than 20mer, the separation selectivity is minimal. Both ion-exchange and reversed-phase HPLC suffers from slow mass transfer of oligonucleotides in the stationary phase. Improved oligonucleotides separation (up to ~60mer) can be achieved using non-porous sorbents [9–11]. However, the relatively low capacity of the sorbents limits the amount of oligonucleotides that can be purified using columns packed with non-porous sorbents [7].

Because of these difficulties, alternative methods for RP-HPLC purification of oligonucleotides are often used. ‘‘Trityl on’’ purification utilizes the hydrophobicity of the dimethoxytrityl (DMT) protecting group from the DNA synthesis [12]. The DMT group protects the 5′-deoxyribose hydroxyl group during the coupling and oxidation steps (Fig. 1). To assist in RP-HPLC ‘‘trityl on’’ purification, the DMT group is not cleaved after last synthesis step. The resulting crude mixture contains the full length ‘‘trityl on’’ oligomer as well as ‘‘trityl off’’ shorter failure sequences. The product, which contains the hydrophobic trityl group is highly retained by RP-HPLC and therefore is easily separated from ‘‘trityl off’’ failure sequences. After purification, the trityl group is cleaved from the product with acid. If chemically resistant polymeric stationary phases for RP-HPLC are used, this detritylation step can be performed on-column (after washing off failure sequences) with dilute trifluoracetic acid. Subsequent flushing with an acetonitrile–buffer mixture elutes the purified detritylated oligonucleotide.

‘‘Trityl on’’ purification is relatively quick and typically yields ~95% oligonucleotide purity for 25mer. The remaining impurities result from small amounts of shorter failure sequences that also contain the DMT group. The DNA synthesis cycle (Fig. 1) starts from a mononucleotide anchored to a controlled pore glass (CPG) solid support via the 3′-hydroxyl group. After initial detritylation of the 5′-hydroxyl group the coupling step is performed and the next mononucleotide is added to the CPG in the liquid phase. Although coupling is very efficient, a small portion of the anchored chain may fail to react. To avoid further elongation of this failure sequence oligonucleotide, the 5′-hydroxyl is ‘‘capped’’ by reaction with acetic anhydride. This side route of DNA synthesis cycle is a prime source of impurities. These shorter chains are correct sequence oligonucleotides, which have been prematurely halted.

A second source of failure products derives from the inefficient detritylation step in which the 5′-OH group is incompletely deblocked, or when the capping reaction is not performed quantitatively. Both of these failure sequences continue elongation in the next cycle of DNA synthesis (Fig. 1), but because one or more mononucleotides were ‘‘skipped’’ in the synthesis cycle, these oligonucleotides differ from the desired target sequence. Consequently, these mismatch oligomers could be a source of a false

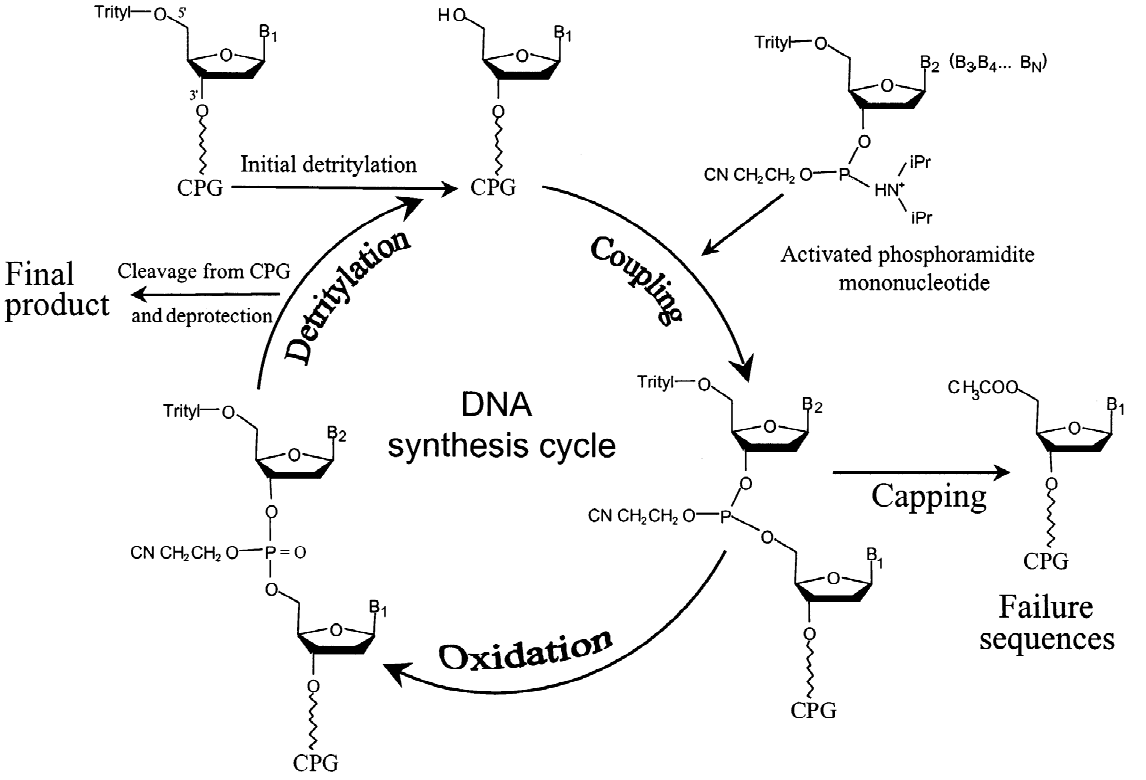

Fig. 1. Phosphoramidite chemistry DNA synthesis cycle. After the last step of synthesis the final product and failure sequences are cleaved from solid support (controlled pore glass, CPG). For further explanation see Introduction.

PCR signal if used as PCR primers. Because these oligonucleotide mismatch sequences are elongated to the last step of synthesis, they contain the DMT group. As a result they cannot be easily separated by RP-HPLC from the desired target oligomer. Another source of ''trityl on'' impurities could be due to depurination of the target oligonucleotide during the DNA synthesis.

Despite these drawbacks, ''trityl on'' RP-HPLC is the most frequently used method for oligonucleotide purification. The main limitation with HPLC purification is the time required. Because HPLC is not a parallel technique, it is not capable of automated purification of hundreds of samples a day. Also, the danger of oligonucleotide cross contamination due to HPLC column memory effect needs to be taken into account.

Reversed-phase solid-phase extraction (SPE) has the promise to solve this bottleneck. Polystyrene sorbents have been used for SPE oligonucleotide purification because of their great pH resistance [13–15]. However, notwithstanding the usefulness of SPE oligonucleotide purification, the published methods sometimes suffer from low yields of target oligonucleotide.

In this work we describe the ''trityl on'' purification protocol for a Waters Oasis HLB 96-well extraction plate, each well packed with 30 mg of divinylbenzene–*N*-vinylpyrrolidone copolymer particles. Oasis HLB sorbent is a novel hydrophilic–lipophilic balanced material with greater capacity and extraction reproducibility than silica-based materials [16–18]. 96-Well extraction plates containing 30 mg of sorbent are well suited for high-throughput purification of 0.2 μM scale oligonucleotide synthesis. The choice of appropriate wash and elution solvents and their pH was found to be crucial for quantitative recovery of oligonucleotides. Similarly to other polymer-based sorbents, Oasis HLB is compatible with pH range 1–14. Relatively small

particle size (30 μm or smaller) of the Oasis HLB sorbent brings advantages for oligonucleotide purification over other SPE products. We discuss the effect of adsorption kinetics on sample breakthrough in the load and wash steps.

Oligonucleotide purity was determined by capillary gel electrophoresis (CGE). The high separation power of CGE even at overloading conditions allows tracking of minor impurities, which are otherwise not detectable. We found that separation performance of conventional RP-HPLC packed with porous sorbents does not provide adequate separation power for monitoring oligonucleotide purity. Slow mass transfer of DNA in stationary phase is the main reason for low separation efficiency. The columns packed with non-porous sorbents [7] are usually preferred for DNA separations, despite of their low sample loading capacity. Here we utilized the XTerra MS C_{18} column packed with 2.5 μm porous sorbent as an alternative to CGE and RP-HPLC on non-porous sorbents. The XTerra column gave us excellent separations for oligonucleotides up to 30 bases long. The ion-pair mode of RP-HPLC with the XTerra column was used for oligonucleotide ''trityl off'' purification and purity monitoring.

2. Experimental

2.1. Instrumentation

The HPLC Alliance 2690 separation module with a 996 photodiode array detector (Waters, Milford, MA, USA) were used. Columns were thermostated in a Nestlab Endocal circulating water bath (Nestlab Instruments, Newington, USA). Capillary electrophoresis was performed using a Waters Quanta 4000E capillary electrophoresis system; linear polyacrylamide gel filled capillaries were prepared in the laboratory according to Ref. [19]. HPLC and CGE data were acquired using Waters Millennium[32] software. SPE was performed using a Millipore vacuum pump Model XX55 000 00 (Millipore, Bedford, MA, USA), and a Waters extraction plate vacuum manifold. Oligonucleotide samples were evaporated in a Pierce Reacti-Therm III heating module under a stream of helium using Reacti-Vap™ III (Pierce, Rockford, IL, USA).

2.2. Materials

Methanol, acetonitrile, glacial acetic acid and triethylamine were purchased from J.T. Baker (Phillipsburg, NJ, USA). Urea, *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED), formamide and acrylamide polyacrylamide gel electrophoresis (PAGE) 40% solution were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Tris, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) and calf spleen phosphodiesterase II were purchased from Sigma (St. Louis, MO, USA); trifluoracetic acid (TFA) was obtained from Pierce. For HPLC and solvent preparation water from a Milli-Q plus ultrapure water system (Millipore) was used.

Phosphorothioate oligonucleotide (25mer) was purchased from Hybridon (Milford, MA, USA). All other oligonucleotides were obtained from Midland Certified Reagent (Midland, TX, USA).

Quantitation and mass balance of oligonucleotide in load, wash, 2% TFA wash, and elution fraction from the SPE plate was performed by RP-HPLC using a SymmetryShield RP8, 50×3.9 mm, 5 μm cartridge column (Waters). For the elution recovery study we used a 20×2.1 mm HPLC column packed in the laboratory with 5 μm Oasis HLB sorbent. XTerra MS C_{18} packing material, 2.5 μm particle size was packed into a 75×4.6 mm column and used for ''trityl off'' separations of synthetic oligonucleotides.

All electrophoretic separations were performed using a 31 cm (effective length 24 cm)×75 μm I.D.×365 μm O.D. capillary. The capillary was filled with 13% linear polyacrylamide buffer containing 15% formamide, 7 *M* of urea and 100 m*M* Tris–TAPS buffer, pH 8.3 [19]. Separation was performed at 15 kV, electrokinetic injection at 10kV for 3–8 s. Separation temperature was 30°C.

2.3. Oligonucleotide SPE purification protocol for 30 mg Oasis HLB 96-well extraction plate

We developed a method for parallel purification of the 96 oligonucleotide samples (0.2 μ*M* synthesis scale) using the Oasis HLB 96-well extraction plate. If desirable, Oasis HLB 1-ml cartridges packed with

30 mg can be used instead of the extraction plate. The protocol consists of five consecutive steps:

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

(2) Sample loading: Dissolve lyophilized 0.2 μ*M* oligonucleotide crude synthesis in 1 ml of 0.1 *M* TEAAc, pH 8 and apply solution to plate. Flow solution by gravity through the sorbent bed (3–4 min).

(3) Washing out the failure sequences: Wash failure sequences from the extraction plate using a weak mobile phase. For phosphorodiester (natural) oligonucleotides, use 1 ml of 14% acetonitrile in 0.1 *M* TEAAc, pH 8. Phosphorothioate oligonucleotides require a stronger eluent: use 20.0% acetonitrile in 0.1 *M* TEAAc, pH 8. To increase purity of oligonucleotide product further, a second wash (1 ml) of washing solvent can be applied.

(4) Detritylation on sorbent: Detritylate target product with 1 ml of 2% trifluoracetic acid. It takes 3–4 min 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.

(5) 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. Lyophilize sample to remove traces of TFA and TEAAc from the DNA sample.

3. Results and discussion

3.1. Effect of flow-rate on oligonucleotide SPE breakthrough

The typical use for the Oasis HLB plate is to clean up drug molecules from plasma proteins and other contaminants. Since the application described here uses significantly larger molecules (molecular mass of 25mer phosphorothioate oligonucleotide is ~7700 g/mol), we were concerned that the smaller diffusion coefficient might adversely affect performance. In particular, flow-rate can significantly affect loading and elution for these compounds. We applied all of the crude 0.2 μ*M* product from a synthesis (~1 mg of 25mer) to the sorbent. This amount is below the sorbent's static mass capacity. The sample was loaded in 0.1 *M* TEAAc buffer, which serves as an ion-pairing agent and improves oligonucleotide retention. Slightly basic buffer, pH 8 was chosen to avoid spontaneous detritylation of the crude oligonucleotide during the sample processing.

As seen from Fig. 2A, we found that significant sample breakthrough occurs at higher sample loading flow-rates. When loading 1 mg of sample at flow-rate 4 ml/min, nearly half of the phosphorothioate 25mer was unretained on the sorbent. We observed that breakthrough decreases as the loading flow-rate decreases (Fig. 2A). Insignificant oligonucleotide loss was found by loading at a flow-rate of 0.25 ml/min (gravity flow). Similar breakthrough at higher flow-rates was found for the 30mer oligodeoxythymidine [$(dT)_{30}$]. On the other hand, negligible breakthrough was observed for 15mer oligonucleotides $(dA)_{15}$, $(dC)_{15}$ and $(dT)_{15}$ (data not shown). These results suggest that the reason for breakthrough is the slow kinetics of mass transfer in stationary phase.

Similar results have been previously described by other researchers; sample breakthrough during loading seems to be general feature of SPE procedures designed for ''trityl on'' purification [13–15]. Multiple reloading of sample on SPE cartridge was recommended to increase sample extraction efficiency.

To avoid excessive sample losses in the load and wash steps as well as to eliminate the sample reloading we used gravity flow for further experiments. It takes approximately 4 min to load 1 ml of sample, which seems to be an adequate price for enhanced SPE recoveries. We speculate that slow diffusion of larger oligonucleotides limits the accessibility of the sorbent's internal surface for sample molecules. This results in overloading of the sorbent's outer surface and leads to sample breakthrough. In other words, decreasing the loading flow-rate enhances accessibility of sorbent surface and improves extraction performance.

An alternative approach to increase the sorbent surface accessibility is to shorten the diffusion path by using a smaller particle size. We packed prototype 96-well plates with 30 mg of 9 μm Oasis HLB sorbent. The diffusion path is substantially shorter than with 30 μm particles used in the standard

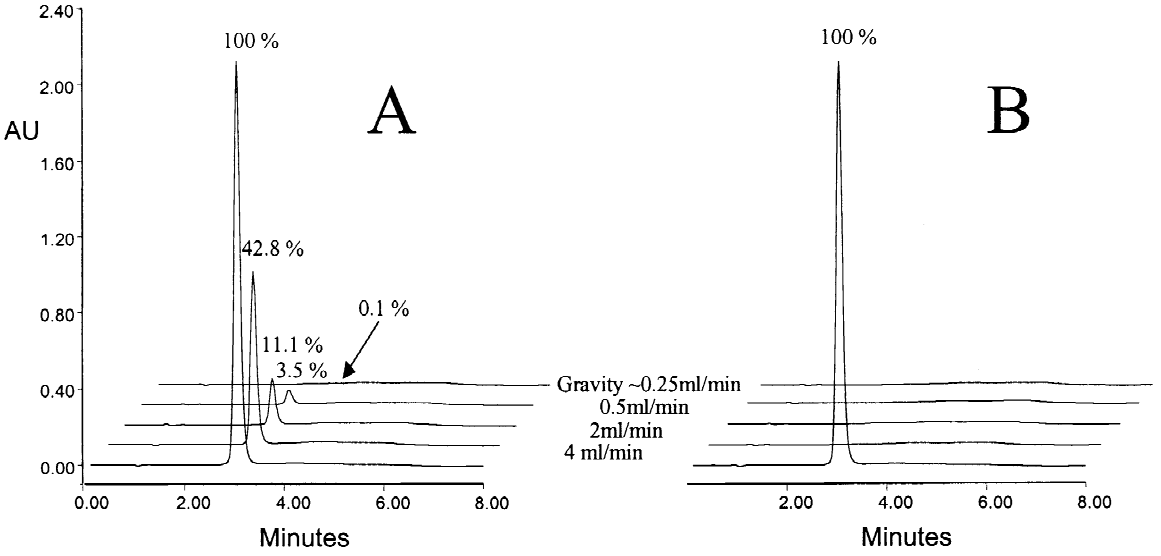

Fig. 2. Effect of sample loading flow-rate on SPE breakthrough. (A) SPE 96-well plate packed with 30 mg of 30 μm Oasis HLB; (B) SPE 96-well plate packed with 30 mg of 9 μm Oasis HLB sorbent. A 1-ml volume of 25mer phosphorothioate oligonucleotide (1 mg/ml) in 0.1 *M* TEAAc, pH 8 was loaded onto Oasis HLB plate wells at designated flow-rate. Breakthrough was measured by HPLC and related to the direct injection of original solution.

Waters Oasis HLB 96-well extraction plates or other competitive products. We repeated the experiments to determine the effect of flow-rate using the 9 μm prototype extraction plate. No breakthrough was detected even at the highest loading rate of 4 ml/min (Fig. 2B).

3.2. Washing and elution SPE conditions

The key step in oligonucleotide purification is to selectively wash the ''trityl off'' failure sequences from the SPE sorbent while keeping the ''trityl on'' oligonucleotide adsorbed to the sorbent. We used detritylated full length target oligonucleotides as a ''trityl off'' model impurity for the wash experiment. Retention behavior of ''trityl on'' and ''trityl off'' oligonucleotides were evaluated by using a prototype 20×2.1 mm I.D. HPLC column packed with 5 μm Oasis HLB sorbent. The column length and particle size of packing was different from those of SPE plate, but the selectivity of sorbent is identical. Two different sets of washing mobile phase strength were evaluated: one for natural phosphorodiester DNA and the second for chemically modified more hydrophobic phosphorothioate DNA. Selected washing conditions are shown in Table 1. Wash mobile phase strength could be further modified for shorter oligonucleotides (Table 1) or for different oligo chemical modifications.

We evaluated the elution mobile phase strength using the same 20×2.1 mm I.D. HPLC column. We found that nearly 100% of the oligonucleotide was eluted as a narrow symmetrical peak with 20% acetonitrile in 0.1 *M* TEAAc (pH 7). However, oligonucleotide recovery decreased when we treated oligonucleotide on-column with 2% TFA (simulated detritylation step). An experiment was performed as follows: 10 μg of oligonucleotide was injected onto a column that was equilibrated with 0.1 *M* TEAAc, pH 8. Subsequently, 2% TFA was pumped through the column. Next, a step gradient with stronger mobile phase was performed (see below). We found that after the TFA wash, the sample elutes as a broad zone with an elution volume of several milliliters. Oligonucleotide recovery varied between 50 and 80% when using 20–50% of acetonitrile in 0.1 *M* TEAAc, pH 7. This experiment suggests that TFA is causing precipitation of oligonucleotide on the column. Less than 10% of the injected oligonucleotide was recovered using 100% acetonitrile. Although

Table 1
Recommended conditions for oligonucleotide ''trityl on'' purification using Oasis HLB 96-well extraction plates (ACN=acetonitrile)

	Wash conditions	Detritylation	Elution conditions
Phosphorodiester; <12mer[a]	1 ml of ACN–0.1 *M* TEAAc, pH 8 (10:90)	1 ml of 2% TFA in water	1 ml of ACN–0.36 *M* TEAAc, pH 11.3 water buffer (10:90)
Phosphorodiester; 12–30mer	1 ml of ACN–0.1 *M* TEAAc, pH 8 (14:86)	1 ml of 2% TFA in water	1 ml of ACN–0.36 *M* TEAAc, pH 11.3 water buffer (10:90)
Phosphorothioate (PS); <12mer[a]	1 ml of ACN–0.1 *M* TEAAc, pH 8 (15:85)	1 ml of 2% TFA in water	1 ml of ACN–0.36 *M* TEAAc, pH 11.3 water buffer (10:90)
Phosphorothioate; (PS); 12–30mer	1 ml of ACN–0.1 *M* TEAAc, pH 8 (20:80)	1 ml of 2% TFA in water	1 ml of ACN–0.36 *M* TEAAc, pH 11.3 water buffer (10:90)

[a] For oligomers <12mer, these conditions are guidelines. For your specific sequence, specific optimization may be required.

pure acetonitrile is the strongest eluent, recovery of oligonucleotide decreased due to the poor DNA solubility in the organic solvent.

To elute the sample from the column quantitatively, we employed a high-pH mobile phase. Using the 0.36 *M* TEAAc buffer, pH 11.3 without any organic additive we found that a major portion (~80%) of adsorbed oligonucleotides was eluted from column. If 10% acetonitrile in 0.36 *M* TEAAc, pH 11.3 was used, nearly 100% recovery for phosphorodiester oligonucleotides was obtained. Gradient step eluted the sample as a narrow zone, with a peak elution volume ~0.25 ml. For elution of more retained phosphorothioate oligonucleotide, 20% acetonitrile in TEAAc buffer was required.

Most of the ''trityl on'' purification procedures published in the literature use as a loading and washing mobile phase solution of NH_4OH. However, when we loaded a mixture of ''trityl on'' and ''trityl off'' 25mer oligonucleotides in 5% NH_4OH on an Oasis HLB extraction plate, we observed 80% and 62.4% breakthrough for ''trityl off'' and ''trityl on'' oligonucleotide, respectively. It is apparent that high-pH solvents behave as a high-elution-strength mobile phase. Repeated loading of the oligonucleotides solution was necessary to achieve better adsorption of ''trityl on'' oligonucleotide on cartridge. Therefore, contrary to other published protocols [13–15], we strongly recommend loading the oligonucleotide samples in 0.1 *M* TEAAc, pH 8. Also, with Oasis HLB sorbent, we recommend using acetonitrile solutions as a washing mobile phase (Table 1) to obtain the best purity of target DNA product, instead of solution of ammonium hydroxide [13–15].

3.3. SPE purification of crude oligonucleotides

The developed load, wash and elution conditions were employed for SPE purification of several oligonucleotides. Lyophilized crude oligonucleotides (0.2 μ*M* synthetic scale) were dissolved directly prior to use in 0.1 *M* TEAAc buffer, pH 8. Oasis HLB extraction plates containing either 30 μm or 9 μm sorbent were used. The purification protocol is described in Experimental. For loading of fractions onto the 9 μm extraction plate, a vacuum was used. During the detritylation step, vacuum was disconnected after loading 2% TFA. Detritylation reaction was performed for 5 min, then vacuum was resumed and the remainder of trifluoracetic acid was drawn through the cartridge.

A SymmetryShield RP8, 50×3.9 mm column was used for HPLC monitoring of all fractions from SPE. A 6-min acetonitrile gradient from 10% to 100% in 0.1 *M* TEAAc, pH 7 was used for phosphorodiester (PO) oligonucleotides, and a 4-min gradient from 30% to 90% acetonitrile was used for phosphorothioate (PS) oligonucleotide. Mass balance for selected oligonucleotides is shown in Table 2. For the plate containing 30 μm sorbent, we did not observe breakthrough for any of the 15mers (data not shown), while breakthroughs of the 25- and 30mers were 4.8 and 1.2%, respectively. The prototype 9 μm plate resulted in 0.8% breakthrough of 25mer and no breakthrough for 30mer when using a 4 ml/min loading flow-rate. We detected sample losses for the 25- and 30mers from the washing step (Table 2), but no breakthrough for any of the 15mers, even though these shorter oligonucleotides are less retained on

Table 2
Mass balance of oligomers in purification process using Oasis HLB 96-well extraction plate packed with 30 mg of 30 μm particle size sorbent

Oligonucleotide sequence (5′–3′)	Oligonucleotide length	Target oligonucleotide in SPE fraction		
		Load breakthrough (%)	Wash breakthrough (%)	Elution recovery (%)
TTT TTT TTT TTT TTT	15mer	0.0	0.0	95.3
CTC TCG CAC CCA TCT-CTC TCC TTC T	25mer	4.8	29.5	61.9
CTC TCG CAC CCA TCT-CTC TCC TTC T	25mer PS[a]	0.0	1.4	89.3
TTT TTT TTT TTT TTT-TTT TTT TTT TTT TTT	30mer	1.2	16.8	81.1

[a] Phosphorothioate DNA.

Oasis sorbent. We found that an additional wash with a second milliliter of the same mobile phase did not further elute the ''trityl on'' 25- and 30mer oligonucleotides. We tried modifying the wash procedure using a lower strength mobile phase, but this modification did not lower the breakthrough significantly. Therefore, we decided to sacrifice quantitative recovery for the final product purity using the original procedure (Table 2). We found a smaller, but still noticeable sample breakthrough (for 25- and 30mers) with the prototype plate containing 9 μm sorbent. Because longer oligonucleotides are more affected one can expect that >30mer oligomers will give generally lower yields in purification on the Oasis HLB extraction plate.

Negligible sample losses were detected in the detritylation step (not shown in Table 2). The elution fraction in Table 2 represents the final purification yield (related to the original concentration of ''trityl on'' oligonucleotides). The lowest yield was 61.9%, other oligonucleotide recoveries were between 80 and 95%. Critical comparison of performance of existing and prototype Oasis HLB extraction plates reveals lower breakthrough in load and wash step for the prototype plate. This performance was obtained with high flow-rate using a vacuum manifold. This reduces total purification time for 96 samples to 5–10 min compared with 30–45 min for gravity flow purification using the standard plate containing 30 μm sorbent. Purity of oligonucleotides was comparable for both SPE plates (Table 3).

Oligonucleotide purity prior to and after SPE purification was estimated by CGE. All N-1 failure products were clearly separated from full-length oligonucleotide. Fig. 3 shows a typical example of crude purified 25mer phosphorothioate oligonucleotide. The major portion of failure sequences was removed from the sample; remaining contaminants originates mostly from ''trityl on'' failure sequence impurities.

3.4. Oligonucleotides purification by ion-pair RP-HPLC

Micro-pellicular non-porous sorbents has been shown to have the best performance for HPLC separation of oligonucleotides and double-stranded DNA (dsDNA) molecules. Slow mass transfer of DNA in the stationary phase disfavors porous sorbents due to low HPLC separation efficiency, or at least requires excessively long analysis times to obtain comparable performance [7]. On the other hand, non-porous stationary phases have low capacity, which limits their usefulness for semi-preparative purifications.

The general approach for oligonucleotide separation optimization by RP-HPLC calls for small particle size, large pore or non-porous particles, enhancement of oligonucleotide diffusion rate by increased separation temperature, and slow mobile phase flow-rates. C_{18} surface chemistry was found to be most successful for RP-HPLC oligonucleotide separation [7–12]. We compared performance of four columns: Symmetry C_{18}, 150×3.9 mm, 5 μm;

Table 3
Purification of phosphorodiester and phosphorothioate (PS) DNA oligonucleotides using Oasis HLB 96-well extraction plates

Oligonucleotide length (sequence 5′–3′)	Crude purity (%)	30 μm extraction plate		9 μm extraction plate	
		Final purity (%)	Yield (%)	Final purity (%)	Yield (%)
15mer (TTT TTT TTT TTT TTT)	89.1	96.0	95.3		
15mer (AAA AAA AAA AAA AAA)	72.6	89.9	83.8		
15mer (CCC CCC CCC CCC CCC)	77.2	89.9	89.3		
25mer (CTC TCG CAC CCA TCT CTC TCC TTC T)	80.7	91.6	61.9	95.1	62.2
25mer PS[a] (CTC TCG CAC CCA TCT CTC TCC TTC T)	85.0	93.1	89.3	94.4	91.1
30mer (TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT)	79.0	93.2	81.1	93.9	96.0

[a] Phosphorothioate DNA.

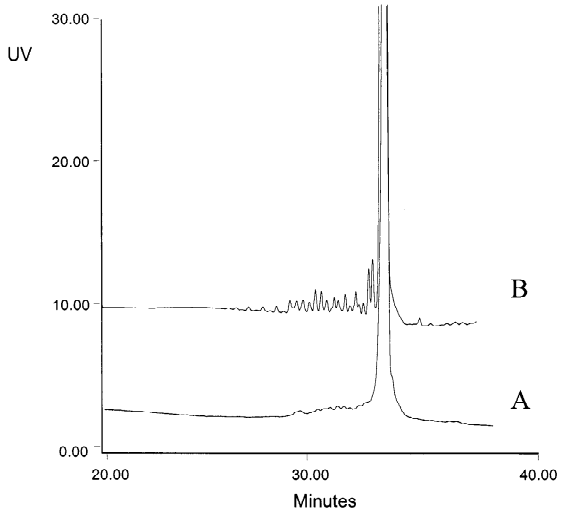

Fig. 3. CGE electropherograms of purified (A) and crude synthesis (B) 25mer phosphorothioate oligonucleotide. Estimated purity before and after purification was 85% and 93.1%, respectively. Separation conditions: 31 cm (effective length 24 cm)×75 μm I.D. capillary, filled with 13% T linear polyacrylamide gel, 7 *M* urea, 15% formamide, 100 m*M* Tris–TAPS, pH 8.3; 15 kV run; 3 s injection at 10 kV. Detection wavelength was set to 270 nm. Separation temperature was 30°C. [T=(g acrylamide+ g *N,N′*-methylenebisacrylamide)/100 ml solution].

Symmetry300 C_{18}, 150×3.9 mm, 5 μm; prototype Oasis HLB, 150×3.9 mm, 5 μm; and XTerra MS C_{18} 75×4.6 mm, 2.5 μm. Separation of oligodeoxythymidine ladder $(dT)_{8-30}$ was performed at ~50°C, using flow-rates from 0.25 to 1 ml/min, and shallow gradients. The oligodeoxythymidine ladder was prepared in the laboratory by 5′-exonuclease separate digestion of $(dT)_{10}$, $(dT)_{15}$ and $(dT)_{30}$. Partially hydrolyzed oligonucleotides were pooled after enzymatic reaction. Shallow gradients were employed to obtain good separation, while keeping acceptable peak widths. We applied a gradient slope of 0.25% of acetonitrile per ml of mobile phase. As a consequence of the shallow gradient, the separation time could be very long. Therefore the choice of initial strength of mobile phase is important. 10% acetonitrile in 0.1 *M* TEAAc buffer, pH 7 was used as an initial mobile phase for all C_{18}-based columns; for the Oasis HLB column the initial mobile phase strength was 12% acetonitrile.

Fig. 4 shows how reduced flow-rates improve sample mass transfer in stationary phase and therefore enhance the chromatographic performance. Three chromatograms of $(dT)_{15}$ and $(dT)_{30}$ oligomer mixture acquired on the 5 μm Oasis HLB 150×3.9 mm column. Relative peak width decreases with decreasing flow-rate, resulting in an improvement of

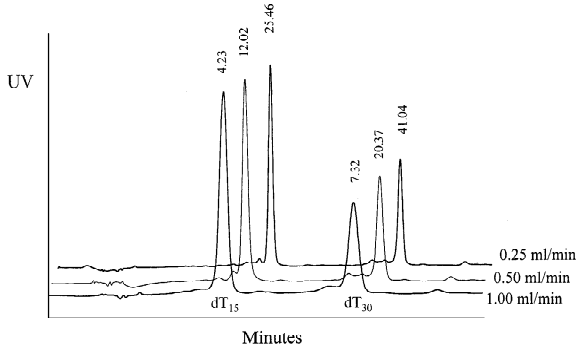

Fig. 4. Effect of mobile phase flow-rate on column peak capacity. Gradient elution was adjusted accordingly to keep peak elution volume constant. Slow flow-rate decreases peak relative width, which results in improvement of column peak capacity. Separation was performed on a 150×3.9 mm column packed with 5 μm Oasis HLB sorbent. Gradient started from 12% of acetonitrile in 0.1 *M* TEAAc, pH 7; gradient slope was adjusted to the flow-rate – 0.25% of acetonitrile per ml of mobile phase. Column was heated to 51.5°C, UV detection wavelength was set at 270 nm.

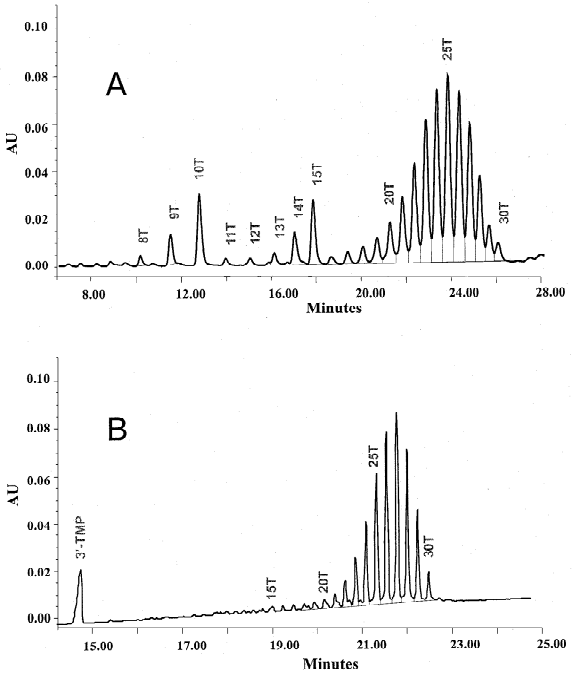

Fig. 5. Separation of oligodeoxythymidine ladder by (A) RP-HPLC (10-, 15- and 30mer oligoT digested by exonuclease); 75×4.6 mm, 2.5 μm XTerra MS C_{18}, 36 min gradient from 10 to 14.5% acetonitrile in 0.1 *M* TEAAc, pH 7, flow-rate 0.5 ml/min, column temperature 51.5°C, UV detection at 255 nm. (B) CGE separation of exonuclease-digested 30mer oligoT; capillary 31 cm (effective length 24 cm)×75 μm I.D., filled with 13% linear polyacrylamide in 7 *M* urea, 15% formamide, and 0.1 *M* Tris–TAPS, pH 8.3. Running voltage was 15 kV, capillary temperature 30°C, UV detection at 270 nm.

the column peak capacity [20]. Analogous behavior was found with the 2.5 μm XTerra MS C_{18} column. Most interestingly, no significant difference in performance was found with Symmetry C_{18}, 5 μm, and Symmetry300 C_{18}, 150×3.9 mm, 5 μm, columns. Larger 300 Å pores did not provide an advantage over common 100 Å pores, which suggest that the size of oligonucleotide fragments is not crucial for their diffusion throughout sorbent pores. Taking into account the fact that the single-stranded 30mer fragment can be pictured as a very flexible rod of ~100 Å length and ~20 Å width, which is comparable with a pore size of 100 Å Symmetry packing, this finding is rather surprising.

Separation of the oligonucleotide ladder was obtained with all columns even for the longest 29mer and 30mer fragments. We were able to achieve the shortest analysis time and the best separation on the XTerra column because of the smaller particle size of packing (shorter diffusion path). The separation performance is comparable to the one obtained by CGE (Fig. 5). This HPLC method can be used for monitoring of crude/purified oligonucleotide purity and for small-scale ''trityl off'' purification (up to 0.05 μ*M* synthesis in single injection). High-purity product can be isolated from crude synthetic mixture without the need for further sample detritylation or desalting.

4. Conclusion

We have developed a method for fast purification of DNA oligonucleotides using Oasis HLB 96-well extraction plates. Sorbent capacity is sufficient for purification of 0.2 μ*M* DNA synthetic scale. ''Trityl on'' oligomers were slowly loaded on the plate in 0.1 *M* TEAAc buffer. Sample loading speed was found to be crucial for purification yield. ''Trityl off'' failure sequences were washed with mobile phase containing acetonitrile in TEAAc buffer, pH 7. Complete detritylation of target oligomer was

achieved on cartridge by washing with 2% TFA. The elution of the product was performed using 10% of acetonitrile in TEAAc buffer, pH 11.3. High pH was found to improve recovery of DNA from the SPE sorbent. The typical purity of oligonucleotides obtained was 90–95% with recovery of target compound of 60–96%. Extraction plates packed with small particle size sorbent (9 μm instead of regular 30 μm) minimized sample breakthrough and allowed for increased speed of oligonucleotides purification.

Ion-pair RP-HPLC separation of detritylated DNA oligonucleotides was shown to be useful for small-scale (0.05–1 μ*M*) DNA purification. We demonstrated good separation up to 30mer using the XTerra MS C_{18} column packed with 2.5 μm porous sorbent. RP-HPLC is capable of replacing CGE for purity analysis of crude synthetic oligonucleotides.

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References

[1] A.A. Padmapriya, J. Tang, S. Agrawal, Antisense Res. Dev. 4 (1994) 185.
[2] Z. Zhang, J.Y. Tang, Curr. Opin. Drug Discovery Dev. 1 (1998) 304.
[3] W.J. Warren, G. Vella, Mol. Biotechnol. 4 (1995) 179.
[4] Y. Kato, Y. Yamasaki, A. Onaka, T. Kitamura, T. Hashimoto, J. Chromatogr. 478 (1989) 264.
[5] W.A. Ausserer, M.L. Biros, BioTechniques 19 (1995) 136.
[6] G. Jilge, K.K. Unger, U. Esser, H.-J. Schafer, G. Rathgeber, W. Muller, J. Chromatogr. 476 (1989) 37.
[7] C.G. Huber, J. Chromatogr. A 806 (1998) 3.
[8] T. Uesugi, K. Sano, Y. Uesawa, Y. Ikegami, K. Mohri, J. Chromatogr. B 703 (1997) 63.
[9] C.G. Huber, P.J. Oefner, G.K. Bonn, J. Chromatogr. 599 (1992) 113.
[10] C.G. Huber, P.J. Oefner, E. Preuss, G.K. Bonn, Nucleic Acids Res. 21 (1993) 1061.
[11] C.G. Huber, P.J. Oefner, G.K. Bonn, Anal. Biochem. 212 (1993) 351.
[12] T.L. Hill, J.W. Mayhew, J. Chromatogr. 512 (1990) 415.
[13] Oligopure: Instruction for Use, Document No. 69157 (rev. A), Hamilton, Reno, NV, USA, 1994.
[14] User Guide to DNA Purification, Glen Research, Glen Research, Sterling, VA, USA, 1999.
[15] Oligonucleotide Purification Cartridge: Oligonucleotide Purification Protocol, Perkin-Elmer, Applied Biosystems Division, USA, 1999.
[16] E.S.P. Bouvier, D.M. Martin, P.C. Iraneta, M. Capparella, Y.F. Cheng, D.J. Phillips, LC·GC 15 (1997) 152.
[17] E.S.P. Bouvier, P.C. Iraneta, U.D. Neue, P.D. McDonald, D.J. Phillips, M. Capprella, Y.F. Cheng, LC·GC 16 (1998) S53.
[18] Y.F. Cheng, U.D. Neue, L. Bean, J. Chromatogr. A 828 (1998) 273.
[19] A. Belenky, D.L. Smisek, A.S. Cohen, J. Chromatogr. A 700 (1995) 137.
[20] J.M. Davis, J.C. Giddings, Anal. Chem. 55 (1983) 418.