DuPont Water Solutions
Life Sciences and Specialties

Purification of oligonucleotides with polymeric reverse phase HPLC resins

Introduction

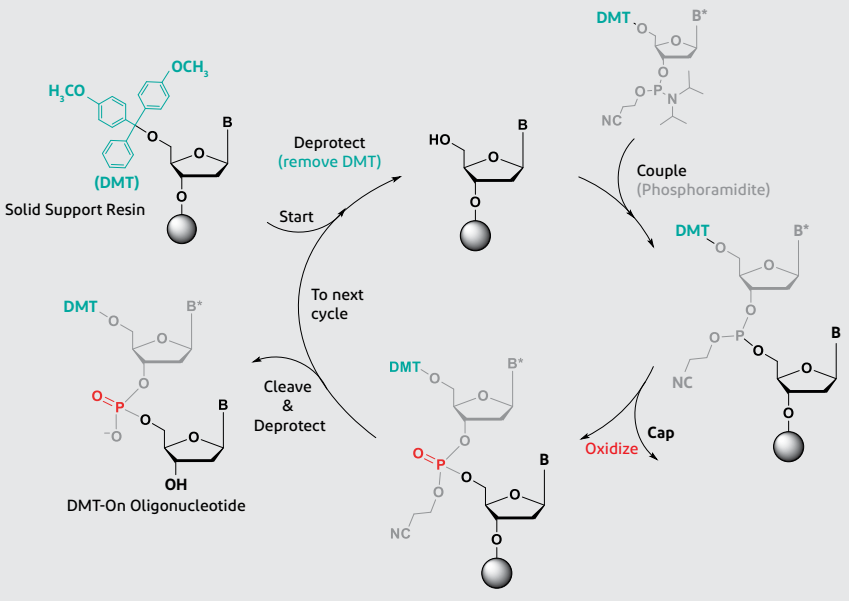
Oligonucleotides of DNA and RNA are important for therapeutic, diagnostic, and research uses. Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) is frequently chosen for preparative chromatography when high purity oligonucleotides are desired. The oligonucleotide is purified by selective adsorption and desorption to the reverse phase resin through a hydrophobic interaction mechanism. By changing the composition of the mobile phase, RP-HPLC can be designed for either trityl-on or trityl-off purification. In this paper, we describe the strategies for purification of oligonucleotides by these two routes and outline the impact that the resin and method selection can have on the separation.

Synthesis of Oligonucleotides

Nearly all synthetic oligonucleotides are made by solid phase synthesis utilizing the phosphoramidite process developed by Caruthers in 1985.[1] The process entails the sequential buildup of the oligonucleotide chain through stepwise nucleoside phosphoramidite addition, typically using an automated oligonucleotide synthesizer. An example chemical process scheme is shown in Scheme 1.

A phosphoramidite monomer with a 5′-dimethoxy trityl (DMT) protecting group (or another type of trityl protecting group) is coupled to the growing oligonucleotide chain & then oxidized to form the phosphate oligonucleotide backbone.

Other modifications to the oligonucleotide can be introduced into the process by adding or changing the process steps. For example, sulfurization, capping or other modifications are often done after the coupling step. When the desired sequence length has been made, the oligonucleotide chain is deprotected and cleaved from the support.



Scheme 1. Solid phase phosphoramidite oligonucleotide synthesis process.

Since oligonucleotide synthesis is a sequential process, the theoretical yield of the target oligonucleotide decreases as the length of the sequence increases if the step efficiency is anything less than 100%. It is estimated that each synthesis cycle has an efficiency of about 98.5-99%.[2] As Table 1 shows, even a high step efficiency of 99% can lead to an overall theoretical yield of only 61% after 50 cycles. The sequence impurities can be difficult to remove; however, is a robust purification technique like RP-HPLC that can be used for a wide range of oligonucleotides of varying sequences and lengths.

The sequential solid phase oligonucleotide synthesis technology is suitable for oligonucleotides up to about 150nt in length,[3] with more opportunity for failure sequences from side reactions and raw material impurities with each step. Because failure sequences can be detrimental to the target specificity, these failure sequences should be removed by purification prior to formulation.

Table 1. Impact of step efficiency on target oligonucleotide yield.

	Step efficiency	
	99%	98.5%
Oligonucleotide length	Theoretical yield of target	
20-mer	82%	74%
50-mer	61%	47%
100-mer	37%	22%
150-mer	22%	10%

Purification methods

The choice of purification methods will depend on the oligonucleotide properties and application requirements, but the options are typically standard desalting, cartridge purification or HPLC purification.

Standard desalting involves passing the oligonucleotide mixture through a size exclusion column after cleavage from the solid support. This step separates the salts from the oligonucleotides through a size exclusion mechanism where the larger species (oligonucleotides) will pass more quickly through the column. This technique is a coarse purification technique that does not effectively remove failure sequences. It should be used only for applications that are not sensitive to the presence of failure sequences.

Cartridge purification, also known as cartridge desalting, removes salts and other hydrophilic species by binding the full-length oligonucleotide sequence with a hydrophobic DMT group onto the resin, while hydrophilic and capped failure species are washed through. The DMT-On oligonucleotide can then be eluted and recovered. Often, this step is coupled with a subsequent on column detritylation step removes the trityl protecting group and elutes the DMT-Off target oligonucleotide. While cartridge purification does not effectively eliminate all oligonucleotide impurities, the oligonucleotides purified by cartridge purification are useful for many diagnostic and research purposes. Unlike standard desalting, cartridge purification effectively removes capped failure sequences.

HPLC chromatography is designed to combine the removal of salts, capped failure sequences, and chemically-similar failure sequences (e.g. N-1, etc.) from the target oligonucleotide sequence. The resulting oligonucleotides are suitable for high purity applications, such as therapeutics. These separations can be done with the DMT protecting group 'on' or 'off' and can use reverse phase or anion exchange resins. Reverse phase chromatography with DMT-On is a preferred route since it is very effective at separating the DMT-On target oligonucleotides from the DMT-Off failure sequences and other impurities. Anion exchange chromatography often requires extensive method development that is unique to the oligonucleotide sequence, while reverse phase HPLC methods tend to be quite similar from molecule to molecule due to the DMT group driving the interaction with the resin.

This publication will focus on HPLC purification methods using reverse phase resins. For more information about DuPont™ products for cartridge purification, please see *Guide to Cartridge Purification of Oligonucleotides with DuPont™ AmberChrom™ Resins* (Form No. 45-D04513).

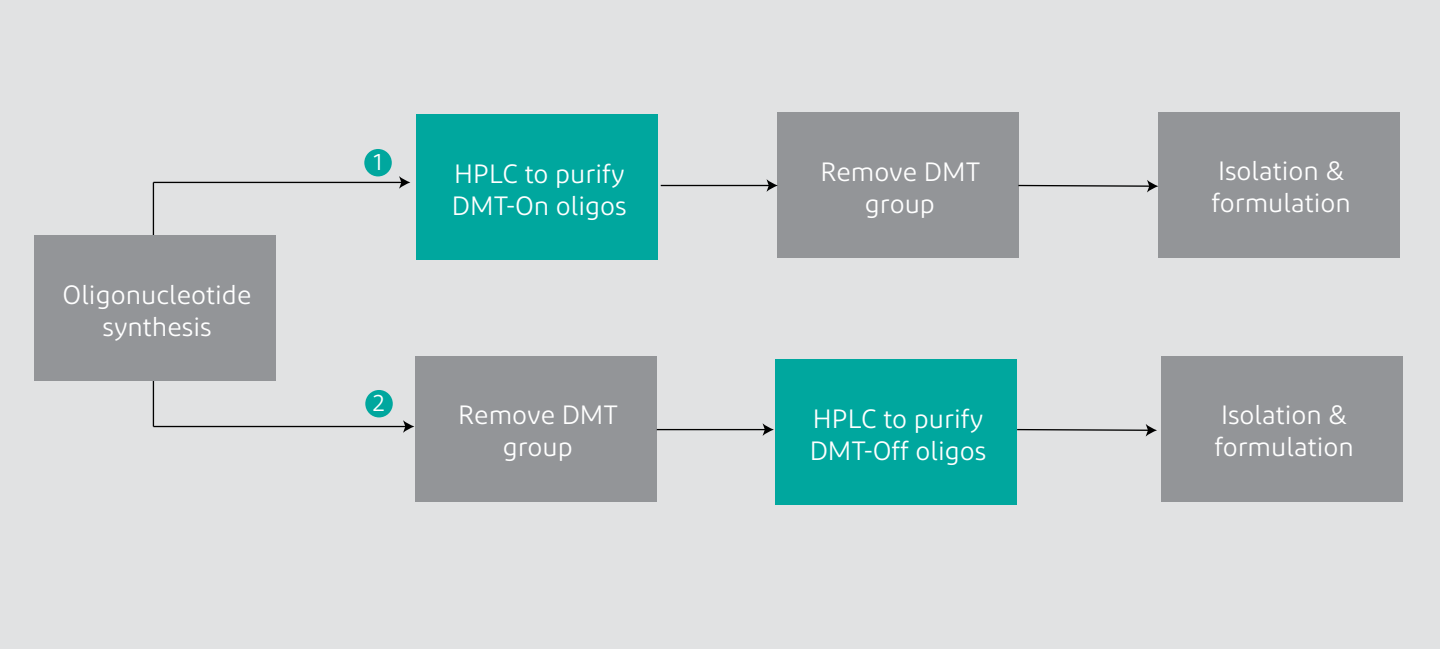
Reverse phase HPLC purification using DuPont™ AmberChrom™ XT chromatography resins

DuPont™ AmberChrom™ XT chromatography resins are ideally designed for oligonucleotide purification. These resins are rigid, macroporous, hydrophobic polymeric resins with excellent chemical and mechanical stability to enable robust and reproducible separation with high process throughput. The XT products within the AmberChrom™ chromatography resin family were designed with a particle structure that can withstand up to 60 bar pressure, which provides consistent performance at high flow rates.

As mentioned earlier, there are two routes by which oligonucleotides are typically purified with a reverse phase resin, as shown in Scheme 2.

1. **DMT-On HPLC purification –** The DMT protecting group provides the oligonucleotide with a hydrophobic nature that attracts it to the hydrophobic resin. Often, this purification strategy is termed DMT-On, or Trityl-On purification. Through proper selection of process conditions and the mobile phase, the target DMT-On sequence is separated from other closely related impurities, such as DMT-On impurities (N±1) and capped DMT-Off failures sequences, through differential partitioning between the mobile phase and the resin during chromatography.

2. **DMT-Off HPLC purification –** Some process developers prefer to deprotect the oligonucleotide prior to purification (DMT-Off or Trityl-Off purification). In this instance, the reverse phase separation requires an ion pairing agent in the mobile phase. The ion pairing agent has both hydrophobic and cationic character and will allow the anionic oligonucleotide to adsorb to the hydrophobic resin. Again, through proper selection of process conditions and the mobile phase, the target DMT-Off sequence is separated from closely related DMT-Off impurities through differential partitioning between the mobile phase and the resin.



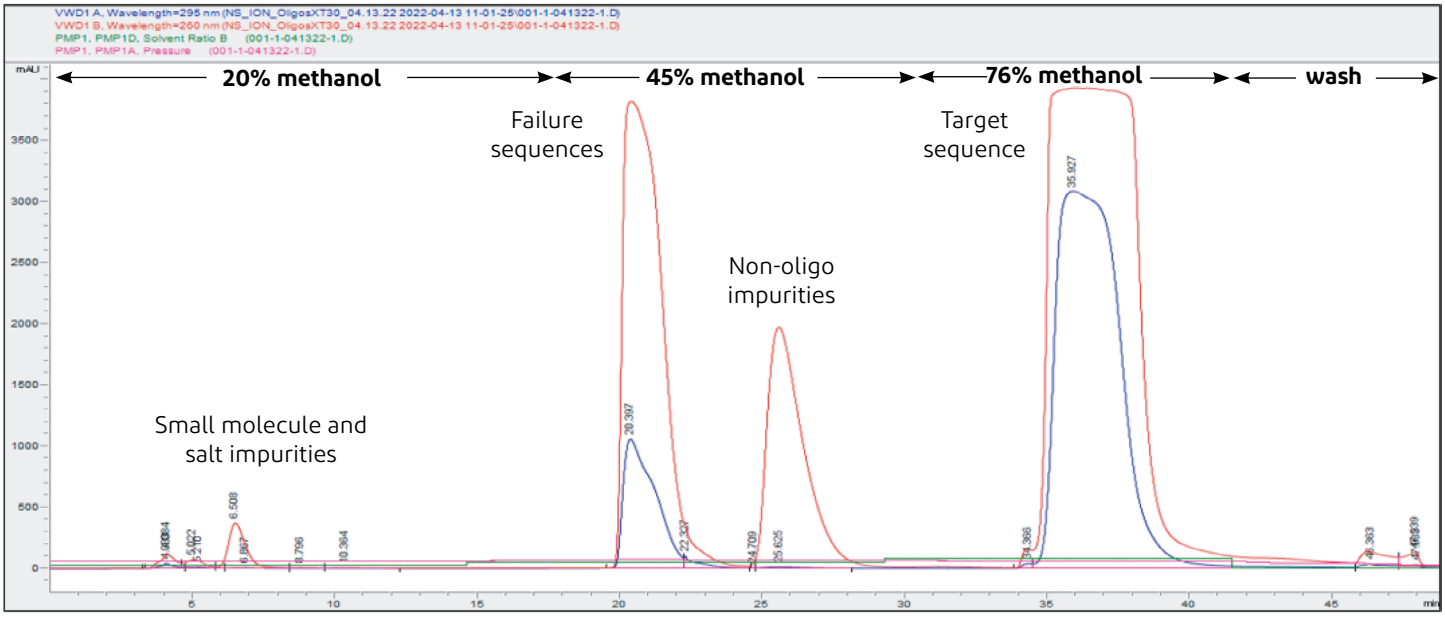
Scheme 2. Routes to downstream purification of synthetic oligonucleotides using reverse phase chromatography.

DMT-On RP-HPLC purification

In DMT-On RP-HPLC purification, the crude eluate from the synthesis column is loaded directly onto the resin column. The ammonia, salts, and other small hydrophilic molecules will flow through the column, while the DMT-Off (e.g. capped failure sequences) and DMT-On sequences adsorb to the resin through a hydrophobic interaction mechanism. The DMT-Off oligonucleotides are weakly held and can be removed with an elution buffer with low solvent content (~25-50%) that will also elute other weakly bound species. The column is now left with only the DMT-On oligonucleotides, which are a combination of the desired target and other DMT-On impurities (e.g. N±1). These DMT-On oligonucleotides can be eluted with a higher ratio of solvent (>50%). This type of purification can be done with a stepwise elution, a gradient elution, or a combination of both depending on the impurities to separate. Common solvents include acetonitrile, methanol and ethanol. Elution solvents that are less hazardous and more sustainable are under investigation.[4] Figure 1 shows the stepwise purification of a crude eluate feed of a 20-mer antisense oligonucleotide loaded directly onto a column with DuPont™ AmberChrom™ XT30 chromatography resin. The small molecule and salt impurities elute from the column with 20% methanol, while other components are still bound. Increasing the methanol concentration to 45% elutes DMT-Off capped failure sequences and other non-oligonucleotide impurities (e.g. benzamide). Finally, the DMT-On full length sequence is eluted with 76% methanol. A final high methanol cleaning wash is included to remove any tightly bound species.

This DMT-On purification strategy is very robust and can be applied across a wide range of oligonucleotides sequences. The presence of the DMT group on the oligonucleotide sequence dominates the adsorption onto the resin, making removal of molecules without it quite straightforward.

Figure 1. Purification of 20-mer DMT-On antisense oligonucleotide (ASO) crude feed with AmberChrom™ XT30 using stepwise elution with methanol.



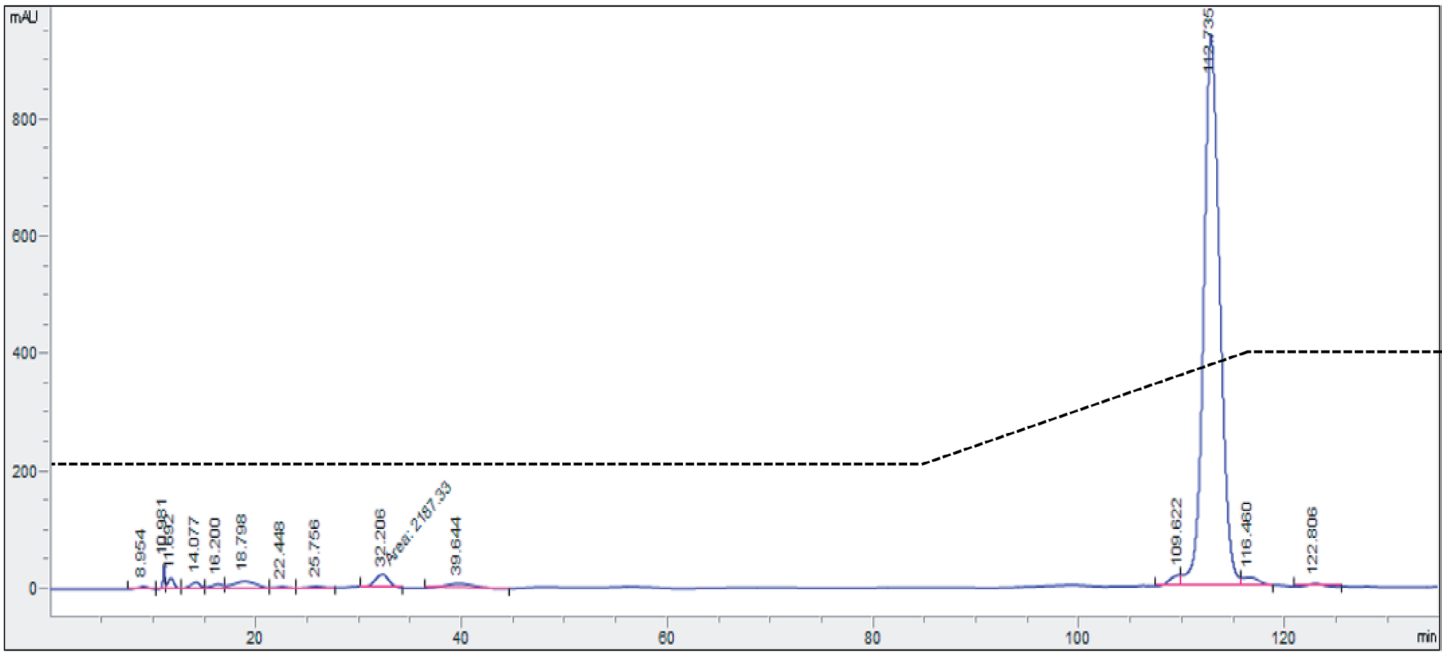
DMT-On 20-mer ASO feed 31.2mg/mL. Column: DuPont™ AmberChrom™ Profile™ XT30 4.6x250mm; 35°C; 100.0 µL injection; Buffer A: 0.2 M NaOAc, Buffer B: 0.2 M NaOAc in MeOH. Method: 20% B for 3CV, 45% B for 3CV, 76% B for 2.5CV followed by a 100% methanol wash for 1.5CV. Flow rate: 0.85mL/min. UV traces at 260nm (red) and 295nm (blue).

DMT-Off RP-HPLC purification

The second route for reverse phase purification uses a combination of ionic and reverse phase interactions to accomplish the separation in a DMT-Off purification design. This process uses a reverse phase resin and an amphoteric ion pairing agent, which has both cationic and hydrophobic character, as a component of the mobile phase. The ion pairing agent interacts with both the anionic phosphate groups on the oligonucleotide and the hydrophobic surface of the resin to enable adsorption. This method is very commonly used for analytical separations but can also be used for preparative purifications when otherwise difficult-to-remove impurities are present. There are a variety of ion pairing agents that can be used to tune the oligonucleotide resin interaction to accomplish a difficult separation.

Figure 2 shows the purification of a 25-mer DMT-Off DNA oligonucleotide using triethylammonium acetate (TEAA) ion pairing agent with DuPont™ AmberChrom™ XT20 chromatography resin. The separation is done with an elution profile that leverages the advantages of both isocratic and gradient chromatography[5] to provide the oligonucleotide as a concentrated, high purity elution fraction. The isocratic portion of the chromatogram shows good separation of early-eluting impurities such as salt and small-molecule impurities, while the gradient portion separates the closely eluting oligonucleotide impurities (N±1).

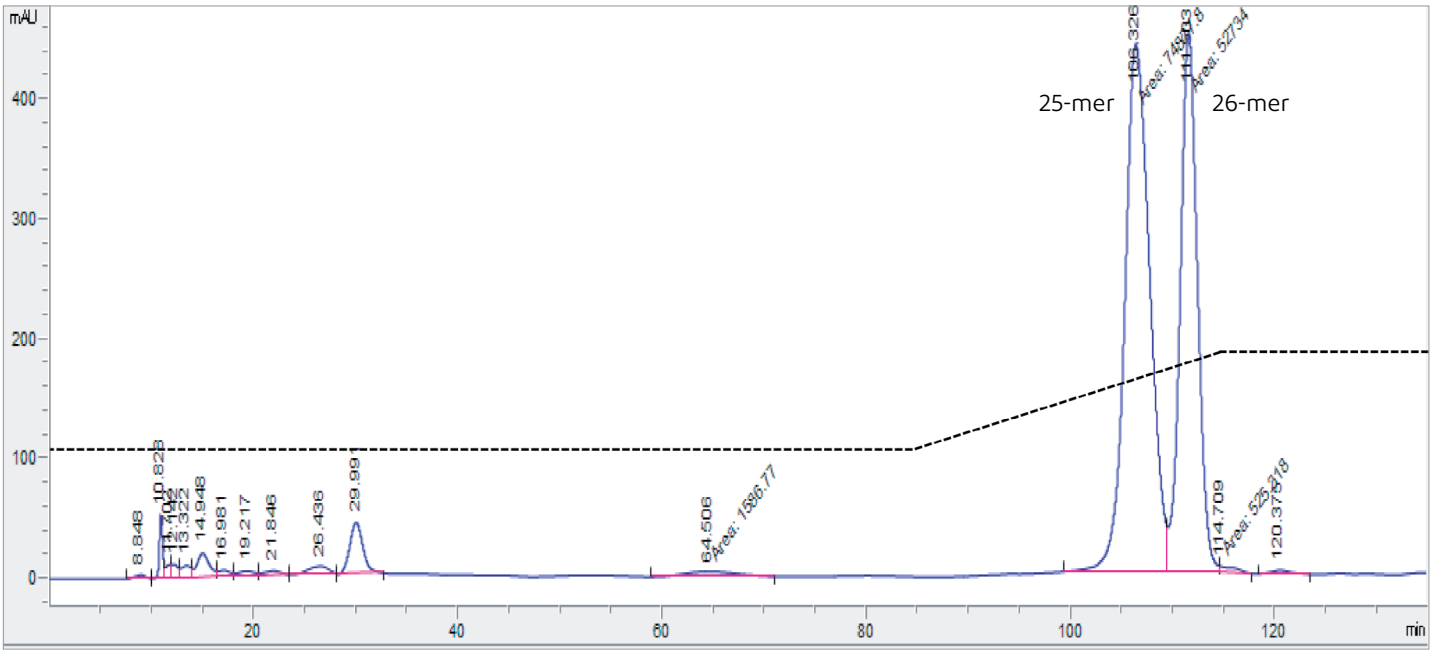
Figure 2. Purification of 25-mer DMT-Off phosphodiester DNA oligonucleotide crude feed using TEAA ion pairing agent.



DMT-Off 25-mer phospodiester DNA. Sequence: GAA CCT GAT GTG AAA CCT GAT GTG C, crude purity 87%. Column: DuPont™ AmberChrom™ Profile™ 4.6x250mm; 60 °C; Injection: 75 µL injection of 95 µM solution in 0.1 M TEAA; Buffer A: 0.1 M TEAA in Water, Buffer B: 0.1 M TEAA in CH_3CN. Method: 10% B for 8 CVs, then ramp from 10% to 13% B over 3 CVs, then 13% B for 2 CVs. Flow rate: 0.4mL/min.

Figure 3 shows the purification of a mixture of 25 and 26-mer DMT-Off DNA oligonucleotides using TEAA ion pairing agent. The separation is also done by combining an isocratic elution with a gradient elution to provide excellent resolution of the different length oligonucleotides. This separation shows the ability of DuPont™ AmberChrom™ XT20 chromatography resin to resolve N+1/N-1 impurities.

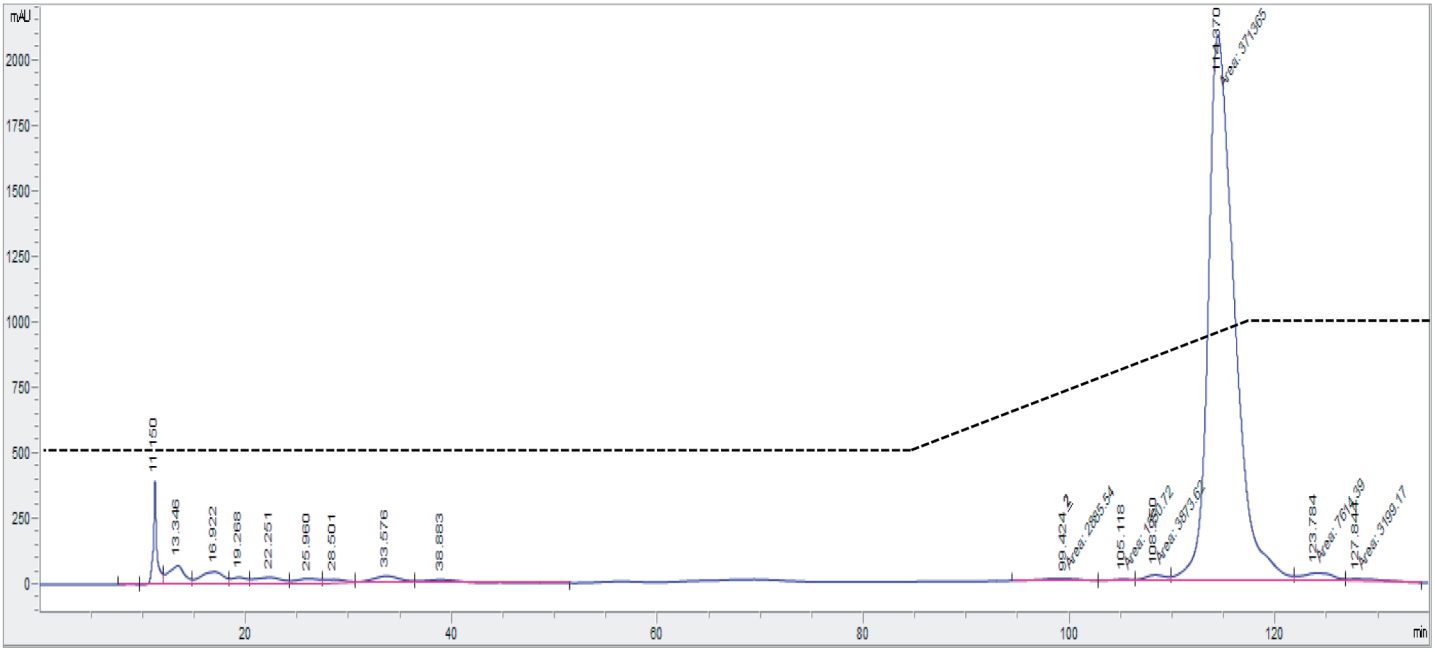
Figure 3. Purification of 25 and 26-mer DMT-Off phosphodiester DNA oligonucleotide crude mixture using TEAA ion pairing agent.



DMT-Off 25 and 26-mer phosphodiester DNA mixture. Sequence: GAA CCT GAT GTG AAA CCT GAT GTG C and GAA CCT GAT GTG AAA CCT GAT GTG CG; crude purity 53% 25-mer, 37% 26-mer and 10% other; Column: AmberChrom™ XT30 4.6x250mm; 60°C; Injection: 75 µL injection of 71.25 µM 25mer + 23.75 µM 26mer in 0.1 µM TEAA; Buffer A: 0.1 M TEAA in Water, Buffer B: 0.1 M TEAA in CH_3CN. Method: 10% B for 8 CVs, then ramp from 10% to 13% B over 3 CVs, then 13% B for 2 CVs. Flow rate: 0.4mL/min.

The versatility of AmberChrom™ XT20 chromatography resin to purify oligonucleotides of various designs is further illustrated in Figure 4, which shows the purification of a crude 41-mer DMT-Off RNA oligonucleotide using TEAA ion pairing agent. This separation shows the excellent versatility of AmberChrom™ XT20 to purify oligonucleotides of longer lengths and with RNA sequences.

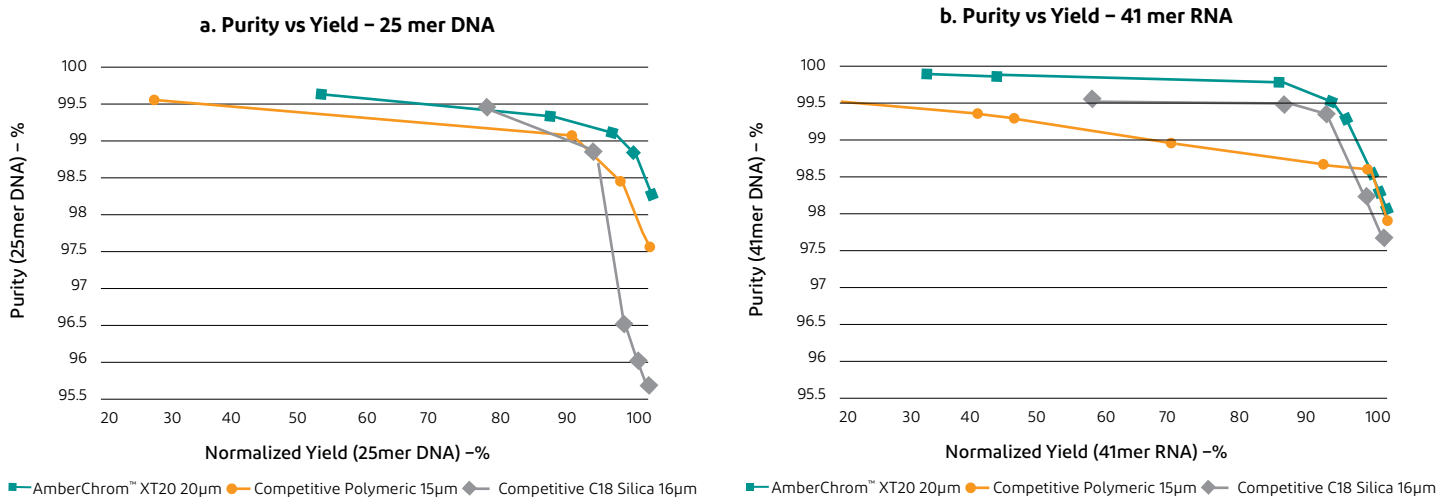
Figure 4. Purification of mixture of 41-mer DMT-Off phosphodiester RNA oligonucleotide crude feed using TEAA ion pairing agent.



DMT-Off 41-mer phosphodiester RNA. Sequence: UAA UUU CUA CUA AGU GUA GAU CAU CAC CGC CAU UGC CAG CC, crude purity 85%. Column: DuPont™ AmberChrom™ Profile™ XT30 4.6x250mm; 60°C; Injection: 100 µL injection of 95 µM solution in 0.1 M TEAA; Buffer A: 0.1 M TEAA in Water, Buffer B: 0.1 M TEAA in CH_3CN. Method: 10% B for 8 CVs, then ramp from 10% to 13% B over 3 CVs, then 13% B for 2 CVs. Flow rate: 0.4 µL/min.

Most chromatographic separations trade off yield and purity since it is difficult to get complete baseline separation due to overlapping peaks of closely related species. This means process designers decide whether to err on the side of purity at the expense of yield or whether to give up a bit of purity in favor of yield. The goal is to maximize both as much as possible. Purity-Yield curves were obtained by HPLC analysis of collected fractions from the chromatograms above. A comparison of the purity at a given yield can be seen in Figure 5 below, with each reverse phase resin under optimized conditions. The data show that DuPont™ AmberChrom™ XT20 chromatography resin can provide higher purity and yield even when compared to smaller particle sized competitive products, indicating that the AmberChrom™ polymer composition and structure are well-suited for oligonucleotide purification.

Figure 5. Purity vs Yield curve for purification of a) 25-mer DNA and b) 41-mer RNA with AmberChrom™ and comparison with other reverse phase resins



Separation Columns: 4.6x250mm; 60°C; Injections: 100 µL injection of 95 µM solution in 0.1 M TEAA; Buffer A: 0.1 M TEAA in Water, Buffer B: 0.1 M TEAA in CH_3CN. AmberChrom™ and Polymeric method: 10% B for 8 CVs, then 10 to 13% B for 3 CVs, then 13% B for 2 CVs. Flow rate: 0.4 µL/min. Silica gradient conditions: 6% B for 8 CVs, then ramp from 6% to 9% B over 3 CVs, then 9% B for 2 CVs.

Analytical ion exchange: DNAPac™ PA200 4x250mm; 80 °C; Injections: 100 µL of collected fraction; Buffer A: 75% 25 mM Na_2HPO_4, 25 % CH_3N, Buffer B: 75% (25 µM Na_2HPO_4,+ 0.4M $NaClO_4$) 25% CH_3CN. DNA method: ramp from 13% to 53% B over 7.5 CVs, then 100% B for 2 CVs, 0.9 µL/min; RNA method: 25 to 65% B over 7.5 CVs, then 100% B for 2 CVs, 0.9mL/min.

Conclusions

DuPont™ AmberChrom™ chromatography resins are shown to be very effective reverse phase resins in both the DMT-On and DMT-Off purification of DNA and RNA oligonucleotides. This performance is why AmberChrom™ chromatography resins have been the go-to resins for preparative scale purification of oligos for applications such as diagnostics, primers and therapeutics for 10+ years. And, unlike RP silica, the AmberChrom™ polymeric resins are stable across the extremes of pH (1-14), resist pressures up to 60 bars, temperatures (up to 60 °C) and are inert to common buffers and solvents. The robustness of the AmberChrom™ polymeric matrix and the selectivity generated by the resin design enable reliable separations over a long lifetime.

References:

1. Caruthers, M.H., 1985. Gene synthesis machines: DNA chemistry and its uses. Science, 230(4723), pp.281-285.
2. Behlke, M. 1999, Choosing The Right Method Of Purification For Your Oligos, Bioprocess Online, viewed 24 Oct 2022, <https://www.bioprocessonline.com/doc/choosing-the-right-method-of-purification-for-0001>
3. LeProust, E.M., Peck, B.J., Spirin, K., McCuen, H.B., Moore, B., Namsaraev, E. and Caruthers, M.H., 2010. Synthesis of high-quality libraries of long (150mer) oligonucleotides by a novel depurination controlled process. Nucleic acids research, 38(8), pp.2522-2540.
4. Yabré, M., Ferey, L., Somé, I.T. and Gaudin, K., 2018. Greening reversed-phase liquid chromatography methods using alternative solvents for pharmaceutical analysis. Molecules, 23(5), p.1065.
5. Schellinger, A.P. and Carr, P.W., 2006. Isocratic and gradient elution chromatography: a comparison in terms of speed, retention reproducibility and quantitation. Journal of Chromatography A, 1109(2), pp.253-266.

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