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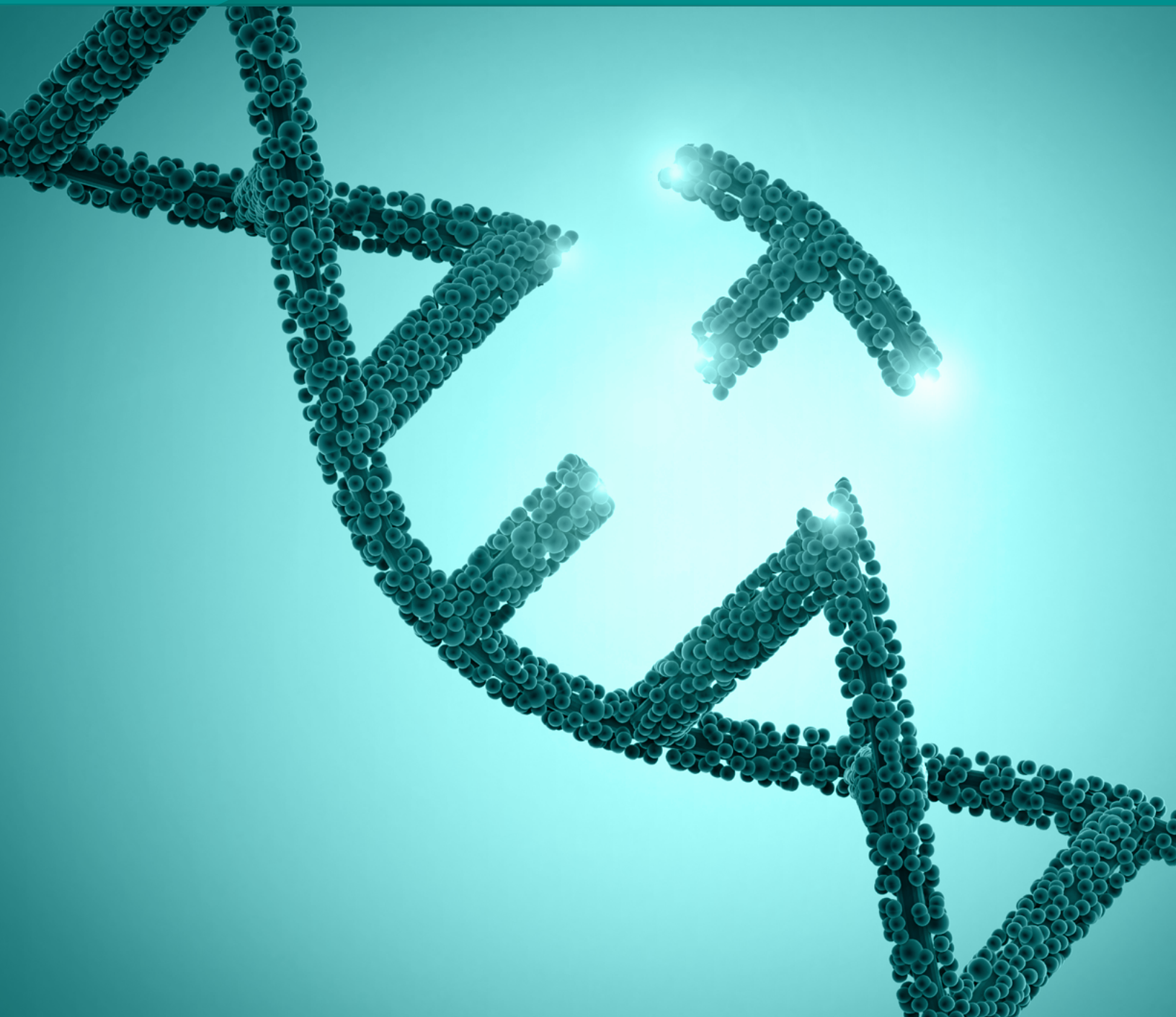
Purification of single guide RNA oligonucleotides with DuPont™ AmberChrom™ chromatography resins



Introduction

Genetic engineering is an emerging field with profound implications to food and health. Gene editing with CRISPR-Cas9 has gained widespread adoption in these areas because the highly specific binding interaction between a guide RNA (gRNA) molecule and the target DNA enables precise modifications across a broad range of genomes. As the technology develops and is directed towards a variety of applications, demand for purified gRNAs will increase. This paper, describes a general preparative-scale reverse phase chromatography method for crude synthetic gRNA that can be used for commercial-scale purification of large quantities of gRNA.

CRISPR-Cas9 Gene Editing

The CRISPR-Cas9 gene editing technique, first described in 2012, is an elegantly simple method for modifying genomic DNA.[1] The technique relies on the interaction between the genomic DNA, a Cas9 nuclease, and an RNA oligonucleotide which 'guides' the Cas9 nuclease to the target location on the DNA. The guiding RNA oligonucleotide, also called guide RNA, is comprised of a CRISPR RNA (crRNA) sequence that is complementary to the target DNA and a trans-activating CRISPR RNA (tracrRNA) sequence that acts as a scaffold for the Cas9 nuclease enzyme.

Once the gRNA-Cas9 complex is formed, the Cas9 nuclease initiates a double stranded break in the DNA, allowing subsequent DNA modifications, such as repair, end-joining, base editing, or prime editing.

In nature, crRNA and tracrRNA exist as separate molecules and work together to form the gRNA. In engineered gRNA, researchers have combined the crRNA and tracrRNA molecules into a single molecule, termed single guide RNA (sgRNA) to distinguish it from native forms. The fused sgRNA is often just referred to as gRNA. The tracrRNA and crRNA molecules can vary in length but are typically 70-100 nt and 20 nt respectively.

Synthesis of Oligonucleotides

Nearly all synthetic oligonucleotides, including gRNAs, are made using solid phase synthesis utilizing the phosphoramidite process developed by Caruthers in 1985.[2] The process entails the sequential synthetic assembly of the oligonucleotide chain through stepwise nucleoside phosphoramidite addition, typically using an automated oligonucleotide synthesizer. An example chemical process scheme is shown in Figure 1.

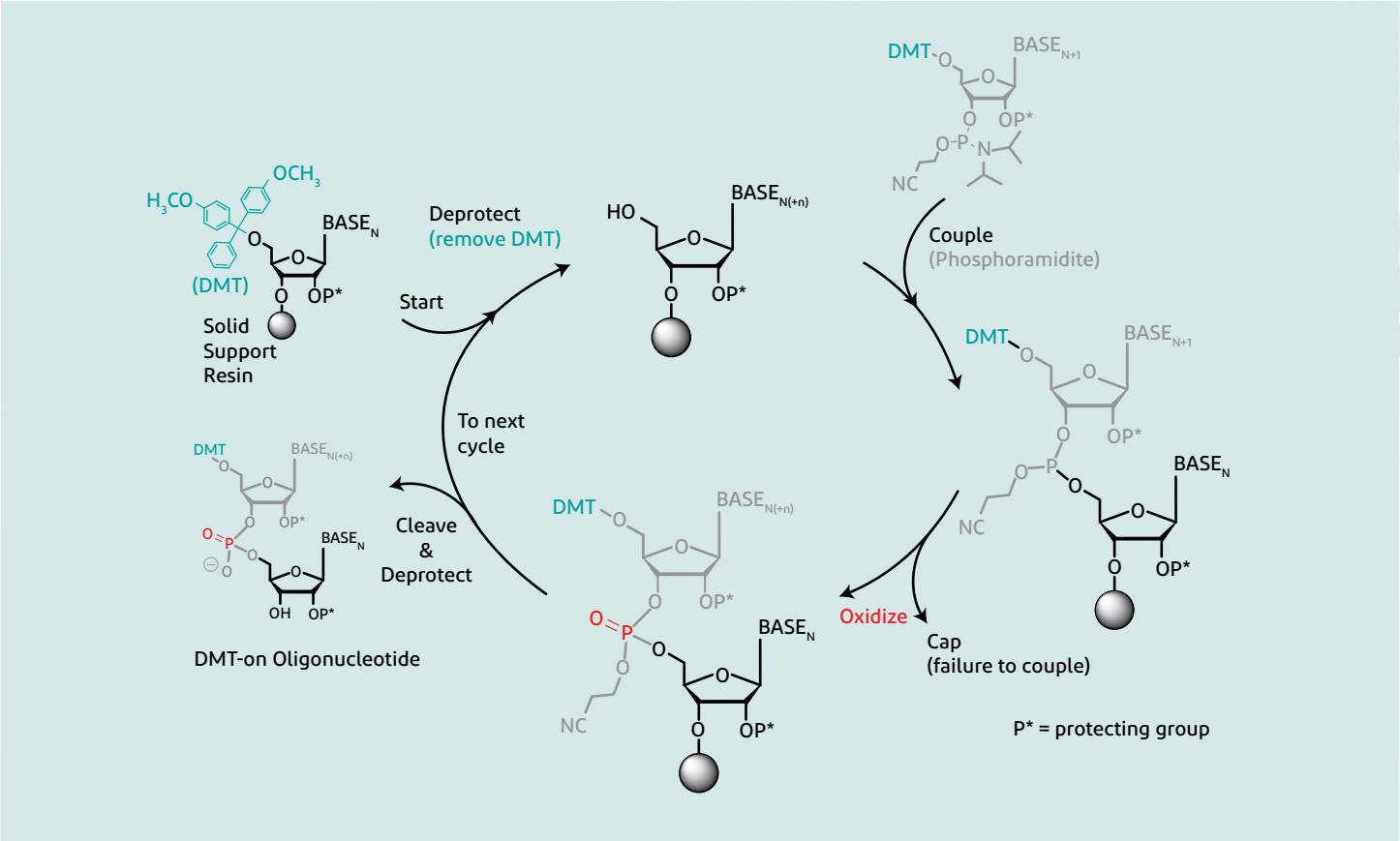


Figure 1. Solid phase oligonucleotide synthesis process for RNA.

The process begins with deprotection of the trityl protecting group, followed by coupling with a phosphoramidite. Coupling failures are capped to prevent continued chain growth. Oxidation of the phosphite ester to the phosphate ester ensures that the oligonucleotide is ready to continue the cycle. Other modifications to the oligonucleotide can be introduced by adding or changing the process steps. For example, sulfurization is done after the coupling step. When the desired sequence length has been reached, the oligonucleotide chain is deprotected and cleaved from the support.

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%. The sequential solid phase oligonucleotide synthesis technology is suitable for oligonucleotides up to about 150 nt in length, with increased risk of introducing failure sequences from side reactions and raw material impurities with each step. It is estimated that each synthesis cycle has an efficiency of about 98.5-99%.[3] As Table 1 shows, even a high step efficiency of 99% can lead to an overall theoretical yield of only 37% after 100 cycles. Because failure sequences can be detrimental to the target specificity, they should be removed by purification prior to formulation. The sequence impurities can be difficult to remove; however, a robust purification technique like reverse phase HPLC (RP-HPLC) can be used for a wide range of oligonucleotides of varying sequences and lengths.

Table 1: Impact of step efficiency on oligonucleotide target 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%

Reverse Phase High Performance Liquid Chromatography Purification Strategies

Reverse Phase High Performance Liquid Chromatography is frequently chosen for preparative chromatography when high purity oligonucleotides are desired. The oligonucleotide is purified through selective adsorption and desorption to the reverse phase resin based on relative differences in hydrophobicity, which makes the reverse phase technique especially useful in separating closely related species. DuPont™ AmberChrom™ XT chromatography resins are ideally designed for this purpose. These chromatographic resins are made of hydrophobic polymers with excellent chemical and mechanical stability capable of withstanding the solvents employed in oligonucleotide synthesis and have a pore size well-suited to the size of oligonucleotides.

There are two manufacturing process strategies by which oligonucleotides are typically purified with a reverse phase resin:

- **DMT-On HPLC Purification –** The 5′-dimethoxytrityl [DMT] protecting group provides the oligonucleotide with a hydrophobic handle that attracts it to the hydrophobic resin. Often, this purification strategy is termed DMT-On, or Trityl-On, or, in the case of monomethoxytrityl capping, MMT-On. For convenience, this class will be collectively referred to as DMT-On purification. Through proper selection of process conditions and the mobile phase, the target DMT-On sequence is separated from non-oligonucleotide impurities, DMT-Off failure sequences, and other DMT-On impurities through differential partitioning between the mobile phase and the resin. In some cases, an ion pairing agent can be used as a component of the mobile phase.

- **DMT-Off HPLC Purification –** Some process developers prefer to deprotect the oligonucleotide and remove the DMT group prior to purification. In this instance, the reverse phase separation requires an ion pairing agent in the mobile phase. The ion pairing agent is a molecule that has both hydrophobic and cationic character and will facilitate the adsorption of the anionic oligonucleotide to the hydrophobic resin. Again, through proper selection of process conditions and the mobile phase, the target DMT-Off sequence is separated from non-oligonucleotide impurities and closely related DMT-Off impurities through differential partitioning between the mobile phase and the resin.

DMT-On Purification of sgRNA using DuPont™ AmberChrom™ XT Chromatography Resins

In DMT-On 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), some reaction byproducts, and DMT-On sequences adsorb to the resin. The DMT-Off oligonucleotides are weakly held and can be removed with a low-strength elution buffer; often this solvent will also elute other weakly bound species. The column is now left with only the DMT-On oligonucleotides, which can be eluted with a higher ratio of solvent. This type of purification can be done with a stepwise elution, a gradient elution, or a combination of both. Common solvents include acetonitrile, methanol, ethanol, and 'greener' options that are also being explored.[4]

Figure 2 shows the gradient elution purification of a crude 91-mer sgRNA feed loaded directly onto a DuPont™ AmberChrom™ Profile™ XT20 HPLC column. The small molecule and salt impurities elute from the column at low concentrations of Buffer B (<25% B), while the target DMT-On remains bound. Increasing the concentration of the elution buffer up to 50% B elutes the DMT-On target sequence, which appears as a double peak due to differences in elution behavior of the diastereomers of the target product. Consistent with the projections from Table 1, the purity of the 91-mer in the crude material was measured to be 29.8%. The small, broad peak that elutes at about 13 column volumes corresponds to very hydrophobic oligonucleotide impurities, which likely contain multiple DMT groups due to branching side reactions during synthesis.

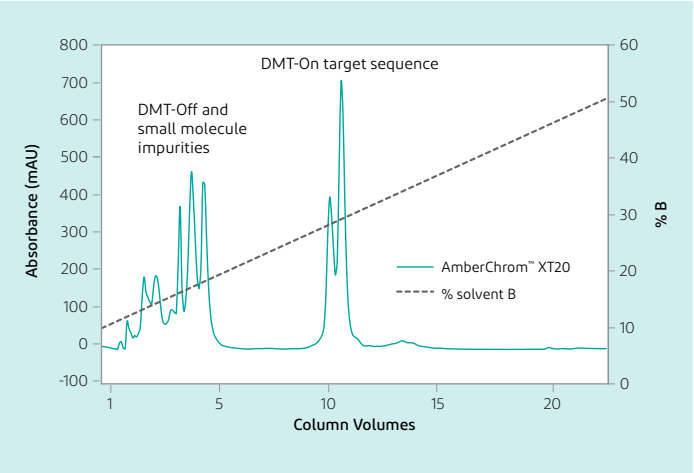


Figure 2. Purification of 91-mer DMT-On sgRNA crude feed with DuPont™ AmberChrom™ XT20 chromatography resin using a gradient elution with acetonitrile.

Column: DuPont™ AmberChrom™ Profile™ XT20 HPLC column 4.6x250mm; 60°C; 100µL injection of 1.7 mg/mL 91-mer sgRNA feed; Buffer A: 0.1 M TEAA in water, Buffer B: 0.1 M TEAA (aq) in 90% ACN (Acetonitrile). Method: 10%-50% B over 22CV (Column Volumes). Flow rate: 0.8 mL/min. UV trace at 260 nm.

It is common to need to do method development to find conditions that will effectively purify new oligonucleotides.

We're here to help. Feel free to reach out to the DuPont application team for tips and suggestions.

See Preparative Packing Guidance for DuPont™ AmberChrom™ XT20 and AmberChrom™ XT30 chromatography resins for instructions on how to pack columns and calculate the amount of dry resin needed to fill a column.

Contact us: www.dupontwatersolutions.com/life-sciences

Scale-up of Reverse Phase HPLC Purification of sgRNA using DuPont™ AmberChrom™ XT Chromatography Resins

Numerous groups are exploring sgRNA for its potential use in therapeutic applications. As such, there is a need for methods to generate larger quantities of purified sgRNA for testing or clinical studies. This entails scaling up both synthesis and purification processes, using the same techniques practiced at the bench scale.

HPLC chromatography is usually very straightforward to scale. Chromatographic scaleup is typically done by expanding the column width, while keeping the column length and linear velocity constant. Concurrently, the amount of crude material loaded onto the column is typically increased to provide a process with suitable product throughput for a manufacturing environment.

Figure 3 shows the high-loading purification of the same crude 91-mer sgRNA feed loaded directly onto a DuPont™ AmberChrom™ Profile™ XT20 HPLC column via the same gradient elution process described earlier. In this case, the loading on the resin column was 17 mg/mL, or 42.5 mg of crude sgRNA oligonucleotide, at 29.8% full length product purity. The chromatogram shows the process of load, wash, and elute. After the loading step, a wash was implemented to remove weakly held impurities and then the product was eluted from the column via a linear gradient. The components of the elution from Figure 2 can be observed in the chromatogram in Figure 3, with DMT-Off failure sequences eluting early in the gradient, the target DMT-On sgRNA oligonucleotide eluting toward the middle of the gradient, and more hydrophobic species, likely due to branched impurities containing multiple DMT groups, eluting last.

Fraction analysis from the target product peak, taken from approximately 80-100 minutes during the chromatography run in Figure 3, shows excellent resolution of the target product from impurities and full-length product (FLP) purity exceeding 99.8% in some fractions. Figure 4 shows a yield-purity analysis of the fraction pool, in which the impact of purity on the yield of pooled fraction products can be seen. This pool analysis shows that by combining the various fractions, very high purity pools can be recovered in high yield from the low purity crude. The regulatory agencies do not provide quantitative guidance on the purity required for therapeutic oligonucleotides, and the purity level examples listed in Figure 4 are for demonstration purposes.[5]

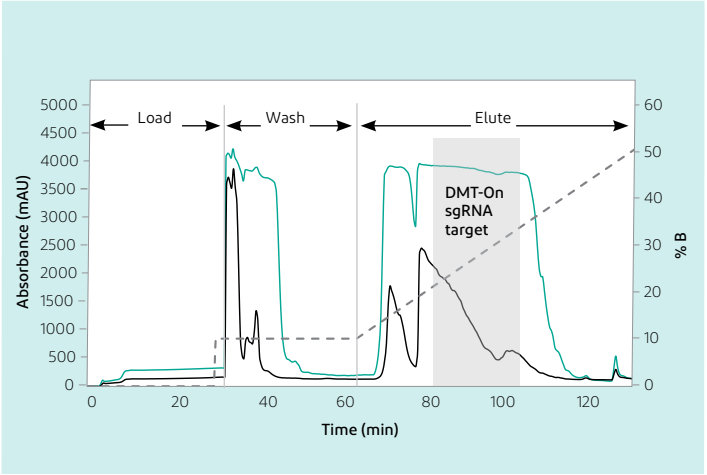


Figure 3. Purification of 91-mer DMT-On sgRNA crude feed with DuPont™ AmberChrom™ XT20 using a gradient elution with acetonitrile.

Column: DuPont™ AmberChrom™ Profile™ XT20 HPLC column 4.6x150mm; 60°C; Buffer A: 0.1 M TEAA in water, Buffer B: 0.1 M TEAA (aq) in ACN. Method: load 25 mL of 1.7mg/mL 91-mer gRNA, wash with 10% B for 11 CV, then 10%-50%B over 22 CV. Flow rate: 0.8mL/min. UV traces at 260nm (green) and 295nm (black).

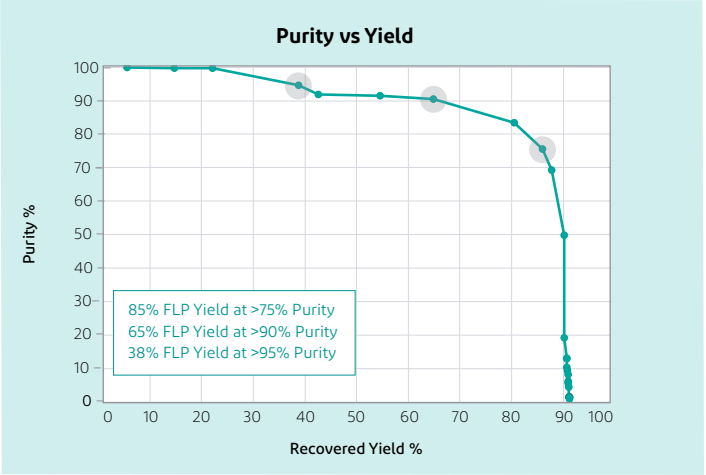


Figure 4. Yield-purity pool analysis from high-loading chromatographic run of 91-mer sgRNA with DuPont™ AmberChrom™ XT20 chromatography resin.

Analytical Column: Waters™ XBridge™ 10x50 mm, 3.9 mL; 60°C; Buffer A: 0.1 M TEAA, Buffer B: 0.1 M TEAA in CH^3CN. Method: 10-50% B in 22 CV. Flow rate: 0.8 mL/min.

The data generated from the crude 91-mer sgRNA purification can be used to estimate the productivity for larger scale chromatography processes. Table 2 models a process in which an 11 cm ID (Internal Diameter) HPLC column is loaded with the 91-mer crude gRNA feed at 17 g/L. An oligonucleotide purification process with an 11-cm column with 2 L of DuPont™ AmberChrom™ XT20 chromatography resin would yield an estimated 8.6 g of product at 75% purity and 3.9 g at >95% purity.

Table 2. Hypothetical scenario of sgRNA yield at production scale.

Column size (ID)	11 cm
Resin volume (L)*	2.0
Resin mass (kg)*	0.5
Crude oligonucleotide loaded (g)	34
Oligonucleotide mass in crude (g)	10.13
>75% Purity yield (g)**	8.6
>90% Purity yield (g)**	6.6
>95% Purity yield (g)**	3.9

* Estimated DuPont™ AmberChrom™ XT20 chromatography resin density = 260 g/L in. this mobile phase; Bed height = 21 cm.

** Assumptions: Single column load/elute, crude purity of 29.8% FLP (Full Length Product)

Finally, the design of larger scale processes should consider other operating factors, such as pressure drop in the column, which can impact the throughput and efficiency of the purification process. The pressure required to move liquid through a packed bed of resin is guided by many factors including the column dimensions, liquid viscosity, particle size, packing density, and compressibility of the resin. Figure 5 shows the pressure drop across a packed bed of AmberChrom™ resin across a range of flow rates in a dynamic axial compression (DAC) column. The linear nature of the line indicates that the bed is stable, and these resins do not compress under these typical operating conditions. AmberChrom™ XT20 resin exhibits similar properties to XT30 and these pressure-flow characteristics will be comparable.

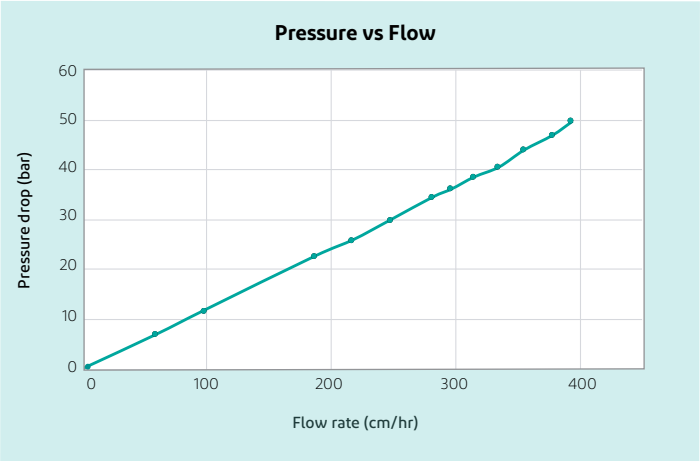


Figure 5. Pressure-flow curve for DuPont™ AmberChrom™ XT30 chromatography resin in a DAC column.

Column: Sartorius AG Hipersep™ Prochrom 110 DAC column, 11 cm ID x 21 cm H. Test conditions: 50% EtOH in water, 23°C, 60 bar piston pressure.

References:

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3. LeProust, Emily M., Bill J. Peck, Konstantin Spirin, Heather Brummel McCuen, Bridget Moore, Eugeni Namsaraev, and Marvin H. Caruthers. "Synthesis of high-quality libraries of long (150mer) oligonucleotides by a novel depurination controlled process." Nucleic Acids Research 38, no. 8 (2010): 2522-2540.
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5. FDA draft guidance. Clinical pharmacology considerations for the development of oligonucleotide therapeutics. Jun 2022. (FDA-2022-D-0235)

Summary

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

All AmberChrom™ XT chromatography resin products used in this paper are available for online purchase for research use at www.dwsadvantage.com/us/life-sciences.html. Products are available in convenient, ready-to-use, pre-packed columns, or as bulk media for users who prefer to pack their own columns. Bulk resins for clinical trials and commercial manufacturing are available directly from DuPont. A column packing guide, Preparative Packing Guidance for DuPont™ AmberChrom™ XT20 and AmberChrom™ XT30 Chromatography Resins, is available on the DuPont website in the Resource Center.

All HPLC analyses were performed on an Agilent™ 1260 Infinity II HPLC.

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