



Nucleic acid therapeutics applications notebook

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Introduction

THERAPEUTIC NUCLEIC ACIDS MARKETPLACE

Over the last 15–20 years, development of nucleic acid therapeutics has exploded from a modest but dedicated effort by specialty research and development organizations into a widespread enterprise fully embraced by all major pharmaceutical firms. This explosion is supported by the discovery of Ribonuclease H (RNase H) function for antisense oligonucleotides, and the more recent realization that cellular mechanisms for control of gene expression and transcriptome regulation can be manipulated to produce therapeutics for ailments that were previously considered unresponsive to drugs. Recently, options for therapeutics with exquisite selectivity also provide the opportunity for new therapeutic modes and intellectual properties. For example, Macugen successfully passed clinical trials and release protocols to become the first approved aptamer therapeutic. This success encourages further efforts and opportunities for therapeutic nucleic acid developers.

THERAPEUTIC NUCLEIC ACID MODELS

Several models of nucleic acid therapeutics have been described, including antisense oligodeoxynucleotides; ribonucleic acid (RNA) interference; immunostimulatory nucleic acids; microRNA (miRNA) (along with other noncoding RNA modes); and aptamers.

Antisense oligonucleotides are nucleic acid therapeutics that are injected into a person for distribution to target organs by various means. From there, these therapeutics recruit RNase H to degrade the resulting deoxyribonucleic acid (DNA)/RNA hybrids, thus minimizing translation of the target messenger RNA (mRNA).

There are various models in which the active pharmaceutical ingredient (API) is mainly RNA. The first, short interfering RNA (siRNA) employs specific short (19–23 base) RNA sequences that are incorporated into a cellular complex RNA-induced silencing complex (RISC) that functions to degrade RNA complementary to the siRNA. This results in diminution of a very specific subset of cytosolic mRNA that is targeted by the administered sequence, severely limiting the expression of the target gene product.

Immunostimulatory RNA (isRNA) is a mode that employs sequence specific motifs, some of which are very simple (e.g., CpG where the C is not methylated). Introduction of the specific RNA sequence motif induces a cellular immune system directing degradation of foreign nucleic acids. This is mediated by the cellular toll-like receptors (TLRs), TLR7, 8, and 9.

miRNA is another RNA model that employs a series of cellular complexes to degrade mRNA in a sequence-specific fashion. This is almost certainly the pathway that is conscripted by the siRNA approach, but represents a mechanism whereby the *cell* controls expression and turnover of specific mRNA. Many miRNA families described regulate different functions in plants and animals, including neogenesis and malignant transformations. The sources of the endogenous miRNA in humans include highly conserved noncoding regions on chromosomes (initially thought to be junk DNA) and sequences inserted in specific genes. There are other noncoding RNA (ncRNA) models, with important ncRNA motifs being reported regularly. These may arise from endogenous variations on the miRNA process described above.

Aptamers are synthetic nucleic acids selected to interact with specific structures—such as cell surface receptors, proteins, and other chemical signatures—in a highly specific manner. They are typically modified with protecting groups (2'-O-methyl phosphorothioate linkages, DNA bases, sometimes inverted 3'-3' linkages, and conjugation to polyethylene glycol). Most therapeutic targets for aptamers have been extracellular, so they operate in the circulatory system where these protecting groups prolong their circulatory half-life.

BIOSEPARATION SOLUTIONS FOR NUCLEIC ACID THERAPEUTICS

Thermo Scientific™ offers solutions for the purification and analysis of essentially all classes of oligonucleotides and their modifications. Purification efforts are supported by both high-capacity Thermo Scientific DNASwift™ SAX-1S Monolith Columns (5 × 150 mm) and DNAPac™ PA-100 Semi-Preparative Columns (9 × 250 mm and 22 × 250 mm). These are housed in bioinert column bodies to prevent column fouling by corrosion of stainless steel frits and tubing, and typically employ either PEEK™ or Titanium high-performance liquid chromatography (HPLC) systems. The solutions described in this notebook include: 1) analysis of phosphorothioate oligonucleotides; 2) analysis of single-stranded (ss) and double-stranded (ds) RNA for therapeutic applications;

3) anion exchange-HPLC-electrospray ionization mass spectrometry (ESI-MS) of derivatized oligonucleotides; 4) preparative and analytical separations of nucleic acid diastereoisomers; 5) coupling of anion-exchange HPLC to ESI-MS using automated desalting with a Titanium-based LC system; 6) separation and analysis of RNA containing aberrant linkage isomers (not resolved by single-stage ESI-MS alone, including identification of the position of the aberrant linkages); 7) analysis of nucleoside mono-, di-, and triphosphates; and 8) useful information for new method development.

THERMO SCIENTIFIC AND DIONEX INTEGRATED SYSTEMS

Dionex™ Products are now a part of the Thermo Scientific brand, creating exciting new possibilities for scientific analysis. Now, leading capabilities in LC, ion chromatography (IC), and sample preparation are together in one portfolio with those in MS. Combining Dionex's innovation in chromatography with Thermo Scientific's leadership position in MS, a new range of powerful and simplified workflow solutions is now possible.

These Thermo Scientific integrated solutions can expand your capabilities and provide tools for new possibilities:

- IC and MS
- LC and MS
- Sample preparation and MS



Analysis of nucleic acids

Nucleic acid therapeutics applications notebook

Analysis of Phosphorothioate Oligonucleotides

Phosphorothioate (PS) linkages are introduced into oligonucleotides to restrict the susceptibility of therapeutic oligonucleotides (ONs) to degradation by plasma and tissue nucleases. A very large fraction of therapeutic ONs harbor one or more PS linkages, so their properties must be thoroughly understood for effective method development.

Anion-exchange chromatography on DNAPac columns allows resolution of ONs that differ in some cases by only one atom. One example of this includes analysis of antisense ONs. These typically employ phosphorothioate linkages. Phosphorothioates are ONs in which one of the oxygen bound only to the phosphorous atom is replaced with a sulfur atom. The introduction of the sulfur atom results in a chiral center at the phosphorus atom, so each linkage harbors two configurations and forms diastereoisomers. An ON with 15 bases can harbor 14 linkages and thus, 2^{14} (16,384) possible diastereoisomers. This results in fairly broad peaks. In the example presented here (Figure 1), dT₁₅ phosphorothioate, previously purified by reversed-phase chromatography, was analyzed at pH 8 and 12.4 using NaClO₄ eluent.

At pH 8, the components elute with broad peaks and are poorly resolved. The major peak represents the full length, fully substituted phosphorothioate. The broad plateau eluting prior to the major peak represents n-x and incompletely thioated ONs.

At pH 12.4, the peaks eluting earlier than the major component are much better resolved, and represent incompletely phosphorothioated components which lack the sulfur atom at one or more linkages. These elute as partially resolved peaks between 7 and 15.5 min. Thus, the peaks labeled here as All PS, 1 PO, and 2 PO differ from one

another by only one (All PS vs. 1 PO and 1 PO vs. 2 PO) or two (All PS vs. 2 PO) atoms.

The broad peaks suggest that some of the 16,384 diastereoisomers may be resolved from one another. In some cases, only one or two PS linkages are employed, so the diastereoisomers from these ONs may be resolved.

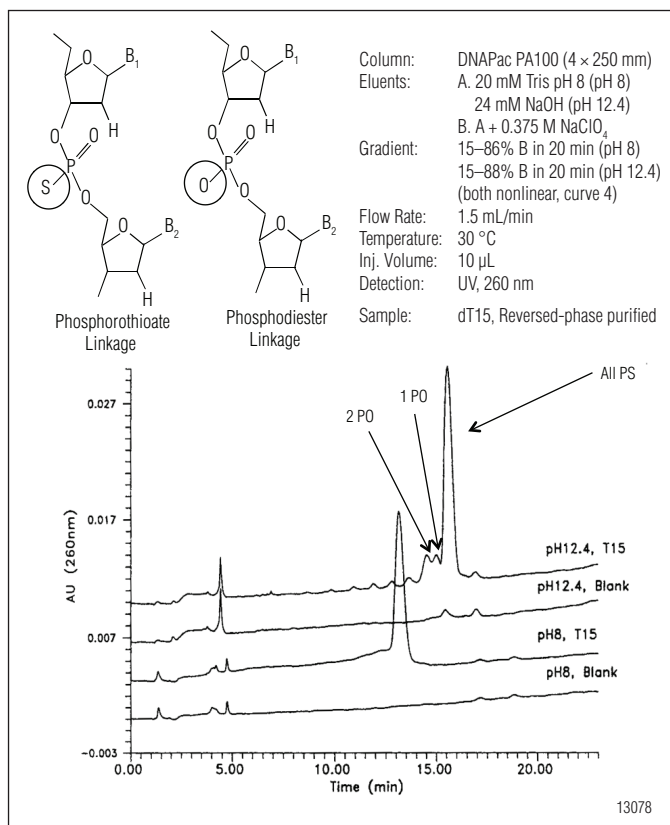


Figure 1. Resolution of phosphorothioate oligonucleotides with incomplete thiolation.

In Figure 2, purification of phosphorothioate diastereoisomers is shown. A 21-base sequence—the sense strand of an eGFP RNAi sample as both RNA and as DNA—is separated on a DNASwift SAX-1S column. This is a hybrid monolith, where anion-exchange nanobeads, with chemistry similar to that of the DNAPac PA200 column, are attached to the monolith surface.

In this example, two phosphorothioate (PS) linkages are inserted at linkages 6 and 14 in the sequence. When separated on the DNASwift (or DNAPac) columns, the four DNA components are resolved into three peaks, and the four RNA components are all resolved.

Since the DNASwift column is designed with capacity similar to porous-bead based anion exchangers (but with better peak shape), this separation can be scaled up for lab-scale purifications.

Here, all four components from the RNA sample were isolated, desalted, and subjected to ESI-MS, where each component produced the mass of the full-length ON with both PS linkages. Since this sequence does not self-associate, this observation demonstrates that the diastereoisomers are resolved.

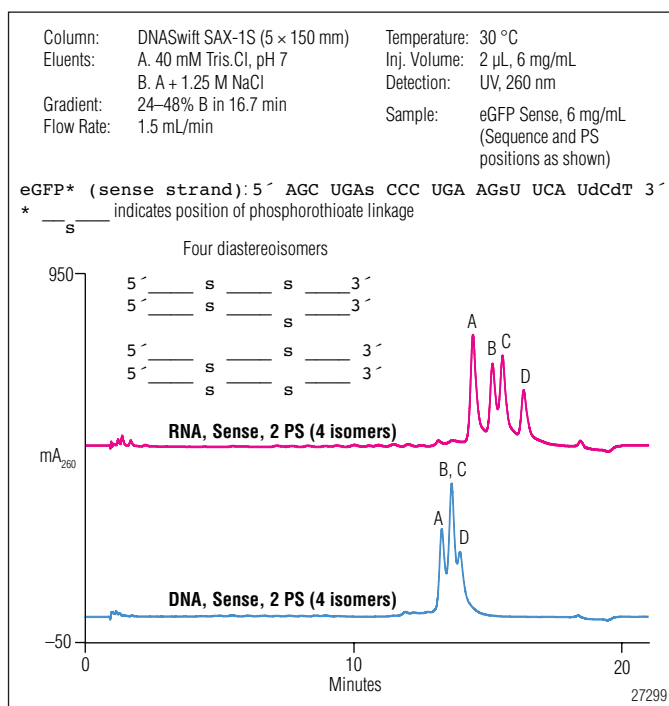


Figure 2. Separation of isobaric diastereoisomers on the DNASwift SAX-1S column.

Analysis of ss- and dsRNA

The discovery that short dsRNA species can effect exquisite control of the expression and turnover of RNA in animals, plants, and protists led to an explosive increase in the development of potential RNA therapeutics. The applications presented here underline the crucial role the DNAPac plays in RNA therapeutic analysis and meeting the needs of developers preparing pharmaceuticals in this rapidly growing field.

This new class of pharmaceuticals consists of two complementary ssRNAs of 19–21 bases that are annealed to form a single dsRNA. In practice, excess ssRNA of

either strand is considered an impurity, and so it must be characterized.

RNA and DNA differ by only one atom per nucleotide. This difference allows them to adopt slightly different solution conformations, and thus slightly different interactions with the stationary phases. Figure 4 demonstrates that DNA tends to elute significantly earlier than RNA at pH 7. RNA might be expected to elute later at a very high pH (> 12), where the 2' hydroxyl will begin to ionize, but not at pH 7 as shown. Hence, the solution conformation influences the ON interaction with the stationary phase.

RNA degrades at pH values as low as 8, hence, pH values above 8 have been considered as a poor choice for the separation of RNA ONs. However, pH-induced RNA degradation is a slow process, and does not usually occur in the time frame for ON analysis on column. To evaluate the RNA degradation by pH during chromatography, an RNA sample was run at pH 11 using different flow rates to change the oligoribonucleotide residence time on the column.

In the example presented here (Figure 3), the number of peaks and relative peak area for both RNA and DNA remained essentially unchanged for residence times from 7 to 21 min, indicating a lack of on-column degradation for that time period even at this very high pH, representing a thousand-fold increase in hydroxide concentration over pH 8. Further, control of extensive hydrogen bonds, such as those formed in G-tetrad ladders of considerable length, may require pH values up to 12.4 or temperatures > 95 °C. On the lower pH end, depurination becomes more likely as the pH falls to ≤ 6. Since depurination will lead to strand scission, pH values ≤ 6.5 are not typically recommended for ON AEC.

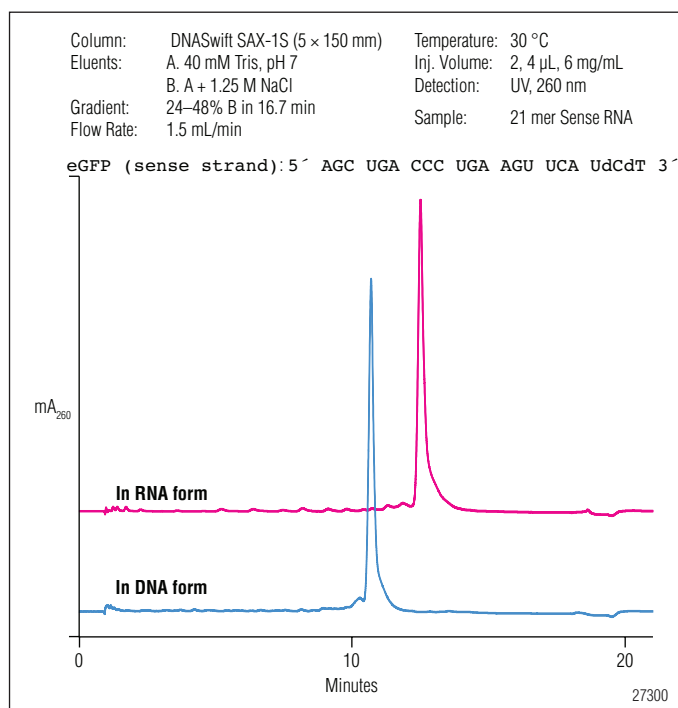


Figure 3. Relative retention of RNA and DNA on a DNASwift monolith column.

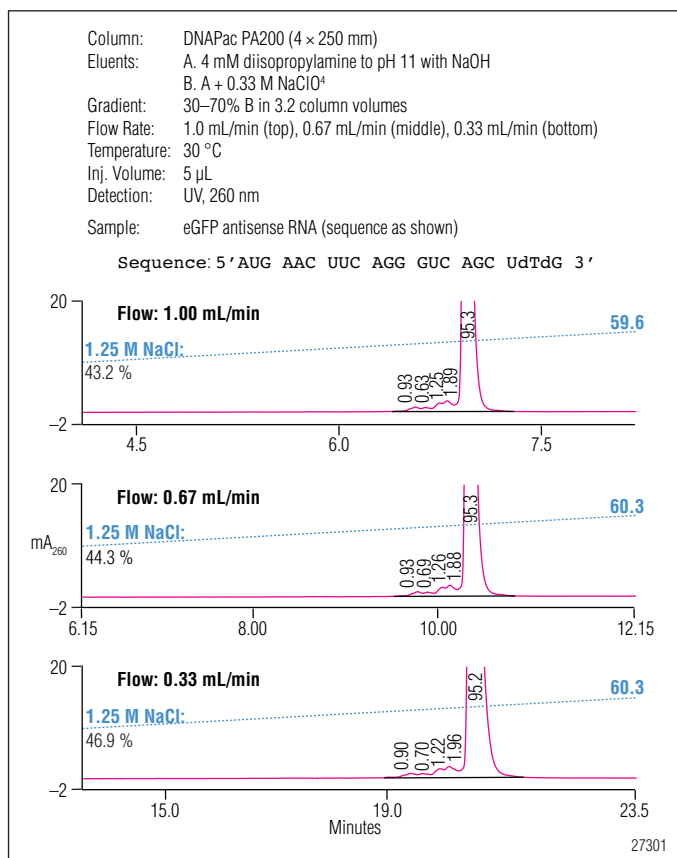


Figure 4. On-column stability: effect of high pH on eGFP antisense RNA. Peak labels indicate relative area (area %) at 7, 10.5, and 20.5 min residence time; pH 11.

DNAPac and DNASwift columns are useful for the analysis of both ss- and dsRNA. The use of elevated temperatures to control hydrogen bonding, within or between ONs, is a widely used technique. Pellicular anion-exchange chromatography of dsRNA and DNA/RNA hybrids can also benefit from careful selection of temperature during chromatography. As shown in Figure 5, two complementary RNA strands, and the duplex formed after annealing them together, were individually analyzed on a DNAPac PA200 column at pH 7 and 30 °C. Under these conditions, the two ssRNA were well resolved from one another, and both were resolved from the duplex. In this example, a small molar excess of the antisense strand was present, and the excess appeared as a small peak eluting at the position of the antisense ssRNA. This is typical of many efforts to prepare dsRNA; this separation technique demonstrates an easy titration method of the two ssRNA components to molar equivalence.

By modifying chromatographic temperature, conditions to better resolve each component may be obtained. As the temperature is increased, the elution positions of the two ssRNAs and the dsRNA will increase. This optimizes the resolution of all three components for the new RNA pharmaceuticals, allowing ssRNA impurity characterization.

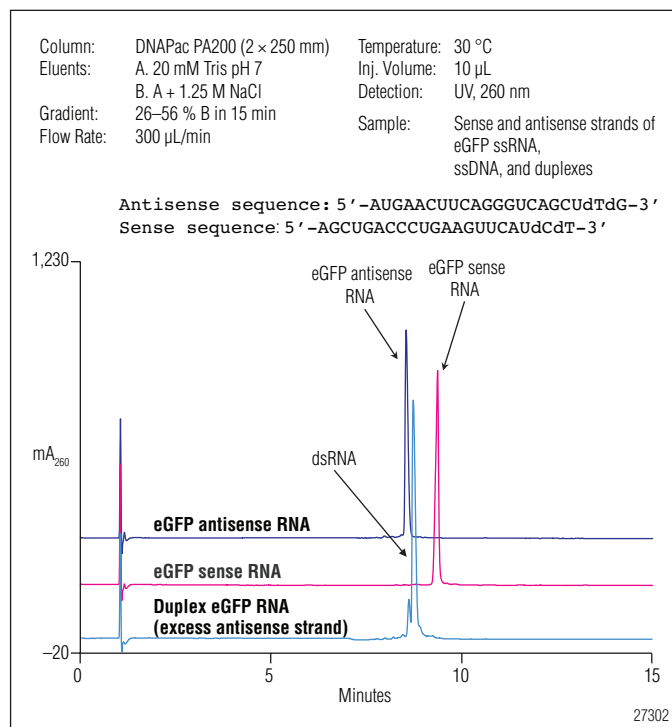


Figure 5. Resolution of duplex, ssRNA at 30 °C on the DNAPac PA200 column: 325–750 mM NaCl in 17.2', 300 µL/min, pH 7.

Figure 6 shows that the retention of each RNA component has increased, thereby exchanging the relative elution order of the ds- and antisense ssRNA strands.

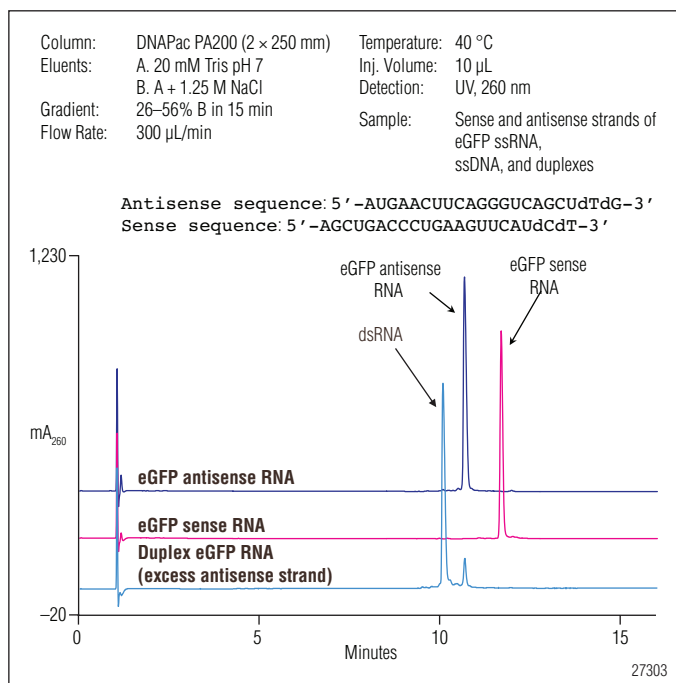


Figure 6. Resolution of duplex, ssRNA at 40 °C on the DNAPac PA200 column: 325–750 mM NaCl in 17.2', 300 µL/min, pH 7

As seen in Figure 7, at 50 °C, the retention of each RNA component has increased, causing the two ssRNAs (sense and antisense) to elute closer together.

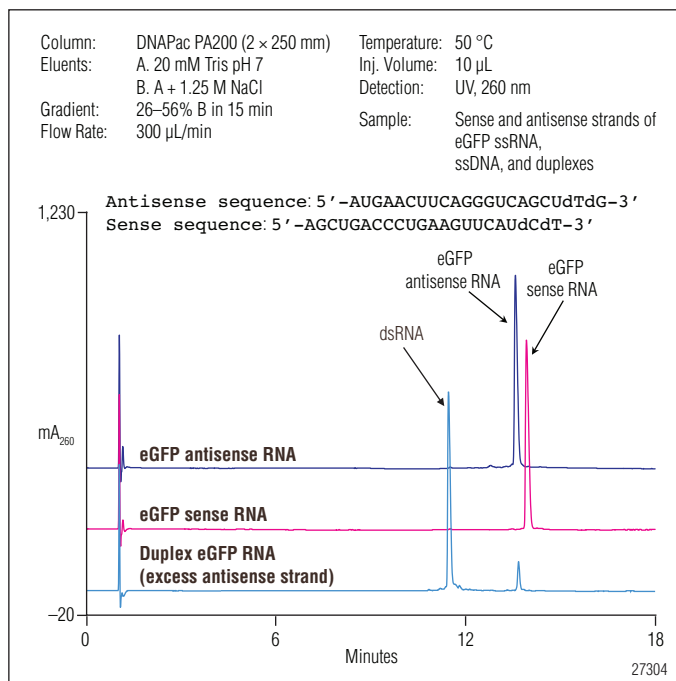


Figure 7. Resolution of duplex, ssRNA at 50 °C on the DNAPac PA200 column: 325–750 mM NaCl in 17.2', 300 µL/min, pH 7

At 60 °C, it is seen that the retention of each RNA component has increased further, causing the elution order of the two ssRNAs (sense and antisense) to reverse.

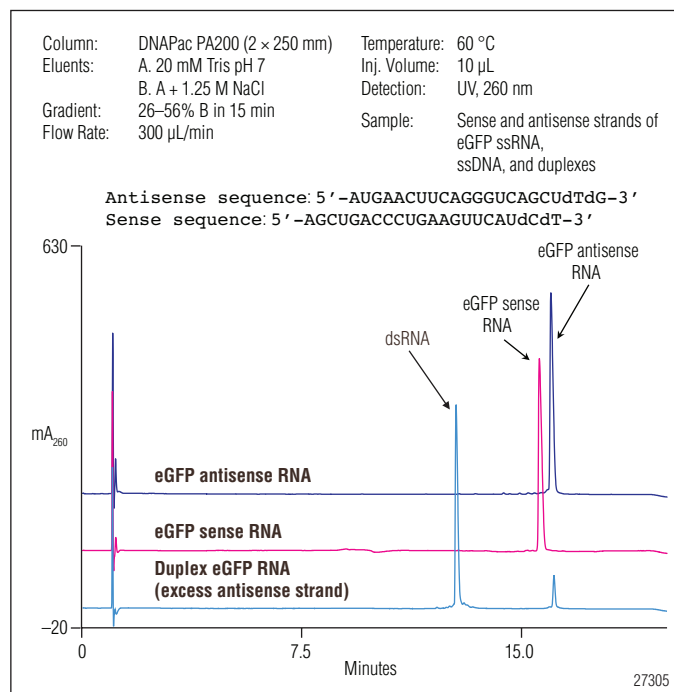


Figure 8. Resolution of duplex, ssRNA at 60 °C on the DNAPac PA200 column: 325–750 mM NaCl in 17.2', 300 µL/min pH 7.

At 70 °C (Figure 9), the retention of each RNA component has again increased, causing the two ssRNAs (sense and antisense) to elute more and further apart.

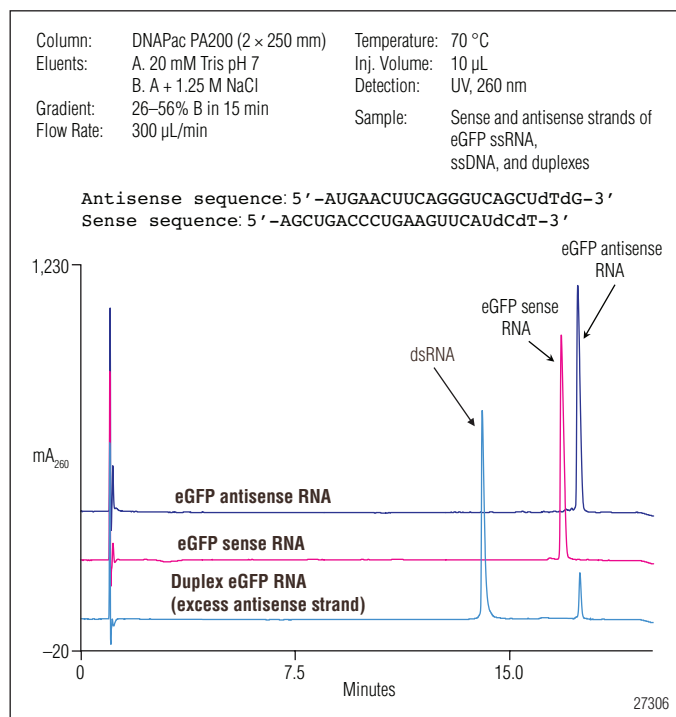


Figure 9. Resolution of duplex, ssRNA at 70 °C on the DNAPac PA200 column: 325–750 mM NaCl in 17.2', 300 µL/min, pH 7.

At 80 °C (Figure 10), the retention of each RNA component has increased, and the duplex has begun to melt (causing both ssRNAs—sense and antisense—to appear in the duplex chromatogram). Therefore, sense and antisense strands are less well resolved at 80 °C than at 70 °C.

This series of temperature assays (30–80 °C) reveals the ability to optimize the separation of both ssRNAs from each other and from the duplex. The 80 °C trace also reveals

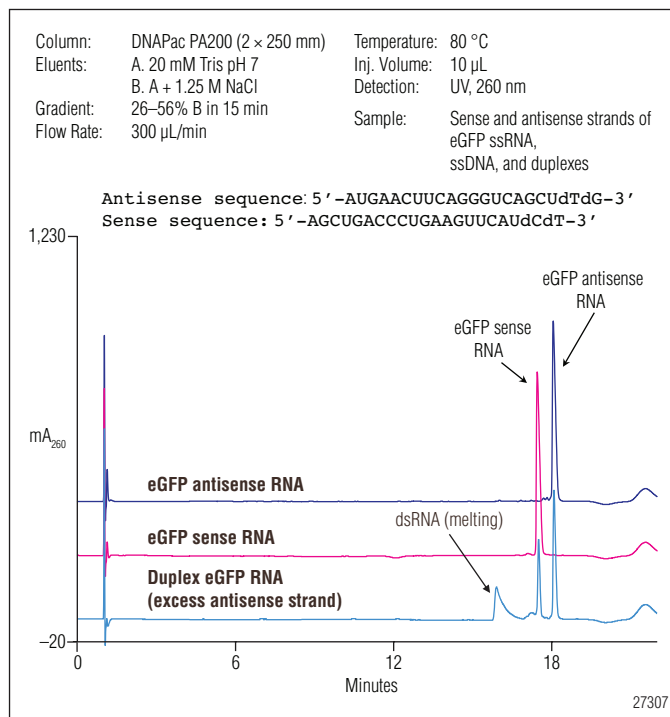


Figure 10. Resolution of duplex, ssRNA at 80 °C on the DNAPac PA200 column: 325–750 mM NaCl in 17.2', 300 µL/min, pH 7.

a difference in the peak areas for the sense and antisense strands in the duplex preparation due to partial melting of the duplex. This peak area difference allows a direct assessment of the relative molar inequivalence of the sense and antisense strands. Using the molar extinction coefficients and the peak areas of each strand, the user can calculate the amount (in this case, of sense strand) required to prepare a perfect 1:1 molar ratio for duplex formation.

Anion-Exchange ESI/MS of Oligonucleotides— Analysis of Impurities in the Target Oligonucleotide Product

Mass spectrometry capabilities have been applied to AE-separated ONs after manual desalting on C18 cartridges by ESI and MALDI-TOF approaches. These techniques are valuable for the identification of ON impurities. ESI was shown to provide superior results compared to simple MALDI-TOF techniques, especially for longer ONs. A high-throughput method for automated desalting of IP-RPLC-separated ONs was developed by Novatia LLC. Here, this method is adapted to automatically desalt anion-exchange-separated ONs. The automated system employs the Thermo Scientific Dionex UltiMate 3000 Titanium LC system equipped with the WPS-3000 TBFC fraction-collecting autosampler as shown in Figure 11. With this system, ONs are applied to DNAPac or DNASwift pellicular AE columns, and eluted using a salt gradient. Peaks detected by absorbance are collected to unique positions in the autosampler, and the collected fractions automatically desalted on an Acclaim® reversed-phase guard column using an ion-pair reagent and methanol as eluents. The desalted sample may be collected and submitted for ESI-MS at a later time, or immediately directed to the ESI-MS inlet.

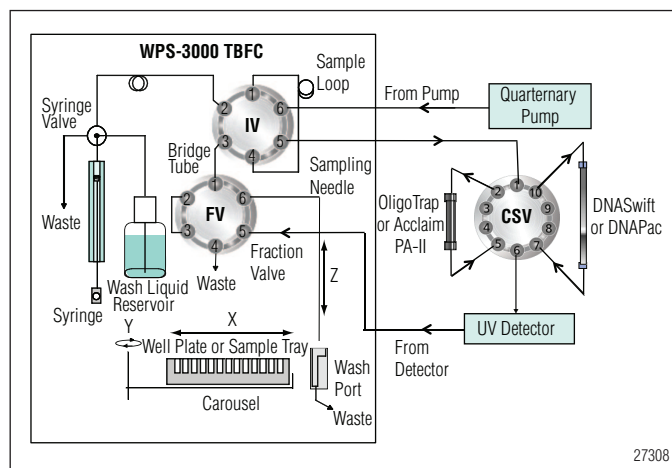


Figure 11. Automated purification and desalting using a well-plate sampler with injection, fraction collection, and column selection valves.

In the example presented here (Figure 12), an oligodeoxynucleotide (ODN) was purified on a DNAPac PA200 column using NaCl as eluent and at pH 12, to examine the purity of a DNAPac-purified ON.

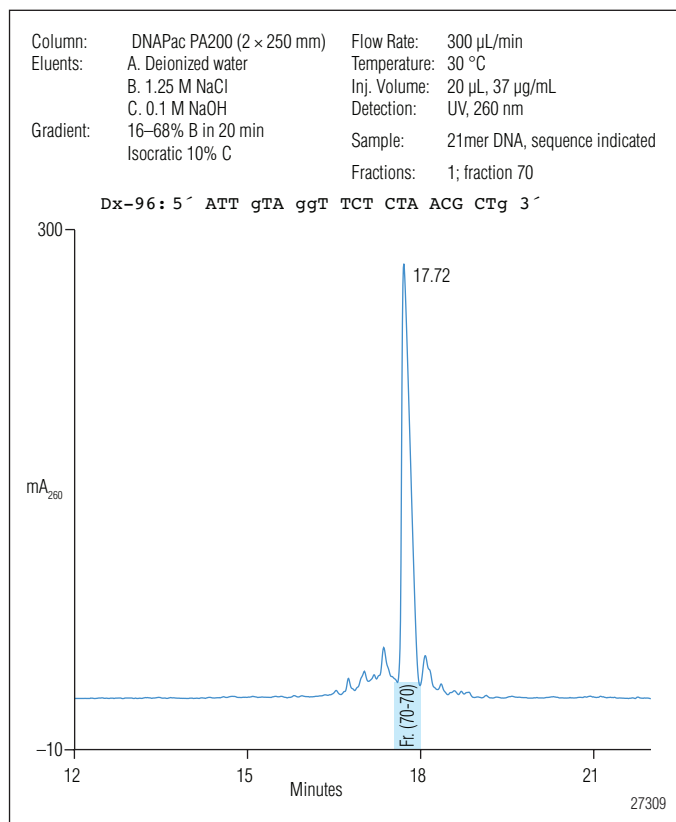


Figure 12. Purification of a 21-base oligonucleotide, $MW = 6427$. 80–195 mM NaClO₄ in 15 min, pH 12.

As shown in Figure 13, the purified ODN sample was desalted on an Acclaim 4.6 × 50 mm guard column before submission to ESI-MS.

The starting flow rate of an ion-pair eluent system (as shown in Figure 13) was 0.7 mL/min. The blue trace indicates the output of a high-sensitivity conductivity detector, and shows elution of the salt from the sample in the first 20 s. At 0.7 min, the eluent switches from 1.5% to 40% CH₃OH in the ion-pair eluent, and the flow is reduced to 0.3 mL/minute for possible introduction into the MS. The ON elutes between 1.7 and 2.0 min, and the initial conditions are restored to re-equilibrate the cartridge at 2.3 min.

When the UltiMate WPS-3000 TBFC was used, the sample was collected back into the autosampler instead of being directed into a mass spectrometer; thus, a flow rate reduction during ODN elution (for MS compatibility) was optional.

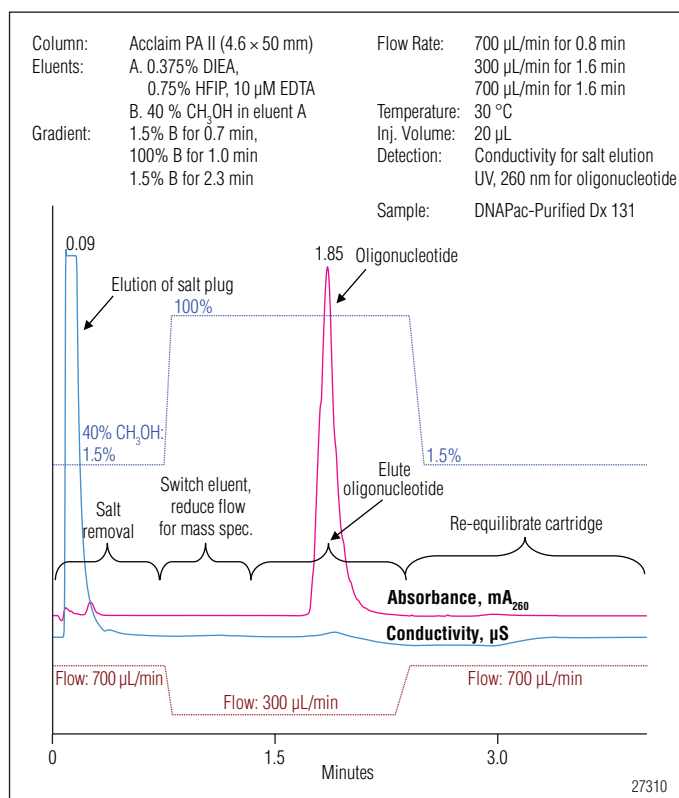


Figure 13. Oligonucleotide desalting on an Acclaim PA-II column; IP-RPLC retention, oligonucleotide elution with CH₃OH.

The ProMass deconvolution peak report indicates the presence of three nucleic acid components and a sodium adduct. This purified ODN's two contaminants include an n-C and an n-A—both at < 5% of the intensity of the full-length ODN. Thus, the DNAPac-purified ODN harbored small amounts of two different n-1 impurities, which were identified by AXLC-ESI/MS.

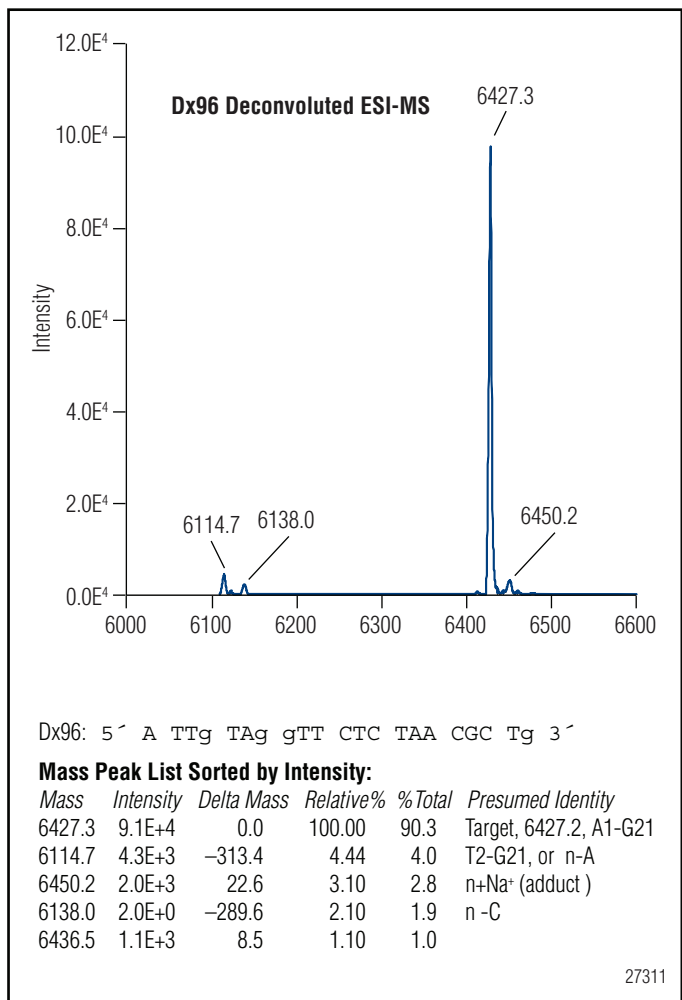


Figure 14. The (deconvoluted) spectra of the sample in Figure 13. The ESI-MS shows impurities in Dx96 primary peak.

Anion-Exchange ESI/MS of Oligonucleotides— Analysis of Oxidation Products of Purified Biotinylated Oligonucleotides

To further demonstrate the separation, desalting, and ESI-MS process, a biotinylated oligodeoxynucleotide was selected with the sequence shown. This ODN was purified on a 2 × 250 mm DNAPac PA200 column using a NaCl gradient at 300 μL/min at pH 12. Under these conditions, the full-length sample appears to resolve from its degradation products and the ON failure sequences (Figure 15).

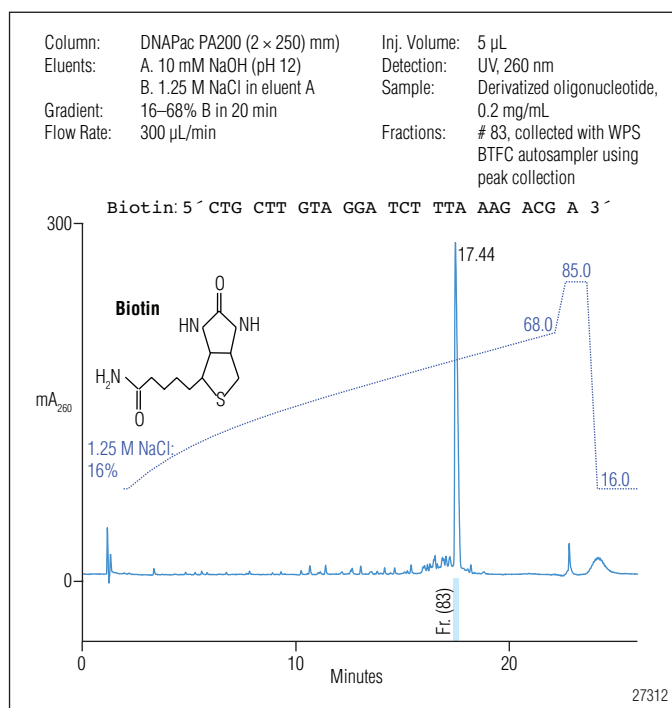


Figure 15. DNAPac purification and desalting of Dx-131 (5'-biotin).

After desalting, the sample is submitted to ESI-MS analysis, and the raw and deconvoluted spectra are shown in Figure 16.

As seen, the biotinylated ODN appears to be free of ON-derived contaminants, and only a small amount of sodium adduction is observed. However, an impurity is observed having a mass of ~16 amu larger than that expected for the biotinylated 25 mer.

The deconvolution peak report generated by ProMass software highlights the unexpected mass, and proposes an addition of oxygen to the purified ODN, indicating an oxidation. Since biotin oxidation is possible, this oxygen is proposed to be due to the oxidation of the biotin moiety, possibly from separation at pH 12.

Hence, this system shows effective purification of a modified ODN, its subsequent and automated desalting, and successful ESI-MS analysis revealing a possible oxidation.

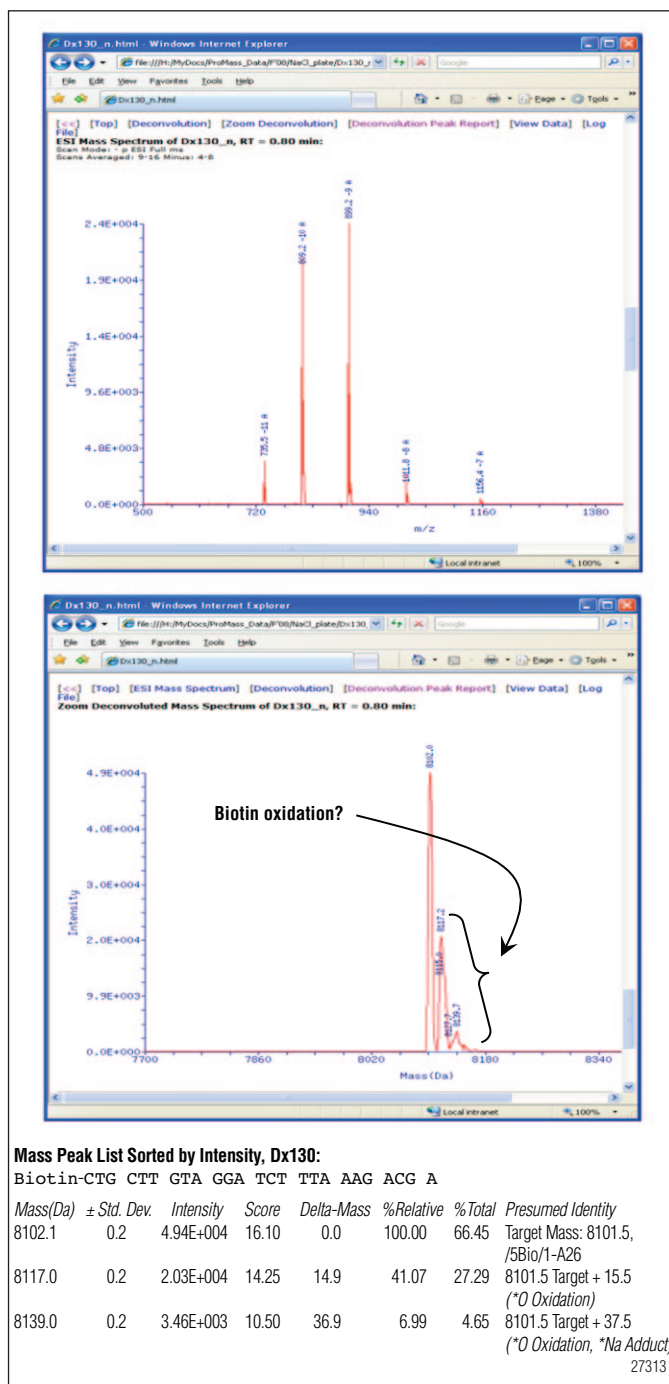


Figure 16. ESI-MS of desalted Dx-131 (5'-biotin) with raw and deconvoluted spectra, and MS Peak report.

Analysis of 2', 5'-Linkage Isomers in RNA and DNA, and Elucidation of Aberrant Linkage Position

The use of RNA as a therapeutic tool has been demonstrated for both ds- (RNA interference and regulatory microRNA) and ss- (such as aptamer) forms. This has stimulated great interest and led to numerous efforts to prepare oligoribonucleotides (ORNs) for therapeutic applications. Unlike DNA, RNA harbors a 2' hydroxyl moiety; under conditions used for synthetic nucleic acid release and deprotection, this can result in a phosphoryl-migration, producing 2', 5'-linkages. These aberrant linkages are known to inhibit certain human nucleases and polymerases, and so agencies responsible for regulating RNA therapeutic development require demonstration of their presence and linkage position.

In addition, some commercial preparations of RNAi libraries employ RNA and DNA with 2', 5'-linkages, so methods to confirm their presence are also needed. The presence of 2', 5'-linkages does not change the ionic character, hydrophobicity, or mass of the RNA or DNA. Thus, commonly employed analyses were not considered adequate by the regulatory agencies. However, ONs with 2', 5'-linkages have been shown to influence ON solution conformation; so, to the extent that the presence of these linkages impact this conformation, they may also influence their interaction with stationary phases.

Here, a DNASwift SAX-1S high-capacity monolith column is used to resolve and purify 12 ORN samples with zero to two 2', 5'-linkages (Figure 17). Using NaCl as the eluent, resolution of a single-sequence RNA with 2', 5'-linkages to different positions within the sequence, was

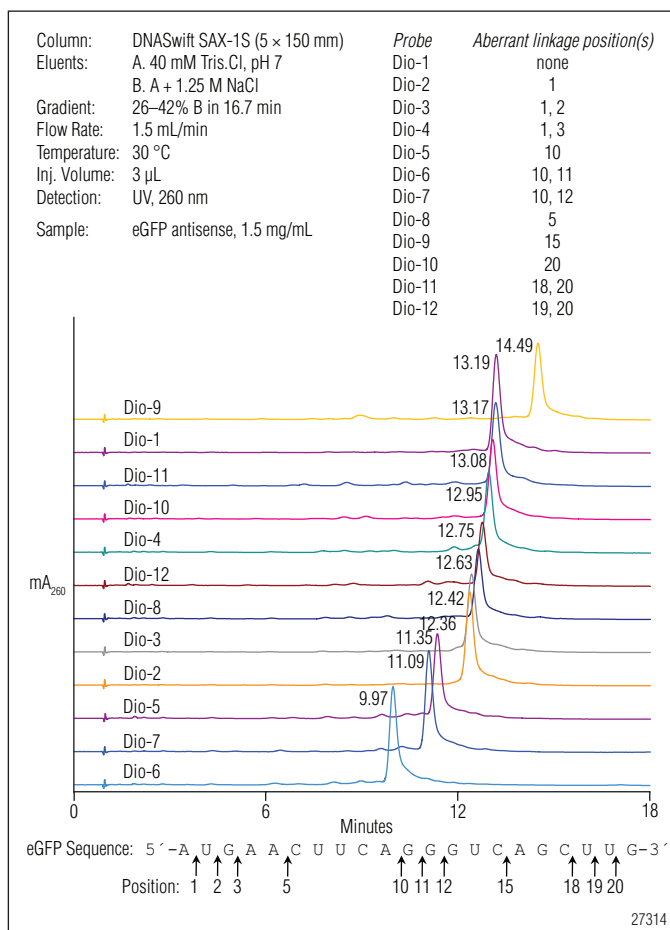


Figure 17. DNASwift resolution of RNA linkage isomers. 4.5 µg injected, 325–575 mM NaCl in 10 CV.

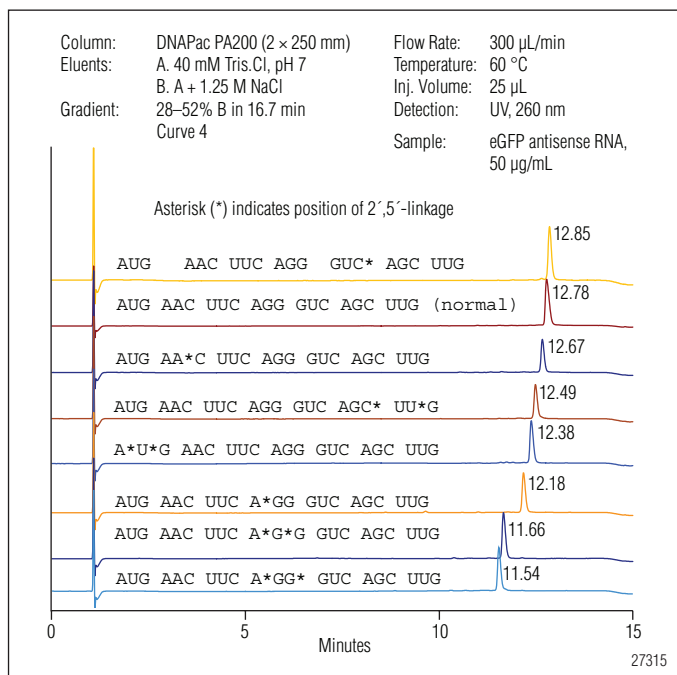


Figure 18. DNAPac PA200 column purity check of RNA, 21 mers.

remarkably similar to that obtained on the DNAPac PA200 column, revealing that several of these linkage isomers are resolved from one another and also from the ORN lacking the aberrant linkages.

While partial resolution of the ORN harboring the aberrant linkages at one position from those harboring them at different positions is useful, it does not constitute a definitive analysis for the presence of these aberrant linkages. However, these linkages are resistant to cleavage by phosphodiesterase-II [Calf-Spleen exonuclease, PDAse-II (E.C.: 3.1.16.1)], so digestion with PDAse-II will produce digestion products reflective of their position within the sequence.

As seen in Figure 19, DNAPac PA200 column chromatography of PDAse-II-treated, mixed-base RNA 21 mers containing one or two aberrant 2'-5' linkages at different positions in the RNA, results in specific, differentially retained degradation products. The chromatographic elution

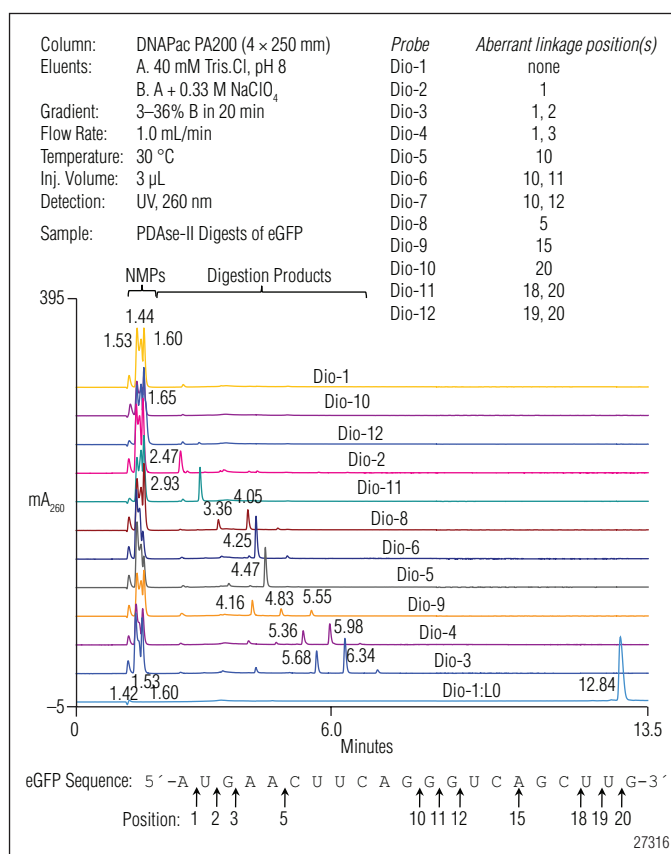


Figure 19. Elution of PDAse-II digestion products. PDAse-II fails to cleave at 2', 5'-linkages leaving partially digested products.

position is indicative on the length of the digested fragment. This allows a definitive demonstration of the presence of these linkages. However, it does not allow elucidation of the position of these aberrant linkages.

In order to establish the position of the aberrant 2', 5'-linkages in ORNs, the following test was repeated. First, the authors used the DNASwift hybrid monolith column to purify ORNs with and without aberrant linkages. Then, their purity was verified and they were digested with PDAse-II. The digestion products were then purified and desalted using the automated protocol on an Acclaim PA-II column, and the desalted digests were examined by ESI-MS.

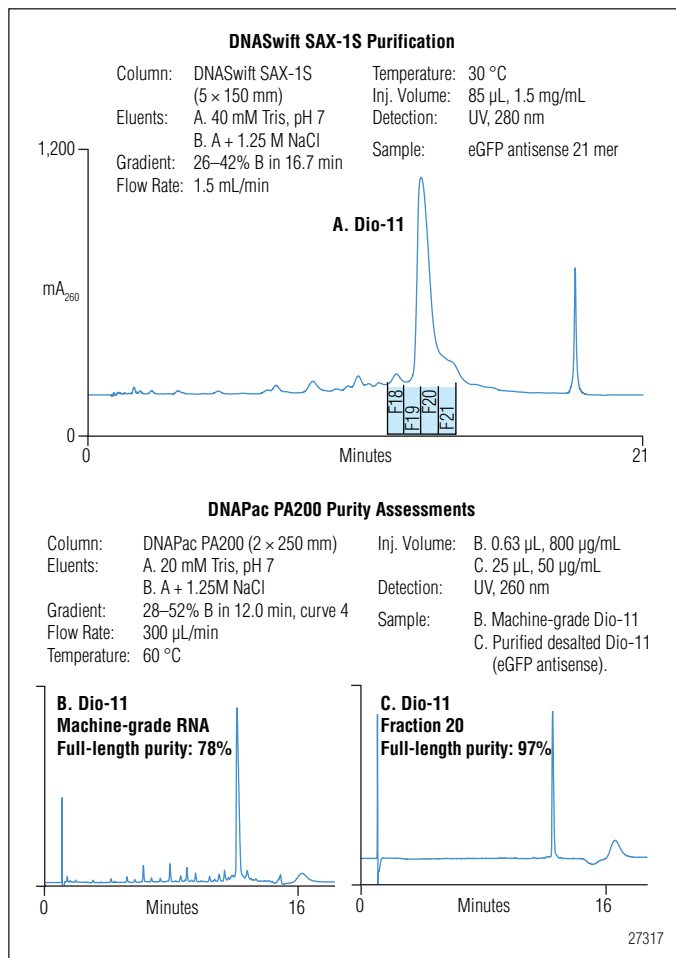


Figure 20. RNA purification using the DNASwift SAX-1S column

The DNASwift purification of one of the ORNs is shown in Figure 20.

In Figure 21, the desalted, full-length ORN is desalted (top panel) and examined by ESI-MS (bottom panel) to verify purity. Only the full-length mass and two sodium adduct masses are observed.

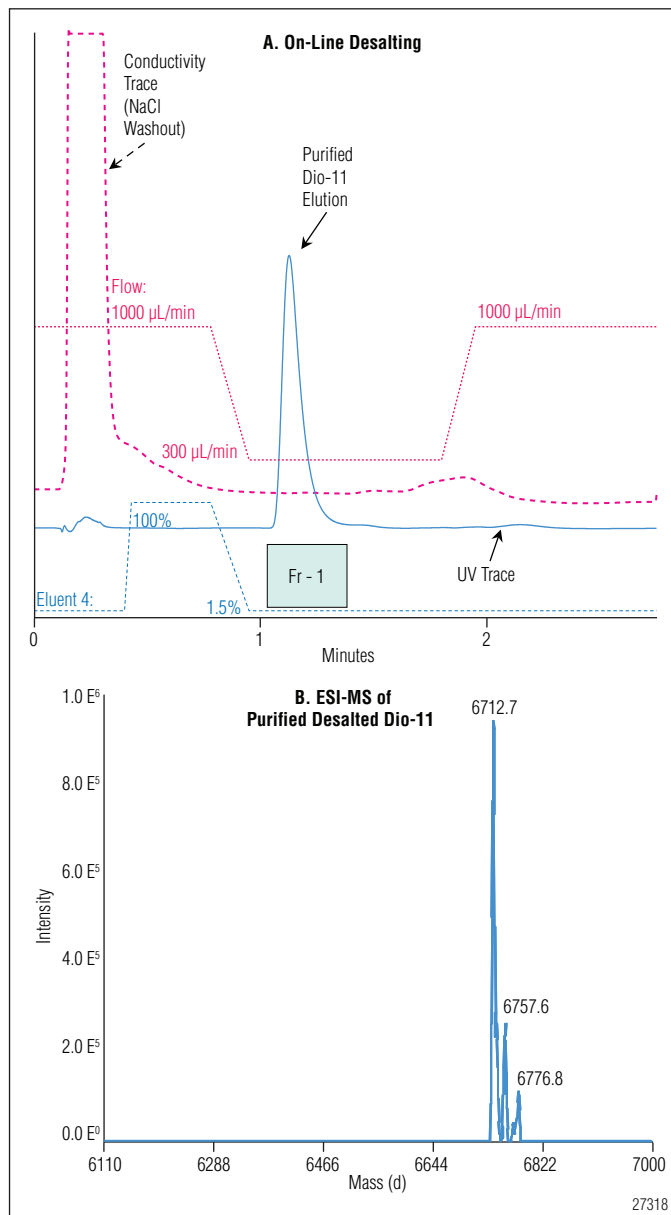


Figure 21. RNA desalting using the Acclaim PA-II guard column.

This sample, amongst several others harboring aberrant linkages, was then treated with PDase-II

The PDase-II digestion products were subsequently purified on a 4 × 250 mm DNAPac PA200 column with NaClO₄ eluent at pH 7, and several fragments were collected

from ORNs known to harbor aberrant linkages at specific positions in the sequence (Figure 22).

These were then desalted using the Acclaim PA2 column with reversed-phase eluents instead of ion-pair eluents, and the desalted fragments were submitted to ESI-MS analyses.

The desalting protocol and an example mass raw spectrum (not deconvoluted) reveal a molecular mass of 1327.2 amu (Figure 23). This is consistent with a base composition of A₂U₁G₁. Given the ORN sequence, the only position in which these bases are contiguous comprises the four bases at the 5' end of the sequence. This indicates the aberrant linkage to be at position 1 or at 1 and other positions in the first four bases.

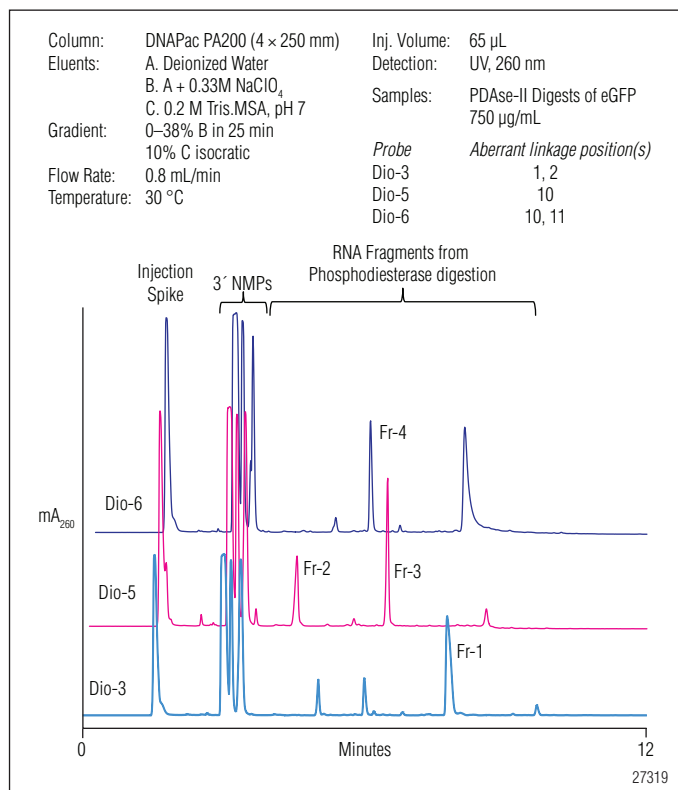


Figure 22. DNAPac purification of PDase-II digests of eGFP anti-sense RNA.

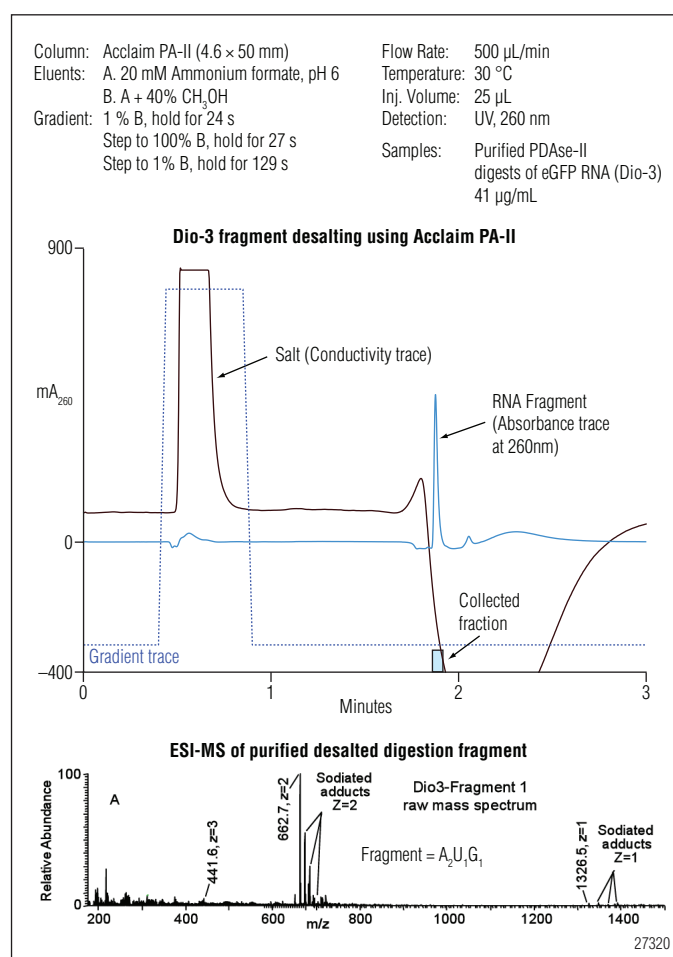


Figure 23. Oligonucleotide fragment desalting.

Table 1 summarizes the data for the samples purified on the DNASwift column, digested with PDase-II; and products purified on the DNAPac PA200, desalted on the Acclaim PAII column, and evaluated by ESI-MS. The table aligns the ESI-MS mass assignments with the sequence and positions of the 21-base RNA at each aberrant linkage. As summarized here, fragments from two to four bases were observed, each with base compositions consistent with the 2'-linked base, and one (or two) bases to the 3' side of the aberrant linkage. In cases where a single aberrant linkage was inserted, fragments were either two or three bases long. In cases where two aberrant linkages were present, fragments

of three to four bases were revealed. Further, cases of two aberrant linkages *separated by a normal linkage* include Dio-7 and Dio-11 (aberrant linkage at the 3' end). In each case, the fragments represent the latest-eluting component and comprised 4-base RNAs. Dio-11 occurs at the 3'-end, so this fragment terminates with a 3'-hydroxyl group instead of a phosphate. The fragment base compositions allows sequence inference from the (known common) parent RNA sequence, thereby confirming that assignment of the positions of the 2', 5'-linkages in RNA samples can be deduced from the ESI-MS-derived base composition and the sequence (unless the sequence harbors frequent repeats).

Name	Fragment Number	Sequence and Position of Digest Fragment	Fragment ID	Mass
Dio1:	none	5' -AUG AAC UUC AGG GUC AGC UUG -3'	None (only NMPs)	-
Dio3:	Fr 1	5' - <u>A*U*G</u> AAC UUC AGG GUC AGC UUG -3'	ApUpGpAp,	1327.2
Dio5:	Fr 2	5' -AUG AAC UUC A*GG GUC AGC UUG -3'	ApGp	691.2
Dio5:	Fr 3	5' -AUG AAC UUC A*GG GUC AGC UUG -3'	ApGpGp	1037.2
Dio6:	Fr 4	5' -AUG AAC UUC A*G*G GUC AGC UUG -3'	ApGpGp	1037.2
Dio7:	Fr 5	5' -AUG AAC UUC A*GG* GUC AGC UUG -3'	ApGpGpGp	1382.2
Dio8:	Fr 6	5' -AUG AA*C UUC AGG GUC AGC UUG -3'	ApCpUp	958.1
Dio9:	Fr 7	5' -AUG AAC UUC AGG GUC* AGC UUG -3'	CpAp	652.1
Dio11:	Fr 8	5' -AUG AAC UUC AGG GUC AGC* UU*G -3'	CpUpUpG-OH	1200.2

Table 1. Identification of phosphodiesterase-II RNA fragments (and resulting mass) based on known sequence. Elution on DNAPac PA200 column. eGFP antisense strand: 5' -AUG AAC UUC AGG GUC AGC UUG -3'. Modifications: Underlined residues have 2'-5' linkages. **Emboldened** bases indicate identified fragments.

Analysis of Nucleoside Mono-, Di-, and Triphosphates

The analysis of the nucleoside triphosphates in PCR reaction mixtures allows verification that the dNTPs (or NTPs for RNA amplification protocols) have not degraded. Analysis of amplification cocktails after amplification can reveal which component may have limited the amplification, and also reveal possible product inhibition. Here, a linear gradient is used to elute all 24 dNTPs and NTPs found in RNA and DNA amplification mixes at 23–27 °C.

The optimum temperatures for RNA and DNA components with this gradient were observed to be different: 23 °C for DNA components, and 27 °C for RNA components. Minor changes to tubing length, mixer design, and column oven may contribute to somewhat different results for different chromatography systems.

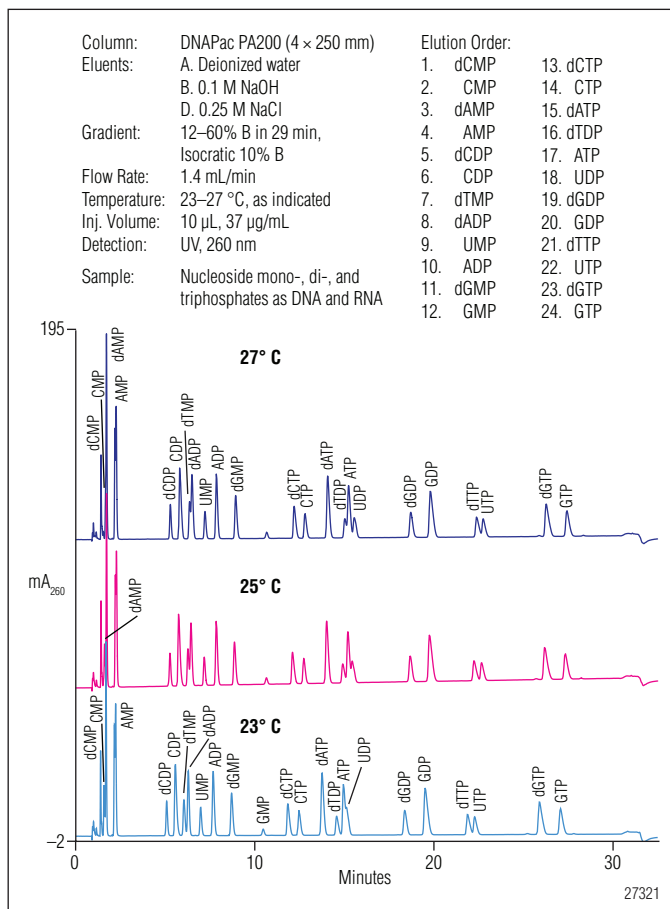


Figure 24. Analysis of nucleoside phosphates: effect of temperature using the DNAPac PA200 column.

Method Development Using Anion-Exchange Chromatography for Nucleic Acids

This section demonstrates method development for nucleic acid separations through nucleic acid interactions with anion-exchange stationary phases.

The development of ONs to be used for pharmaceutical applications require critical analyses. This analysis is supported by Dionex anion-exchange chromatography columns that were designed to employ ON chemistry for selectivity optimization.

In Figure 25, a linear salt gradient was used to elute a mixed-base 25 mer sequence. The top panel is with NaCl eluent and the bottom panel uses NaClO₄ as the eluent salt. In both panels, increasing eluent pH produces a pronounced increase in retention of the ON. This is due to the ionization of the tautomeric oxygen on G and T bases (T shown as inset). This ionization confers a formal increase in negative charge for each T and G as the pH increases between 9 and 11 (this does not occur if the ON contains only C and A). Since different ONs often contain different proportions of G and T, their pH-dependent retention increases will differ. This

allows users with sequence or composition information to predict eluent pH values likely to produce resolution between ONs eluting near one another at neutral pH.

While the effects of pH are qualitatively similar in these different eluents, perchlorate-based systems elute ONs at much lower concentrations, and use lower gradient slopes (5 mM/mL vs. 15 mM/mL for NaCl) without significant loss of resolution. Perchlorate is also a chaotropic salt, so it influences hydration of both the ON and stationary phase. This tends to minimize the effect of hydrophobic interactions between the nucleobases and the phase. Therefore, the use of NaClO₄ tends to encourage retention primarily on net charge. Similarly, reducing the pH will minimize the charge on the nucleobases. Thus, reducing the pH with NaClO₄ eluent will tend to encourage retention bases primarily on ON length. For DNAPac and DNASwift columns, pH represents a more powerful influence for control of retention, while salt form exerts a smaller influence.

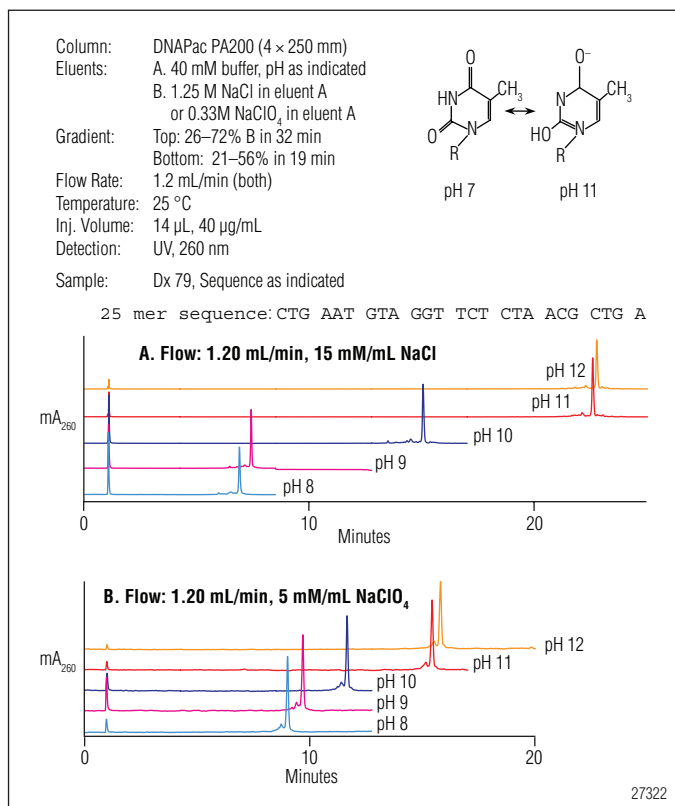


Figure 25. Effect of eluent salts on pH-induced retention using the DNAPac PA200 column and showing the effects of pH and salt form on single-stranded nucleic acid retention.

Some ONs used for critical applications harbor more hydrophobic characteristics. These require a more specific approach for critical analyses. Like NaClO₄, the addition of solvent to the eluent will tend to reduce interactions between the nucleobases and the stationary phase.

In Figure 26, pH-dependent retention in NaCl eluent on the PA200 column is compared in the presence and absence of 20% CH₃CN. With this solvent, pH-dependent retention persists, but overall retention is significantly reduced. The effect of solvent also impacts pH-dependent retention, in that ON elution time increases slightly when the pH shifts from 11 to 12 without solvent, but decreases with the programmed pH shift in 20% solvent. This effect is not observed when perchlorate eluents are used without solvent.

An important use of high pH eluents is control of Watson-Crick or other hydrogen-bonding interactions. For most ONs, these bonds are overcome at pH values above 10.5 or 11, and eventually GC or poly-G hydrogen bonds are theoretically denatured at pH 12.4. Hence, use of high pH is also a useful method for controlling unwanted inter- and intrastrand interactions during chromatography.

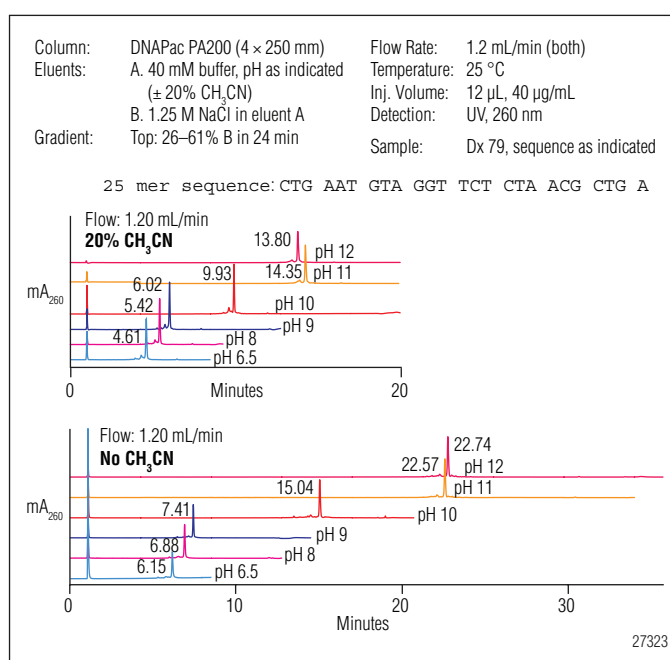


Figure 26. Influence of solvent on DNAPac PA200 retention: NaCl eluents.

Oligonucleotides may be modified with chemical derivatives for use as capture probes or as specific detection beacons. These are often very hydrophobic, and can introduce additional hydrophobic interactions that influence chromatographic peak shape. These need to be well controlled for critical analyses.

To demonstrate the utility of salt form for control of peak shape, a 25 nt sample without and with one or more common derivatives was analyzed on a DNAPac PA200 column with NaCl and NaClO₄ eluents (Figure 27).

As seen in the top panel of Figure 27, NaCl eluent were used without solvent to separate these very hydrophobic samples. The peak width values (at half-height [PW_{1/2}], in minutes) reveal PW_{1/2} values of 0.275 ± 0.07 min. However, the same samples separated with NaClO₄-based eluents produced PW_{1/2} values of 0.064 ± 0.007 min. In this case, use of NaClO₄ eluent reduced the peak width parameter to < 25% of that observed using NaCl eluents.

While use of such hydrophobic derivatives is not required for many ON assays, and often hydrophobic derivatives have no effect on ON peak shape, Figure 27 illustrates a simple method to control peak shape when hydrophobic derivatives must be used.

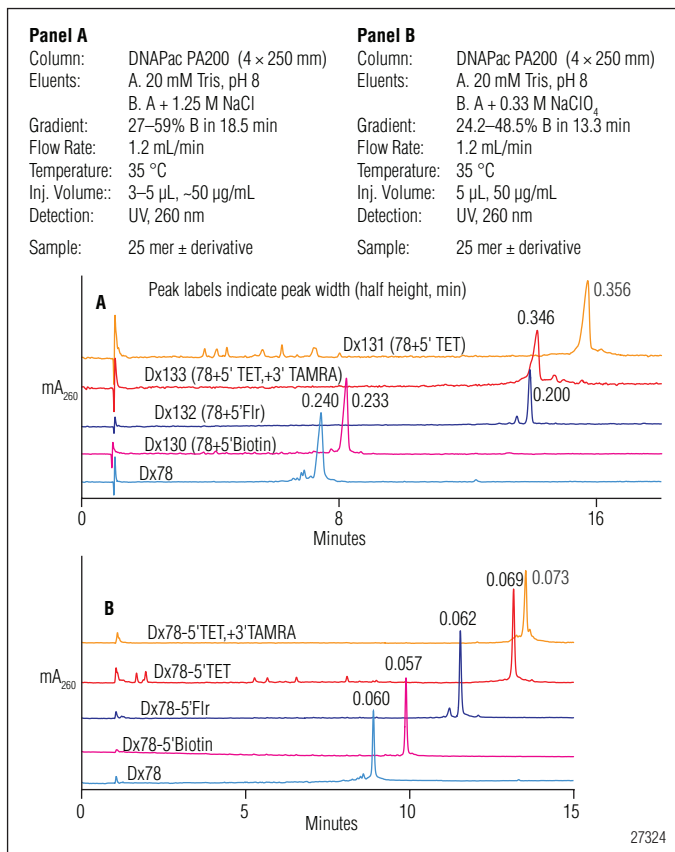


Figure 27. Effect of salt form on hydrophobic DNA peak width: NaCl vs. NaClO₄.

As in the previous example, ONs modified with very hydrophobic chemical derivatives for use as capture probes or as specific detection beacons introduce additional hydrophobic interactions that influence chromatographic peak shape. As a second example of peak shape control, a 25 nt sample without and with one or more common derivatives was analyzed on a DNAPac PA200 column with NaCl eluents with and without 20% acetonitrile as solvent (Figure 28).

In the top panel of this example, eluents without solvent were used to separate these very hydrophobic samples.

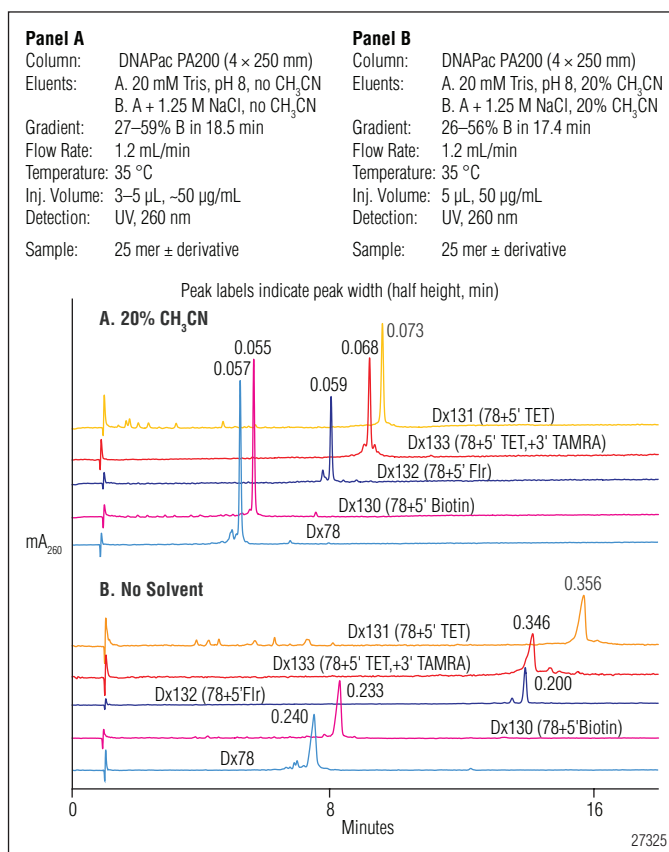


Figure 28. Effect of solvent derivatized on peak width.

The peak width values (at half-height, [PW]_{1/2}) in minutes) reveal PW_{1/2} values of 0.275 ± 0.07 min. However, the same samples separated with 20% CH₃CN produced PW_{1/2} values of 0.062 ± 0.008 min. In this case, use of solvent eluent reduced the peak width parameter to < 25% of that observed using NaCl eluents.

While use of such hydrophobic derivatives is not required for many ON assays, and often hydrophobic derivatives have no effect on ON peak shape, Figure 28 illustrates a second method to control peak shape when hydrophobic derivatives must be used.

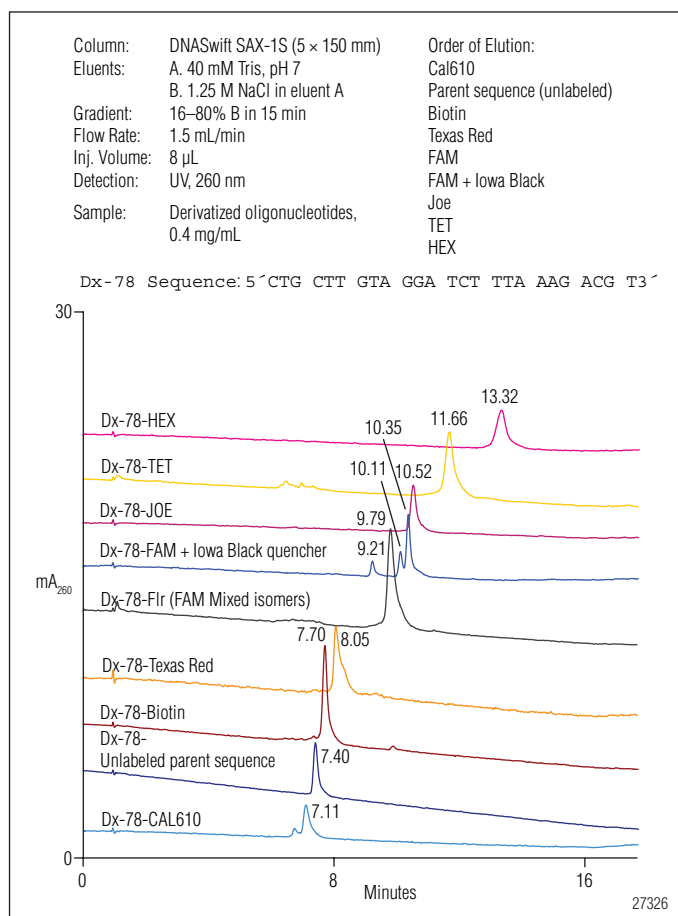


Figure 29. DNASwift oligonucleotide probes: fluorophore series.

Like the DNAPac columns, the DNASwift hybrid monolith column also resolves ONs harboring hydrophobic probe structures from one another, and from the parent sequence lacking the derivatives. In Figure 29, 3 µg samples were separated using a steep (survey) gradient on NaCl at pH 7. Each of the ONs bearing the derivatives are resolved from one another and all, except the ONs with Cal610 or biotin, are resolved from the parent sequence without modifications. As demonstrated, when using DNAPac columns, a less steep gradient will better resolve the ON derivatives, and addition of a small amount of solvent, use of NaClO₄, or even use of higher temperature with a column oven, will improve peak shape.

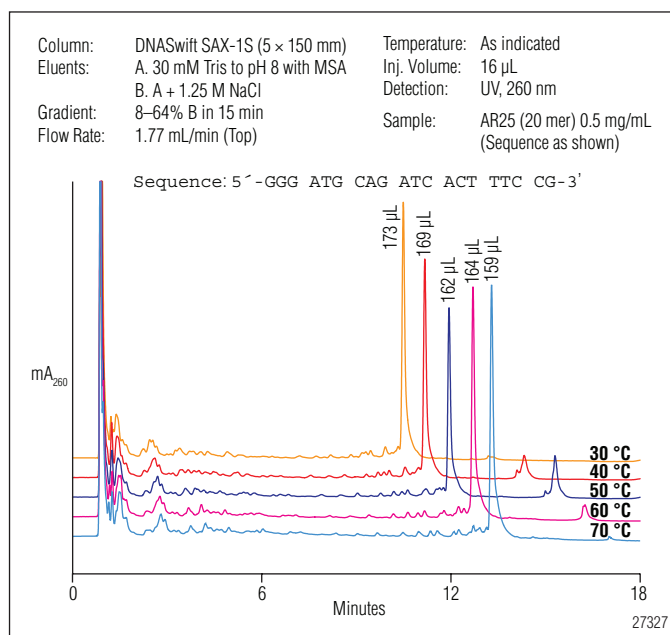


Figure 30. Effect of temperature on DNASwift PW_{1/2} and resolution.

In Figure 30, a 20-base ON was examined on a DNASwift SAX-1S hybrid monolith column at different temperatures. Adequate separation is observed at 30 °C, but increasing the temperature increases retention and reduces peak width (improving resolution), especially of the components eluting near the full-length AR25 20 mer. This illustrates the advantages of high-temperature ON chromatography. Another advantage is that operation above the melting temperature of the ON eliminates possible intra- and interstrand hydrogen bonding.

Since use of low pH, NaClO₄, and solvent all tend to reduce the influence of nucleobases on retention, combining these three conditions should tend to promote elution primarily in order of ON length, and this is a common goal during analysis.

To demonstrate this effect, 21 ONs with alterations at 5' and 3' ends, and containing 21–25 bases were separated on a DNAPac PA200 column at pH 6.5, using NaClO₄ eluents with 20% CH₃CN. The earliest- and latest-eluting components for each ON length were overlaid together on this set of chromatograms. The components of each length are at least partially resolved from the components of every adjacent length, even though they all have nearly identical sequence. This demonstrates that ONs with related base sequences are resolved from one another primarily on the number of bases under these conditions.

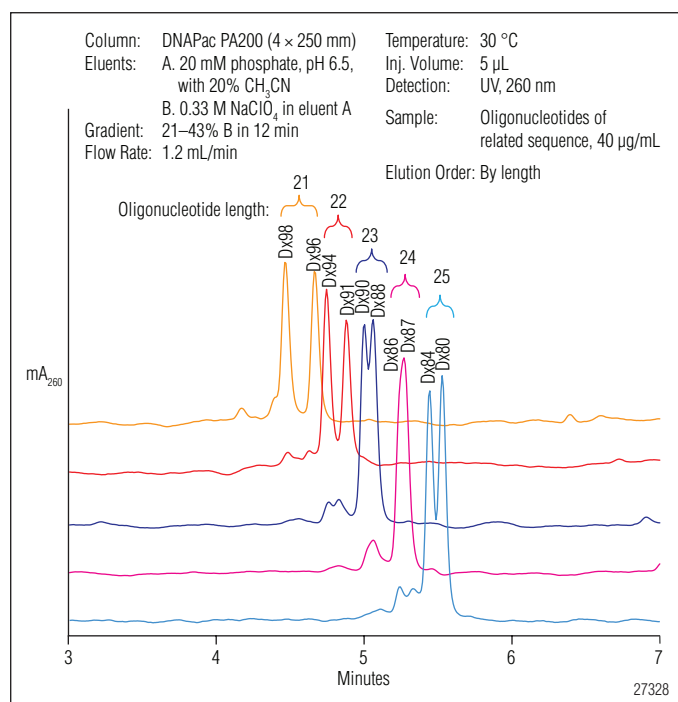


Figure 31. Oligonucleotide elution based primarily on length using the DNAPac PA200 column. Excerpted from *Analytical Biochemistry* **2005**, 338, 39–47.

As mentioned earlier, often, unwanted hydrogen-bonding within- or between-oligonucleotide strands can interfere with chromatographic separations regardless of the separation mode. With anion-exchange chromatography, high pH or high temperature can be used. In this case where the pH must remain low, unwanted hydrogen bonding interactions can be controlled either by increased temperature, or by the addition of chaotropic agents, such as 6 M urea or 25% formamide. These agents increase the eluent viscosity, and thus the operating pressures, so their use requires some care to ensure that rapid increases in flow (and thus very high pressure) do not damage the instrument.

Since the increase in pH increases ON retention, and since the retention increases depend on the number of T and G bases. Changes in pH can also influence retention order. In the cases where two closely related ONs must be resolved, for example n and n-1 sequences, changes in pH can allow control of selectivity.

In Figure 32, the effect of pH on selectivity with two 23-base ODNs (sequences provided) in NaCl, and NaClO₄ eluent systems is demonstrated. Both ONs exhibit dramatically increased retention with increasing pH. This is due to the ionization of the tautomeric oxygen on each G and T as the pH increases (T shown). These two ONs also exhibit pH-dependent elution order reversals in each salt. With NaCl, the elution order reverses between pH 9 and 10. With NaClO₄, the reversal is between pH 9 and 11 (coelution at pH 10). The extent of the retention difference between the ONs is more dramatic in NaCl than in NaClO₄. This derives from the chaotropic nature of NaClO₄, that suppresses hydrophobic interactions, contributing to the separation in NaCl.

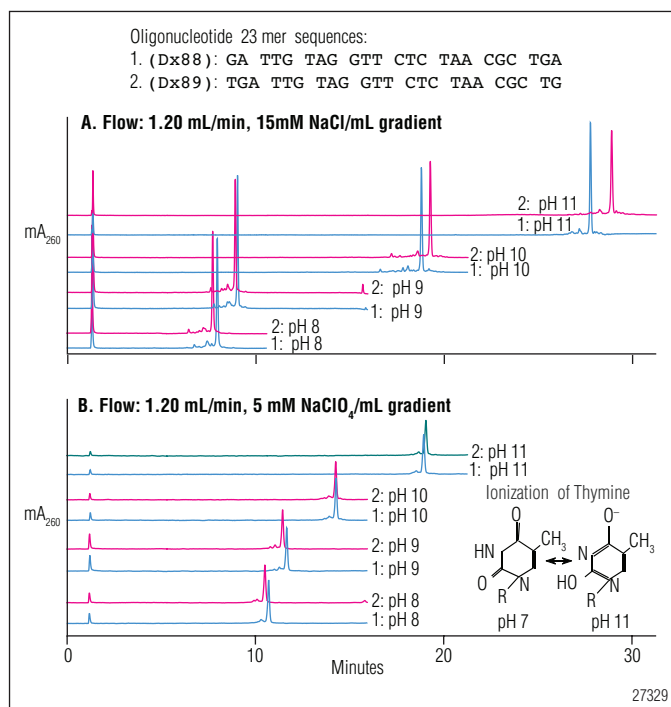


Figure 32. Effect of salt form on pH-dependent selectivity.

IP-RPLC does elute ONs based on their size, but does not support facile control of selectivity, and does not resolve many ONs having identical mass, such as RNA linkage isomers, or diastereoisomeric ONs, even when coupled to single-stage MS.

Because ON retention is profoundly influenced by pH between 6 and 12.4, the authors have developed a method to quickly and reliably deliver eluents at pH values in that range. While it is possible to employ eluent selection valves to deliver premade eluents at various pH values, these are cumbersome to execute as each eluent must be separately prepared and multiple valve systems must be employed. The method developed is a programmable system with quaternary eluent proportioning. This allows use of one pair of eluents for pH control, and the other pair of eluents to provide a salt gradient. Usually, the gradient eluents are 1) deionized water, and 4) a salt solution (e.g., 1.25 M NaCl or 0.33 M NaClO₄). Eluent 2 and 3 are used for pH programming. Eluent 2 is 0.2 M NaOH, and eluent 3 is TAD buffer, a combination of Tris-base, 2-amino-2-methyl-1-propanol, and di-isopropylamine, each at 0.2 M, and adjusted with methane sulfonic acid (MSA) to pH 7.2. These buffers harbor pK values ranging from pH 8 to 11. While HCl is typically used to adjust pH for eluents, it was found that HCl-adjustment of this buffer set tends to promote microbial growth, while MSA tends to inhibit it. Proportioning between eluents 2 and 3 allows delivery of a target pH. Generally, a fixed fraction of the total delivered eluent (e.g., 20%) is used to generate the desired pH, but this system also allows pH gradient formation.

An advantage of this system is that the salt form can be quickly changed by preparing a single new eluent (e.g., 0.33 M LiClO₄). To determine the proportion of each of the pH-generating eluents, the authors prepared a standard curve from the pH delivered by each proportion (0–20%) of the two eluents. The pH is monitored with a calibrated pH detector placed in the eluent line after the column and UV detector. Since three buffers are used, the relationship of proportion to pH is not linear. Each buffer contributes a distinct pH curve, and these are designed to overlap. Our pH vs. relative proportion data is converted into the standard curve using a sixth-order polynomial regression using Microsoft Excel, and the data with the regression line printed to use for day-to-day experiments. In most of the authors' work,

sodium salts are used which are known to influence pH measurements; also, relatively high salt concentrations are used, usually between 50 mM and 1 M. To examine the effect of sodium ion on pH measurement, the pH measurements were repeated using salt concentrations of 100, 400, and 800 mM. It was found that the pH vs. eluent 3 to eluent 4 proportion is essentially identical under each condition. Figure 33 illustrates a standard curve prepared with NaCl

This figure also provides the Excel-derived sixth-order polynomial equation, which is then employed to calculate the proportions of eluents 2 and 3 to deliver a desired eluent pH. To verify that the desired pH is correctly delivered, we employ a calibrated, in-line pH electrode. Using this system one can program a desired pH with eluents 2 and 3, and use eluents 1 and 4 to prepare simple or complex multistep gradients.

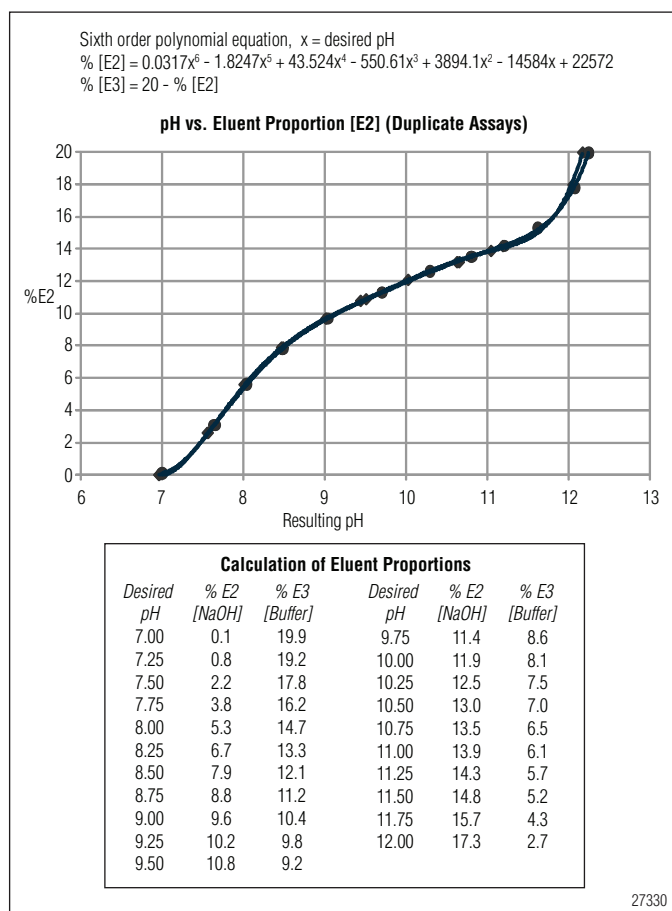


Figure 33. Dial-a-pH standard curve: pH vs. eluent proportion using 0.2 M NaOH vs. 0.2 M TAD. TAD = 0.2 M Tris, 0.2 M, 2-amino-2-methyl-1-propanol, 0.2 M di-isopropylamine, pH 7.2.



Application notes

Nucleic acid therapeutics applications notebook

High-Resolution Analysis and Purification of Oligonucleotides with the DNAPac™ PA-100 Column

INTRODUCTION

The DNAPac™ PA-100 anion-exchange column is specifically engineered to provide unit-base resolution of synthetic oligonucleotides to 60 bases and beyond. This column is packed with a pellicular anion-exchange resin in which quaternary amine-functionalized microbeads are bound to a 13- μ m diameter nonporous polymeric substrate (see Figure 34). The rapid mass transport characteristics of this resin result in higher resolution oligonucleotide separations than are possible with traditional macroporous resins or reversed-phase columns. Additionally, the DNAPac PA-100 can be operated routinely under strongly denaturing conditions, including high temperature or high pH. Anion-exchange separations under denaturing conditions are particularly useful for the resolution of oligonucleotides with high guanine (G) content or with regions of complementary sequence.

In this application note, methods for the separation of various synthetic oligonucleotides on the 4-mm diameter DNAPac PA-100 analytical column are presented. Included are pH 8.0 and pH 12.4 gradient programs. Also presented are strategies for the scale up of analytical separations to semipreparative purifications using a 22-mm diameter column.

EQUIPMENT

Dionex DX 500 HPLC system consisting of:

GP40 Gradient Pump

AD20 UV/Visible Absorbance Detector

LC20 Chromatography Enclosure

Dionex PeakNet Chromatography Workstation

REAGENTS AND STANDARDS

Deionized water, 17.8 M Ω -cm resistance or better

Sodium hydroxide solution, 50% w/w, low carbonate

Tris(hydroxymethyl)aminomethane (molecular biology grade)

Sodium chloride

Sodium perchlorate monohydrate

Disodium ethylenediaminetetraacetate (EDTA), dihydrate (molecular biology grade)

pd(A)₁₂₋₁₈, (Pharmacia Biotech Inc., Piscataway, New Jersey, USA)

pd(A)₄₀₋₆₀, (Pharmacia Biotech Inc.)

pd(G)₁₂₋₁₈, (Pharmacia Biotech Inc.)

-20 sequencing primer, crude 17-mer, (Genosys Biotechnologies, Inc. Woodlands, Texas, USA)

CONDITIONS

Columns:	DNAPac PA-100 analytical, 4 × 250 mm DNAPac PA-100 guard, 4 × 50 mm DNAPac PA-100 semipreparative, 22 × 250 mm
Eluent A:	Deionized water
Eluent B:	0.20 M NaOH
Eluent C:	0.25 M Tris-Cl, pH 8.0
Eluent D:	Option 1: 1.0 M NaCl Option 2: 2.0 M NaCl Option 3: 0.375 M NaClO ₄
Flow Rate:	1.5 mL/min, except where noted
Inj. Volume:	200 μ L
Detection:	UV, 260 nm

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent A: Deionized H₂O

Vacuum degas 1 L of deionized water.

Eluent B: 0.20 M NaOH

Dilute 10.4 mL of low carbonate 50% w/w sodium hydroxide to 1 L with degassed, deionized water. Pellets of NaOH are coated with a layer of sodium carbonate and are not suitable for eluent preparation.

Eluent C: 0.25 M Tris-Cl, pH 8.0

Dissolve 30.28 g of tris(hydroxymethyl)amino-methane in 800 mL of deionized water. Adjust the pH to 8.0 by the addition of approximately 10 mL of concentrated HCl. Add deionized water to a final volume of 1 L and vacuum degas the solution.

Eluent D, Option 1: 1.0 M NaCl

Dissolve 58.45 g of sodium chloride in 800 mL of deionized water. Add deionized water to a final volume of 1 L and vacuum degas the solution.

Eluent D, Option 2: 2.0 M NaCl

Dissolve 116.9 g of sodium chloride in 800 mL of deionized water. Add deionized water to a final volume of 1 L and vacuum degas the solution.

Eluent D, Option 3: 0.375 M NaClO₄

Dissolve 52.67 g of sodium perchlorate monohydrate in 800 mL of deionized water. Add deionized water to a final volume of 1 L and vacuum degas the solution.

0.5 M EDTA, pH 8.0

Add 93.1 g of disodium ethylenediaminetetraacetate dihydrate to 400 mL of deionized water. While stirring, add NaOH (approximately 10 g of pellets) until the EDTA is completely dissolved and the pH is stable at 8.0. Add deionized water to a final volume of 500 mL and autoclave to sterilize.

TE Buffer, pH 8.0

10 mM Tris-Cl, pH 8.0
1 mM EDTA, pH 8.0

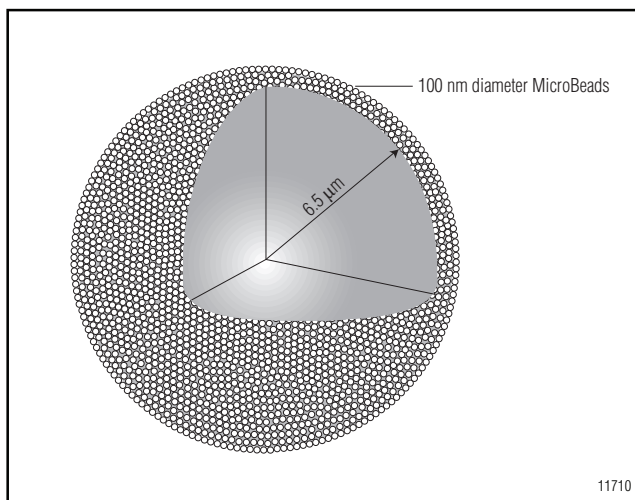


Figure 34. Pellicular structure of the DNAPac PA-100 resin.

SAMPLE PREPARATION

pd(A)₁₂₋₁₈, pd(A)₄₀₋₆₀, and pd(G)₁₂₋₁₈

Each lyophilized DNA homopolymer sample was dissolved in TE buffer to produce a 1-mg/mL solution. Before injection, DNA was diluted to the appropriate concentration with deionized water. The “p” in the chemical symbol indicates that the oligomers are phosphorylated.

-20 Sequencing Primer

The lyophilized, unpurified synthetic oligonucleotide was suspended in TE buffer. To remove particulate matter, the sample was centrifuged at 12,000 x g for 5 minutes and the supernatant was transferred to a fresh tube. The DNA concentration was determined by measuring the absorbance at 260 nm and applying the manufacturer’s suggested conversion factor of 1.0 A₂₆₀ = 30 μg/mL. The oligonucleotide was diluted with deionized water to the indicated concentration before injection.

RESULTS AND DISCUSSION

High-Resolution Separations at pH 8.0

The high resolving power of the DNAPac PA-100 can be demonstrated by the analysis of synthetic DNA homopolymers. Typical results for the analysis of 1 μg of pd(A)₁₂₋₁₈ are shown in Figure 35. A gradient of 100 to 450 mM NaCl in the presence of 25 mM Tris-Cl pH 8.0 was used to elute the pd(A) oligonucleotides.

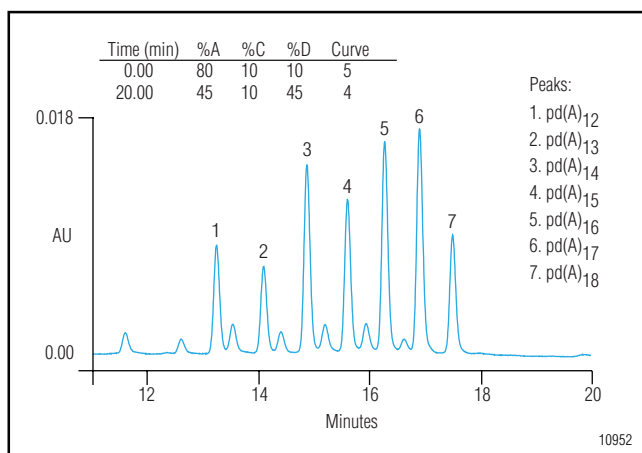


Figure 35. Separation of 1 µg of pd(A)₁₂₋₁₈ at pH 8.0 with a 100 to 450 mM NaCl gradient. Small peaks represent dephosphorylated impurities (see text). Eluent D, Option 1 was used: 1.0 M NaCl.

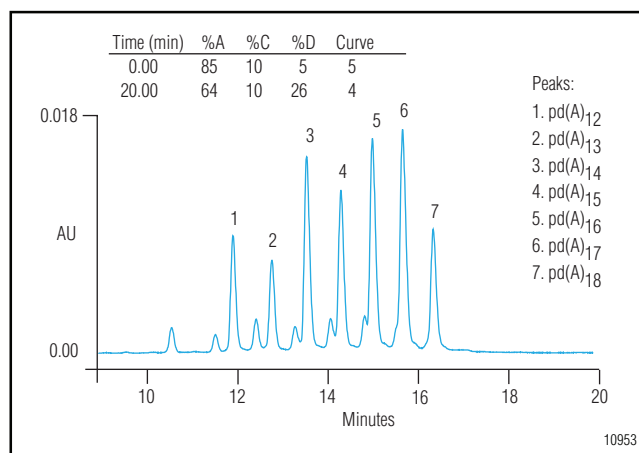


Figure 36. Separation of 1 µg of pd(A)₁₂₋₁₈ at pH 8.0 with a 19 to 98 mM NaClO₄ gradient. Slightly different selectivity relative to NaCl elution is observed (see Figure 35). Eluent D, Option 3 was used: 0.375 M NaClO₄.

The sample was injected at a relatively low NaCl concentration of 100 mM to ensure efficient binding of the oligonucleotides to the column. The pd(A) homopolymer mixture was resolved into seven main peaks, which represent the seven phosphorylated oligonucleotides described by the manufacturer. Also detected were seven minor peaks, which coeluted with the d(A)₁₂₋₁₈ oligonucleotides produced by dephosphorylation of the pd(A)₁₂₋₁₈ sample with alkaline phosphatase (data not shown).

DNA can be eluted from the DNAPac PA-100 by a variety of anions other than chloride, including acetate,¹ bromide,² and perchlorate.¹ The use of NaClO₄ as an eluent has been thoroughly investigated.¹ Perchlorate is particularly useful for the analysis of phosphorothioate DNA, in which sulfur has been substituted for non-bridging oxygen on the phosphate backbone. Since this sulfur substitution results in an increased affinity of DNA for the DNAPac resin, conventional salt gradients such as 0–2 M sodium chloride often will not elute phosphorothioates.^{1,2} For the pd(A)₁₂₋₁₈ sample, gradient elution with NaClO₄ rather than NaCl produced similar chromatographic results (see Figure 36). Relative to NaCl, much lower concentrations of NaClO₄ are needed for DNA elution.

A somewhat different NaCl gradient was used for analysis of a pd(A)₄₀₋₆₀ oligonucleotide mixture (see Figure 37). In addition to the 21 expected peaks, at least one additional minor peak was detected. Since these

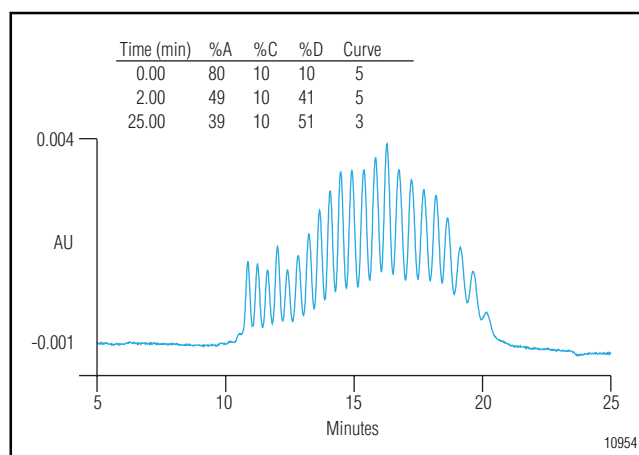


Figure 37. Separation of 1.5 µg of pd(A)₄₀₋₆₀ at pH 8.0 with a shallow 410 to 510 mM NaCl gradient. The sample was injected at 100 mM NaCl to ensure efficient binding of the DNA to the column. Eluent D, Option 1 was used: 1.0 M NaCl.

oligonucleotides are longer and possess a stronger negative charge than the pd(A)₁₂₋₁₈ species, higher NaCl concentrations are needed for elution. After sample injection at 100 mM of NaCl, the NaCl concentration was stepped to 410 mM over 2 minutes. A shallow (410 to 510 mM) NaCl gradient over 23 minutes was used for elution of the DNA. This gradient strategy couples the high resolving power of shallow gradients with short run times.

High pH Separations

Self-complementary sequences or poly-G stretches can result in intra- and intermolecular associations of oligonucleotides. These associations can prevent efficient oligonucleotide separation under non-denaturing conditions. The DNAPac PA-100 can be operated at high temperature (to 90 °C) or at high pH (to pH 12.4) for the separation of samples that contain such sequences. As an example, the attempted separation of pd(G)₁₂₋₁₈ at room temperature with a 100 to 900 mM NaCl gradient at pH 8.0 is shown in Figure 38. The poly-G sequence composition causes the sample to elute as one broad, diffused band. At pH 12.4, hydrogen bonding is abolished. As a consequence, the seven primary pdG homopolymers are resolved at pH 12.4, as are a number of minor contaminating species (see Figure 39). Relative to pH 8.0, an additional negative charge is present on the bases G and T at pH 12.4. This extra charge provides additional separation selectivity because it causes oligonucleotides with high levels of G and T to elute later than sequences low in G and T.

Analytical to Semipreparative Scale Up

DNAPac PA-100 columns are available in diameters of 9 mm and 22 mm for semipreparative applications. Analytical separations on the 4-mm diameter column can be scaled directly to the larger columns, so that methods development for semipreparative applications can be performed rapidly on the analytical column with small amounts of sample. For direct scale up of analytical gradient chromatography, a constant column volume scaling rule should be observed: *The number of column-volumes of eluent delivered over the duration of the gradient should remain constant.* To demonstrate scale up of a method from the 4-mm diameter to the 22-mm diameter column, an analytical separation was developed for the “-20 primer,” a crude synthetic 17-mer with the sequence 5'-GTAAAACGACGGCCAGT-3'. A 7.5 to 124 mM NaClO₄ gradient over 20 minutes at pH 8.0 was used for separation of the primer from the various failure sequences in the sample. Figure 40a shows an analytical separation of 1.0 µg of this sample on the 4-mm diameter column.

The objective of the scale up exercise was the transfer of this analytical method from the 4-mm diameter column at a flow rate of 1.5 mL/min to the 22-mm diameter column at 10.0 mL/min.

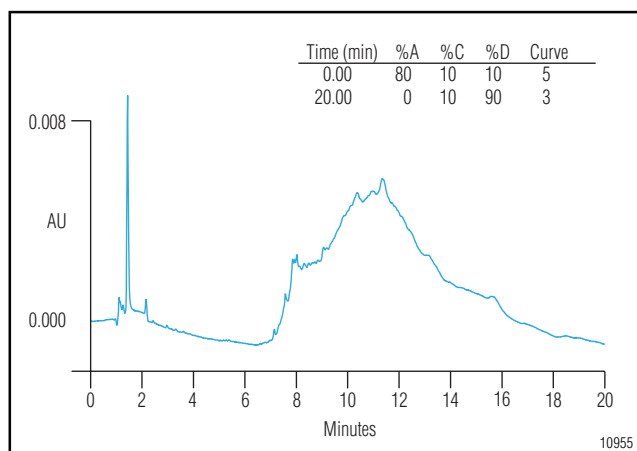


Figure 38. Attempted separation of 3 µg of pd(G)₁₂₋₁₈ with a NaCl gradient at pH 8.0. Self-complementary sequences or poly-G regions in DNA can prevent oligonucleotide separation under non-denaturing conditions. Eluent D, Option 1 was used: 1.0 M NaCl.

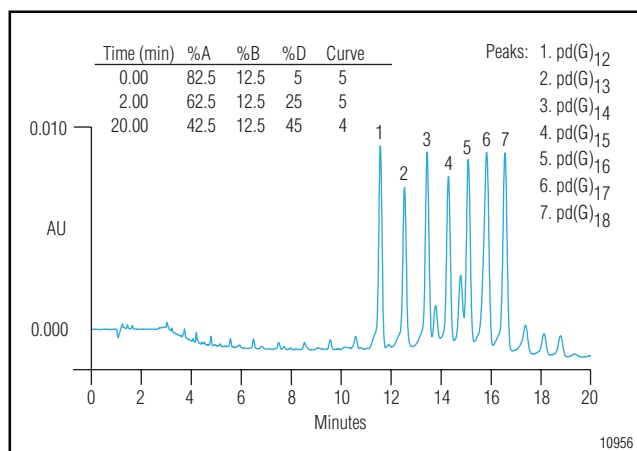


Figure 39. Resolution of pd(G)₁₂₋₁₈ homopolymers at pH 12.4 with a 500 to 900 mM NaCl gradient. The sample was injected at 100 mM of NaCl. At high pH, hydrogen bonding between poly-dG sequences is eliminated. Eluent D, Option 2 was used: 2.0 M NaCl.

The analytical method was modified in the following three ways to accomplish the transfer:

- 1) The flow rate of the analytical method was decreased from 1.5 mL/min to 0.33 mL/min. A 10.0-mL/min flow rate on the 22-mm diameter column is equivalent to a 0.33-mL/min flow rate on the 4-mm column because flow rate scales with the cross-sectional area of the column:

$$10.0 \text{ mL/min} \times \frac{\pi(2 \text{ mm})^2}{\pi(11 \text{ mm})^2} = 0.33 \text{ mL/min} \quad \{1\}$$

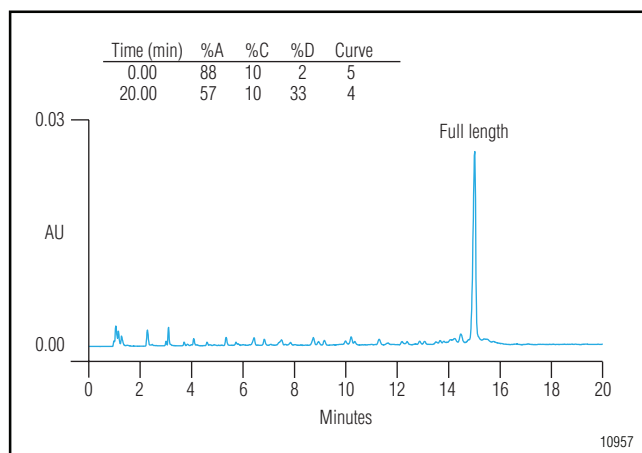


Figure 40a. Separation of the -20 primer from synthesis failure sequences. A 7.5 to 124 mM NaClO₄ gradient was used for analysis of the 1- μ g sample. Eluent D, Option 3 was used: 0.375 M NaClO₄.

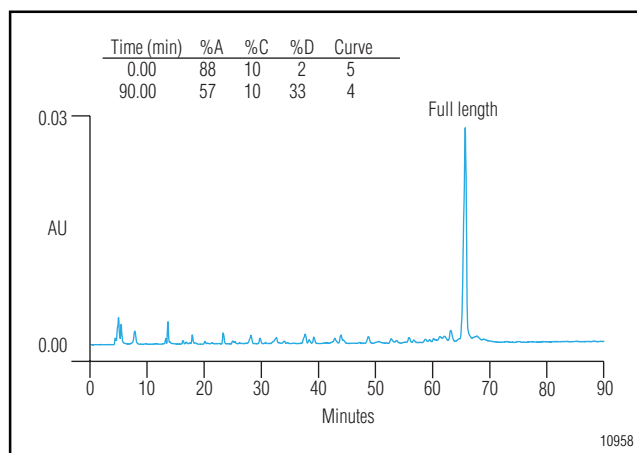


Figure 40b. Separation of 1 μ g of the -20 primer with a 90-minute NaClO₄ gradient delivered at 0.33 mL/min. Note the similarity between this separation and the 20-minute separation shown in Figure 40a. Eluent D, Option 3 was used: 0.375 M NaClO₄.

- 2) The duration of the analytical method was increased to 90 minutes. The constant column volume scaling rule dictates that the 20-minute gradient at 1.5 mL/min is equivalent to a 90.9-min gradient at 0.33 mL/min:

$$20 \text{ min} \times \frac{(1.5 \text{ mL/min})}{(0.33 \text{ mL/min})} = 90.9 \text{ min} \quad \{2\}$$

Separation of 1 μ g of DNA on the 4-mm diameter column with a 90-minute gradient at a flow rate of 0.33 mL/min is shown in Figure 7b. Nearly identical chromatograms were obtained in Figures 7a and 7b, which demonstrates the scaling fidelity of separations on the DNAPac PA-100.

- 3) The final step in the scale up was to transfer this 90-minute separation from the 4-mm diameter to the 22-mm diameter column. For this transfer, the gradient used for Figure 40b was delivered to the 22-mm diameter column at a flow rate of 10.0 mL/min. Figure 40c shows the separation of 30 μ g of DNA by this method. The chromatography scales in a highly predictable manner, so that analytical methods can be transferred easily to preparative applications.

Oligonucleotide Purification

Injections up to approximately 10 μ g of crude synthetic DNA yield sharp, symmetric peaks on the analytical 4-mm diameter column. On the 9-mm and 22-mm diameter columns, upper load limits for analytical chromatography are approximately 50 μ g and 300 μ g, respectively. Purification of larger samples is possible by

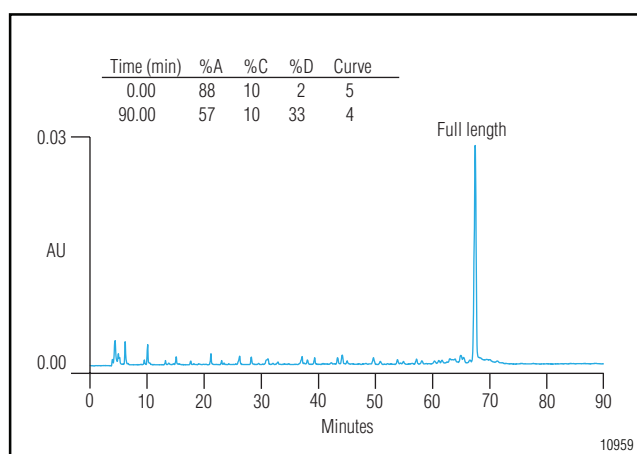


Figure 40c. Scale up of the -20 primer analytical separation to the 22-mm diameter DNAPac column. A 30- μ g sample was analyzed at a flow rate of 10.0 mL/min. Eluent D, Option 3 was used: 0.375 M NaClO₄.

overloading the analytical capacity of the DNAPac column. The full-length oligonucleotide can elute as a very broad peak under overload conditions. However, a highly pure full-length oligonucleotide typically is present throughout this peak. Figure 41a shows an example of overload purification of 150 μ g of the -20 primer on the 4-mm diameter analytical column. The separation was performed with a 90-minute NaClO₄ gradient at a flow rate of 0.33 mL/min, exactly as in the analytical separation in Figure 7b. The target oligonucleotide appears as an intense 3-minute peak beginning at about 61 minutes. Three 1-minute fractions were collected as shown in Figure 41b and

reanalyzed (see inset for fraction #2). The overall purity of the oligonucleotide collected from 61 to 64 minutes was > 98%. For another example of overload purification, a 1-mg purification of crude primer DNA to > 97% purity on the 4-mm diameter DNAPac has been demonstrated in reference 1.

CONCLUSION

By using a variety of eluent salts, highly efficient separations of synthetic DNA are possible on the DNAPac PA-100. The DNAPac pellicular anion-exchange resin provides higher resolution separations of single-stranded DNA than macroporous resins or reversed-phase columns. Analytical and semipreparative chromatography additionally can be performed under denaturing conditions for the separation of difficult samples. High-resolution analytical chromatography can be transferred directly to the 9-mm and 22-mm diameter columns for semipreparative applications.

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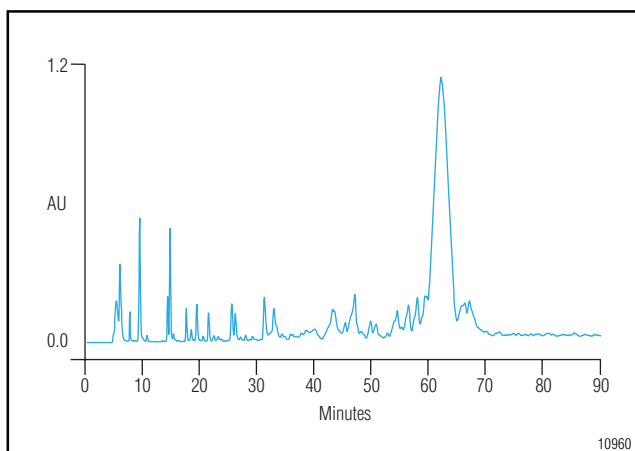


Figure 41a. Purification of 150 µg of the -20 primer on the 4-mm diameter column. The full-length primer elutes as a broad peak under these overload conditions. Chromatographic conditions are identical to those used for Figure 40b.

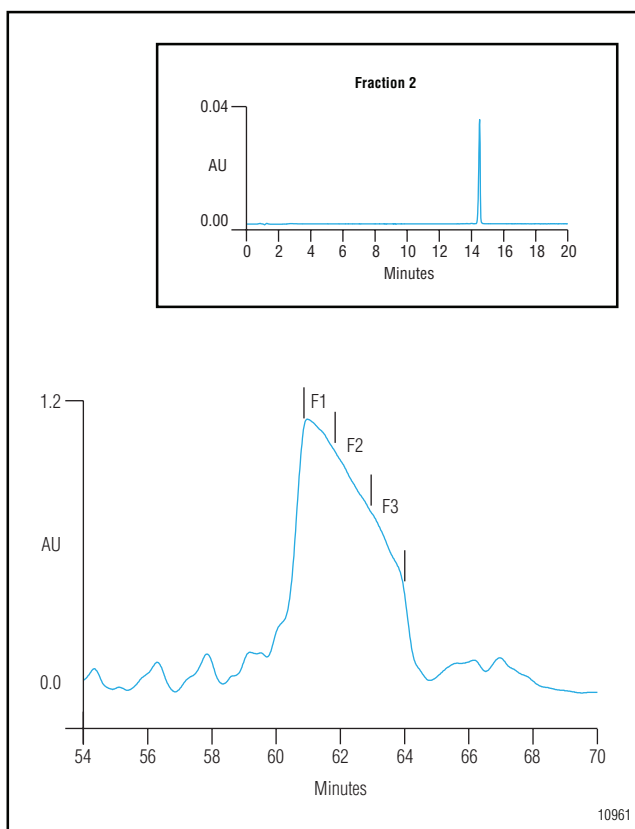


Figure 41b. Expanded view of full-length primer peak from Figure 41a. Fractions are indicated. Purity of pooled fractions was >98%. Inset: rechromatography of fraction 2.



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Nucleic acid therapeutics applications notebook

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- Capable of n-1, n+1 resolution for oligonucleotides
- Compatible with solvent, high pH, and high temperatures
- Provides easy scale up

Additional column technologies within the DNAPac family include the DNASwift SAX-1S Monolith anion-exchange column, which provides exceptionally high purity and yields of oligonucleotides. This column combines DNAPac and monolith technology to provide exceptionally high resolution and capacity for oligonucleotide purification, making it the ideal column for therapeutic and diagnostic research. All of these nucleic acid columns provide high resolution of full length from n-1, n+1, and other failure sequences that may not be possible with other columns on the market.



Column Selection Guide for Oligonucleotide Separations

Columns for Oligonucleotide Separations

Column	Target Applications	Base Matrix Material	Substrate Crosslinking	Latex Crosslinking	Capacity	Recommended Eluents	Recommended Flow Rate	Solvent Compatibility	Maximum Backpressure	pH Range
DNAPac PA100	High resolution separations of single and double stranded DNA or RNA oligonucleotides	13µm diameter nonporous substrate agglomerated with alkyl quaternary ammonium functionalized latex 100nm MicroBeads	55%	5%	40µeq	Hydroxide	1.5mL/min	0–100%	4000psi (28MPa)	2–12.5
DNAPac PA200	High resolution separations of single and double stranded DNA or RNA oligonucleotides	8µm diameter nonporous substrate agglomerated with alkyl quaternary ammonium functionalized latex 130nm MicroBeads	55%	5%	40µeq	Hydroxide, acetate/hydroxide	1.2mL/min	0–100%	4000psi (28MPa)	2–12.5
DNASwift	High resolution separations for purification of oligonucleotides	Monolith; polymethacrylate substrate agglomerated with quaternary amine functionalized latex	N/A	N/A	50mg, of a 20 mer oligonucleotide	NaClO ₄ and NaCl	0.5–2.5mL	Most Common Organic Solvents	1500psi	6.0–12.4



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Nucleic acid therapeutics applications notebook

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