DNAPac PA200
DNAPac PA200 RS

065036 Revision 03 • October 2015

Thermo SCIENTIFIC

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Product Manual

for

DNAPac PA200 Analytical Columns
(4 x 250mm, P/N 063000)
(2 x 250mm, P/N 063425)

DNAPac PA200 Guard Column
(4 x 50mm, P/N 062998)

DNAPac PA200 FAST 2mm Column
(2 x 50mm, P/N 063423)

DNAPac PA200 Semi-Prep Column
(9 x 250mm, P/N 063421)

DNAPac PA200 Semi-Prep FAST 9mm Column
(9 x 50mm, P/N 063419)

DNAPac PA200 PREP Column
(22 x 250mm, P/N 088781)

DNAPac PA200 Prep FAST 22mm Column
(22 x 50mm, P/N 088780)

Rapid Separation (RS) UHPLC Formats

DNAPac PA200 RS High Resolution Column
(4.6 x 250mm, P/N 082510)

DNAPac PA200 RS Analytical Column
(4.6 x 150mm, P/N 082509)

DNAPac PA200 RS Fast Analytical Column
(4.6 x 50mm, P/N 082508)

Revision History:
Revision 02, February, 2013, Rebranded for Thermo Scientific. Added information for new product formats.
Revision 03, October, 2015, Converted special part numbers to standard part numbers.

Safety and Special Notices

Make sure you follow the precautionary statements presented in this guide. The safety and other special notices appear in boxes.

Safety and special notices include the following:

 **SAFETY**	***Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.***
 **WARNING**	***Indicates a potentially hazardous situation which, if not avoided, could result in damage to equipment.***
 **CAUTION**	***Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. Also used to identify a situation or practice that may seriously damage the instrument, but will not cause injury.***
 **NOTE**	***Indicates information of general interest.***
IMPORTANT	***Highlights information necessary to prevent damage to software, loss of data, or invalid test results; or might contain information that is critical for optimal performance of the system.***
Tip	***Highlights helpful information that can make a task easier.***

Contents

1. Introduction

1.1 DNAPac PA200 AND PA200 RS

The Thermo Scientific™ DNAPac™ PA200 AND PA200 RS (Rapid Separation UHLC) anion exchange columns are designed specifically to provide fast, high-resolution separations of single stranded nucleic acids. The DNAPac PA200 and PA200 RS:

- Achieve N, N-1 resolution over a wide range of oligomer lengths;
- Perform separations under a variety of denaturing conditions.
  - High temperature, pH 8 or below.
  - High pH (12.4) at 30° or below.
  - Use of denaturing eluents (e.g., Guanidine·HCl).
- Utilize pH control to optimize selectivity for specific oligonucleotides.

The packing material is composed of 130 nm quaternary amine functionalized NanoBeads™ bound to a 4 (RS) or 8 µm solvent compatible, non porous substrate. Benefits of this design include rapid mass transport, higher loading capacity than conventional non-porous materials, and good durability. The result is a scalable column with resolution better than non-scalable anion exchange columns that use 2µm resins.

WARNING

DNAPac PA200 columns employ polymer end fittings. For use at pressure below 5000 psi, PEEK fitting and ferrules are effective. However, at pressure above 5000 psi, PEEK fittings are likely to slip, resulting in excess "dead volume" or leaks. The polymeric end fittings are NOT compatible with stainless steel (SST) fittings and ferrules. Without extreme care, use of SST ferrule will break PEEK end fitting components, and void the column warranty. Thermo Fisher recommends use of Viper® tubing for all UHPLC columns, and offers biocompatible Viper tubing in PEEK lined fused silica http://www.dionex.com/en-us/products/accessories/reagents-accessories/nano-viper-fingertight/lp-81337.html, and MP35N. The MP35N kit for UltiMate 3000 Bio RS systems (6841.2301) includes 3 MP35N Viper capillaries (one each of 1x 0.10 x 250 mm, 0.10 x 350 mm, and 0.18 x 550 mm (I.D. x L)).

		DNAPac PA200	DNAPac PA200 RS
Resin Characteristics:	Particle Size: Pore Size:	8 µm non porous	4 µm non porous
	Cross-linking:	55%	55%
	Ion exchange capacity:	~14 µeq/mL	~20 µeq/mL
Latex Characteristics:	Functional Group:	quaternary ammonium ion	quaternary ammonium ion
	Latex Diameter:	~130 nm	~130 nm
	Latex Cross-link:	5 %	5 %
Operational limits:	pH range:	4-10 unrestricted eluents 2.5-4 and 10-12.5, (Operation at these pH values require co-ion concentration to be more than equimolar with hydroxide at high pH or H+ at low pH)	
	Temperature:	5 - 85°C	
	Pressure:	4,000 psi	10,000 psi
	Organic Solvent Limit:	100% acetonitrile or methanol for cleaning	
	Typical eluents:	High purity water (18.2 megohm-cm), sodium chloride, sodium perchlorate, buffers, sodium acetate and sodium hydroxide	

1.2 Bio UHPLC System (without Columns)

Table 1 System Components Recommended for DNA Analysis

Basic Gradient System	Standard Gradient System
Solvent Rack	Solvent Rack with degas option
Gradient BioRS (UHPLC) Pump with Degas	Dual-Gradient Bio RS (UHPLC) Pump
Standard thermostatted column compartment	RSLC Thermostatted Column Compartment
Variable Wavelength Absorbance Detector (VWD)	Diode Array Absorbance Detector with pH and Conductivity Monitor, and/or Mass Spectrometer
Manual injection valve or Autosampler	Thermostatted Well Plate Sampler with Fraction collection

1.3 Short Column Use

A short (4.0- or 4.6- x50mm) column can be used for analytical purposes if resolution of the target oligonucleotides is sufficient. For conversion from a standard 50mm or 250mm long column, employ the same flow rate, and scale the gradient time proportional to the change in column length. For example to scale a 25 min gradient on a 250mm long column to a 50mm long column, reduce the gradient time to one fifth (50mm/250mm) of the original gradient time, or 5 minutes. . In the case of converting a 4.0mm ID column to a 4.6mm ID column, use the ratio of cross sectional area of the two columns. For example, to convert a 1.0 mL/min method on a 4.0mm ID column to a 4.6mm ID column multiply the flow-rate by the ratio of cross sectional areas (e.g., [1.0mL/min x (16.62/12.57)] = 1.32 mL/min).

1.4 DNAPAC PA200 and PA200 RS-4µm Anion Exchange Columns

	Part Number	Product Description
HPLC Formats	063000	DNAPac PA200, Analytical (4 x 250mm)
	062998	DNAPac PA200, Guard (4 x 50mm)
	063425	DNAPac PA200, Analytical 2.0x250mm
	063423	DNAPac PA200, Fast Analytical 2.0x50mm
	063421	DNAPac PA200, Semi-Prep 9.0x250mm
	063419	DNAPac PA200, Fast Semi-Prep Analytical 9.0x50mm
	088781	DNAPac PA200, Prep 21.2x250mm
	088780	DNAPac PA200, Fast Prep 21.2x50mm
UHPLC Formats	082508	DNAPac PA200 RS-4µM, Analytical (4.6 x 250mm)
	082509	DNAPac PA200 RS-4µM, Analytical (4.6 x 150mm)
	082510	DNAPac PA200 RS-4µM, Fast Analytical (4.6 x 50mm)

1.5 DNAPac PA200 Column Family

There are two varieties of columns in the DNAPac PA200 column family; standard HPLC and UHPLC compatible. Both are non-porous anion exchangers that provide high-resolution oligonucleotide separations. The choice of column depends upon the goal of the separation. The DNAPac PA200 consists of an 8µm substrate particle with ~130 nm functionalized NanoBeads. This column is available in a variety of formats (See PNs in Section 1.4) and should be used when higher capacity is required via scale-up to semi-preparative formats is anticipated. Both versions have been manufactured to provide greater stability to high pH, and to elevated temperature, although the combination of these conditions is NOT recommended.

The DNAPac PA200 RSconsists of a 4µm substrate particle with ~130 nm functionalized NanoBeads. These columns are UHPLC compatible and provide higher resolution than the DNAPac PA200 but can be operated at substantially higher pressures.

2.Operation and System Requirements

2.1 System Requirements

Oligonucleotide separations with the DNAPac PA200 and PA200 RS columns are optimized for use with INERT chromatography systems, such as the Thermo Scientific UltiMate BioRS UHPLC chromatograph. The key issue is that the sample and eluent flow paths from reservoir to detector are of materials that are not corroded by halide salts.

There are several configurations for the BioRS system. Each configuration offers multiple sampling options; however, consistently reproducible quantification and an absence of disturbing artifacts are achieved best using an autosampler and "full loop" injection mode. Optimal reproducibility of retention times can be achieved by regulating the temperature of the column using a column oven or thermal compartment. Control of this and many other chromatographs is best performed with the Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System.

Figure 1 Oligonucleotide System Configuration

Eluent Rack with Degasser
Gradient Pump
Absorbance Detector
Thermal Compartment
Autosampler

Chromeleon Chromatography Data System

2.2 System Operation Requirements

The oligonucleotide analysis systems should be configured with Thermo Scientific modules to provide the following attributes:

1. All components of the fluid path are chemically inert, to eliminate poisoning of the column.
2. Mobile phase components are kept under helium or nitrogen to minimize out-gassing (bubble formation) in the detector cell. On-line degassing of eluents may be provided with the eluent degas options in Thermo Scientific pump and eluent rack modules.
3. Accurate reproducible flow and gradient generation at settings between 0.02 and 5.0 mL/min.
4. Minimal contribution to the background signal by contaminants from the system and reagents.
5. Thermostatted column compartment for consistent temperature control of the chromatography columns.

Minimal system volumes (employ minimum tubing length and Viper® connecting fittings for best results). For 4.0-4.6mm ID columns, liquid line inside diameter (ID) should be ≤ .007" (0.18mm) before the injection valve and ≤ 0.004" (0.1mm) from the injection valve to the detector. For 2.0-2.1mm operation, liquid line ID should be ≤ .004" (0.1mm) before the injection valve, and ≤ 0.003" (0.075mm) from the injection valve to the detector. PEEK or MP35N tubing is preferred as they are considered inert.

2.3 DNAPAC PA200 Column Operational Parameters

Table 2 Column Operational Parameters

pH Range:	pH = 4-10 (unrestricted eluents)		
	pH = 2.5- 4, and 10-12.5: Operation at these pH values require co-ion concentration (e.g., Cl^- or ClO_4^- at high pH and Na^+ or NH_4^+ at low pH) to be at more than equimolar with hydroxide at high pH or more than equimolar with H^+ at low pH.		
Temperature Limit:	≤ 85°C		
Pressure Limit:	PA200 (8µm)	4,000 psi	
	PA200 RS-4µm	10,000 psi	
Organic Solvent Limit:	100% Acetonitrile, or methanol, if required for cleaning.		
Chaotrope Limit:	30% formamide, 6 M Urea. Guanidine HCl has been successfully used as a *denaturing eluent*.		
	Note: Use of these chaotropes will increase back pressure, and reduce column lifetime.		
Typical Eluents:	High purity water (18 megohm-cm), sodium chloride, sodium perchlorate, buffers, sodium acetate and sodium hydroxide.		
Detergent Compatibility: **CAUTION**	Nonionic, cationic or zwitterionic detergents. ***Do not use anionic detergents. Anionic detergents will bind irreversibly to the column.***		
Capacity: **DNAPac PA200 (11µeq./mL)**	2 x 50 mm:	2.2	µEq.
	2 x 250 mm:	11	µEq.
	4 x 50 mm:	6.9	µEq.
	4 x 250 mm:	34	µEq.
	9 x 25 0mm:	175	µEq.
	21.2 x 25 0mm:	970	µEq.
Capacity: **DNAPac PA200 RS** **(14 µEq/mL)**	2.1 x 150 mm:	7.3	µEq.
	2.1 x 250 mm:	12	µEq.
	4.6 x 50 mm:	12	µEq.
	4.6 x 150 mm:	35	µEq.
	4.6 x 250 mm:	58	µEq.

3. Purity Requirements for Chemicals

Reliable and reproducible results require eluents that are prepared consistently and are free from impurities.

3.1 Deionized Water

The de-ionized (DI) water, used to prepare eluents, should be Type I reagent grade water with a specific resistance of 18.2 megohm-cm. The water should be free from ionized impurities, organics, microorganisms, and particulate matter. Ultra Violet (UV) treatment in the water purification unit is recommended. Follow the manufacturer's instructions regarding the replacement of ion exchange and adsorbent cartridges. All filters used for water purification must be free from UV absorbing components. Contaminated water in eluents causes high background signals, gradient artifacts, and even sample degradation due to nucleases arising from microbial contamination.

3.2 Inorganic Chemicals

Inorganic chemicals of reagent grade or better should always be used to prepare ionic eluents. Whenever possible, inorganic chemicals that meet or surpass the latest American Chemical Society standard for purity should be used. These products will include detailed lot analyses on their labels.

3.3 Solvents

Solvents can be added to the ionic eluents used in DNAPac PA200 and PA200 RS-4µm columns to modify the ion exchange process. The solvents used must be free from ionic impurities; however, since most manufacturers of solvents do not test for ionic impurities, it is important that the highest grade of solvents available be used. Currently, several manufacturers are making "ultra high" purity solvents that are compatible with HPLC and spectrophotometric applications. These "ultra high" purity solvents will usually be of sufficient purity to ensure that your chromatography is not affected by ionic impurities in the solvent. We have obtained consistent results using *High Purity Solvents* manufactured by Burdick and Jackson or *Optima Solvents* by Fisher Scientific.

When using an ionic eluent with solvent, column generated back pressure will depend on the solvent used, the concentration of the solvent, the ionic strength of the eluent, the temperature and the flow rate applied. The column backpressure will also vary if the composition of the water-solvent mixture varies. The practical backpressure limit for the DNAPac PA200 is 4,000 psi (27.6 MPa), and for the PA200 RS-4µm is 10,000 psi (69.0 MPa). The DNAPac PA200 and PA200 RS-4µm can withstand common HPLC solvents in a concentration range of 0-100%. Solvents and water should be premixed in concentrations which allow proper mixing by the gradient pump and to minimize out-gassing. Ensure that all of the inorganic chemicals are soluble in the highest solvent concentration to be used during the analysis.

Solvent / water mixtures are usually specified with a volume to volume basis. If a procedure requires an eluent of 90% acetonitrile, prepare the eluent by adding 900 mL of acetonitrile to an eluent reservoir. Then add 100 mL of deionized water or eluent concentrate to the acetonitrile in the reservoir. Using this procedure to mix solvents with water will ensure that a consistent true volume/volume eluent is obtained. Where the specific gravity of each eluent components is known, gravimetric preparation of v/v mixtures will provide even better reproducibility. Premixing water with solvent will minimize the possibility of outgassing, which causes bubble formation between the column and the detector cell. If you choose to use pump proportioning to mix eluents containing solvents with those that do not, the eluent degas option for the pump is highly recommended. Alternatively, pre-degassing the eluents, and covering the eluent reservoir with Helium gas to limit gas dissolution into the eluents will help prevent out-gassing.

4. Quality Assurance

The chromatograms in this section were obtained using a calibrated system that meets the operational parameters listed in Section 2. Different systems will differ slightly in performance due to slight variations in column sets, system void volumes, liquid sweep-out times, different component volumes, and laboratory temperature.

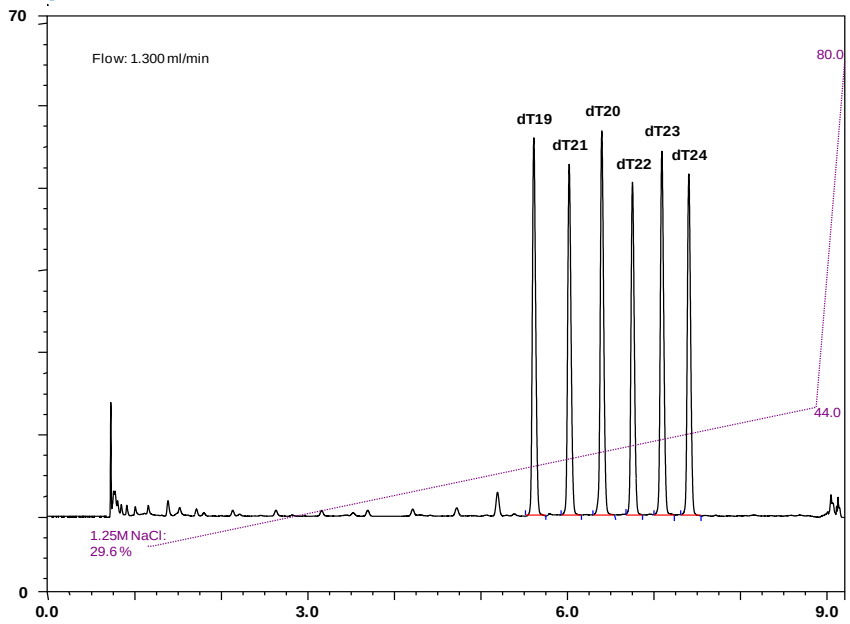
4.1 Certificate of Performance – Resin Batch Testing

Each batch of resin used for packing the DNAPac PA200 family columns is tested to ensure reliable performance and resolution. Separations of dT_{19-24} with DNAPac PA200 columns, packed with both production and test resins, are compared. This procedure ensures that resins with the highest quality are used, and produces consistent column performance.

Column:	DNAPac PA200 RS-4µm (4.6x150mm)
Flow rate:	1.20 mL/minute
Eluent 1:	20 mM Tris pH 8
Eluent 2:	20 mM Tris pH 8, 1.25M NaCl
Detection:	Absorbance (260 nm)
Injection:	10 µL
Storage Solution:	1.0M NaCl in 20mM Tris pH 8
Sample:	dT_{19-24} 5 µg each / mL
Gradient :	

Time	%1	%2	Comments
0.0	70.6	29.6	Equilibration solution
7.67	44.0	56.0	Gradient ramp 350 – 550 mM NaCl (4 column volumes)
10.11	20.0	80.0	Column wash start
11.11	20.0	80.0	Column Wash end
11.2	70.4	29.6	Re-Equilibration solution
14.12	70.4	29.6	End equilibration

Figure 1 Certificate of Performance



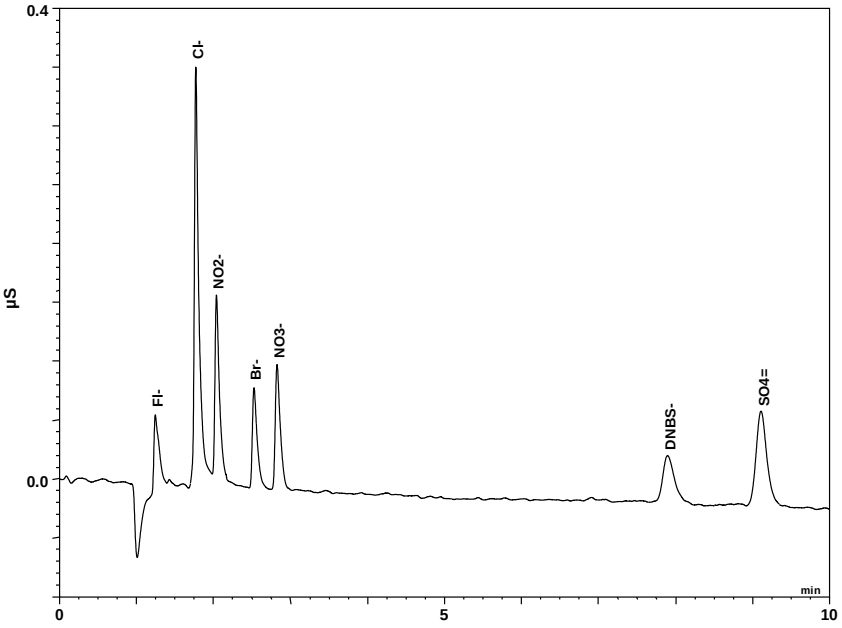
4.2 Production Test Chromatograms

To guarantee that all DNAPac PA200 analytical columns meet high quality and reproducible performance specification standards, all columns undergo the following production control test. Because gradient separation is not an accurate test for determining column capacity and packing quality, an isocratic separation of seven inorganic anions is employed to measure individual column performance utilizing a sodium carbonate, bicarbonate eluent.

The retention time of sulfate is used to measure the capacity of the column. Peak efficiency and peak symmetry of sulfate are used to measure the packing quality of the column. Retention times and resolution of chloride, nitrate and dinitrobenzene sulfonate are used to measure the overall selectivity of the column.

Column:	DNAPac PA200 RS 4.6x150mm
Eluent 1:	1.7mM $NaHCO_3$ 1.8 mM Na_2CO_3
Flow rate:	1.12 mL/minute
Detection:	Suppressed Conductivity using 4mm ASRS300, recycle mode, 12mA current
Sample:	7 Anion DNAPac standard
Injection Volume:	6 µL
Storage Solution:	1.0M NaCl in 20 mM Tris pH 8

Figure 2 Production Test Chromatogram



5. Method Development

5.1 Sample Cleanup

This table lists some sample preparation and matrix removal guidelines, for oligonucleotide samples, prior to injection onto the DNAPac PA200 column.

Table 3 Sample Preparation and Matrix Removal Guidelines

Matrix Interferent	Effect	Possible Removal
Halides	High concentrations of salts in the sample will affect the retention time of analytes	Dialysis, dilution, ethanol precipitation, cleanup with Reversed-phase cartridges.
Anionic Detergents	Will bind irreversibly to the column	Dialysis, dilution, solid phase extraction using the OnGuard RP Cartridge

5.2 Elution Order

The native elution order of oligonucleotide bases from the DNAPac PA200 phase using linear gradients of NaCl or $NaClO_4$ is as follows:

DNA > RNA (DNA is more retained than RNA)

5.2.1 Homopolymer Series:

pH 8: G > C > T > A
pH 12: G > T > C > A

NOTE

Poly-G will form extensive tetrad ladders at pH values below ~10.5. These are not readily disrupted, even at 85°C in salt solutions. Also, each G or T base (U in RNA) contributes a formal negative charge from their tautomeric oxygen at pH 12.

5.2.2 Heteropolymer Series:

Elution is influenced by the base composition (especially % G +T), terminal base sequence, pH, solvent concentration, and eluent salt. At pH 12 each T or G base contributes a negative charge from tautomeric oxygen atoms; as pH shifts from 10.5 – 12.5, hydrogen bond interactions decrease causing base-pair denaturation. This yields the expected chromatographic patterns.

5.3 Effect of Salt Type on Oligonucleotide Elution

5.3.1 Eluent Strength

Sodium perchlorate ($NaClO_4$) and sodium chloride (NaCl) are the two eluent salts used most commonly with DNAPac columns. Sodium perchlorate is a stronger eluent than sodium chloride, so a higher concentration of sodium chloride than of sodium perchlorate is required for any given separation. For example, typically ~0.2M $NaClO_4$ will elute a 75-base oligonucleotide at pH 8, while ~0.7M NaCl would be required.

5.3.2 Loading Capacity

Column loading capacity is the maximum amount of a given oligonucleotide that can be loaded onto the column before the peak shape starts to deteriorate. Column loading capacity is affected by the salt type. The stronger the salt, the lower the loading capacity. Thus, use of $NaClO_4$ would result in peak broadening at a lower sample loading concentration than a NaCl eluent.

5.4 Gradient slope

Phosphodiester oligonucleotides generally exhibit good peak shape when the gradient slope is ~15 mM/mL (NaCl) or ~5 mM/mL ($NaClO_4$). Higher values will generally result in shorter run times, but result in poorer resolution. Conversely lower values may produce improved resolution, but also require longer run times.

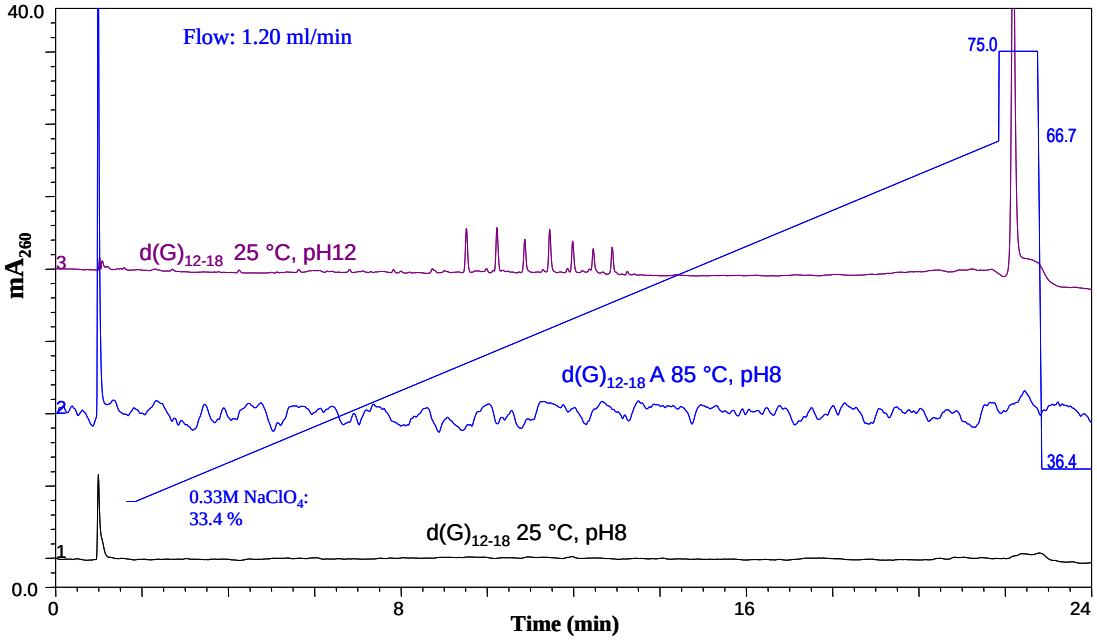
5.5 Effect of pH and Solvent on Oligonucleotide Chromatography

Use of elevated pH offers two advantages over chromatography at neutral pH. First, elevated pH allows control of hydrogen bonding interactions. At pH 11 and above, (pH 12.4 is the recommended upper limit for the DNAPac columns), Watson-Crick and poly-G hydrogen bonds break. Hence, at high pH chromatographic analysis of oligonucleotides with self-complementary sequences results in sharp, well-resolved peaks. Second, for each Thymine (T) and Guanine (G) residue, an increase in oligonucleotide charge is generated with rising pH values due to ionization of the tautomeric oxygen on these bases. Between pH 9 and 11, oxyanion formation on these bases increases retention of oligonucleotides in proportion to the number of T and G residues on the molecule. This offers opportunity to control of oligonucleotide selectivity with eluent pH.

5.5.1 Effect of pH on Hydrogen Bond Interactions

In the figure below, the chromatography of a PdG_{12-18} sample at 25°C and pH 8 (bottom trace) reveals the absence of identifiable peaks. Under these conditions, poly-G tracts **form** tetrad ladders with 8 hydrogen bonds between each set of bases. Chromatography at 85°C (pH 8, middle trace) dramatically increases the detector noise compared to pH8 and 25°C, but still no identifiable peaks are eluted. However, at 25°C and pH 12 each of the sample components are clearly eluted, and completely resolved from all the other components.

Figure 3 Controlling H-bond interactions in poly-G Tracts: Comparison of High pH and High Temperature



5.5.2 Effect of pH on Retention

The next figure illustrates the influence of pH on oligonucleotide retention. An oligonucleotide with base composition of $G_6C_5A_5T_9$ was eluted with a gradient of NaCl over 30 minutes at pH 6.5 to 12. Between pH 9 and 11, a substantial increase in retention is observed. As shown in Figure 2, this is due to the formation of an oxyanion on the tautomeric oxygen on each G and T (see chemical transition to the right).

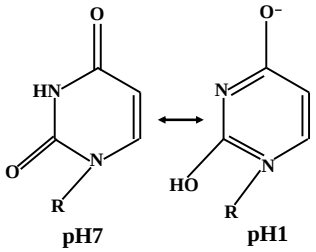
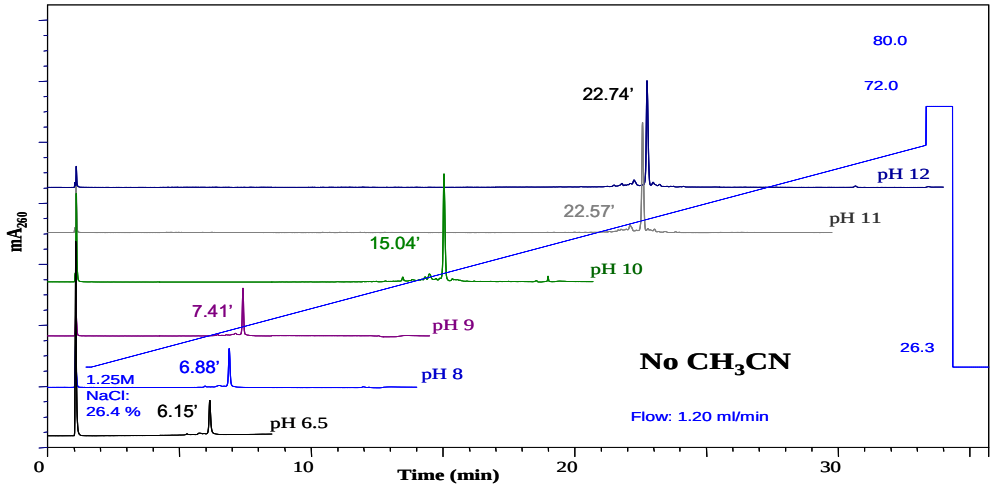


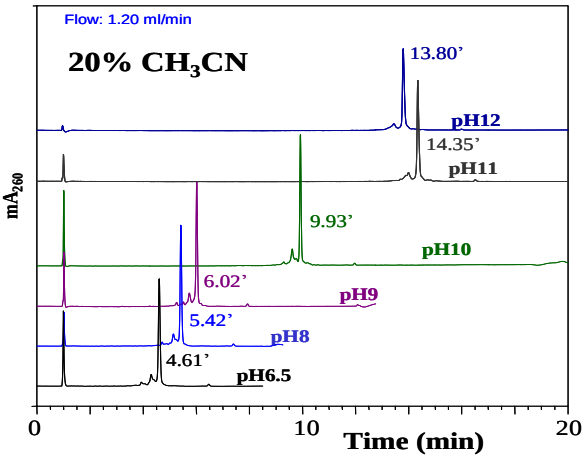
Figure 4 Effect of pH on Retention



5.5.3 Effect of Solvent on Retention

As shown in the next figure, addition of acetonitrile to the eluent will mask some of the native selectivity of the DNAPac PA200, and reduce retention of oligonucleotides. In some cases, resolution of closely spaced or co-eluting oligonucleotides may be assisted by adding solvent. These effects can be seen clearly by comparing Figure 4 with Figure 5. The scales have been aligned to make this comparison easier.

Figure 5 Effect of Solvent on Retention



5.5.4 Effect of pH on Selectivity

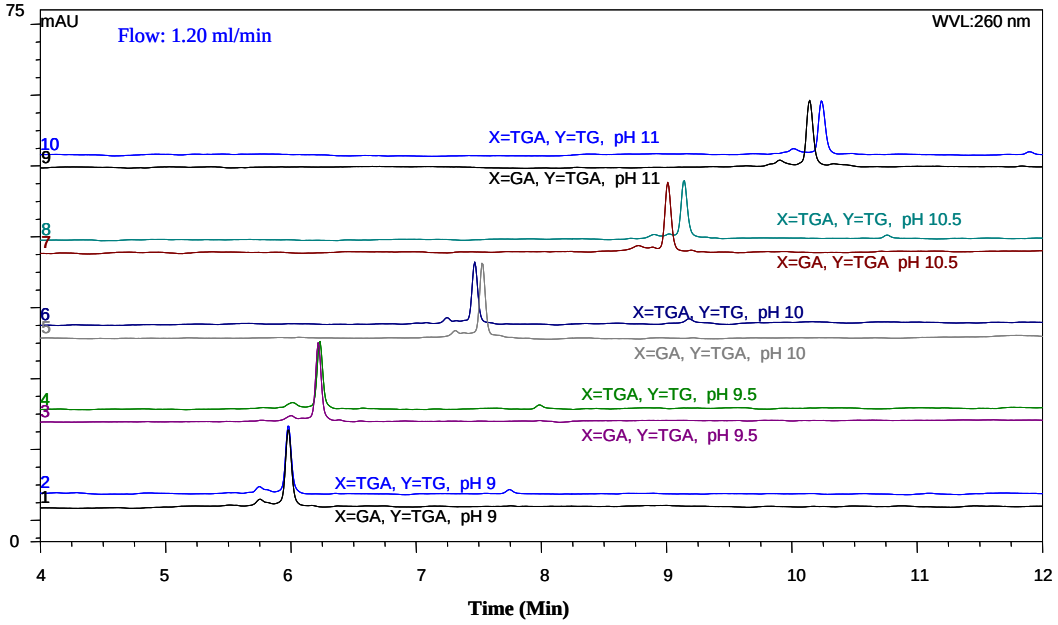
The figure below illustrates the influence of pH on oligonucleotide selectivity on the DNAPac PA200. Here the elution patterns of two 23-base oligonucleotides differing only in their 5’ and 3’ terminal bases are compared between pH 9 and pH 11.

The top trace for each pair of chromatograms has an additional T at the 5’ end of the molecule, and lacks the 3’ A. The oligonucleotide chromatographed in the bottom trace lacks the 5’ T, but has the 3’A. At pH 9 - 9.5 (bottom 2 pairs of traces) these oligos are unresolved.

At pH 10, the 5’ TG-3’G oligonucleotide is eluted earlier than the 5’ G-3’ GA oligonucleotide, and the two are only partially resolved. However, at pH 10.5 and 11 this elution order is reversed, due to the relative contributions of T and A to retention at these pH values.

The base composition of these 23-base oligos is 5’ X-$G_4C_4A_3T_7$-Y 3’, and optimal resolution is observed at pH 10.5.

Figure 6 Effect of pH on Selectivity



5.6 Effect of Temperature on Oligonucleotide Retention

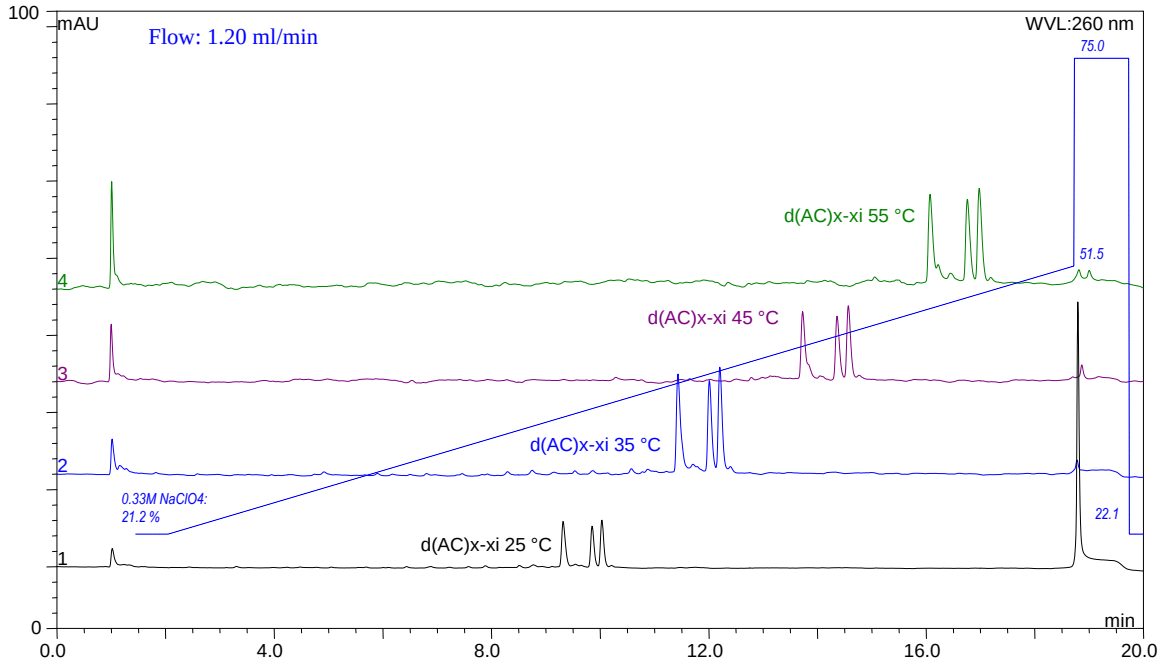
Elevated temperature is often used to limit or eliminate Watson-Crick, and poly-G hydrogen bonding within, and between oligonucleotides that have self-complementary sequences.

NOTE

Thermo Scientific does NOT recommend combining the use of elevated temperatures with high pH elution systems. Such conditions will accelerate degradation of the DNAPac PA200 stationary phase.

At relatively low pH, 9 or below, increased temperature may have mixed effects on nucleic acid retention. Nucleoside monophosphates and very short, 2-3 base, oligonucleotides may exhibit decreased retention times at elevated temperatures. Nucleoside triphosphates and oligonucleotides greater than a few bases long usually exhibit increased retention as the temperature increases. The figure below illustrates the influence of increased temperature at constant pH (8) on a DNAPac PA200. As the chromatographic temperature increases, retention of the oligonucleotides also increases, in this case by an average of ~ 2.7 min per 10°C.

Figure 7 Effect of Temperature on Retention of Oligonucleotides $d(AC)_{10-11}$: pH 8, 5 mM $NaClO_4$ / mL



5.7 Effect of Terminal Base on Selectivity

5.7.1 Selectivity in Sodium Chloride (NaCl) Gradients.

The influence of the 5' and 3' terminal base on retention is shown below for NaCl eluents on a DNAPac PA200. These examples consist of mixed-base oligodeoxynucleotide (ODN) 25 mers with identical sequence except for the 3' and 5' terminal bases. The samples are chromatographed at pH 9, 10 & 11.

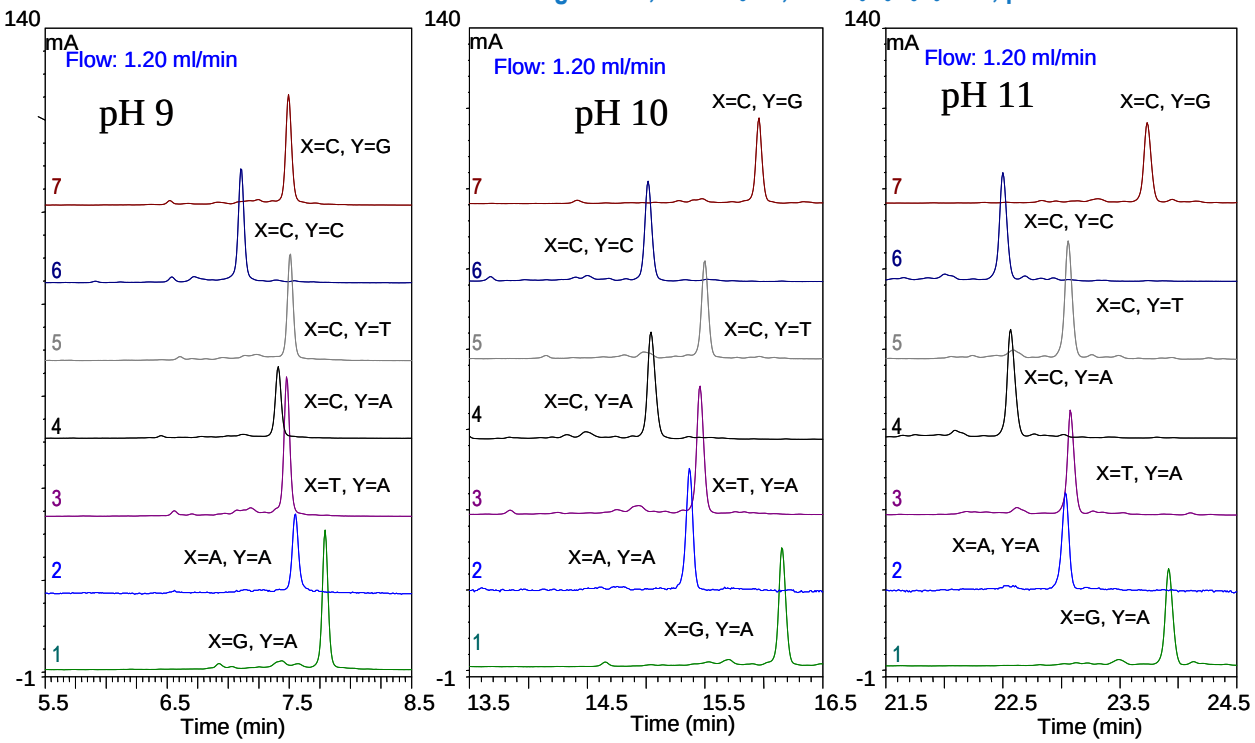
In each panel, the top 4 traces show elution of ODNs with identical 5' termini, and altered only at the 3' base. The bottom 4 traces show elution of ODNs with identical 3' termini, and altered only at the 5' base. The middle trace is common to both sets.

Using NaCl as the salt, all of the ODNs differing only at the 3' base are resolved at pH 9 or 10. The ODNs differing only at the 5' base are also at least partially resolved at pH 9 or 10. In each case, C contributes the least to retention at either the 5' or 3' end, and G contributes the most.

The relative contributions of A and T at either end are pH dependent. At pH 9, A contributes more than T but at the higher pH values it contributes less.

Addition of solvent (e.g., CH_3CN) tends to reduce retention, and minimize hydrophobic interactions. This may in some cases improve selectivity and resolution, (figure not shown).

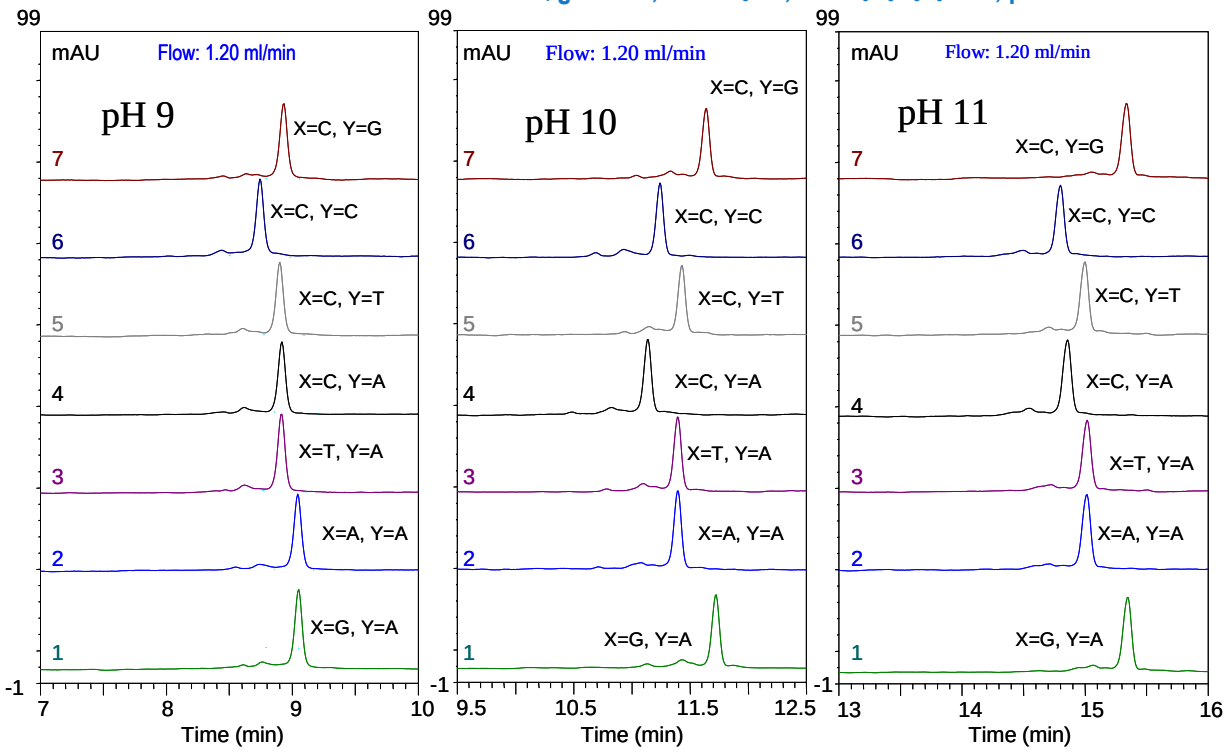
Figure 8 Effect of pH on Retention (by Terminal Base): 15 mM/mL NaCl gradient, 0% CH_3CN, 5'X-$G_6C_3A_5T_9$ -Y3', pH 9-11



5.7.2 Selectivity in Sodium Perchlorate ($NaClO_4$) Gradients

When NaCl eluent is replaced with $NaClO_4$ on a DNAPac PA200 (see below), the retention differences are less pronounced, and the effect of pH on retention is also reduced. However, all of the ODNs with 3' base substitutions are again resolved, and those with 5' substitutions are at least partially resolved, at pH 9 or 10. Addition of solvent to $NaClO_4$ eluent will reduce retention and minimize hydrophobic interactions, resulting in smaller selectivity changes due to terminal base differences at these pH values.

Figure 9 Effect of pH on Retention (by Terminal Base): 5 mM/mL $NaClO_4$ gradient, 0% CH_3CN, 5'X-$G_6C_3A_5T_9$ -Y3', pH 9-11



5.8 Application-Specific Mobile Phase Recommendations

From the observations detailed in the preceding sections, the following suggestions can be made:

5.8.1 Eluent Systems Minimizing Base-Specific Retention

For synthetic ONs where the goal is to evaluate purity, determine the coupling efficiency, or purify the full-length component from "n-1" and "n+1" impurities in the sample, eluent systems minimizing base-specific retention would produce the best results. Hence, eluents containing ~ 20% solvent, and $NaClO_4$ as eluent salt using pH 6-6.5, where pH-induced ionization is minimal, would be the logical choice.

5.8.2 Eluent Systems Maximizing Base-Specific Retention

When multiple possible ONs of similar length in the same solution must be resolved, eluents maximizing base-specific retention would provide the best probability of success. Examples of such samples include: Identification of all primers in a multiplex PCR amplification cocktail, QA / QC of multiple primers in amplification-based diagnostic kits, identification of the different components in "n-1" or "n+1" impurities when troubleshooting nucleic acid synthesis protocols, or identifying unknown contaminants in synthetic ONs possibly harboring residual protecting groups, linkage and/or stereoisomers, hydrophobic linkers, or fluorescent derivatives . For these applications, use of NaCl as eluent salt, without solvent, and at pH values between 8.5 and 11.5 would be more likely to produce the desired separations.

5.8.3 Exploitation of Interactions Between the stationary phase and ODN Derivatives

When hydrophobic ODN derivatives are present, their contribution to selectivity may be exploited. Examples of such derivatives include numerous fluorescent dyes, and hydrophobic tags such as C-6 linkers or the "Trityl" group used to protect the oligo from unwanted base additions at each elongation step during ODN synthesis. Since these hydrophobic moieties tend to result in peak tailing at ambient temperatures, elevation of the temperature (to 45-60 °C) will be helpful in restoring or maintaining the excellent peak shape on DNAPac columns. In these cases we do not recommend use of pH values above 8.5, unless absolutely necessary, as the combination of elevated pH and temperatures will promote column degradation.

6. Applications

6.1 Denaturing Conditions for Control of Secondary Structure

Single-stranded nucleic acids may contain inter-, and/or intra-, strand hydrogen bonding. Such interactions, if sufficiently strong, result in spurious peaks and a general inability to distinguish between the oligonucleotide components in the sample. There are three common methods to restrict these interactions; high temperature, addition of chaotropic agents such as urea or formamide, and use of high pH. Both the temperature used and the concentration of chaotropic agent used depend upon the extent of hydrogen bonding. For pH, values between pH 11-12.4 are effective at controlling both Watson-Crick, and non Watson-Crick oligonucleotide interactions.

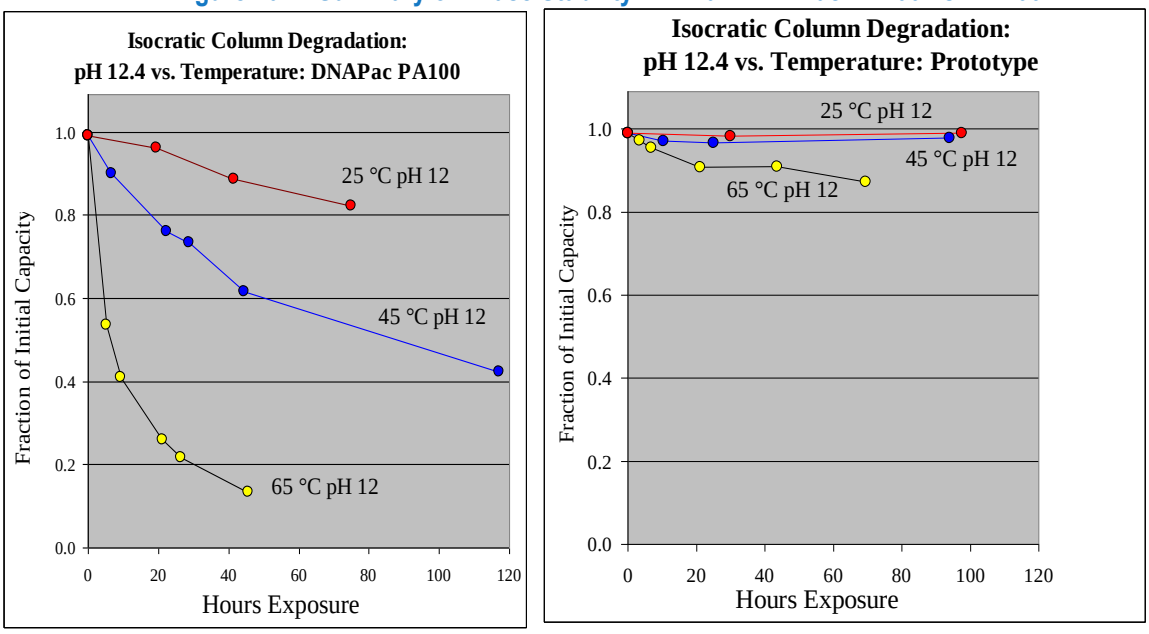
While the DNAPac PA200 can be used with any of the above methods for controlling secondary structure, there are certain considerations that should be taken into account when deciding which approach to use:

a) The use of a chaotropic agent, such as formamide or urea, tends to reduce the lifetime of the column.
b) The use of elevated temperature *and* elevated pH will accelerate DNAPac column degradation.
c) Elevating the temperature of the DNAPac PA200 will increase the retention time of the oligonucleotide. This means that a greater proportion of the eluent with higher salt will be required to elute the oligonucleotide, and thus the amount of salt eluting with it will be increased.
d) Increasing the pH of the eluent will also generally increase retention of oligonucleotides, but in a manner that allows control of oligonucleotide selectivity.

6.2 Effect of High Temperature and High pH on Column Lifetime

The combination of both high temperature and high pH reduces the useful life of the DNAPac columns, as shown in the figure below. **DNAPac PA100** is more susceptible to column degradation when the combination of high temperature and high pH are employed. However, even the DNAPac PA200 shows some phase degradation when operated at 65°C and pH 12, and this combination is not recommended. The PA200 8µm attributes apply to the PA200 RS-4µm column.

Figure 10 Summary of Phase Stability in Alkali: DNAPac PA100 vs. PA200



6.3 Phosphodiester Analysis

6.3.1 Sodium Perchlorate Eluent Systems

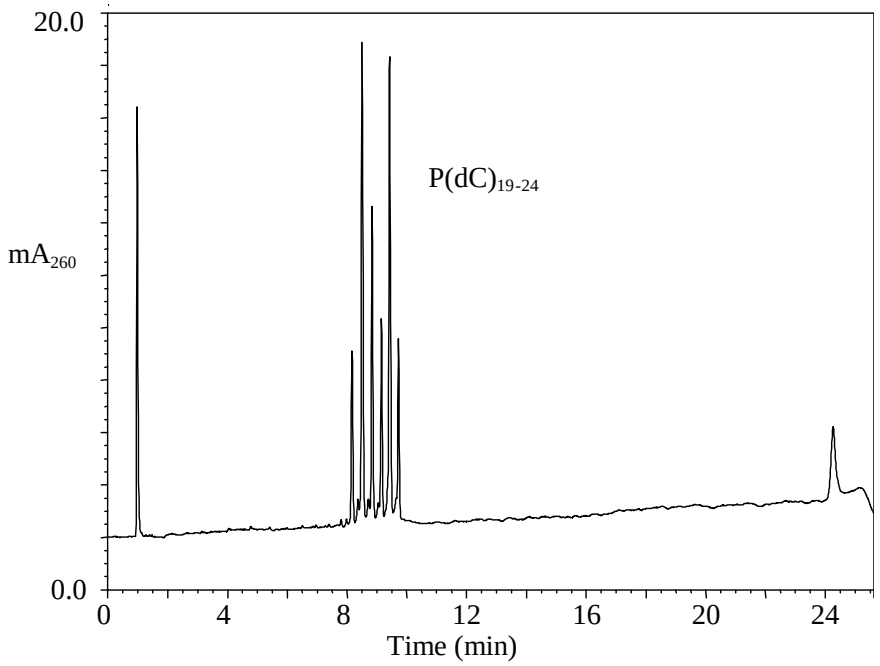
The following separation represents a good starting guideline for developing sodium perchlorate ($NaClO_4$) based methods for longer oligonucleotides.

In this example, phosphorylated deoxycytosine oligomers, 19 – 24 bases long, were injected onto a DNAPac PA200 column and eluted according to the conditions listed below. At pH 8, this gradient is effective for resolving the "full-length" oligonucleotide phosphodiesters, up to 25 bases long, from the n-1 components.

The same gradient of 5 mM $NaClO_4$ per mL of eluent can also be used to resolve full length from n-1 components between pH 8 and pH 12, using other buffers, e.g., AMPS, Na_3PO4, etc.

Conditions: 22 minute gradient from 70 to 202 mM $NaClO_4$ in 20 mM Tris buffered eluent at pH 8.
Flow rate: 1.2 mL/minute.
Injection volume: 6 µL.
Sample: 1 A_{260}/mL solution of phosphorylated deoxycytosine oligomers, Temperature: 25°C

Figure 11 N, N-1 Separation of Phosphorylated Deoxycytosine Oligomers

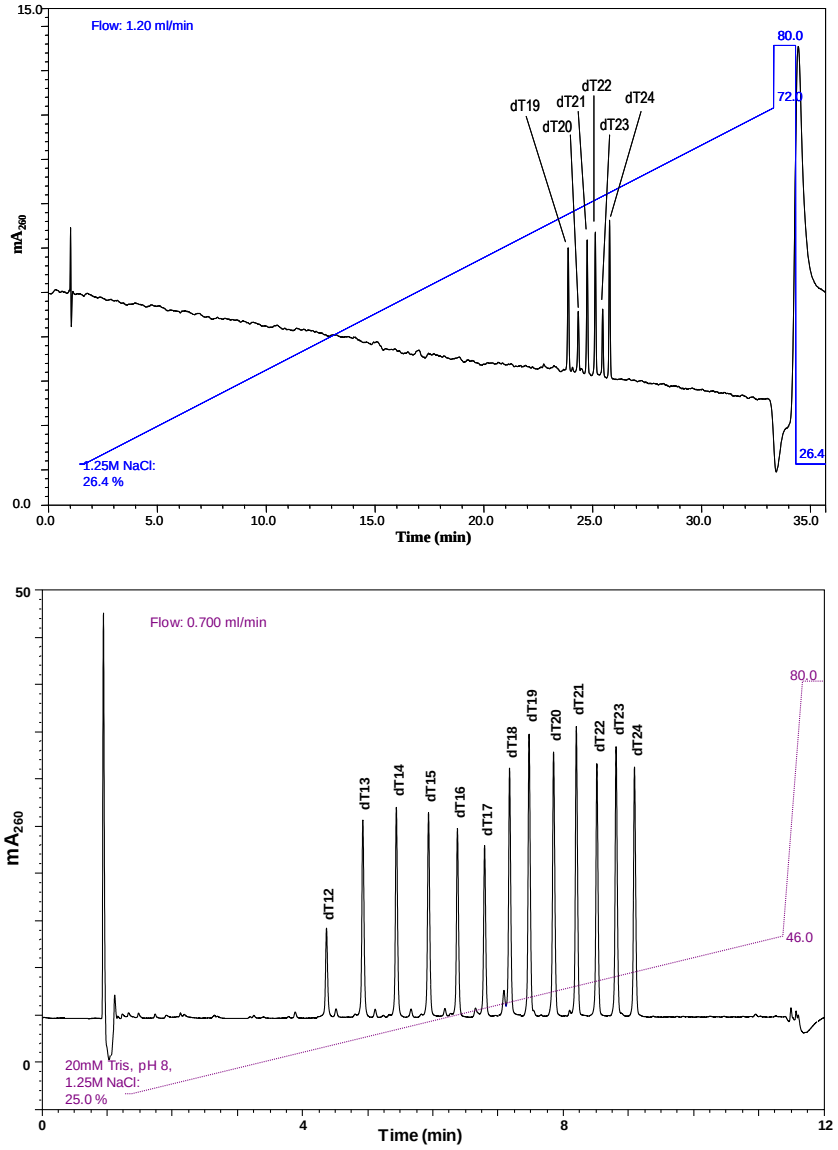


6.3.2 Sodium Chloride Eluent Systems

The following separation represents a good starting guideline for developing sodium chloride (NaCl) based methods for longer oligonucleotides.

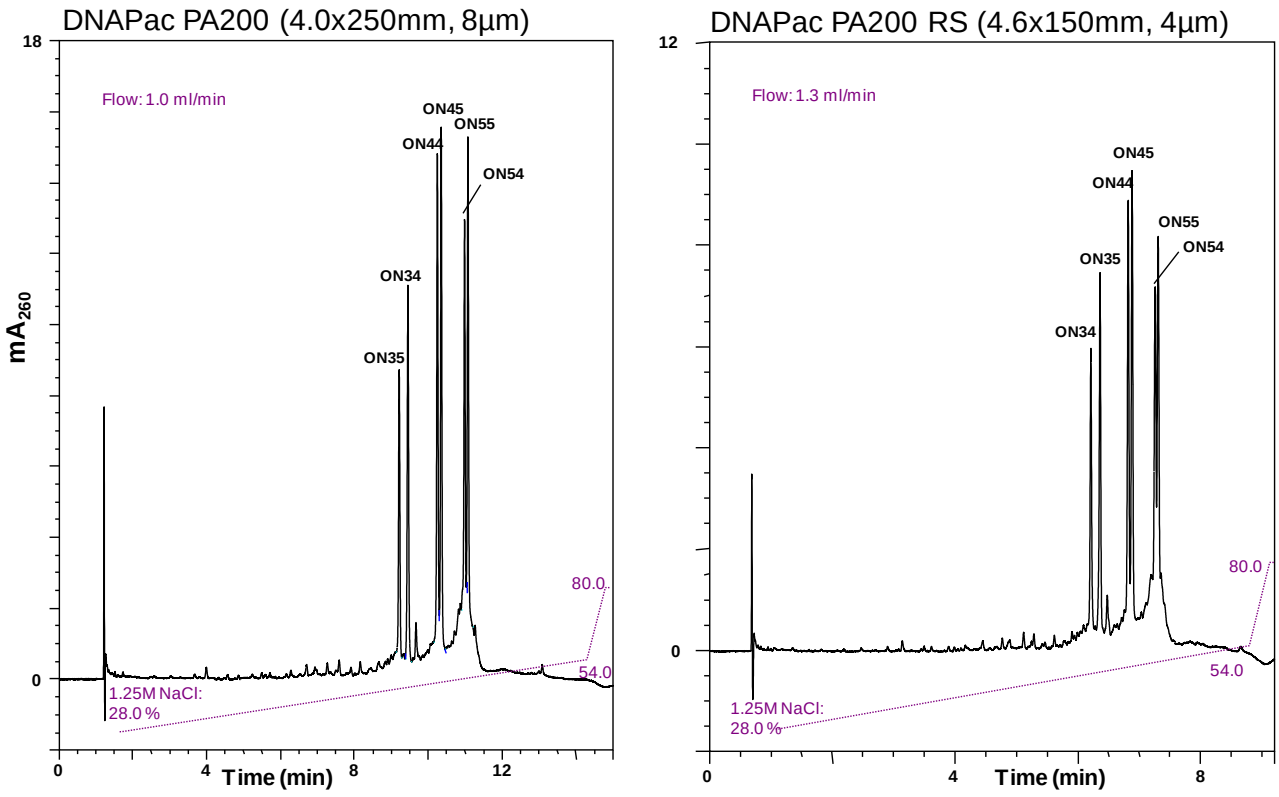
Here, deoxythymidine oligomers 19 – 24 bases long were injected onto a DNAPac PA200 column and eluted using a gradient of 330 – 900mM NaCl in 20m*M* NaOH (pH 12.4) at 1.2mL/min. At pH 12, this gradient resolves the "full-length" oligonucleotide phosphodiesters, up to 25 bases long, from the n-1 components. The same salt gradient (15 mM NaCl / mL eluent) also resolves full length from n-1 components between pH 8 and pH 12, using other buffers (e.g., Tris, AMPS, Na_3PO_4, etc). As shown in the second chromatogram below, faster and better resolution of this set and shorter oligonucleotides is accomplished with the 4.6x150mm DNAPac PA200 RS-4µm column using a shorter, more *optimized* NaCl elution gradient.

Figure 12 Resolution of full length oligodeoxythymidines on the DNAPac PA200 columns



In the example below, the 8µm 4x250mm and 4µ 4.6x150mm columns are compared using a linear gradient of 450-625mM NaCl in 4 column volumes at 1.12mm/min. In this example the 4µm column delivers essentially equivalent resolution for these six oligonucleotides in less than 70% of the time required for the longer 8µm column.

Figure 13 DNAPac PA200 and PA200RS-4µm resolution of oligonucleotides up to 55 bases long.

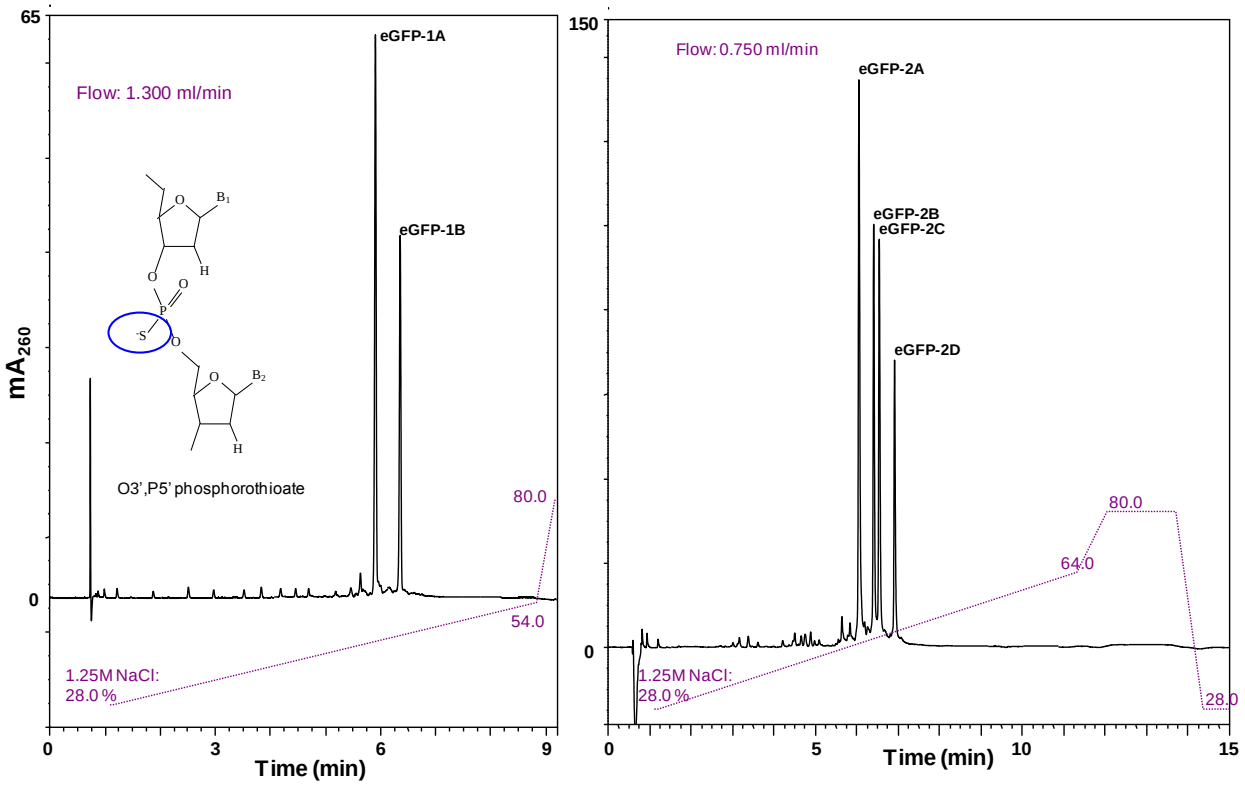


6.4 phosphorothioate oligonucleotide analysis

A common modification employed to confer nuclease resistance to therapeutic nucleic acids is the replacement of a non-bridging oxygen in the phosphodiester linkage with a sulfur atom. Since the replacement can occur in either of two positions, this introduces chirality to the phosphorus atom. Under appropriate conditions, the DNAPac PA200 columns resolve the resulting diastereoisomers.

If there is one phosphorothioate linkage, one pair of isomers are eluted, as in the left chromatogram below. When two such linkages are present two pairs of isomers may be resolved, as in the right chromatogram below. When all of the linkages harbor phosphorothioate linkages, a single, very broad peak is observed. Use of elevated temperatures (e.g., ≥ 60 °C) at neutral pH values, or elevated pH (≥ 10) at ~ 30 °C helps minimize resolution of the linkages, improving peak widths of completely phosphorothioated ONs.

Figure 14 Resolution of phosphorothioate oligonucleotide diastereoisomers.

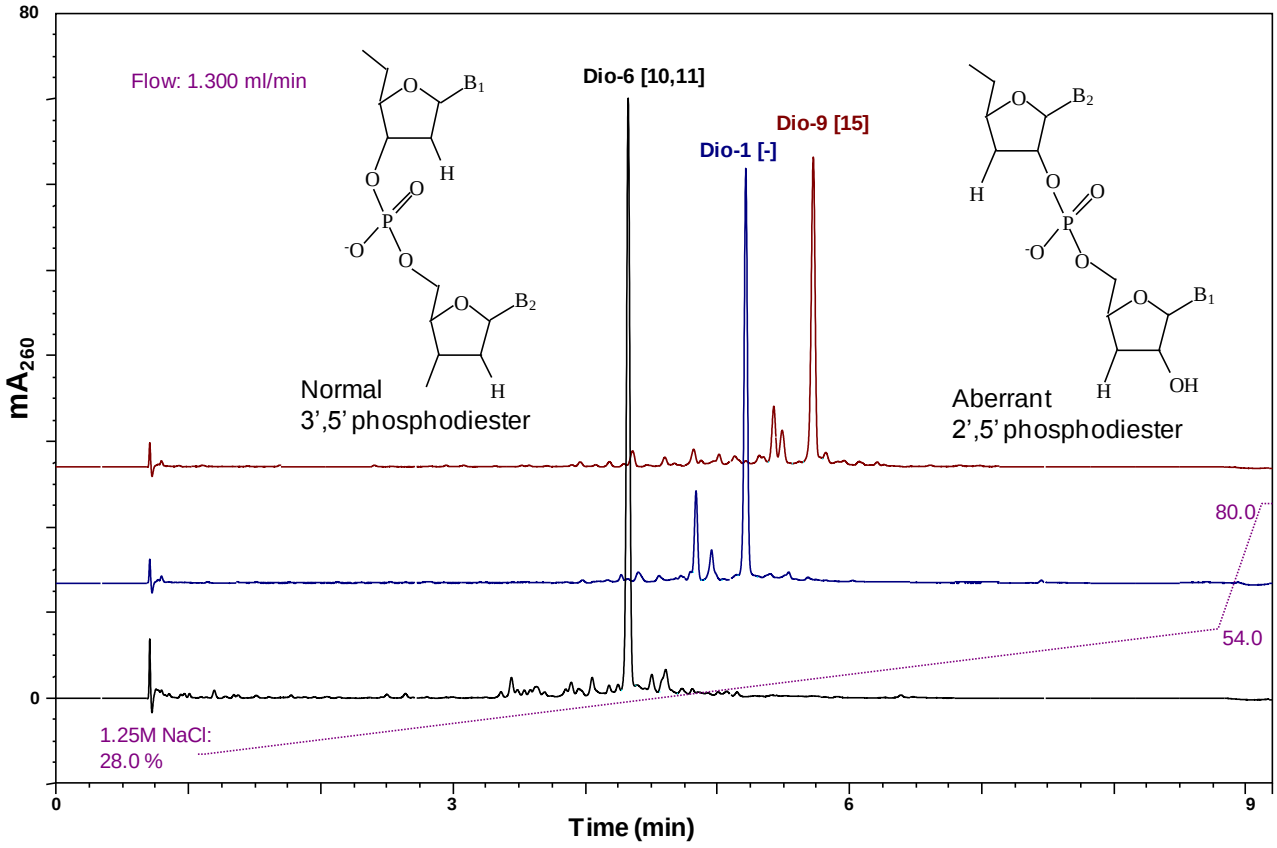


6.5 RNA

RNA in several forms is now used in a variety of therapeutic models. These include both aptamers, relatively long RNA sequences with specific secondary and tertiary structure, and siRNA (short interfering RNA) in double stranded form. Both of these RNA types require specific folding processes, usually accomplished by heat-denaturing and cooling. This process allows the 2´ and 3´ ribose carbons to exchange linkages, resulting in either strand scission or introduction of aberrant 2´,5´-linkages.

Strand scission results in shorter RNA forms that elute early in the chromatographic profile, but the 2´,5´ linkages are of identical length and sequence as the RNA from which they were derived. Identification of the RNA with aberrant linkage requires the ability to resolve the derivative from the source RNA. In the chromatograms below, Dio-1 is the original 21-base RNAwith all normal linkages, Dio-6 is the same RNA with 2´,5´-linkages at positions 10 and 11 in the sequence, and Dio-9 harbors the aberrant linkage at position 15. These are readily resolved in less than 6 minutes on the new DNAPac PA200 RS column, as shown in the figure below.

Figure 15 Resolution of identical-length, identical-sequence oligonucleotides differing by the presence or position (shown in [-] brackets) of aberrant 2´,5´-linkages.



7. DNAPac PA200 Resources

This list is not intended to be comprehensive. However, these resources can be used to determine initial conditions for separations on the DNAPac PA200.

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8. Troubleshooting Guide

The following instructions will help you to locate and eliminate problems traceable to hardware and chemistry issues. It also provides a selection of cleanup and reconditioning procedures that have been found effective by users.
Some problems may be related to parts of your experimental protocol (sample contamination, imprecision during sample transfer, problems during oligonucleotide deprotection, etc.)

8.1 Finding the Source of High System Back Pressure

a) A significant increase in system backpressure may be caused by a plugged inlet frit.
b) Before replacing the inlet bed support assembly of the column, make sure that the column is the cause of the excessive backpressure.
c) Check for pinched tubing or obstructed fittings from the pump outlet, throughout the eluent flow path to the detector cell outlet. To do this, disconnect the eluent line at the pump outlet and observe the backpressure at the usual flow rate. It should not exceed 100 psi. Continue adding components (injection valve, column, and detector) one by one while monitoring the system backpressure. Assuming aqueous eluents without high viscosity components:
   - Addition of the 4x50 mm DNAPac PA200 should add no more than 500 psi back pressure at 1 mL/min.
   - Addition of the 4x250 mm DNAPac PA200 should add no more than 2400 psi back pressure at 1 mL/min.
   - Addition of the 4.6x50 mm DNAPac PA200 RS should add no more than 2500 psi back pressure at 1 mL/min.
   - Addition of the 4.6x150 mm DNAPac PA200 RS should add no more than 5000 psi back pressure at 1 mL/min.
   - Addition of the 4.6x250 mm DNAPac PA200 RS should add no more than 7000 psi back pressure at 1 mL/min.

   No other component should add more than 500 psi (3.5 MPa) to the system back pressure.

NOTE

Inclusion of solvent in eluent will often increase column backpressure by 10-30%.

8.2 Backpressure on Column Has Increased

The DNAPac PA200 (4 x 250 mm) operates at $\leq$ 2400 psi at 1 mL/min in the absence of solvent. Similarly the DNAPac PA200 RS-4µm column operates at $\leq$ 7000 psi at 1 mL/min. If the high backpressure is due to the column, first try cleaning the column (See sction 8.11).

If the high backpressure persists, replace the column bed support (PA200 only, the DNAPac PA200 RS does not support user exchangeable bed supports). Note the color of the original bed support. If the inlet side of the used bed support is discolored (light brown to grey-black for the DNAPac PA200 frits) metal contamination is the primary suspect. Metals in non-inert systems are susceptible to corrosion by the halide eluent systems used on the DNAPac PA200 columns. The presence of metals on the frits suggests that considerable fouling by metals may have occurred. Metal fouling can cause increased backpressure, loss of efficiency, and changes in selectivity on DNAPac columns.

To eliminate metal fouling, Dionex strongly recommends the use of inert “PEEK-“, or Ti and “MP35N-” based pumps and tubing (e.g., Thermo Fisher/Dionex SP, DP or UltiMate BioRS chromatographs).

8.3 Decreasing Peak Retention Times

Eluent contaminants may be decreasing the capacity of the column. Use de-ionized water with a specific resistance of 18.2 megohm-cm. Sterilize by filtering through a 0.2-µm, or 0.45-µm filter. Also, check for changes in eluent concentration and pH. If column cleanup and use of clean fresh eluents fail to restore retention, the column cleanup process, later in this section, should be performed.

8.4 Decreasing Peak Efficiency and Resolution

a) If changes to the system plumbing have been made, check for excess lengths of tubing, larger than normal tubing diameter and leaks.
b) The column may be fouled. Perform a column cleanup procedure.
c) The DNAPac PA200 column may have been subjected to pressures above 4000 psi (34.5 MPa), causing irreversible bed compression. This requires column replacement. For the DNAPac PA200 RS-4µm column, the upper limit is 10,000 psi,

8.5 Poor Peak Efficiency and Resolution

Try alternate eluents such as $NaClO_4$. If this fails to improve matters, try operating under denaturing conditions (see Section 1.1). With the DNAPac PA200 RS at high gradient rates, the data acquisition rate may be too low. Increase the data acquisition rate to at least 25 Hz.

8.6 Unidentified Peaks Appear

a) Intra- or intermolecular oligonucleotide associations may cause unidentified peaks. Operate under denaturing conditions (see Section 1.1).
b) The sample may be degrading. When appropriate, check for nucleotide degradation and dephosphorylation.
c) The eluents may be contaminated. Prepare new eluents using fresh deionized filtered water.

8.7 Decreased Detection Sensitivity

Reduced detection sensitivity may be caused by sample degradation, column degradation leading to increased peak width (lower peak height), or limitations to light throughput in the absorbance detector (e.g., dirty or improperly installed flow cell, near failure of D_2 lamp).

8.8 Column Problems

CAUTION

When used, the guard column protects the main column from contamination introduced by use of a non-inert instrument. To prevent instrument induce contamination, disconnect the guard column only during troubleshooting as described later in this section.

To determine if the column set is the source of the high background replace the column with a section of PEEK or MP35N tubing. If the background reading returns to normal after the columns are replaced then the column was the cause.

8.9 Peak Efficiency and Resolution Are Decreasing

Peak deformations may sometimes be caused by sample matrix. Always have a spare guard available

a) Run a standard separation with the Guard column removed from the system. If the separation improves with the old Guard removed, install a new Guard column. It is quite common to replace the Guard column several times during the lifetime of the analytical column.
b) Verify that 0.01" ID or smaller tubing is installed for all connections between injector and detector (for Dionex tubing, black = 0.010", red = 0.005", and yellow = 0.003" ID. BioViper MP35N tubing is available in diameters between 0.004" (0.10mm) and 0.007" (0.18mm) ID.
c) Verify that the shortest possible length of tubing is installed between the column and detector.
d) Check for proper installation of ferrules on all PEEK tubing, starting with the injector outlet and all other connectors to the detector cell inlet.
e) Check temperature settings in your method and/or the actual temperature in your column oven.
f) The column may be overloaded. Try a smaller injection volume, or reduced sample concentration.
g) If all of the above does not lead to an improved separation, the resin bed of the main separator column has been damaged and column must be replaced.

8.10 System Problems

8.10.1 High Detection Background Caused by the System

a) Verify the problem is not related to the detector or column.
b) Prepare new eluents with freshly filtered deionized water.
c) Rinse all eluent lines with the new eluents (at least 40mL using the priming syringe)
d) If new eluent introduces high background without the column set installed, your deionized water source, or eluent components have become contaminated.

8.10.2 No Peaks, Poor Peak Area Reproducibility or Unexpectedly Small Peak Area.

a) Check the position and filling levels of sample vials in the autosampler.
b) Check injector needle-height setting.
c) Check each line of the schedule for proper injector parameters. Employ full loop methods if other injection modes (partial loop fill) do not provide acceptable reproducibility.
d) Service the injection valve (check for leaks, rotor fragments, or sediments inside the valve)
e) Check sampling needle for bits of vial septa clogging the flow path.

8.10.3 Incorrect or Variable Retention Times

a) Check your eluent preparation procedure for possible errors.
b) Prime the pump if necessary.
c) Measure the flow rate by weighing out the eluent collected during exactly five minutes of flow. Recalibrate the pump if necessary.
d) Set the eluent composition for 100% for each eluent and draw out at least 40mL of eluent from each of the lines to verify pump is primed.
e) Check and/or service the pump's proportioning valve. With the pumping turned off, the flow through the pump outlet tubing (disconnected from the injector) should be zero in all eluent positions. Check this separately for each eluent line.

8.11 Column Cleanup

If contamination of the column, the most common cause of column-contributed high background signal and loss of retention, is suspected the column can be cleaned with the following processes:

8.11.1 High Salt Wash to Remove Ionic Components

This cleanup procedure should be performed when component retention decreases. This solution will protonate most organic acids (1 mM HCl = pH 3). The $NaClO_4$ will still be largely ionized so it will elute the organic acids. The solvent will aid in removing components bound primarily by hydrophobic interactions

a) Rinse column with 15 volumes (~ 12-65 mL) of deionized water.
b) Wash column with 15 volumes (~ 12-65 mL) of a solution containing:
c) 0.4 M $NaClO_4$, 1 mM HCl and 30% CH_3CN
d) Rinse column with 15 volumes (~12-65 mL) of deionized water.
e) Regenerate column with 1 volume (~ 1-5 mL) of high concentration eluent used for column wash at the end of each chromatographic run.
f) Equilibrate column with 2 volumes (~ 2-9 mL) of eluent used at the start of each chromatographic run.

8.11.2 Organic Solvent Wash to Remove Non-Ionic Components

Use this approach when band broadening without loss of retention occurs, or when the background signal is elevated. This solution will elute neutral compounds bound primarily by hydrophobic interactions (e.g., non-ionic detergents).

a) Rinse column with 15 volumes (~12-65 mL) of deionized water.
b) Wash column with 15 volumes (~12-65 mL) of 100% CH_3CN.
c) Rinse column with 15 volumes (~12-65 mL) of deionized water.
d) Regenerate column with 1 volume (~ 1-5 mL) of high concentration eluent used for column wash at the end of each chromatographic run.
e) Equilibrate column with 2 volumes (~ 2-9 mL) of eluent used at the start of each chromatographic run.