

## PRODUCT MANUAL

**DNAPAC PA200 ANALYTICAL COLUMN  
(4 x 250mm, P/N 063000)**

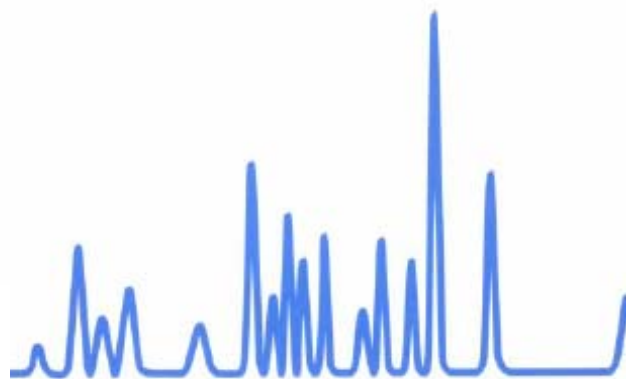
**DNAPAC PA200 GUARD COLUMN  
(4 x 50mm, P/N 062998)**

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## SECTION 1 - INTRODUCTION

### 1.1 DNAPac PA200

The DNAPac PA200 is a pellicular anion exchange column designed specifically to provide high-resolution separations of single stranded nucleic acids. The DNAPac PA200 provides n, n-1 resolution over a wide range of oligomer lengths and can perform separations under a variety of denaturing conditions:

- High temperature, pH 8 or below
- High pH (12.4) at 30° or below

Because of the unique pH stability of the packing material, elevated pH conditions can be used to optimize selectivity for specific oligonucleotides.

The packing material inside the DNAPac PA200 is composed of 130 nm quaternary amine functionalized MicroBeads™ bound to an 8 µm solvent compatible, non-porous substrate. The non-porous substrate design provides rapid mass transport resulting in narrow high efficiency peaks. The low column capacity, typical of non-porous packings, is avoided by agglomerating functionalized MicroBeads to the surface of the substrate particle, resulting in higher loading capacity than is possible with conventional non-porous materials, and good durability. This produces a column with oligonucleotide resolution superior to columns using 2 to 3 µm resins.

#### Resin Characteristics:

Particle Size:	8 µm
Pore Size:	non porous
Cross-linking:	55%
Ion exchange capacity:	~40 µeq/column

#### Latex Characteristics:

Functional Group:	quaternary ammonium ion
Latex Diameter:	~130 nm
Latex Cross-link:	5 %

#### Typical Operating Parameters:

pH range:	4-10 unrestricted eluents 2.5-4 and 10-12.5, (Operation at these pH values require co-ion concentration to be at least equimolar with hydroxide at high pH or H <sup>+</sup> at low pH)
Temperature:	≤85°C
Pressure:	3,000 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 BioLC System (without Columns)

**Table 1: System Components Recommended for DNA Analysis**

Basic Gradient System	Standard Gradient System
BioLC gradient pump, with degas	BioLC gradient pump (degas recommended)
Chromatography oven with injection valve and regulator assembly	Autosampler
	Column Oven
Absorbance detector (D <sub>2</sub> lamp for UV)	Absorbance detector (D <sub>2</sub> lamp for UV)
EO1 eluent organizers	EO1 Eluent organizers

### 1.3 Guard Column Use

A guard column is usually placed before the analytical column to prevent contaminants in the sample from eluting onto the analytical column. The addition of the guard column increases the net column capacity, which translates into an increase of about 20% in the retention times for isocratic runs. If a guard is added to a system running a gradient method that was initially developed for an analytical column alone, the analytes will elute slightly later and usually with slightly better resolution.

### 1.4 DNAPAC PA200 Anion Exchange Columns

Part Number	Product Description
063000	DNAPac PA200, Analytical (4 x 250mm)
062998	DNAPac PA200, Guard (4 x 50mm)

### 1.5 DNAPac Column Family

There are two varieties of columns in the DNAPac column family. Both columns are non-porous anion exchangers that provide high-resolution oligonucleotide separations. The choice of column depends upon the goal of the separation. The DNAPac PA100 consists of a 13  $\mu\text{m}$  substrate particle with 100 nm functionalized MicroBeads. This column is available in a variety of formats and should be used when higher capacity is required and if scale-up to semi-preparative scale separations is anticipated.

The DNAPac PA200 consists of an 8  $\mu\text{m}$  substrate particle with 130 nm functionalized MicroBeads. This column provides higher resolution than the DNAPac PA100. The DNAPac PA200 is operated at a lower flow rate than the DNAPac PA100, thus less eluent is consumed during a run. In addition, the DNAPac PA200 has been manufactured to provide greater stability to high pH at elevated temperature, although this combination is not recommended.

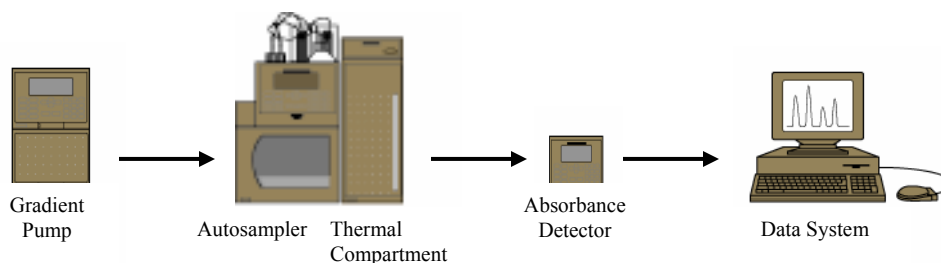
Assistance is available for any problem that may be encountered during the shipment or operation of DIONEX instrumentation and columns through the DIONEX North America Technical Call Center at 1-800-DIONEX-0 (1-800-346-6390) or through any of the DIONEX offices listed in "DIONEX Worldwide Offices."

## SECTION 2 - OPERATION AND SYSTEM REQUIREMENTS

### 2.1 System Requirements

Oligonucleotide separations with the DNAPac PA200 columns are optimized for use with NON-METALLIC systems, such as the Dionex BioLC. The key issue is that the eluent flow path from reservoir to detector is metal-free, because the salts used for oligonucleotide elution attack the metallic components of metallic pumps and tubing. The released metals will irreversibly foul the column.

Each of the possible configurations 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. Reproducibility of retention time results can be enhanced by regulating the temperature of the column using a column oven or thermal compartment.



**Figure 1 Oligonucleotide System Configuration**

### 2.2 System Operation Requirements

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

- a) All components of the fluid path are non-metallic, to eliminate column poisoning.
- b) 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 option on Dionex pump modules.
- c) Accurate reproducible flow and gradient generation at settings between 0.20 and 2.0 mL/min.
- d) Minimal contribution to the background signal by contaminants from the system and reagents.
- e) Thermostated column compartment for consistent temperature control of the guard and separation columns.
- f) Minimal system volumes (employ low volume unions and minimal tubing length).
  - 4-mm operation, liquid line inside diameter (I.D.) should be between .007” and 0.01”.
  - 2mm operation, liquid line inside diameter (I.D.) should be between .003” and 0.005”.In both operations, PEEK tubing is preferred as it does not contribute to metal leaching.

### 2.3 DNAPAC PA200 Column Operational Parameters

**TABLE 2 Column Operational Parameters**

<b>pH Range:</b>	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 <sup>-</sup> or ClO <sub>4</sub> <sup>-</sup> at high pH and Na <sup>+</sup> or NH <sub>4</sub> <sup>+</sup> at low pH) to be at least equimolar with hydroxide at high pH or H <sup>+</sup> at low pH.
<b>Temperature Limit:</b>	≤ 85°C
<b>Pressure Limit:</b>	4,000 psi
<b>Organic Solvent Limit:</b>	100% Acetonitrile, or methanol, if required for cleaning.
<b>Chaotrope Limit:</b>	30% formamide, 6 M Urea. <i>Note: Use of these chaotropes will increase back pressure, and reduce column lifetime.</i>
<b>Typical Eluents:</b>	High purity water (18 megohm-cm), sodium chloride, sodium perchlorate, buffers, sodium acetate and sodium hydroxide.
<b>Detergent Compatibility:</b>	Nonionic, cationic or zwitterionic detergents.

**CAUTION:** Do not use anionic detergents. Anionic detergents will bind irreversibly to the column.

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## SECTION 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 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 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 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. At Dionex, we have obtained consistent results using High Purity Solvents manufactured by *Burdick and Jackson* or *Optima Solvents by Fischer 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, 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). The DNAPac PA200 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. Premixing water with solvent will also minimize the possibility of out gassing which causes bubble formation in the detector cell. If you choose to mix eluents containing solvents with those that do not – the eluent degas option for the pump is highly recommended. As a second choice, pre-degassing the eluents and covering the eluent reservoir with Helium gas to limit gas dissolution into the eluents will help limit out-gassing.

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## SECTION 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 columns is tested to ensure reliable performance and resolution. Separations of dT<sub>19-24</sub> 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.

<b>Eluent 1:</b>	25 mM Tris pH 8			
<b>Eluent 2:</b>	25 mM Tris pH 8, 1.25 M NaCl			
<b>Flow rate:</b>	1.20 mL/minute			
<b>Detection:</b>	Absorbance (260 nm)			
<b>Injection:</b>	25 µL			
<b>Storage Solution:</b>	Eluent 2			
<b>Gradient:</b>	<u>Time</u>	<u>%1</u>	<u>%2</u>	<u>Comments</u>
	0.0	68	32	Equilibration solution
	0.1	68	32	Equilibration solution
	10.1	53.6	46.4	Gradient ramp 400 – 580 mM NaCl
	10.11	35	60	Column wash start
	11.11	35	60	Column Wash end
	11.2	68	32	Re-Equilibration solution
	14.12	68	32	End equilibration

Sample: dT<sub>19-24</sub> 1.5 µg each / mL

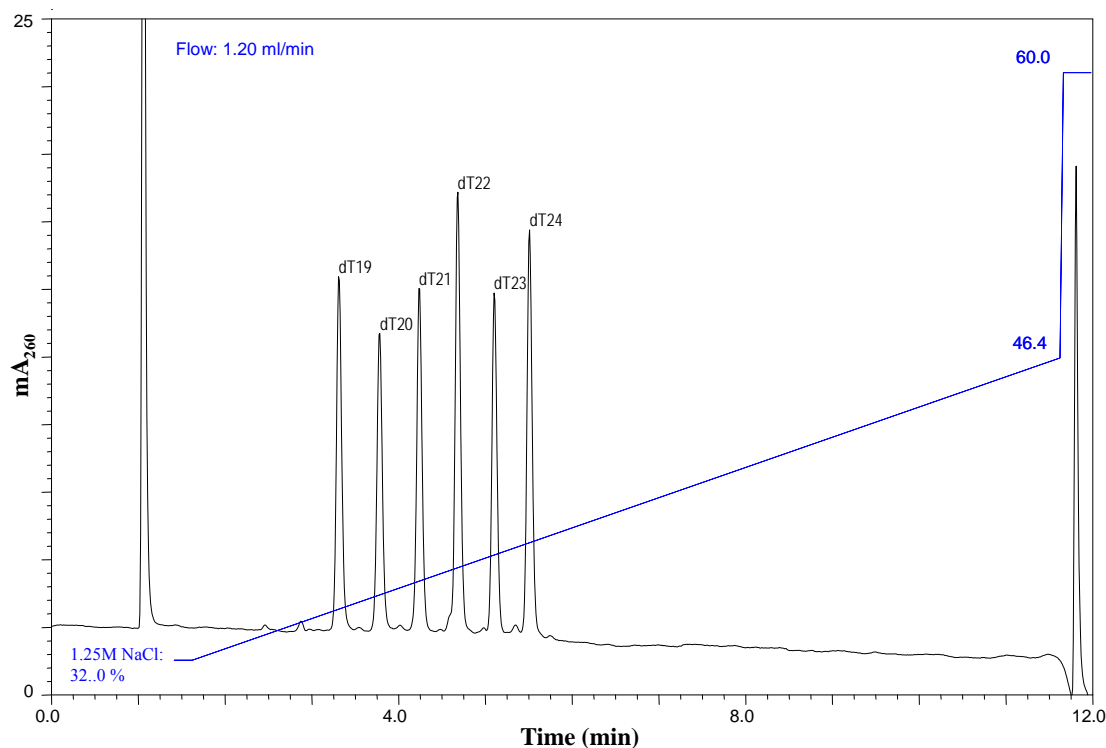


CHART 1 Certificate of Performance

## 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 phosphate are used to measure the overall selectivity of the column.

Eluent 1: 1.28mM NaHCO<sub>3</sub> 1.35 mM Na<sub>2</sub>CO<sub>3</sub>  
Flow rate: 1.00 mL/minute  
Detection: Conductivity (Suppressed)  
Sample: 7 Anion Standard with (1/10 dilution)  
Injection Volume: 25 µL

Storage Solution: 25 mM Tris pH 8, 1.25M NaCl

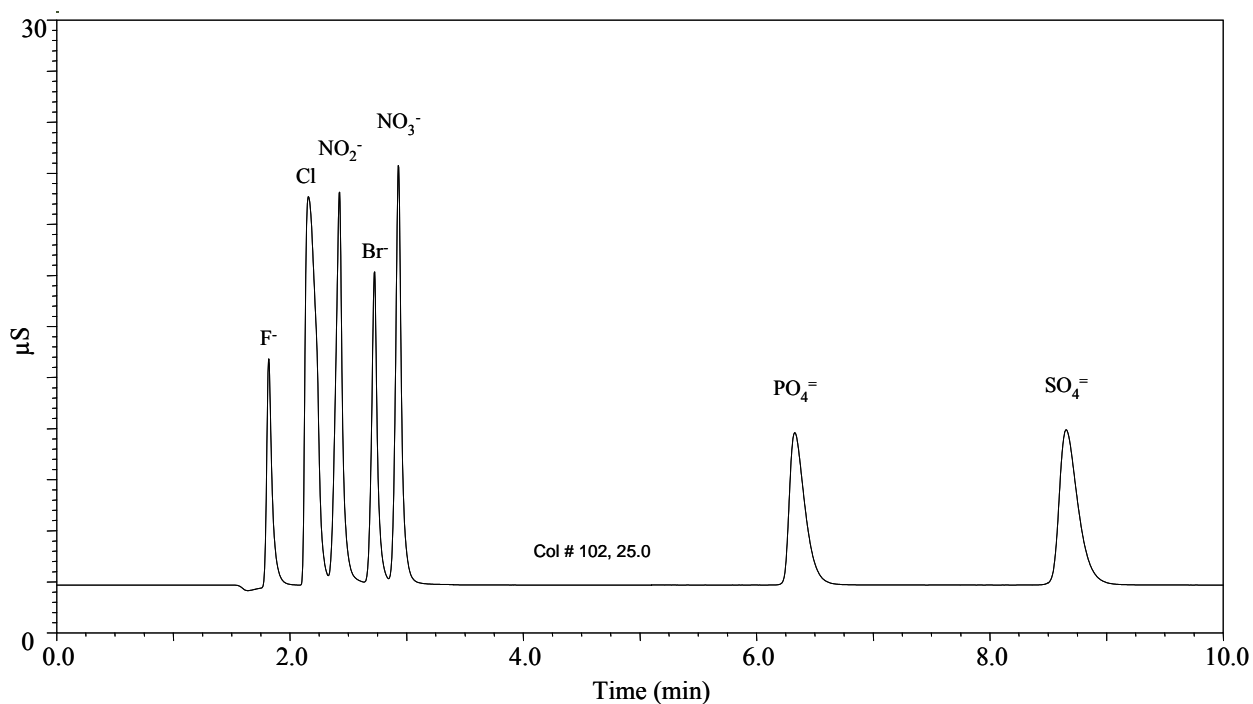


CHART 2 Production Test Chromatogram

## SECTION 5 - METHODS 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<sub>4</sub> is as follows:

**DNA > RNA** (DNA is more retained than RNA)

#### 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.*

#### 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 yielding the expected chromatographic patterns.

### 5.3 Effect of Salt Type on Oligonucleotide Elution

#### 5.3.1 Eluent Strength

Sodium perchlorate (NaClO<sub>4</sub>) 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<sub>4</sub> 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<sub>4</sub> would result in peak broadening at a lower sample loading concentration than a NaCl eluent.

## 5.4 Gradient slope

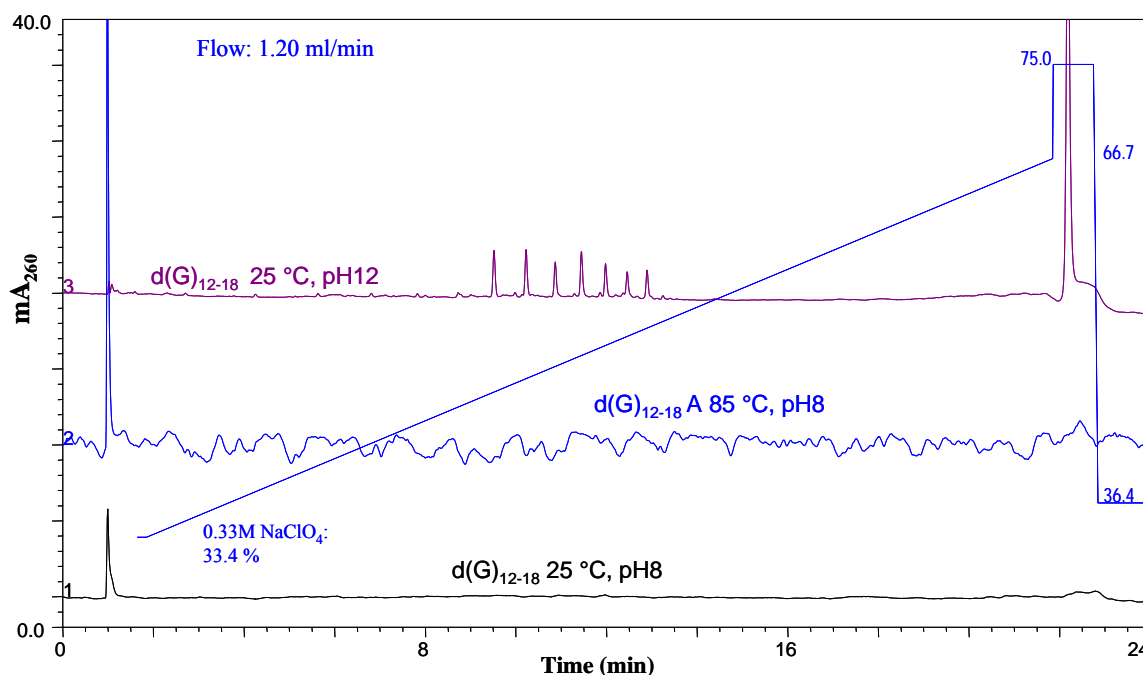
Phosphodiester oligonucleotides generally exhibit good peak shape when the gradient slope is  $\sim 15$  mM/mL (NaCl) or  $\sim 5$  mM/mL (NaClO<sub>4</sub>). Higher values will generally result in shorter run times, but result in poorer resolution. Conversely lower values may produce improved resolution, 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 chart below, the chromatography of a PdG<sub>12-18</sub> 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.



**CHART 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 chart illustrates the influence of pH on oligonucleotide retention. An oligonucleotide with base composition of G<sub>6</sub>C<sub>5</sub>A<sub>5</sub>T<sub>9</sub> 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.

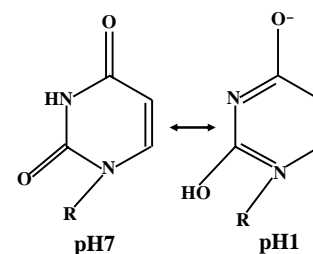


FIGURE 2

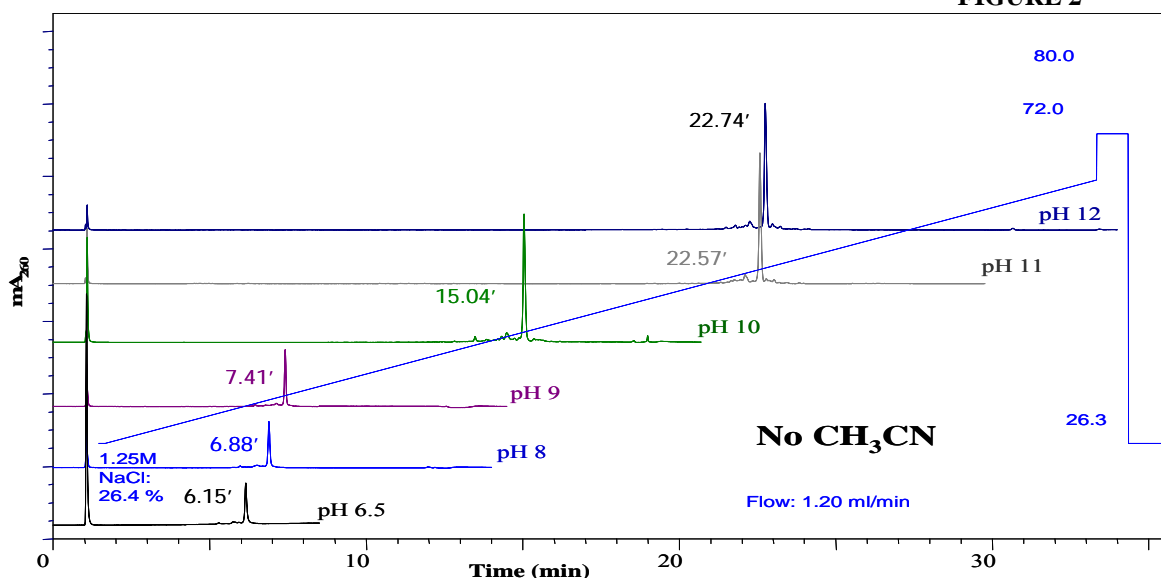


CHART 4 Effect of pH on Retention

### 5.5.3 Effect of Solvent on Retention

As shown in the next chart, 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 Chart 4 with Chart 5. The scales have been aligned to make this comparison easier.

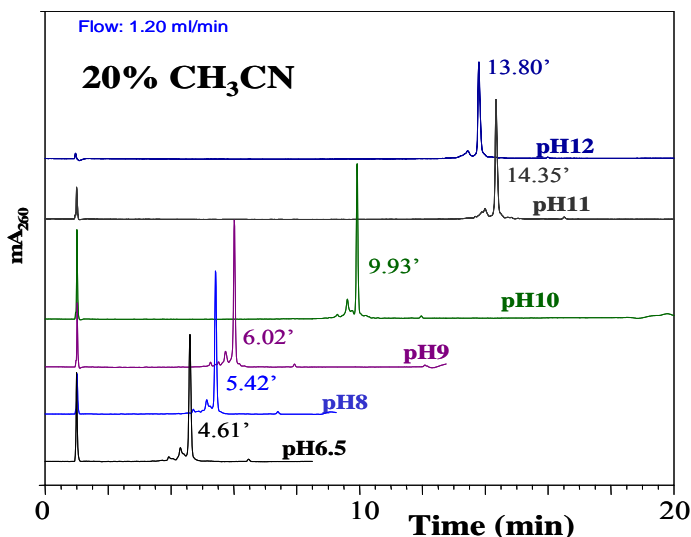


CHART 5 Effect of Solvent on Retention

#### 5.5.4 Effect of pH on Selectivity

The figure below illustrates the influence of pH on oligonucleotide selectivity. 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<sub>4</sub>C<sub>4</sub>A<sub>3</sub>T<sub>7</sub>-Y 3', and optimal resolution is observed at pH 10.5.

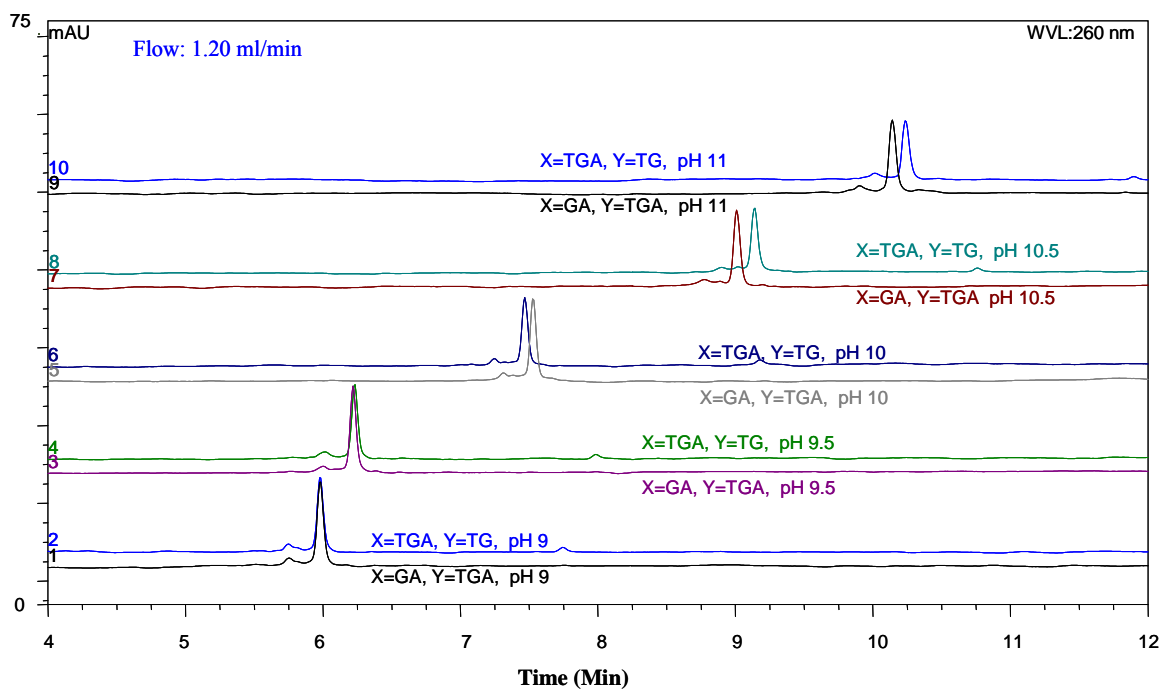


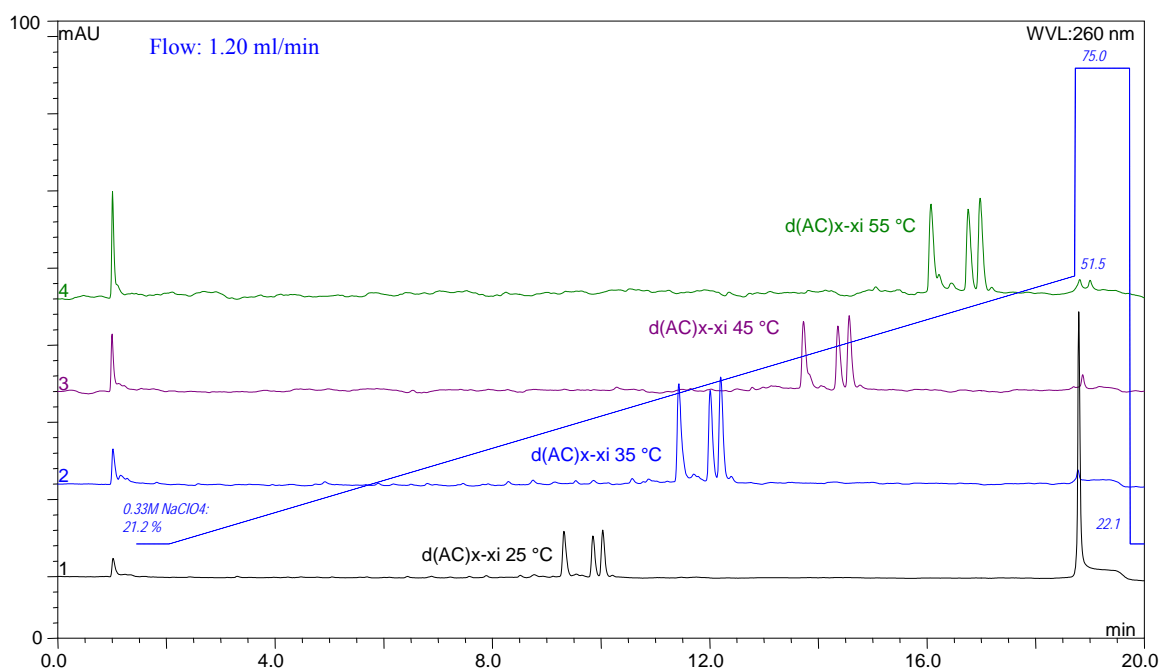
CHART 6 Effect of pH on Selectivity

## 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:** *Dionex 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 chart below illustrates the influence of increased temperature at constant pH (8). As the chromatographic temperature increases, retention of the oligonucleotides also increases, in this case by an average of ~ 2.7 min per 10°C.



**CHART 7 Effect of Temperature on Retention of Oligonucleotides**  
**d(AC)<sub>10-11</sub>: pH 8, 5 mM NaClO<sub>4</sub> / mL**

## 5.7 Effect of Terminal Base on Selectivity

### 5.7.1 Selectivity in Sodium Chloride (NaCl) Gradients

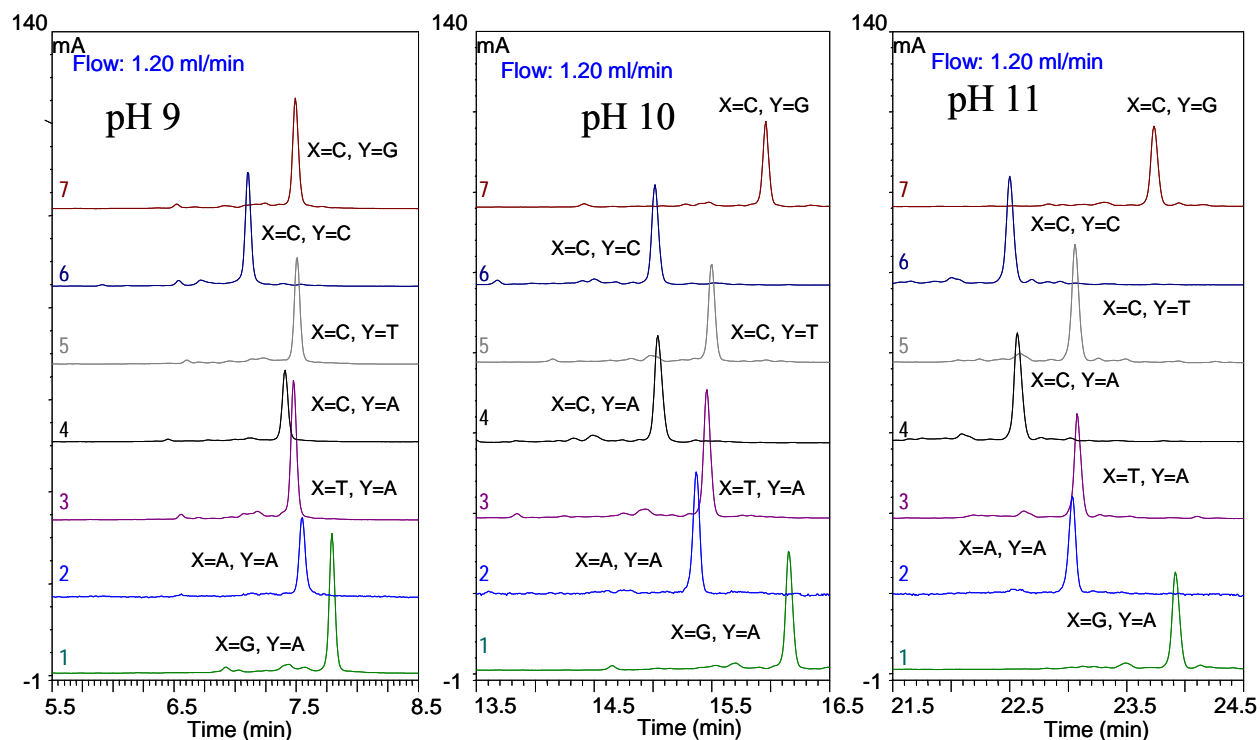
The influence of the 5' and 3' terminal base on retention is shown below for NaCl eluents. These examples consist of mixed-base oligonucleotide (ODN) 25 mers with identical sequence except for the 3' and 5' terminal bases. The samples are chromatographed at pH 9, 10 & 11.

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<sub>3</sub>CN) tends to reduce retention, and minimize hydrophobic interactions. This may in some cases improve selectivity and resolution, (chart not shown).

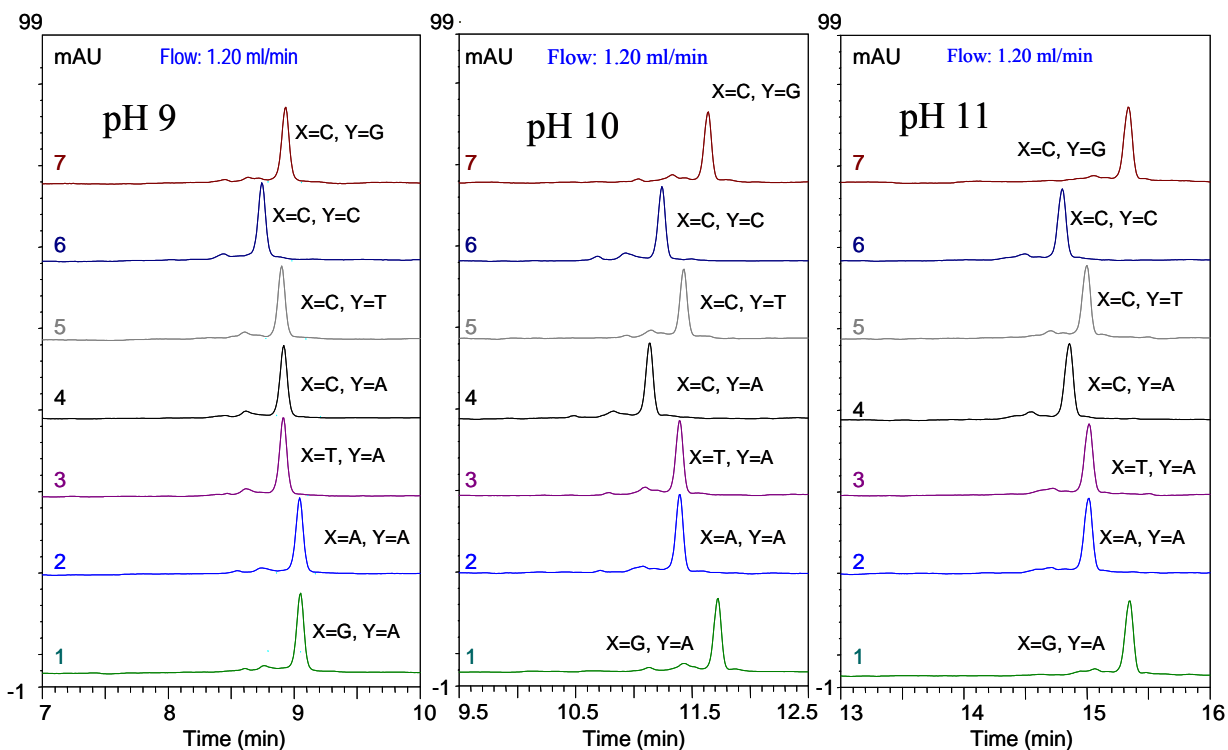


**CHART 8 Effect of pH on Retention (by Terminal Base):**  
15 mM/mL NaCl gradient, 0% CH<sub>3</sub>CN, 5'X-G<sub>6</sub>C<sub>3</sub>A<sub>5</sub>T<sub>9</sub>-Y3', pH 9-11



5.7.2 Selectivity in Sodium Perchlorate ( $\text{NaClO}_4$ ) Gradients

When  $\text{NaCl}$  eluent is replaced with  $\text{NaClO}_4$  (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  $\text{NaClO}_4$  eluent will reduce retention and minimize hydrophobic interactions, resulting in smaller selectivity changes due to terminal base differences at these pH values.



**CHART 9 Effect of pH on Retention (by Terminal Base):**  
5 mM/mL  $\text{NaClO}_4$  gradient, 0%  $\text{CH}_3\text{CN}$ , 5'X-G<sub>6</sub>C<sub>3</sub>A<sub>5</sub>T<sub>9</sub>-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 ODNs 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, solvent-containing NaClO<sub>4</sub> eluent at pH 9 or below, where pH-induced ionization is further minimized, would be the logical choice.

### 5.8.2 Eluent Systems Maximizing Base-Specific Retention

When multiple possible ODNs 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. For these applications, NaCl without solvent, at pH values between 9 and 11 would be more likely to produce the desired separations.

### 5.8.3 Exploitation of Interactions Between The stationary phase and ODN Derivatives

When hydrophobic interactions between some bases and the phase are suspected, interactions between the phase and additional ODN derivatives may also be exploited. Examples of such derivatives include numerous fluorescent dyes, and the “Trityl” group used to protect the oligo from unwanted base additions at each elongation step during ODN synthesis.

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## SECTION 6 - APPLICATIONS

### 6.1 Denaturing Conditions for Control of Secondary Structure

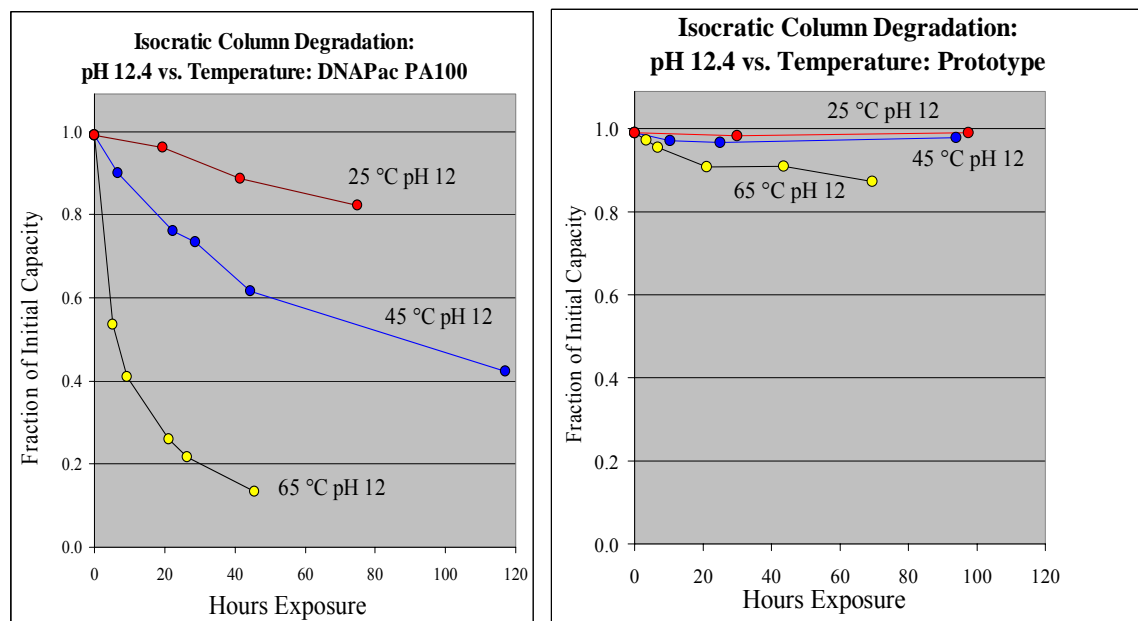
Single-stranded nucleic acids may contain inter-, and/or intra-, strand hydrogen bonding. Such interactions, if sufficiently strong, result in spurious peaks and a general inability to distinguish between the oligonucleotide components in the sample. There are three common methods to restrict these interactions; 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:

- The use of a chaotropic agent, such as formamide or urea, tends to reduce the lifetime of the column.
- The use of elevated temperature tends to reduce the lifetime of the column
- Elevating the temperature of the DNAPac PA200 will increase the retention time of the oligonucleotide. This means that more eluent will be required to elute the oligonucleotide, and thus the amount of salt eluting with it will be increased
- 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 Chart 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.



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

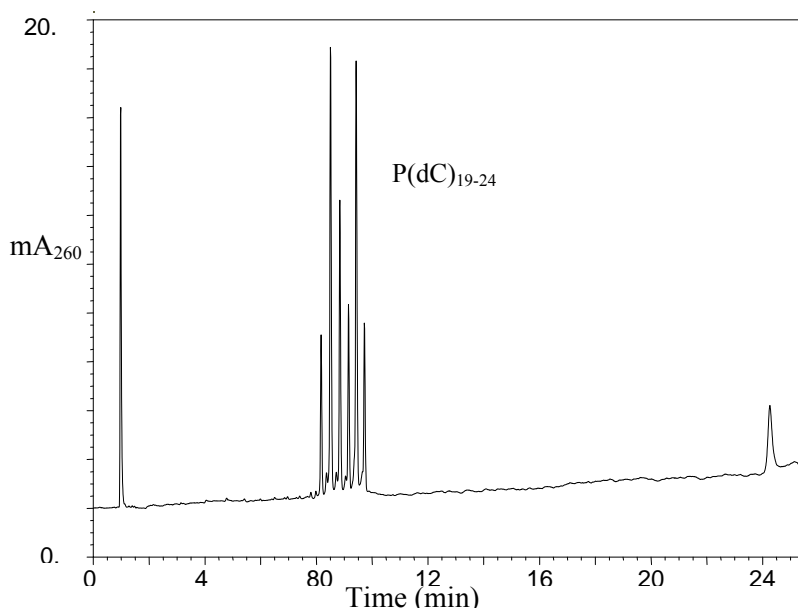
## 6.3 Phosphodiester Analysis

### 6.3.1 Sodium Perchlorate Eluent Systems

The following separation represents a good starting guideline for developing sodium perchlorate ( $\text{NaClO}_4$ ) 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 phosphodiester, up to 25 bases long, from the n-1 components.

The same gradient of 5 mM  $\text{NaClO}_4$  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,  $\text{Na}_3\text{PO}_4$ , etc.



**CHART 11 N, N-1 Separation of Phosphorylated Deoxycytosine Oligomers**

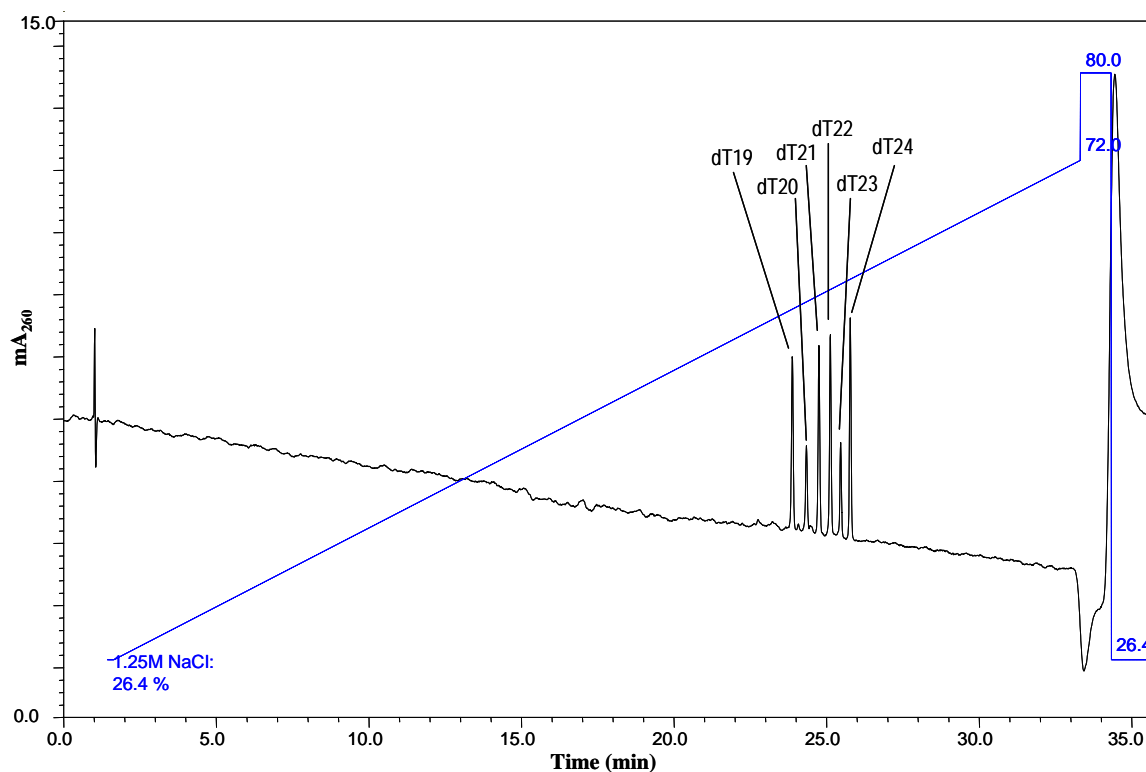
Conditions: 22 minute gradient from 70 to 202 mM  $\text{NaClO}_4$  in 20 mM Tris buffered eluent at pH 8.  
Flow rate: 1.2 mL/minute.  
Injection volume: 6  $\mu\text{L}$ .  
Sample: 1 A<sub>260</sub>/mL solution of phosphorylated deoxycytosine oligomers, Temperature: 25°C

### 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.

In this example, deoxythymidine oligomers, 19 – 24 bases long, were injected onto a DNAPac PA200 column and eluted according to the conditions listed below. At pH 12, this gradient is effective for resolving the “full-length” oligonucleotide phosphodiester, up to 25 bases long, from the n-1 components.

The same gradient of 15 mM NaCl per mL of eluent can also be used to resolve full length from n-1 components, between pH 8 and pH 12, using other buffered eluent systems, e.g., Tris, AMPS, Na<sub>3</sub>PO<sub>4</sub>, etc.



#### CHART 12 N, N-1 Separation of Deoxythymidine Oligomers

Conditions: 31.5 minute gradient of 330 to 900 mM NaCl in 20 mM NaOH (pH 12.4).  
Flow rate: 1.2 mL/minute.  
Injection volume: 4  $\mu$ L.  
Sample: 1 A<sub>260</sub>/mL solution of deoxythymidine oligomers, Temperature: 25°C.

## SECTION 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.

Posters: Available from Dionex Sales Support.

- a) Oligonucleotide purity analysis with an improved anion-exchange phase: Selectivity control using pH and eluent composition. Presented at the Tides 2004 conference in Las Vegas NV, April 25-29, 2004. Authors: J. R. Thayer, *Victor Barreto*, *Christopher Pohl*, Dionex Corporation, Sunnyvale, CA, USA
  - b) Control of oligonucleotide resolution on a new strong anion-exchange column: Optimization using pH and eluent composition. Presented at HPLC 2004 in Philadelphia PA, June 12-18, 2004. Authors: J. R. Thayer, *Victor Barreto*, *Christopher Pohl*, Dionex Corporation, Sunnyvale, CA, USA.
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## SECTION 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 50 psi. Continue adding components (injection valve, column, and detector) one by one while monitoring the system backpressure.
  - Addition of the 4 x 250 mm DNAPac PA200 should add no more than 2400 psi back pressure at 1 mL/min.
  - Addition of the 4 x 50 mm DNAPac PA200 should add no more than 1000 psi back pressure at 1 mL/min.

No other component should add more than 100 psi (0.7 MPa) to the system back pressure.

**NOTE:** *Inclusion of solvent to 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. If the high backpressure is due to the column, first try cleaning the column.

If the high backpressure persists, replace the column bed support. Note the color of the original bed support. If the inlet side of the used bed support is discolored (light brown to grey-black) 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-based” pumping systems (e.g., Dionex GP50), PEEK tubing, and connected fittings.

### 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- $\mu$ m, or 0.45- $\mu$ m 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 column may have been subjected to pressures above 4000 psi (34.5 MPa), causing irreversible bed compression. This requires column replacement.
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## 8.5 Poor Peak Efficiency and Resolution

Try alternate eluents such as NaClO<sub>4</sub>. If this fails to improve matters, try operating under denaturing conditions.

## 8.6 Unidentified Peaks Appear

- a) Intra- or intermolecular oligonucleotide associations may cause unidentified peaks. Operate under denaturing conditions.
- 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

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<sub>2</sub> lamp).

## 8.8 Column Problems

**CAUTION: The guard column protects the main column not only from contamination but also from excessive pressure fluctuations caused by the instrument or by operator errors. Have the guard column installed at all times. To prevent accidental over pressure, disconnect the guard column only during troubleshooting described in this section, or when priming the pump.**

To determine if the column set is the source of the high background replace the column with a section of PEEK 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. For Upchurch/Scivex tubing, yellow = 0.007" ID)
- c) Verify that the shortest possible length of 0.010" I.D. tubing (black) 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<sub>4</sub> 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 (~ 50 mL) of deionized water.
- b) Wash column with 15 volumes (~ 50 mL) of a solution containing:  
0.4 M NaClO<sub>4</sub>  
1 mM HCl  
30% CH<sub>3</sub>CN
- c) Rinse column with 15 volumes (~ 50 mL) of deionized water.
- d) Regenerate column with 1 volume (~ 3 mL) of high concentration eluent used for column wash at the end of each chromatographic run.
- e) Equilibrate column with 2 volumes (~ 6 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 (~ 50 mL) of deionized water.
- b) Wash column with 15 volumes (~ 50 mL) of 100% CH<sub>3</sub>CN.
- c) Rinse column with 15 volumes (~ 50 mL) of deionized water.
- d) Regenerate column with 1 volume (~ 3 mL) of high concentration eluent used for column wash at the end of each chromatographic run.
- e) Equilibrate column with 2 volumes (~ 6 mL) of eluent used at the start of each chromatographic run.