Agilent ZORBAX Bio Series Oligo Column datasheet

Introduction

The ZORBAX Bio Series Oligo column has been developed specifically for the separation and purification of single-stranded oligonucleotides. This datasheet is intended to outline its application to the analytical and preparative chromatography of such compounds. Due to the wide range in size and nature of oligonucleotides, the conditions given are intended to be of general utility and to act as a basis for initial operation. For specific cases, an optimum separation may be achieved by tailoring the gradient profile to the size range of oligonucleotides of interest, especially if a large number of compounds of similar chain lengths are being investigated.

Range Of Utility

The ZORBAX Bio Series Oligo column has been used for the purification of heterodeoxyoligonucleotides with chain lengths in the range of 3 to 48 bases. This is, however, not the theoretical limit of separations that are possible with the Oligo column. It is expected that the separation and purification of oligonucleotides in the 60- to 80-mer range can be achieved by simple modification of the gradient profile (see below). In addition, the column may be used for the separation of oligoribonucleotides.

The column dimensions of the analytical column (6.2 mm ID x 80 mm) allow sample loads of 200-300 micrograms of oligonucleotide per run to be injected with little or no deterioration in resolution or product purity. Oligonucleotides are generally isolated from the ZORBAX Bio Series Oligo column with purities well in excess of 90-95%. This has been demonstrated by autoradiography using ^{32}P labeling. The remaining trace impurity is generally the n-1 oligonucleotide.

Stability

These HPLC columns are based on the patented zirconium oxide-stabilized packing materials used in the ZORBAX GF series of gel filtration columns and the Bio Series Ion Exchange columns. The packing material is stable to basic mobile phases up to pH 8.5 (The lower operating pH limit is 2.5. Below pH 4.5, there is excessive retention of the oligonucleotides). The ligand which confers the specific chromatographic interactions is covalently bonded to the packing to give a stable, bleed-free matrix.

The Bio Series Oligo columns have a high degree of mechanical stability. They can, therefore, be used at high flow rates and operating pressure; the columns are stable up to 300 bar. They are also compatible with rapid changes in ionic strength; thus, short gradients and rapid reequilibration are possible.

Column Performance

Each lot of packing must pass a series of rigorous performance requirements, including stability, capacity and selectivity in the separation of standard oligonucleotides, prior to its release for shipment.

In addition, each column is performance-tested prior to shipment to ensure efficiency and quality. The Quality Control report enclosed with each column gives the result of this testing.

Several sample chromatograms can be found in this guide. Some of these have been generated with readily available samples. It is strongly recommended that new columns be tested with such samples to verify performance and that these tests be made periodically thereafter.

Mobile Phase Selection

Oligonucleotides can be separated and purified on the ZORBAX Bio Series Oligo column under a variety of conditions. This guide describes solvent systems which have been found to provide good separations for a range of oligonucleotides and gives recommendations for conditions of general applicability. Since the optimum gradient and mobile phase conditions may vary for particular separations, suggestions are given for making modifications to the general conditions.

The separation of oligonucleotides is best achieved by increasing solvent strength as a function of time, (gradient elution). Isocratic (single solvent) methods could be developed for specific materials. The conditions would change for each different sample. Changes in ionic strength have the most profound effect on the separation and are employed in creating the gradient. The pH of the buffer and the concentration of the organic modifier also influence the chromatography. To effect a separation, an organic solvent must be present. The most useful organic solvent for the system is acetonitrile; substituting methanol or tetrahydrofuran has a small and negative effect on both resolution and efficiency. The pH of the separation can be varied between 5 and 8.5, although the range between 6 and 7 gives the

best pH compromise between efficiency and retentivity. Increases in the pH result in lower retention.
Retention is dependent upon the chain length of the oligonucleotide. In addition, there is a small effect of its sequence. For homo-oligonucleotides, deoxythymidylates elute before deoxyadenylates. There is a much larger effect on substituting ribose for deoxyribose in the oligonucleotide; adenylates are more strongly retained than the deoxyadenylates.

Nature Of The Buffer

Most of the separations outlined in this guide have been performed with an increasing sodium chloride concentration gradient in a constant mixture of aqueous sodium phosphate buffered at pH 7.0 and acetonitrile. The pH can be varied with little effect between 6 and 7. Substitution of potassium salts for the sodium salts also makes no difference to the separation. The gradient may also be made from volatile buffers such as ammonium acetate for recovery of the oligonucleotide by freeze-drying (lyophilization) rather than the more usual desalting by gel permeation chromatography.

Developing A Separation

Developing chromatographic separations on the Oligo column is similar to other column gradient elution techniques. A basic three-step procedure for gradient method development is detailed below.

- First, run a broad-range gradient of moderate duration. For the Oligo column we recommend using Solvent 1 (20% acetonitrile: 80% 0.02 M sodium phosphate (monobasic) adjusted to pH 7.0) in Reservoir A and Solvent 2 (1.0 M sodium chloride in solvent 1) in Reservoir B. A 90-minute gradient from 35 to 80% B provides a good first run for a sample. The gradient time may be varied from 40-120 minutes depending on oligonucleotide length; longer oligonucleotides require longer gradient times.
- Second, adjust the initial and final compositions of the gradient to just encompass the peaks of interest. This often means altering the initial and final % B to focus on the oligonucleotide of interest. Occasionally, optimized separation requires expansion of the range, concentration and gradient time. For long-chain, highly retained samples Solvent 3 (2.0 M sodium chloride in Solvent 1) may be used in Reservoir B. A higher initial salt concentration will allow a faster purification of the larger oligomers at the expense of resolution of the low molecular weight species.
- The third step of the procedure is adjusting the gradient duration to achieve the desired compromise between resolution and analysis time. In general, increased gradient duration results in increased resolution but at the cost of increased time for analysis. The gradient time may be varied from 40-120 minutes depending on oligonucleotide, and the resolution required.

This procedure is simply a guide and should be pursued only as far as necessary to achieve your separation goals. The chromatograms in this guide are good examples of oligonucleotide separations, but seldom was a full optimization completed. The figures can also be used to shortcut the procedure if your sample is similar to one of the test samples illustrated.

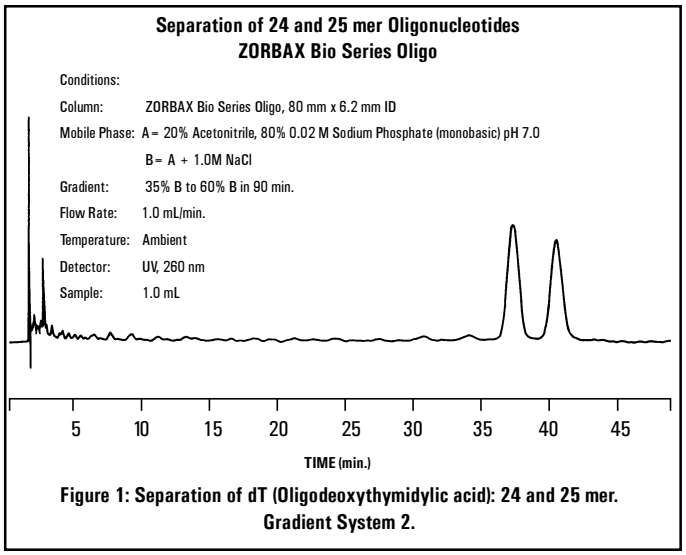

Figure 1: Separation of dT (Oligodeoxythymidylic acid): 24 and 25 mer. Gradient System 2.

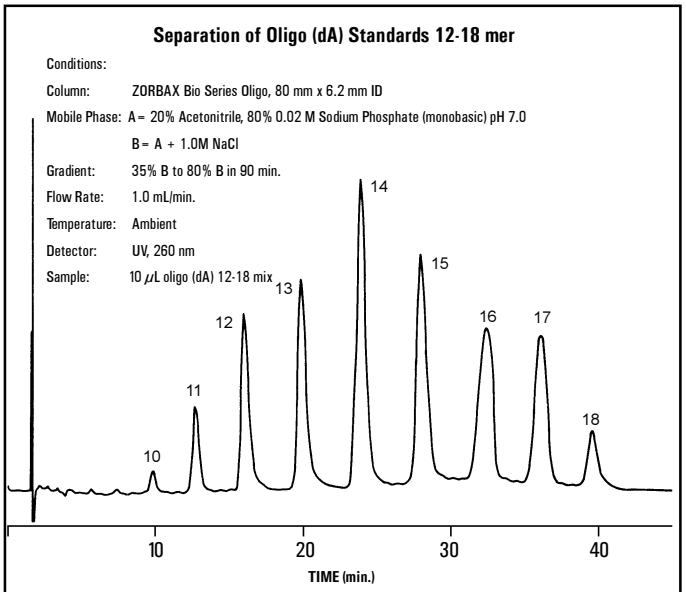

Figure 2: Oligo dA (Oligodeoxyadenylic acid): 12 to 18 mer. Gradient System 3.

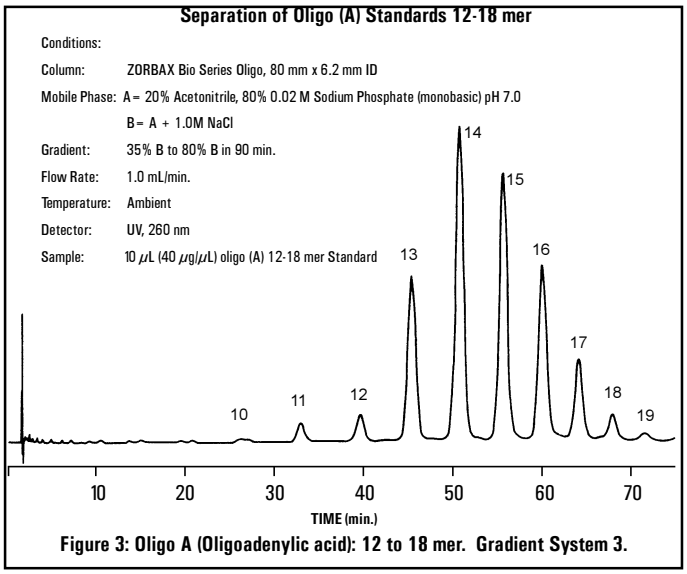

Figure 3: Oligo A (Oligoadenylic acid): 12 to 18 mer. Gradient System 3.

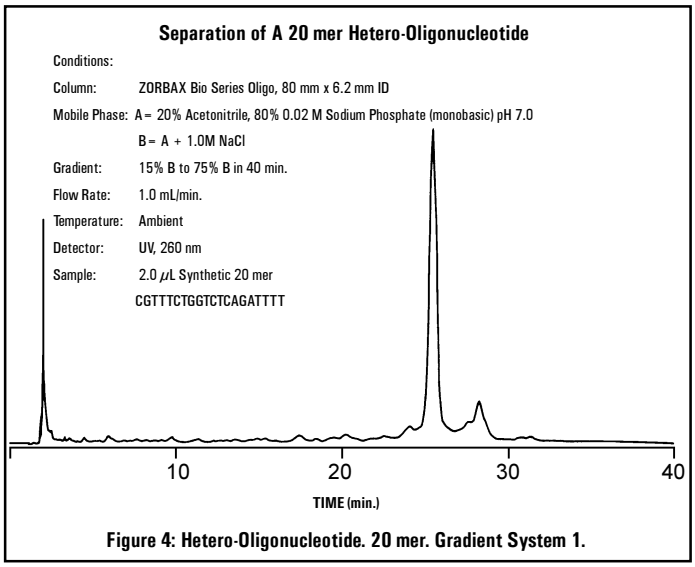

Figure 4: Hetero-Oligonucleotide. 20 mer. Gradient System 1.

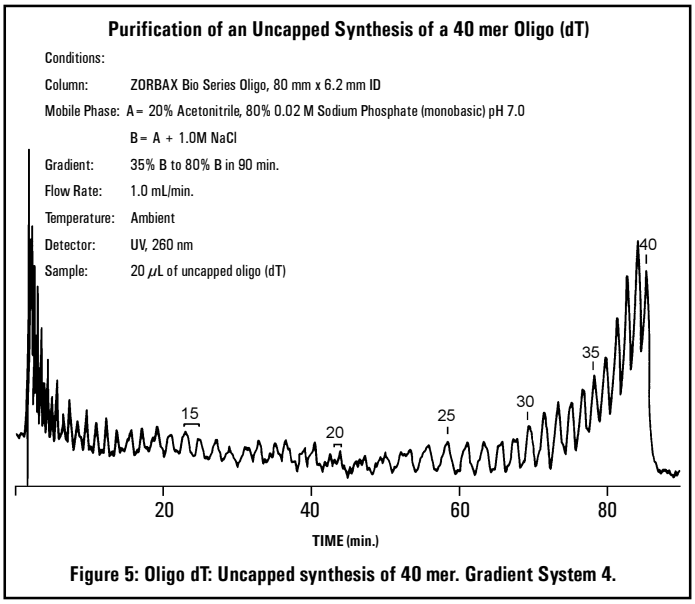

Figure 5: Oligo dT: Uncapped synthesis of 40 mer. Gradient System 4.

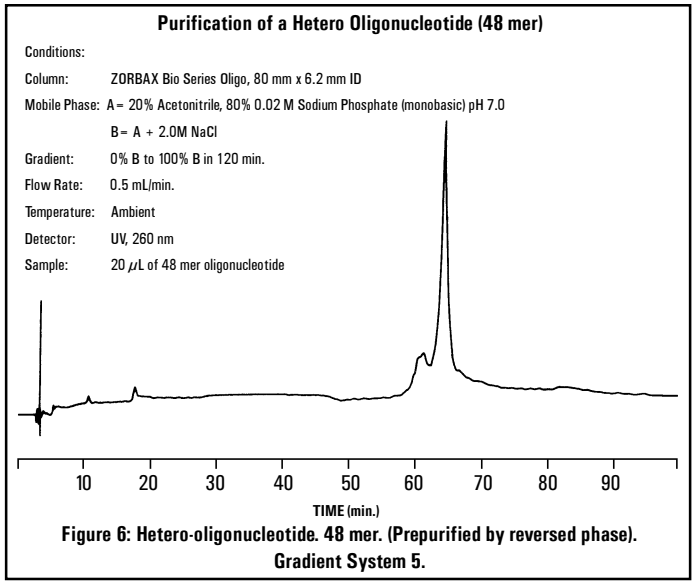

Figure 6: Hetero-oligonucleotide. 48 mer. (Prepurified by reversed phase). Gradient System 5.

Separation Of Oligonucleotides

Examples of typical solvent systems used with Oligo column are summarized in Table 1.

Table 1

GRAD NO.	SOLVENT A	SOLVENT B	GRADIENT RANGE % B	GRADIENT TIME (min)	FLOW RATE mL/MIN
1	1	2	15 TO 75	40	1.0
2	1	2	35 TO 60	90	1.0
3	1	2	35 TO 80	90	1.0
4	1	2	35 TO 80	120	1.0
5	1	3	0 TO 100	120	0.5

Solvent 1: 20% acetonitrile: 80% 0.02 M sodium phosphate (monobasic)
Solvent 2: 1.0 M sodium chloride in Solvent 1
Solvent 3: 2.0 sodium chloride in Solvent 1
All solvents are adjusted to pH 7.0 with 10 M sodium hydroxide before mixing with acetonitrile.
The figures show a number of separations achieved with the solvent systems outlined above.

Separation Problems

There are two characteristic problems which may be seen in the chromatography of oligonucleotides. The first is the appearance of unusual peak shapes. These apparently misshaped peaks are typically broad and negatively skewed (fronting). This is generally due to some interaction between oligonucleotides molecules, generally self-hybridization or looping. These unusual peak shapes do not, however, influence the purity of the collected product. The other observation is that oligonucleotides present in small concentrations, such as minor components arising from failure sequences, can exhibit split peaks (sometimes triplets) in the chromatogram. This peak splitting is not an indication of a column failure or void in the column, but is rather an artifact of the chromatography. This phenomenon is often encountered in this high-resolution chromatographic analysis of oligonucleotides. Collection and rechromatography demonstrate that the apparently different constituents of the oligomer are, in fact, identical. In practice, the only effect of this is a slightly more difficult to interpret chromatogram, although it is still easy to count the number of peaks in the chromatogram to confirm the length of the oligonucleotide in question. This peak splitting phenomenon is more apparent at low concentration. Usually the desired product is present at a higher concentration and does not exhibit this behavior.

Column Equilibration

The column is supplied in methanol. After attaching the column to the liquid chromatograph it should be flushed with 20 mL of HPLC-grade water. Change the mobile phase to the buffer system to be used, and wash the column with 20 mL of the low and high-strength buffers. Finally, reequilibrate the column with 20 mL of buffer at the required starting composition. See the section on column care for details on storage.

Sample Introduction

Samples are usually introduced to the column through a conventional loop valve injector. If it is desired to inject very large volumes of dilute sample, it is possible to pump the sample directly onto a column which is equilibrated with a low-ionic-strength starting buffer composi-

tion; this will ensure complete retention of the sample during the loading phase. The samples should be free of all particulate matter and should, therefore, be filtered.

Unless special techniques are used, the sample volume should be the smallest volume possible, allowing for the mass and concentration of the oligonucleotide to be purified. The maximum volume and sample load will depend upon the elution position of the oligonucleotide, the gradient slope and the degree of resolution required. Typical maximum injection volumes are of the order of 250 microliters to 1 mL. The maximum sample load which will not adversely affect the separation is 200-300 micrograms; larger samples may cause peak broadening which could result in lower purity of the collected fraction.

Column Care

When the column is in frequent use, it is not generally necessary to flush out the mobile phase on a daily basis. If the column is not to be used for several days, it should be flushed first with water to remove the buffer salts and then with methanol or acetonitrile for storage, closing the column with the protective screw fittings provided. Because the mobile phase contains significant proportions of acetonitrile, it is not generally necessary to use antibacterial agents in the mobile phase. Columns should be stored at room temperature. It should be noted that halide salts are corrosive to stainless steel. Alternative buffers such as acetates or sulfates may be used.

The ZORBAX Bio Series Oligo columns can be used with most buffer systems with a pH range of 2.5 to 8.5. Oligonucleotides are not, however, eluted from the column below pH 4.5. Flow rates up to 6 mL/min may be used at operational pressures up to 300 bar.

To Maximize Column Life:

- Filter mobile phase and samples.
- Do not open the column.
- Do not inject sample dissolved in high-concentration salt solutions.
- Do not store in halide buffers.
- Do not allow the column to dry out.
- Flush the column with water before filling with organic solvents for storage.
- Do not use beyond the recommended pH range (2.5 to 8.5).
- Do not freeze the columns.
- Operate the column with the flow in the direction of the flow arrow on its label.
- Do not operate at temperatures above 50 degrees C.

Safety Considerations

Some important points to keep in mind for safe operation with LC components are:

- All points of connection in liquid chromatographic systems are potential sources of leaks. Users of liquid chromatographic equipment should be aware of the potential hazards from such leaks due to the flammability, chemical and biological toxicity of the chosen mobile phases.
- Because of its small size, dry ZORBAX packing is respirable.
- These columns have not been approved for use in processing products for human use.

Ordering Information

	Agilent Part No.
ZORBAX Bio Series Oligo Columns	
6.2 mm ID x 80 mm	820940-901
9.4 mm ID x 250 mm	884973-903
21.2 mm ID x 250 mm	884974-915

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Part No. 820943-301 C