Quality Control and Purification of Oligonucleotides on the WAVE™ Nucleic Acid Fragment Analysis System

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The Transgenomic WAVE™ System employs two methods for performing oligonucleotide separations. The methods are based on size dependent separations for performing failure sequence analysis or size and sequence dependent separations for purification and analysis. The system allows high-throughput quality control of oligonucleotide preparations.

Introduction

Oligonucleotides are arguably the most widely used reagents in modern molecular biology. The quality of synthesized oligonucleotides is especially important because they are used for applications involving the development of new pharmaceuticals or diagnostic tools. It is important to determine the presence of failure sequences or to purify the oligonucleotide from failure sequences or other material. Analysis methods employing rapid separations and significant resolving power would result in important advances for molecular biology and biotechnology. Methods for oligonucleotide analysis and purification must be fast, inexpensive, robust, and sensitive.

The WAVE™ System offers a valuable method for determining the purity of oligonucleotides or for producing pure material. Methodology used for analytical purposes can be scaled to produce material on a semi-preparative or production basis. Since the introduction of DNASep™ nonporous micropellicular packing, chromatographic analyses of oligonucleotides have been obtained within minutes. The packing has properties such as fast mass transfer kinetics, maximum surface accessibility, and fast column regeneration. Ion pairing technology used in combination with liquid chromatography takes advantage of the lipophilic

Fragment	Sequence	Concentration	Retention Time
14 mer	5'-AAA AGT CCG TGA GA-3'	0.28 µg/µL	16.36 min
15 mer	5'-CAA AAG TCC GTG AGA-3'	0.28 µg/µL	17.13 min
16 mer	5'-ACA AAA GTC CGT GAG A-3'	0.31µg/µL	17.78 min

Table 1. Oligonucleotide sequences, concentrations and retention times.

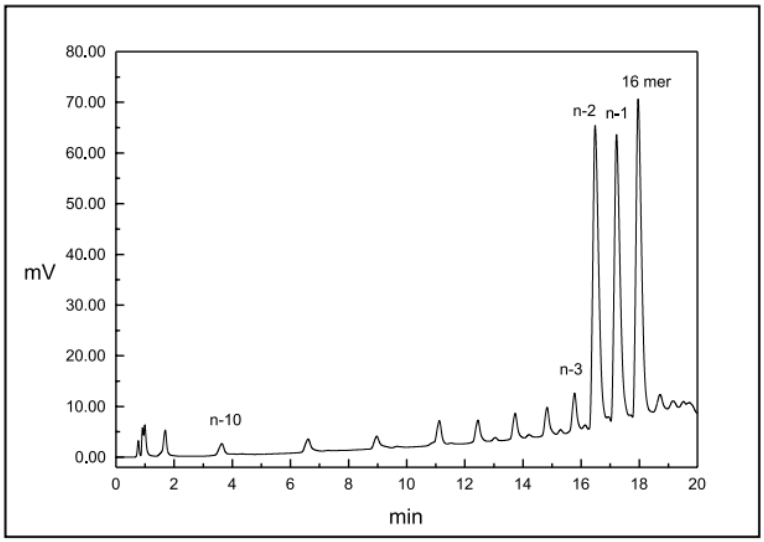

Figure 1. Separation of three oligonucleotides (14, 15, 16 mer). Conditions: Buffer A: 0.025 M tetrabutylammonium bromide (TBuABr), buffer B: 0.025 M TBuABr, 50.0% CH_3CN; 10-90% B in 17 min, 60-100% B in 1 min, 100-10% B in 1 min, T = 60°C, detection UV at 260 nm, injection vol = 5 µL

character of the alkylammonium salts of oligonucleotides. The ion-pairing reagent binds as a cation to the anionic phosphate groups of the oligonucleotide making it a more hydrophobic species. This ion-pair can now be separated on the lipophilic surface of the DNASep™ column matrix by means of a gradient including acetonitrile as the organic solvent. Depending on the ion-pairing reagent used, the separations can be either size dependent or size and sequence dependent.

Examples

Failure sequence analysis

Size dependent separation

By using a proper ion-pairing reagent and column, it is possible to achieve separations strictly according to size. Tetrabutylammonium bromide ion-pairing reagent transforms the nonpolar surface of the DNASep™ column matrix into a dynamic anion exchanger where the oligomers are separated according to their length. Therefore, the separation is based on size and not on base content. Figure 1 shows a separation of three oligonucleotides with sequences shown in Table 1. The elution of the 14-16 mers was confirmed by separation of the individual oligonucleotides. Smaller failure sequence contaminants are also seen in Figure 1.

Sequence independent separations are not limited to short oligonucleotides only, as demonstrated in Figure 2. Three oligonucleotides, i.e., 23, 24, and 25 mers, with sequences shown in Table 2, were separated with the WAVE® System.

Size and sequence dependent separation

By choosing a different ion pairing reagent, the separation of oligonucleotides is based on sequence and size. Advantage is taken of the lipophilic character of oligonucleotides to offer an additional selectivity parameter. This can be important when derivatized oligonucleotides are analyzed. To show the effect, two 30 mer oligonucleotides of different sequence were separated (Table 3). Oligo 1 has a T content of 36.7%, whereas in Oligo 2, the T content is only 30.0%. This difference is enough to separate both oligomers. T is the most hydrophobic base, and therefore T-rich oligomers are retained longer on the column. The order of retention of nucleic acids on the column under these conditions is C < G < A < T.

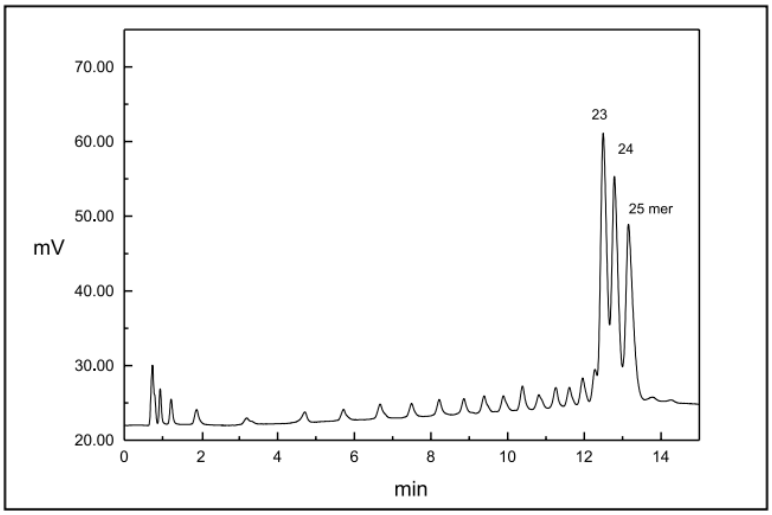

Table 2. Separation of 23, 24 and 25 mers. Buffer A: 0.01 M TBuABr, buffer B: 0.01 M TBuABr, 50% CH_3CN, 40-100% in 20 min, 100% B for 5 min, 100-40% B in 3 min; 0.75 mL/min, UV detection at 260 nm, T = 69C, injection vol = 5µL

Fragment	Sequence	Retention Time
23 mer	5'-TCC GTG AGC AAA AGT CCG TGA GA-3'	12.49 min
24 mer	5'-GTC CGT GAG CAA AAG TCC GTG AGA-3'	12.78 min
25 mer	5'-AGT CCG TGA GCA AAA GTC CGT GAG A-3'	13.15 min

Table 2. Oligonucleotide sequences with retention times.

Quality verification of commercially obtained oligonucleotide

A vendor prepared and purified a 15 mer oligonucleotide with sequence 5'-ACA AAG GTG AGG TTT-3' by HPLC. The exact procedure is proprietary and purification conditions were not available. The oligonucleotide was analyzed with the DNASep™ column under the following conditions: Buffer A: 0.1 M TEAA pH = 7.3, buffer B: 0.1 M TEAA, 25.0% CH_3CN; 10-60% B in 17 min, 60-100% B in 1 min, 100-10% B in 1 min, T = 80°C, detection UV at 260 nm, injection volume = 5 µL. The retention time was 6.46 min (80°C). Figure 3 shows that the HPLC purified oligonucleotide was in fact only about 70% pure. This demonstrates the capability of the WAVE® System to be used for quality control of oligonucleotide sequence procedures.

Discussion

The separation of single-stranded oligonucleotides is dependent on the choice of ion-pairing reagent. The latter converts the stationary phase into a dynamic anion exchanger. The hydrophobic properties of the DNASep™ Column matrix depend on the number of methylene groups in the alkyl chains of the ion-pairing reagent. Short alkyl chains (number of methylene groups per alkyl chain) allow the matrix to partially retain its hydrophobic or reverse-phase properties. Longer alkyl chains provide complete coverage and anion-exchange is the predominant mechanism of separation.

Triethylammonium ions cover the matrix only partially and therefore separation is dependent on both size and base composition. Tetrabutylammonium ions completely cover the matrix, and separation of single-stranded oligonucleotides is size-dependent.

Conclusion

Single stranded oligonucleotide samples from automated synthesizers usually contain many impurities. The purification of oligonucleotides and quality control of the synthesis process is conveniently performed on the WAVE™ System. Changing of the ion-pairing reagent allows for separation based on either oligonucleotide size or sequence content and size.

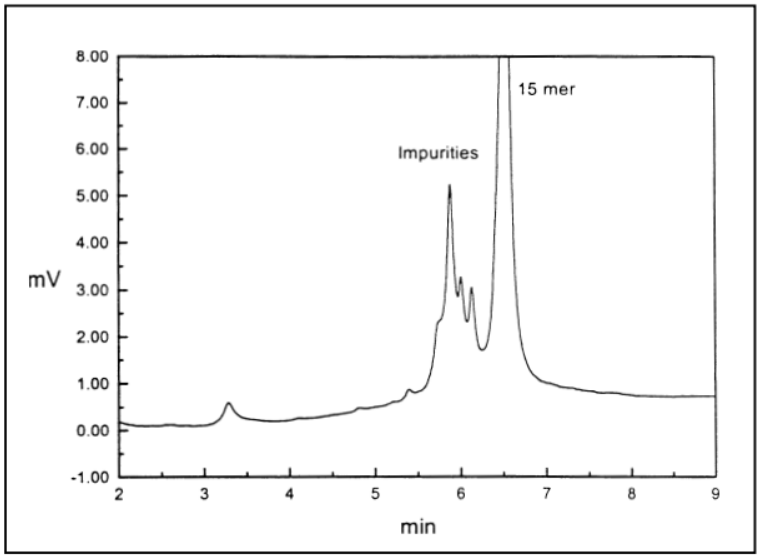

Figure 3. Quality control of an HPLC purified 15 mer oligonucleotide. Buffer A: 0.1 M TEAA, buffer B: 0.1 M TEAA, 25.0% CH_3CN, 10-60% B in 15 min, 60-100% B in 1 min, 100-10% B in 1 min, 10% B for 2 min; T = 80°C, UV detection at 260 nm, injection vol = 5µL

Fragment	Sequence	Retention Time
Oligo 1	5'-TCC TTG ACC ATC TGC TCG TAC TCC TCG TCT-3'	9.45 min
Oligo 2	5'-ACA AAG GTG AGG TTT AAA AGA AGT TTT CTG-3'	9.19 min

Table 3. Oligonucleotide sequences of two 30 mers, along with retention times.

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