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Note

New reversed-phase high-performance liquid chromatographic column for oligonucleotide separation

HIROYUKI MORIYAMA* and YOSHIO KATO
Central Research Laboratory, Tosoh Corporation, 4560 Tonda, Shinnanyo, Yamaguchi 746 (Japan)
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Modern reversed-phase chromatography (RPC) has been widely applied to the separation of nucleobases, nucleosides and nucleotides. Long-chain oligo- and polynucleotides, which are valuable tools in the genetic engineering process, have become easily available because of the rapid developmental of automated DNA synthesizers. The synthesized mixture commonly contains many compounds such as short chains so that oligo- and polynucleotides have to be rapidly and successfully purified from by-products after the synthetic process. It is important to obtain high quality oligonucleotides for gene manipulation.

High-performance liquid chromatography (HPLC) is extensively utilized for separating nucleic acids. For instance, gel filtration chromatography (GFC) is suitable for isolation of large molecules such as DNA fragments[1–3] and ion-exchange chromatography (IEC) is advantageous for separation of a wide range of nucleic acids[4–6]. On the other hand, RPC is also an useful method for separation of nucleic acid samples, mainly small compounds[7–9].

Recently, a new reversed-phase chromatographic column, TSKgel OligoDNA RP (Tosoh, Tokyo, Japan) has become commercially available. This new column, which has a wide pore size and octadecyl groups bonded to silica gel, is designed for separation of medium-molecular-weight compounds such as oligo- and polynucleotides. We have evaluated it in terms of oligonucleotide separation by comparison with a conventional ODS column and the results are now described.

EXPERIMENTAL

HPLC equipment

The instrument consisted of a Model CCPM dual pump equipped with a dynamic mixer, a Model UV-8000 variable-wavelength detector operated at 260 nm and a Model CP-8000 chromatographic integrater (all from Tosoh). Oligonucleotides were separated on stainless-steel columns (15 cm × 4.6 mm I.D. for analytical and 15 cm × 7.8 mm I.D. for semi-preparative measurement) of TSKgel OligoDNA RP and TSKgel ODS-80T_M, a conventional ODS column, at a constant temperature (25°C).

Chemicals and oligonucleotides

Homogeneous oligodeoxyadenylic acids, oligodeoxythymidylic acids and het-

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TABLE I

OLIGONUCLEOTIDE SEPARATION CONDITIONS

Linear gradients from A to B were used. Eluents: A, 5% organic modifier–0.1 *M* ammonium acetate (pH 7.0); B, 25% organic modifier–0.1 *M* ammonium acetate (pH 7.0). The values in parentheses are the increasing percentage of organic modifier in the eluent per minute.

Condition	*Organic modifier*	*TBAH (mM)*	*Gradient time (min)*
Ia	Methanol	–	90 (0.22)
Ib	Acetonitrile	–	90 (0.22)
Ic	Isopropanol	–	90 (0.22)
IIa	Methanol	–	38 (0.66)
IIb	Acetonitrile	–	97 (0.21)
IIc	Isopropanol	–	150 (0.17)
IIIa	Acetonitrile	0	90 (0.22)
IIIb	Acetonitrile	1.25	90 (0.22)
IIIc	Acetonitrile	5	90 (0.22)
IVa	Acetonitrile	0	97 (0.22)
IVb	Acetonitrile	1.25	50 (0.40)
IVc	Acetonitrile	5	36 (0.56)

erogeneous oligonucleotides (primers and linkers) were obtained from Wako Pure Chemicals (Osaka, Japan). Reagent-grade ammonium acetate and HPLC-grade acetonitrile, methanol and isopropanol were obtained from Kishida Chemicals (Osaka,

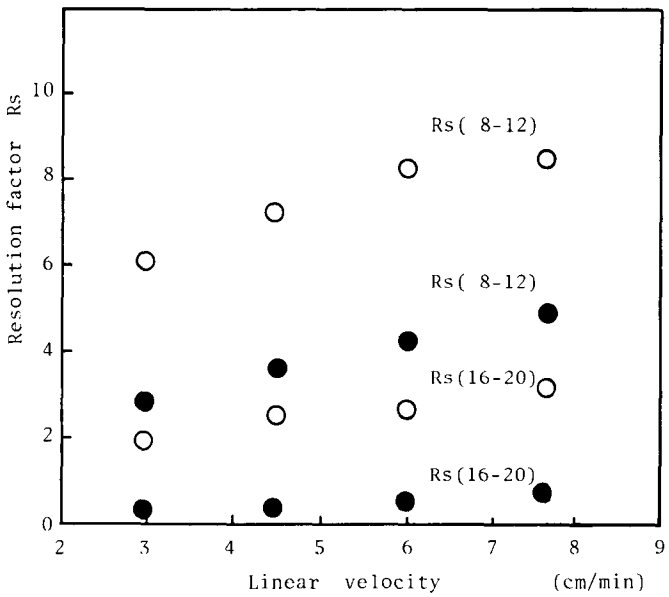

Fig. 1. Comparison of the dependences of the resolution on the linear velocity on TSKgel OligoDNA RP and TSKgel ODS-80T$_M$. The elution condition Ib was used at flow-rates of 0.5–1.25 ml/min with a 90-min linear gradient on TSKgel OligoDNA RP (○) and TSKgel ODS-80T$_M$ (●). Rs(8–12) and Rs(16–20) correspond to the resolutions between 8 and 12 bases and 16 and 20 bases, respectively.

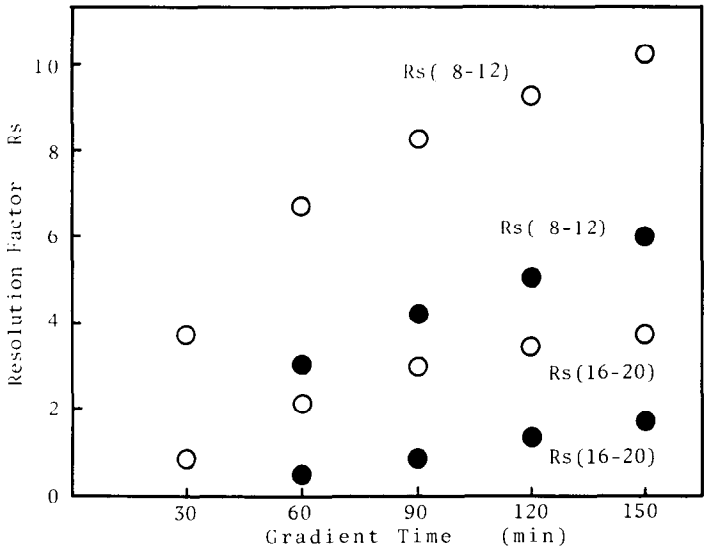

Fig. 2. Comparison of the dependences of the resolution on the gradient time on TSKgel OligoDNA RP and TSKgel ODS-80T$_M$. The elution condition Ib was used at a flow-rate of 1.0 ml/min with gradient times of 30–150 ml/min on TSKgel OligoDNA RP (○) and TSKgel ODS-80T$_M$ (●).

Japan). Tetrabutylammonium hydroxide (TBAH) used for ion-pair chromatography (IPC) was from Tokyo Kasei (Tokyo, Japan).

Chromatographic procedure

Linear gradients of organic modifier, methanol, acetonitrile and isopropanol, from 5 to 25% in 0.1 *M* ammonium acetate (pH 7.0) were employed. For IPC, TBAH was added to mobile phases. All chromatographic conditions are summarized in Table I.

RESULTS AND DISCUSSION

The dependences of the resolution of homogeneous oligodeoxyadenylic acids on linear velocity and gradient time on TSKgel OligoDNA RP and TSKgel ODS-80T$_M$ are shown in Figs. 1 and 2, respectively. In protein separations, the resolution generally increases with increasing flow-rate and gradient time and then becomes constant[10]. A similar dependence was observed in oligonucleotide separation, although the resolutions of large oligomers (16–20 bases) were smaller than those of small oligomers (8–12 bases) under the same conditions on each column. It seems that higher flow-rates and longer gradient times result in dilution of samples and longer separation times. Consequently, linear velocities of 5–7 cm/min (corresponding to flow-rates of 0.75–1 ml/min) and gradient times of 60–120 min seem to be acceptable for large oligomer separation using these ODS columns.

In large molecule separations, the pore size of the packing is also an important factor in obtaining better resolution. The use of packings having large pore sizes (over 20 nm) allows better results compared with small pore sizes[11,12]. In oligonu-

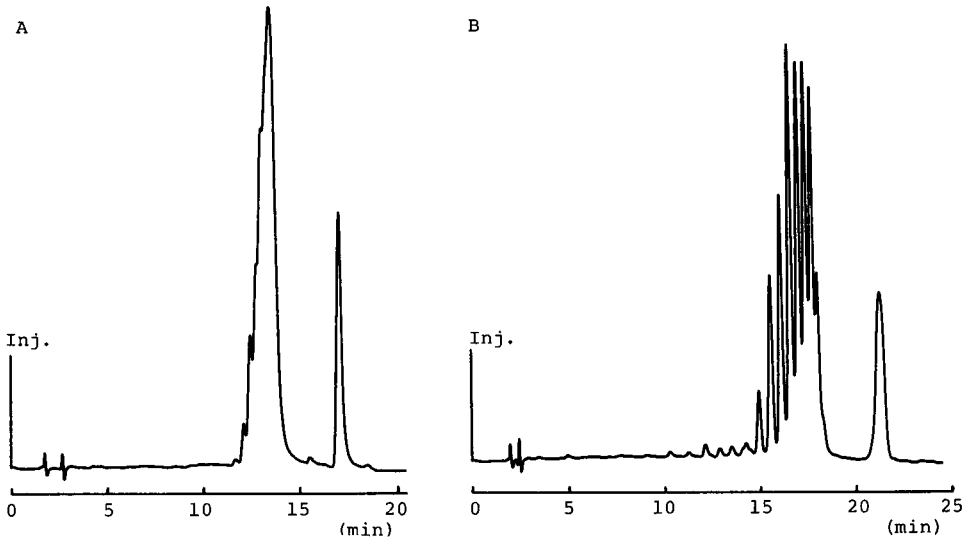

Fig. 3. Chromatograms of a mixture of homogeneous oligodeoxyadenylic acid (12–18 bases) on TSKgel OligoDNA RP and TSKgel ODS-80T_M. The elution condition Ib was used at a flow-rate of 1.0 ml/min with a 90-min linear gradient on TSKgel ODS-80T_M(A) and TSKgel OligoDNA RP(B).

cleotide separation, Makino *et al.*[13,14] reported that large pore sizes (more than 15 nm) resulted in small heights equivalent to a theoretical plate (HETPs) for oligonucleotides but negative effects were obtained on packings having wider pore sizes (over 30 nm). Clearly, TSKgel OligoDNA RP (pore size 25 nm) revealed higher resolution

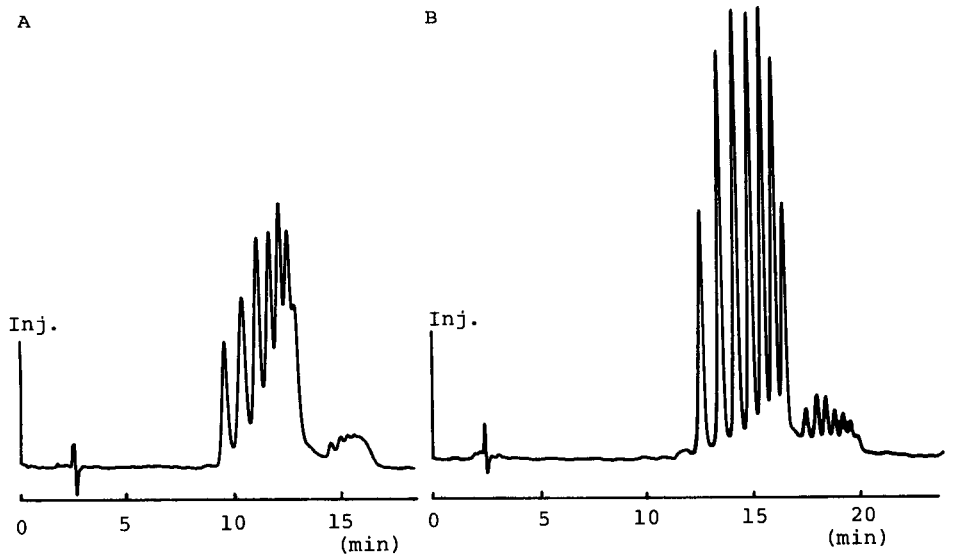

Fig. 4. Chromatograms of a mixture of homogeneous oligodeoxythymidylic acid (12–18 bases) on TSKgel OligoDNA RP (B) and TSKgel ODS-80T_M (A). Elution conditions as in Fig. 3.

TABLE II

EFFECT OF ORGANIC MODIFIER ON THE RESOLUTION BETWEEN THE 16mer AND 20mer OF HOMOGENEOUS OLIGODEOXYADENYLATE

Elution conditions as in Table I, Ia–IIc.

Organic modifier	*Elution conditions Ia–Ic*		*Elution conditions IIa–IIc*	
	Retention time (min)	*Resolution*	*Retention time (min)*	*Resolution*
Methanol	t_R^{16} 64.46 t_R^{20} 67.70	3.62	35.0 36.0	2.36
Acetonitrile	t_R^{16} 14.98 t_R^{20} 15.74	2.41	35.8 36.6	2.27
Isopropanol	t_R^{16} 3.65 t_R^{20} 4.03	0.65	35.5 36.4	2.16

(as shown in Figs. 1 and 2) and better resolution (as shown in Figs. 3B and 4B) compared with the conventional ODS column (pore size 8 nm).

The effect of an organic modifier in the eluent on the resolution was examined. A slight change in the concentration of the organic modifier affects not only the retention time but also the resolution of oligonucleotides in comparison with those of peptides and proteins (Table II). Using the same gradient programme (elution conditions, Ia–Ic), different retention times and resolutions were observed as predicted. On the other hand, the resolutions were almost the same at the same elution positions of the samples (elution conditions, IIa–IIc). Accordingly, the use of acetonitrile as an organic modifier is recommended for oligonucleotide separation when considering the pressure drop.

It is emphasized that ion-pair reversed-phase chromatography (IP-RPC) is a powerful technique for separating oligonucleotides by improving their hydrophobic-

TABLE III

EFFECT OF THE CONCENTRATION OF THE ION-PAIR REAGENT IN THE ELUENT ON THE RESOLUTION BETWEEN THE 16mer AND 20mer OF HOMOGENEOUS OLIGODEOXYADENYLATE

Elution conditions as described in Table I, IIIA–IVc.

Concn. of TBAH	*Elution conditions IIIa–IIIC*		*Elution conditiosns IVa–IVc*	
	Retention time (min)	*Resolution*	*Retention time (min)*	*Resolution*
0	t_R^{16} 14.98 t_R^{20} 15.74	2.41	35.8 36.6	2.27
1.25	t_R^{16} 41.00 t_R^{20} 44.80	7.31	35.5 37.2	5.39
5	t_R^{16} 55.03 t_R^{20} 59.95	9.09	35.3 37.1	6.22

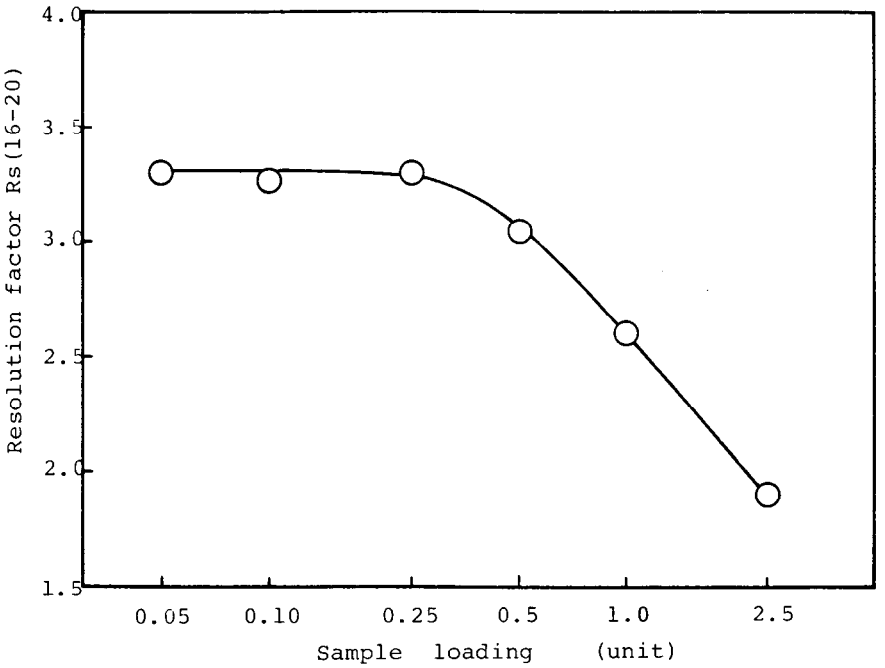

Fig. 5. Dependence of resolution on the loading capacity of oligodeoxyadenylic acid (20 bases) on a semi-preparative TSKgel OligoDNA RP column. The elution conditions were as in Fig. 3 except for the wider column diameter (7.8 mm I.D.) and the higher flow-rate (3.0 ml/min).

ities and permits more effective isolation of samples according to their base numbers[15,16]. The influence of the concentration of the ion-pair reagent, TBAH, in the eluents on the retention time and resolution was investigated (Table III). Both the retention time and resolution increased with increasing amount of TBAH in the eluent with the same gradient system (0.22% acetonitrile/min, elution conditions. IIIa–IIIc). At similar elution positions of the samples, an higher concentration of TBAH in the eluent gave better resolution (elution conditions, IVa–IVc). The technique of oligonucleotide separation with an ion-pair reagent, therefore, is acceptable for improving the selectivities according to the base numbers, but would be not appropriate for purification of samples because it is difficult to remove the ion-pair reagent bonded to the sample. For purification of oligonucleotides, a volatile buffer should be chosen.

The loading capacity of homogeneous oligodeoxyadenylic acid, 20 bases, on a semi-preparative column (7.8 mm I.D.) was examined. As shown in Fig. 5, the resolution remains constant up to at least 0.25 units injected (corresponding to 0.2 mg of sample) and then decreases at greater than 0.25 units. The maximum loading capacity of this column is around 0.2 mg.

Fig. 6 shows the elution profiles of sequencing isomers, octamers, on TSKgel OligoDNA RP. A good separation was obtained in each case, while the attempted isolation between the linker E.coR I and the E.coR V was unsuccessful.

Under the same conditions, two sequencing hexadecamers, which are com-

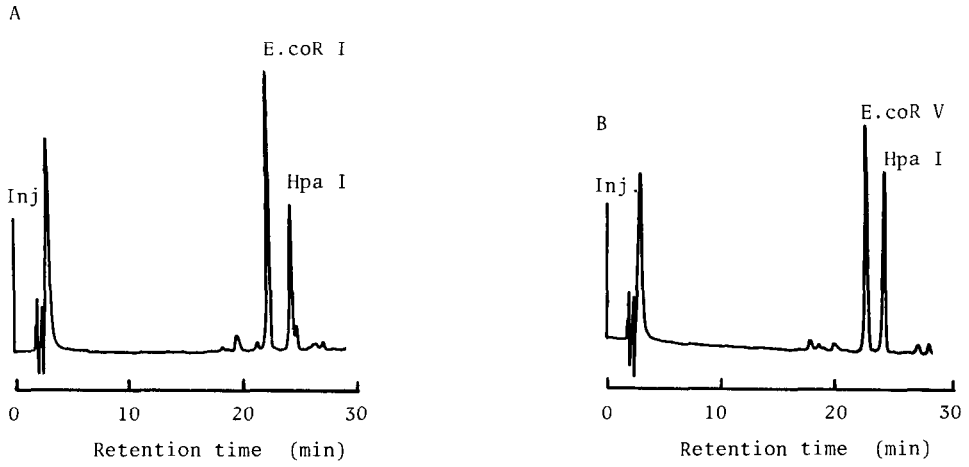

Fig. 6. Separation of sequencing isomers on TSKgel OligoDNA RP. The elution conditions were as in Fig. 3 except for the use of a 120-min gradient time. The samples were (A) linker E.coR I, d(CGAATTCG) and Hpa I, d(CGTTAACG); (B) linker E.coR V, d(CGATATCG) and Hpa I.

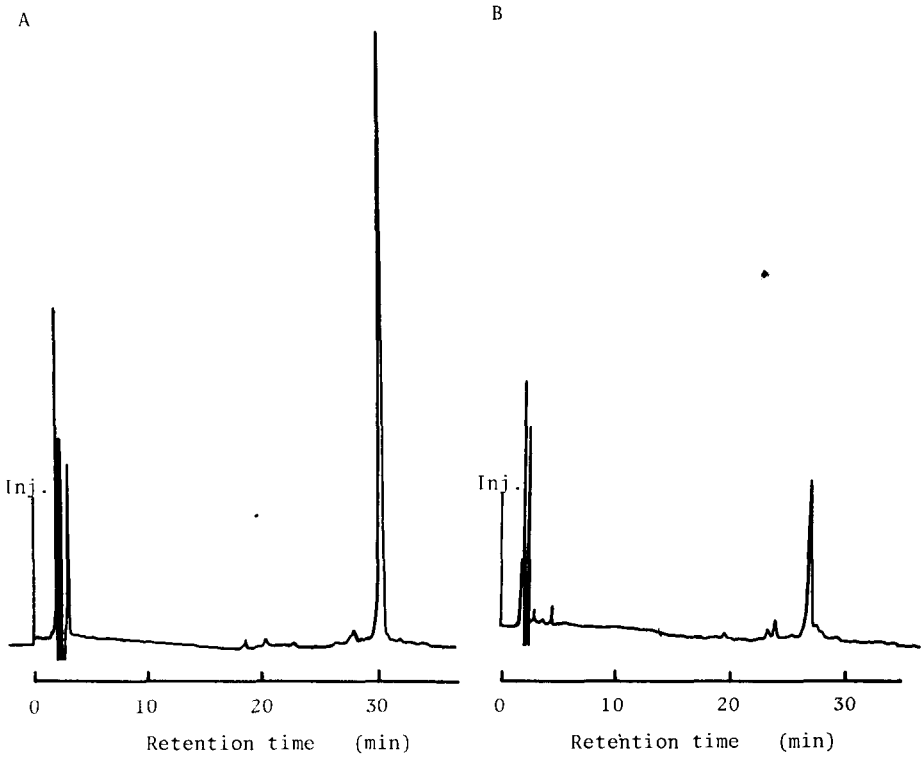

Fig. 7. Separation of sequencing isomers on TSKgel OligoDNA RP. Elution conditions as in Fig. 6. Primers pBR322/E.coR I, d(GTATCACGAGGCCCTT) (A) and M13 forward, d(GCAATTTAACTGTGAT) (B), were used.

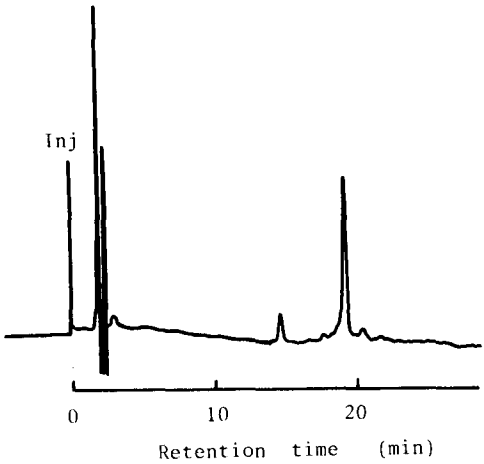

Fig. 8. Chromatogram of commercial heptadecamer on TSKgel OligoDNA RP. Elution conditions as in Fig. 6. The sample was a primer, pBR322/Hind III, d(GTAAAACGACGGCCAGT).

mercially available primers pBR322/E.coR I and M13 forward, were chromatographed as shown in Fig. 7. In spite of the same base numbers, the retention times of the two samples were quite different on account of their inherent hydrophobicities. It is an advantage that, in RPC without an ion-pair reagent, the separation of oligonucleotides is based on their base compositions.

Fig. 8 shows the chromatogram of a heptadecamer, primer pBR322/Hind III, on the analytical TSKgel OligoDNA RP column used to determine the purity of the sample. Small amounts of impurities were recognized.

The evaluation of the new reversed-phase column, TSKgel OligoDNA RP, for oligonucleotide separation was performed. It is concluded that the new column, having a wide pore size suitable for large molecule separation, is more advantageous for the separation of oligonucleotide samples in comparison with the conventional ODS column.

The purification of longer synthetic oligonucleotides on this column is now underway.

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