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Use of Mono Q High-Resolution Ion-Exchange Chromatography To Obtain Highly Pure and Active *Escherichia coli* RNA Polymerase†

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ABSTRACT: A method for the purification of highly pure and active *Escherichia coli* RNA polymerase holoenzyme is described. This method is simple, reproducible, and can be performed at room temperature. The procedure involves the high-performance liquid chromatography of a partially purified RNA polymerase sample on a Mono Q ion-exchange column. Under the conditions used, RNA polymerase holoenzyme is well separated from the core RNA polymerase and other impurities. The purified RNA polymerase contains virtually no impurities as judged by SDS-polyacrylamide gel electrophoresis. The purified RNA polymerase holoenzyme contains the σ^{70} subunit in stoichiometric amounts and is at least 90% active.

The DNA-dependent RNA polymerase of *Escherichia coli* is an important enzyme that plays an essential role in gene expression and regulation. It is a multisubunit enzyme that exists in two forms. Initiation of transcription at defined promoter sites is carried out by RNA polymerase holoenzyme ($\alpha_2\beta\beta'\sigma^{70}\omega$) while elongation and termination are carried out by core RNA polymerase ($\alpha_2\beta\beta'\omega$) (Burgess et al., 1969; Burgess & Travers, 1970).

In order to study RNA polymerase in detail, it is often necessary to obtain highly purified RNA polymerase holoenzyme with the σ^{70} subunit present in stoichiometric amounts (that is, one σ^{70} subunit per core polymerase). The reason for this is that some impurities or even excess core RNA polymerase might interfere with the holoenzyme and therefore complicate the interpretation of results.

Only a few purification procedures can separate the core RNA polymerase from holoenzyme. Chromatography on a single-stranded DNA-agarose column (Nusslein & Heyden, 1972; Lowe et al., 1979) or on phosphocellulose in 50% glycerol (Gonzalez et al., 1977) separates the enzyme into core and holoenzyme-enriched fractions. However, the former method results in RNA polymerase which is only 60-70% saturated with σ^{70}. Although the latter method gives a higher molar ratio of σ^{70} subunit in the holoenzyme fraction, the increased viscosity caused by the high concentration of glycerol in the buffer makes it difficult to perform the chromatography. RNA polymerase holoenzyme can also be reconstituted by adding purified σ^{70} subunit (Lowe et al., 1979) to holoenzyme or core polymerase (Burgess & Jendrisak, 1977). However, it is time-consuming and requires access to purified σ^{70}.

In this paper, we describe a rapid purification method using Mono Q chromatography, which has proven very useful for obtaining highly pure and active *E. coli* RNA polymerase holoenzyme.

Materials and Methods

Materials. E. coli K12 cells (MG1655) were grown on 4 × LB (with 1 × NaCl) in a 10-L fermenter at the University of Wisconsin Biotechnology Center. Cells were harvested at A_{600} = 10 in late log growth phase. About 150 g wet cell paste was obtained and stored at −70 °C before use in the RNA polymerase preparation.

Most reagents were purchased and prepared as in Burgess and Jendrisak (1975). Nucleotides were purchased from

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Boehringer Mannheim Biochemicals. γ-AmNS-UTP[1] (Yarbrough et al., 1978) and T7 D111 DNA were kind gifts from Bruce Beutel and Sigrid Leirmo (Tom Record's laboratory). Plasmid pGR40 (Roe et al., 1984) DNA containing the λPR promoter was purified as described (Maniatis et al., 1982). After digestion of pGR40 DNA with *Pvu*II, an 896-nt fragment containing the λPR promoter was purified by electroelution using an electroeluter from IBI. The concentration of the *Pvu*II DNA fragment (896 nt) was determined by UV scanning with a scanning spectrophotometer.

Buffers and Columns. All buffers and stock solutions were the same as those used for the purification of holoenzyme (Burgess & Jendrisak, 1975). The basic buffer used throughout was TGED: 0.01 M Tris-HCl, pH 7.9 at 20 °C, 5% glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol. Storage buffer was TGED & 0.1 M NaCl with 50% glycerol. The water was filtered by using the Milli-Q system (Millipore). All the buffers, solutions, and Milli-Q water used in Mono Q chromatography were filtered through a 0.22-μm filter prior to use. The ionic strength of buffers and samples was measured before they were loaded on columns.

A 1.5 cm × 30 cm DNA–cellulose column (about 40 mL) was prepared as described (Burgess & Jendrisak, 1975). Sephacryl S-300 was purchased from Pharmacia.

Mono Q HR 5/5 analytical (1 mL) and Mono Q HR 10/10 (8 mL) preparative columns were purchased from Pharmacia and maintained as suggested by the manufacturer. The HPLC system (Beckman) had Model 114M pumps and a Model 421A detector. Samples were applied with a 10-mL Superloop purchased from Pharmacia. We routinely carried out chromatography with back-pressures of 150–300 psi.

Protein Determination. Protein concentrations were determined by UV absorbance using the extinction coefficient data of Lowe et al. (1979). The purity of the RNA polymerase preparation was estimated following polyacrylamide–SDS gel electrophoresis, staining with Coomassie Brilliant Blue R-250, destaining, and scanning as described for σ quantitation below.

Polyacrylamide Gel Electrophoresis. 10% polyacrylamide–SDS stacking gels (Laemmli, 1970) or prepoured 4–20% polyacrylamide–SDS gradient gels, purchased from Integrated Separation Systems, were run until the bromphenol blue marker dye was near the bottom of the gel.

Quantitation of σ^{70} Content. The basic method for determining the σ^{70} content or percent saturation with σ^{70} was as described (Lowe et al., 1979). Polyacrylamide–SDS gels (10%) were stained 4–12 h in 0.075% Coomassie Brilliant Blue R-250 and destained to a background absorbance of 0.05–0.10 at 550 nm and equilibrated. Gels were scanned at 550 nm with a Beckman DU-8 spectrophotometer with a scanning attachment. The slit width of the light beam was 0.1 mm. The scanning rate was 1 or 2 cm/min and the chart speed 2 cm/min. The minimum peak chosen for area determination was 0.1 absorbance unit. Protein peak area was integrated by using the valley–valley method provided by Beckman as part of the DU-8 software package.

Enzyme Activity Assays. A steady-state assay for the RNA polymerase abortive initiation reaction has been described (McClure et al., 1978). The standard abortive initiation assay condition for titration of the λPR promoter by RNA polymerase was used essentially as described [see Figure 1 in Hawley and McClure (1980)] with the use of fluorescent γ-AmNS-UTP in place of radiolabeled UTP. The steady-state rate of abortive initiation was determined by a fluorescence detection method as described by Bertrand-Burggraf et al. (1984). A quantitative assay to measure RNA polymerase activity was performed essentially as described by Chamberlin et al. (1979), with minor modifications. Samples (20 μL) were taken from the reaction mixture at different times and spotted directly onto 2.25-cm-diameter DEAE-cellulose assay disks (Whatman DE-81) which had been prespotted with 20 μL of 0.1 M EDTA. The dried filters were then washed with 5% Na_2HPO_4, water, and ethanol as described by Somers and Pearson (1975). After the filters were dried, they were placed into scintillation vials with 5 mL of scintillation fluid and counted by scintillation spectrometry.

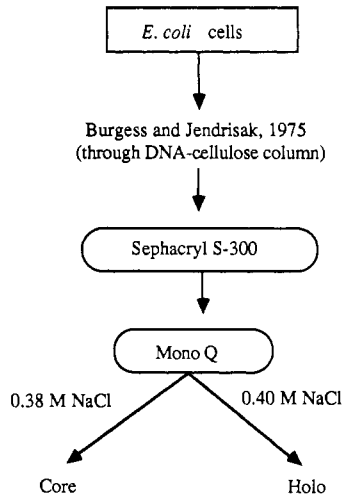

FIGURE 1: Schematic for the purification and fractionation of core and holoenzyme RNA polymerase.

RESULTS

Purification Procedure

A schematic for the purification procedure is shown in Figure 1.

Early Steps of RNA Polymerase Purification. For the early steps in the RNA polymerase purification, we basically followed the procedure of Burgess and Jendrisak (1975) (scaled down to 50 g of *E. coli* K12 cells) with a few modifications listed below. The lysis condition was changed to obtain better cell lysis and therefore better overall yield for RNA polymerase. Twice the amount of lysozyme (260 μg/mL instead of 130 μg/mL) and a 4-fold increase in the amount of sodium deoxycholate (from 0.05% to 0.2%) were used. In eluting RNA polymerase from the DNA–cellulose column, a 0.40 M NaCl step elution was performed instead of a 0.15–1.0 M NaCl linear salt gradient. The reason for this change is to speed the elution process and to obtain a relatively higher concentration of pooled protein. The RNA polymerase eluted from the DNA–cellulose column was pooled (about 20 mL), precipitated with ammonium sulfate (35 g of solid ammonium sulfate/100 mL of eluate), and centrifuged. The precipitate was resuspended in 1 mL of TGED.

Sephacryl S-300 Chromatography. Sephacryl S-300 was used in the place of Bio-Gel A-1.5m (Burgess & Jendrisak, 1975) to improve the flow rate and elution profile reproducibility. A 175 mL (1.5 × 100 cm) Sephacryl S-300 column was equilibrated with 500 mL of TGED + 0.50 M NaCl. The 1-mL sample from the previous section was carefully loaded onto the Sephacryl S-300 column and eluted with TGED + 0.50 M NaCl at 25 mL/h. About 10 mL of RNA polymerase fraction was pooled from the second UV-absorbing peak which

[1] Abbreviations: HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; γ-AmNS-UTP, uridine 5′-triphosphate containing 1-aminonaphthalene-5-sulfonate attached via a γ-phosphoamidate bond.

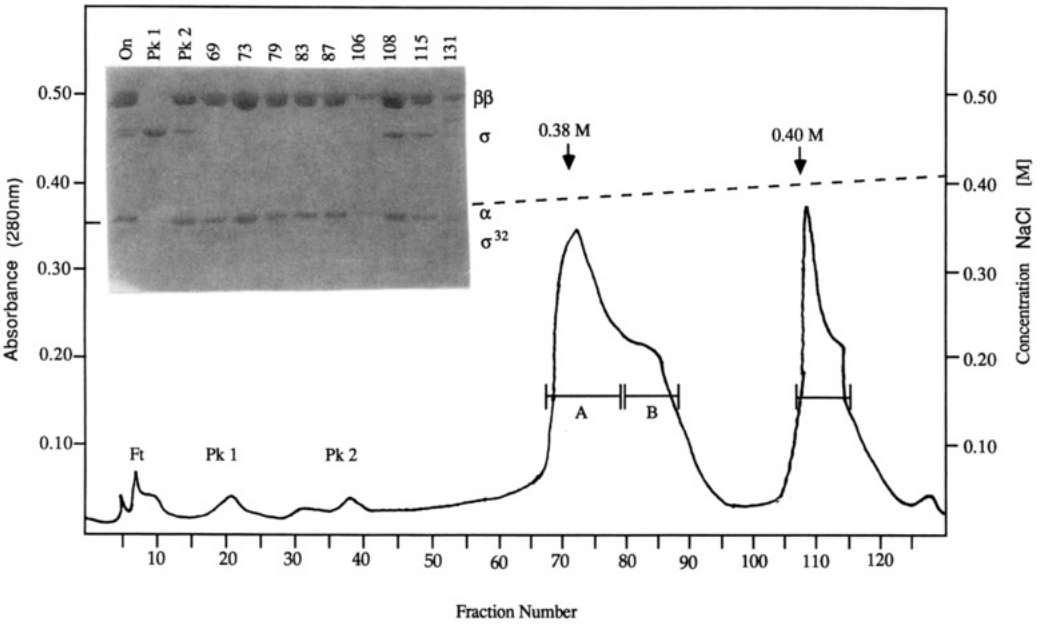

FIGURE 2: Mono Q chromatography of RNA polymerase. "On" fraction is partially purified RNA polymerase pooled from the second peak off of the Sephacryl S-300 column chromatographed as described. The inset shows 7.5 μL of each fraction or pooled peak electrophoresed on Tris–glycine SDS–10% polyacrylamide stacking gels.

Table I: Summary of the Purification of RNA Polymerase

sample	volume (mL)	total protein[a] (mg)	yield (%)	purity (%)	% σ^{70} saturation	holoenzyme[c] activity (%)
"on" Mono-Q	15	11.0	100	85	<36	28 (1)
core (pooled fractions), A + B	5[b]	4.5	41	>99	(–)[d]	NA[e]
holo (pooled fractions)	4[b]	3.0	27	>99	>99	92 (3)

[a] From 50 g wet weight *E. coli* K12 cells. [b] Volume after dialysis vs storage buffer. [c] Data obtained from Figure 4. The number in parentheses indicates the specific activity relative to the "on" sample based on data from Figure 5. [d] As determined from Western blots. [e] Not applicable.

eluted at about one-third column volume. This sample was either dialyzed overnight or diluted with an equal volume of TGED to a final concentration of 0.25 M NaCl in preparation for application to the Mono Q column.

Mono Q Chromatography. A typical protocol to run the 10/10 preparative Mono Q column for a 50-g cell preparation is summarized as follows: (1) Wash stored column with 50 mL of MilliQ water at 1–2 mL/min. (2) Equilibrate with 50 mL of TGED + 0.30 M NaCl for 1 h at 1 mL/min. (3) Load sample (in TGED + 0.25 M NaCl) using the Superloop on ice at 1 mL/min. (4) Wash for 10 min with TGED + 0.30 M NaCl at 1 mL/min. (5) Elute with a shallow gradient from TGED + 0.35 to 0.50 M NaCl in 270 min, at 1 mL/min (increase the gradient time for increased amount of protein loaded). (6) Put the fractions containing peaks of interest at 4 °C immediately. (7) Pool the core and holoenzyme fractions separately, dialyze against storage buffer, and store at −70 °C. (8) Regenerate column by washing with TGED + 1.0 M NaCl for 30 min and then with 100 mL of MilliQ water at 2 mL/min. (9) Prepare the column for storage by applying 30 mL of 20% methanol at 2 mL/min, and store at room temperature.

A typical load for the preparative Mono Q column is 10–20 mg of protein. The back-pressure of the column varied depending on previous usage but usually ran between 150 and 300 psi. A typical example of an elution profile from the Mono Q column is shown in Figure 2. Following several trial runs, we found that the gradient needs to be very shallow in order to attain separation of the core and holo polymerase peaks. The core RNA polymerase peak elutes at about 0.38 M NaCl while holoenzyme elutes at about 0.40 M NaCl. Both peaks elute heterogeneously. An overall summary of the purification and characterization can be found in Table I.

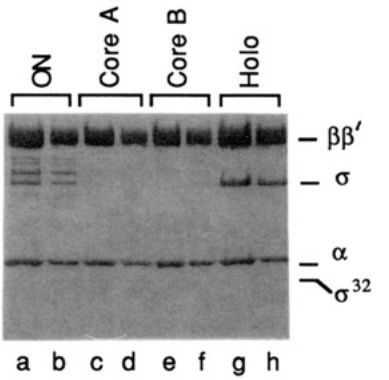

FIGURE 3: Tris–glycine SDS–4–20% polyacrylamide gradient gels. All samples were dialyzed versus storage buffer: (a and b) onput for Mono Q, 3.4 and 1.7 μg, respectively; (c and d) core pool A, 5.0 and 2.5 μg, respectively; (e and f) core pool B, 4.8 and 2.4 μg, respectively; (g and h) holoenzyme pool, 5.0 and 2.5 μg, respectively. The core and holoenzyme pools are indicated in Figure 2.

Some minor modifications in running conditions are necessary when using the 1-mL analytical Mono Q. A typical load of RNA polymerase for this column is 1–5 mg. The flow rate is cut to 0.5 mL/min, and the gradient time may need to be adjusted, depending upon the amount of sample loaded.

Characterization of Purified Enzyme

Purity of the RNA Polymerase. The purity of RNA polymerase before and after the Mono Q column is estimated from an overloaded gels to be about 85% and more than 99% pure, respectively (see Figure 3). Most of the limited impurity, if not all, was eluted either before the core peak or after the holoenzyme peak during the 1.0 M NaCl wash. One

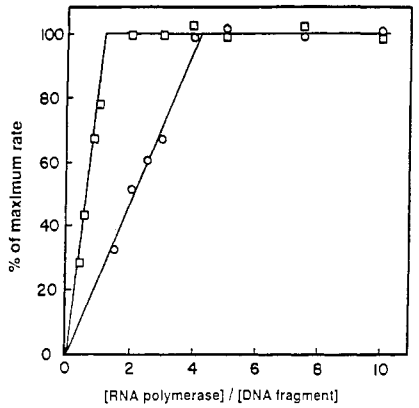

FIGURE 4: Titration of the λP_R promoter with RNA polymerase. The *Pvu*II 896-nt λP_R fragment was present at 1 nM. The rate of pppApU synthesis was measured as described under Materials and Methods and is expressed as a percentage of the maximum rate. The concentration of RNA polymerase is indicated assuming that all proteins present in each fraction are subunits of RNA polymerase. (○) Protein fraction before the Mono Q column; (□) after the Mono Q column.

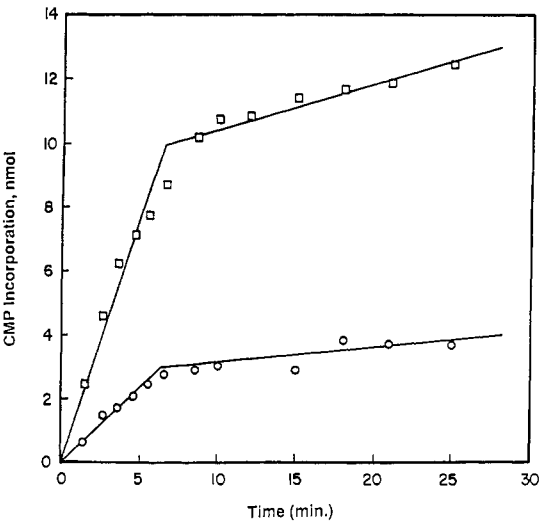

FIGURE 5: Kinetics of T7D111 RNA synthesis by RNA polymerase from samples before the Mono Q column (○) or after the Mono Q column (□), respectively. The assay conditions are described under Materials and Methods. The nucleotide incorporation was calculated for a 1-mL reaction to which 3.0 μg of protein was added.

impurity, which had the size of σ^{70} on polyacrylamide–SDS gels, was eluted early off of the Mono Q column (see Figure 2, peak 1). Lowe et al. (1979) also noticed this polypeptide. Some $\alpha_2\beta$ subassembly also eluted early from the Mono Q column (see Figure 2, peak 2), along with some of the σ^{70}-sized protein seen in the previous peak. In purified core and holoenzyme fractions, in most preparations we detected no bands other than those composing RNA polymerase. These fractions did include the ω subunit, which is not seen on this gel (see Figure 3). We see no differences in the UV spectrum of the purified holoenzyme; it was identical with that reported by Lowe et al. (1979).

σ Saturation in the Holoenzyme Peak. The molar ratio of σ^{70} subunit to the core enzyme was determined. A 100% σ^{70} saturation of a holoenzyme is defined as a molar ratio of 1. The σ^{70} saturation in the holoenzyme peak from the Mono Q column was found to be >95% following scanning and quantitation of the Coomassie-stained polyacrylamide–SDS gels as described under Materials and Methods. On the other hand, the σ^{70} saturation in the sample applied to the Mono Q column was only 36% (see Figure 3). This 36% content of σ^{70} is a slightly overestimated number because there is a σ^{70}-sized polypeptide in the sample as described above.

Enzyme Activity. We determined the RNA polymerase holoenzyme activity quantitatively by two methods as described under Materials and Methods. One was to measure the steady-state rates of abortive initiation using different ratios of protein and promoter-containing DNA fragment. Another was to measure the formation of RNA transcripts from T7 D111 template. Both methods gave the same results (Figures 4 and 5). The percent of active holoenzyme molecules before and after the Mono Q column was 28% and 90% of the maximum possible, respectively.

The latter method, a quantitative assay to measure RNA polymerase activity on T7 DNA, cannot only be used to determine the activity of a holoenzyme but also to determine its rate of elongation as described by Chamberlin et al. (1979). Although the holoenzyme activity after the Mono Q column was increased about 3-fold, its elongation rate was the same as before the column (Figure 4). Therefore, Mono Q chromatography is an effective step to increase the specific activity of holoenzyme, but it has no detectable effect on the elongation properties of the enzyme. It is likely that the factors that affect the elongation, such as NusA protein, were absent from the sample before the Mono Q column. It is known that NusA protein dissociates completely from RNA polymerase at 0.5 M NaCl (Greenblatt & Li, 1981), and it is likely that it was separated before the Mono Q column.

Discussion

It is desirable to carry out the preparation as rapidly as possible in order to obtain highly active enzyme. If necessary, a convenient break point during the procedure is following the ammonium sulfate precipitation step, since at that point the RNA polymerase is stable.

We found that the capacity of both the analytical and preparative Mono Q columns was lower than manufacturer's specifications for general use, both because the onput sample (pooled Sephacryl S-300 peak) is already quite pure and because core polymerase and holoenzyme are separated by less than 0.02 M in the salt gradient (see Figure 2). Therefore, in order to separate the core from the holoenzyme effectively, the gradient cannot be too steep, and the flow rate must be set slow enough to give acceptable resolution, while avoiding unnecessary dilution.

Mono Q chromatography has been used by others for resolving very similar forms of proteins, such as cytosolic and particulate transglutaminase (Hand et al. 1988), even forms of GTP binding proteins indistinguishable on immunoblots (Goldsmith et al., 1988), and different serotypes of botulinum toxin (Woody & DasGupta, 1988).

We often see protein peak heterogeneity in the Mono Q chromatograph of both the core and holoenzyme peaks (see Figure 2). Such protein peak heterogeneity has been described by other groups during purification of proteins. Mulroonay et al. (1988) noted that urease activity eluted as a doublet from a Mono Q column. In some cases, such peak heterogeneity corresponds to different isoforms of proteins (Olsson & Hogstrand, 1986). We find the ω subunit in all the core peaks and holoenzyme peaks. We have found σ^{32} to be present in the leading edge of the core peak (pool A) in wild-type cells (Figure 3 and unpublished results). Since the amount of σ^{32} found in the leading shoulder of the core peak is relatively small, we believe that it is not causing the heterogeneity shown in the chromatogram. We also see heterogeneity in the holoenzyme chromatogram. Upon analysis of various fractions by SDS–polyacrylamide gels and isoelectric focusing, we see nothing that correlates with this heterogeneity. The hete-

rogeneity is almost always seen, although the relative proportions of the multiple peaks vary somewhat among preparations. In some cases, when the holo peaks are rerun, they rerun as single peaks. No obvious difference in enzymatic properties of the peaks has been observed. We favor the existence of moderately stable conformational isomers as an explanation for peak heterogeneity. More work on this subject is in progress.

The recovery of RNA polymerase (both core and holo) from the Mono Q column is between 60 and 65%. Addition of nonionic detergents such as 0.1% NP-40 to the buffer did not improve the recovery.

We have used this method to purify highly active and electrophoretically pure RNA polymerase holoenzyme. The method yields holoenzyme with virtually 100% σ^{70} saturation. We believe this is one of the reasons for its high activity. In fact, the activity of proteins correlates well with their σ^{70} content (see Table I and Figures 4 and 5). The enzyme prepared by this method will therefore be suitable and essential for many biochemical and physical studies where pure and active RNA polymerase is required.

A byproduct of this procedure is pure core RNA polymerase that is free of σ^{70} as detected by Western blot. This pure core enzyme therefore can be used for some special studies. For example, different σ factors can be added to the core to generate holoenzymes with varying specificities.

Mono Q chromatography can be used as a final polishing step in the purification of RNA polymerase. While the early steps can be different from what are described in this paper, a final Mono Q chromatographic step will increase the purity and specific activity of RNA polymerase holoenzyme, since $\alpha_2\beta$ subassemblies, σ^{70}-sized contaminating protein, and core are separated from holoenzyme. We have also found this Mono Q step useful is isolating active holoenzyme from old samples of holoenzyme that have deteriorated over time during storage. We are testing the columns for their ability to resolve different forms of holoenzyme containing not only σ^{70} but also other minor *E. coli* σ family members as well.

Although Mono Q columns are expensive, the cost for each run is relatively low because the columns can be reused many times and are very durable. Some columns which we have used more than a hundred times are still in good condition. Also, the chromatographic conditions are highly reproducible, and the procedure is rapid. Even though the capacity of the column is low for partially purified RNA polymerase, samples may be divided into portions, and several runs can be reproducibly done if a larger RNA polymerase preparation is desired.

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Registry No. RNA polymerase, 9014-24-8.

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