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Purification of bacterial RNA polymerase: tools and protocols

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Summary

Bacterial RNA polymerase is the first point of gene expression and a validated target for antibiotics. Studied for several decades, the *Escherichia coli* transcriptional apparatus is by far the best characterized, with numerous RNA polymerase mutants and auxiliary factors isolated and analyzed in great detail. Since the *E. coli* enzyme was refractory to crystallization, structural studies have been focused on *Thermus* RNA polymerase s, revealing atomic details of the catalytic center and RNA polymerase interactions with nucleic acids, antibiotics, and regulatory proteins. However, numerous differences between these enzymes, including resistance of *Thermus* RNA polymerases to some antibiotics, underscored the importance of the *E. coli* enzyme structures. Three groups published the se long awaited structures in 2013, enabling functional and structural studies of the same model system. This progress was made possible, in large part, by the use of multicistronic vectors for expression of the *E. coli* enzyme in large quantities and in a highly active form. Here we describe the commonly used vectors and procedures for purification of the *E. coli* RNA polymerase.

Keywords

Transcription; RNA polymerase; Expression; Purification

1. Introduction

In bacteria, a single multisubunit RNA polymerase (RNAP) transcribes all genes. The holoenzyme is composed of a (most commonly) α2ββ′ω core, which carries out all types of catalysis but cannot specifically bind to or melt the DNA, and one of σ factors, which recognize distinct promoter elements and facilitate DNA strand separation near the transcription start site. Understanding the regulation of gene expression requires studies of the basic molecular mechanism of RNAP and the ways accessory factors interact with the enzyme during all stages of transcription. Bacterial RNAP is also a validated target for antibiotic therapies, but only two RNAP inhibitors are currently used in clinical practice.

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Among many methods that have been applied to study transcription, structural analysis is indispensable in providing the atomic level of detail required for understanding of catalysis and design of antibiotics targeting the transcription apparatus. After the first structure of *T. aquaticus* RNAP was solved by the Darst lab in 1999 (Zhang et al., 1999), *Thermus* enzymes became the structural model of choice. Many key features have been characterized using these thermophilic RNAPs, including the active site geometry and conformational transitions during the nucleotide addition cycle (Vassylyev et al., 2007b), RNAP interactions with the nucleic acids in initiation (Zhang et al., 2012) and elongation complexes (Vassylyev et al., 2007a), and binding sites for σ (Vassylyev et al., 2002), elongation factors (Tagami et al., 2010), and antibiotics (Belogurov et al., 2009; Campbell et al., 2001; Vassylyev et al., 2005; Vassylyev et al., 2007b).

However, the gap between the structural analysis and the functional studies of bacterial transcription, carried out predominantly with mesophilic enzymes, was expanding. *Thermus* enzymes are an excellent model for the dissection of the catalytic mechanism, which is highly conserved among multisubunit RNAPs. In contrast, RNAP regions distant from the active site are quite divergent, and many species-specific insertions are located in the β and β′ subunits (Lane and Darst, 2010), complicating sequence alignments. Regulatory strategies and auxiliary factors, which in many cases bind to divergent sites, appear to be quite distinct, with *Thermus* species lacking many of the accessory proteins characterized in *E. coli*. *Thermus* RNAPs are resistant to several inhibitors of the *E. coli* enzyme, including fidaxomycin, a recently approved by the FDA antibiotic for treatment of *Clostridium difficile* infections. Finally, even when a ligand binds to both RNAPs, the binding sites could be far apart; for example, the regulator of stringent response ppGpp binds to different sites, and has different effects, on the *E. coli* and *T. thermophilus* enzymes (Artsimovitch et al., 2004; Ross et al., 2013; Zuo et al., 2013).

Ideally, functional and structural studies should be carried out on the same model system. By obtaining the structure of the *E. coli* RNAP, the Murakami (Murakami, 2013), Steitz (Zuo et al., 2013) and Darst (Bae et al., 2013)labs have made this possible. This breakthrough will facilitate mechanistic analysis of transcriptional machinery, aid in improvement of existing RNAP inhibitors, and may lead to development of novel antibiotics which are urgently needed to treat all infections, and those caused by Gram-negative pathogens in particular.

The success in obtaining these structures came from purification of a recombinant *E. coli* RNAP core assembled *in vivo* from co-expressed subunits. The first multicistronic vector (Fig. 1) contained *rpoA*, *rpoB* and *rpoC* genes (encoding the α, β and β′ subunits, respectively) expressed from the T7 gene 10 promoter (Artsimovitch et al., 2003). The wild-type spacer between the *rpoB* and *rpoC* genes, which are likely assembled co-translationally *in vivo*, was maintained and a purification tag was placed at the C-terminus of β′ to purify the complete core, which is assembled in the order of $\alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$. Using this vector, it was possible to purify active RNAP, yet no well-diffracting crystals could be obtained. Surprisingly, a nonessential 91-residue ω subunit proved to be the key missing factor. After it became clear that ω is essential for RNAP regulation by ppGpp (Vrentas et al., 2005), we added *rpoZ* to the *rpoABC* cassette to yield pVS10 (Belogurov et al., 2007), a version that

was used by the Steitz lab (Zuo et al., 2013), a similar construct was used by Darst a co-workers (Bae et al., 2013; Twist et al., 2011) co-expressing ω from a compatible plasmid in the Murakami lab (Murakami, 2013) worked equally well.

These structures will provide a framework for the analysis of the basic mechanism of catalysis and its regulation. In the course of this analysis, construction of many variant RNAPs with substitutions at chosen locations will be indispensable to test molecular models, design enzymes for specific applications, and perhaps to improve the resolution of the structures. Here we describe the commonly used vectors and procedures for purification of the *E. coli* RNAP that will facilitate these studies.

2. Vectors for RNAP expression and mutagenesis

Many vectors are available for mutagenesis, subcloning, and expression of the *E. coli* RNAP subunits. Here, we provide examples of the vectors we are used routinely in several laboratories. Some of these have been published, and all are available upon request.

2.1. Polycistronic vectors

These vectors (*see* Table 1) allow for efficient expression of the RNAP core enzyme, $\alpha_2\beta\beta'\omega$, either from a single vector or from two compatible vectors, one of which carries the *rpoZ* gene encoding the ω subunit.

All *rpo* genes are expressed from T7 gene 10 promoter in a “standard” pET vector background, with T7 terminator downstream from *rpoZ*. The levels of expression follow the order $\omega > \alpha_2 > \beta > \beta'$, thus only the fully-assembled, ω-saturated core is purified when a purification tag is located at the C-terminus of the β′ subunit. Other advantages of the co-overexpression system are (*i*) a streamlined purification protocol; (*ii*) relatively high yields; (*iii*) ability to purify the core enzyme that contains toxic and assembly-defective mutations, such as the deletions of “dispensable regions” (Artsimovitch et al., 2003); and (*iv*) a “ crystallizable” protein (Murakami, 2013; Zuo et al., 2013).

The purified core enzyme is catalytically active on nucleic acid scaffolds but not in promoter-dependent initiation, which requires an initiation factor σ (typically σ^{70}) added *in trans;* see chapter 11 for an example of (a variant) *E. coli* σ^{70} purification protocol.

2.2. Single-subunit vectors

However, these vectors are large (15+ kb) and have very few remaining restriction sites that can be used for subcloning of the altered fragments. Therefore, we carry out mutagenesis in smaller vectors, encoding single subunits or their fragments (*see* Table 2 and Fig. 2).

In these vectors, an *rpo* gene is expressed under control of T7 gene 10 promoter (*rpoA* and *rpoD*), IPTG-inducible P_{trc} promoter (*rpoB* and *rpoC*), or arabinose-inducible P_{BAD} promoter (*rpoZ*).

The single-subunit vectors can be used to express altered subunits alone, most commonly to test their ability to complement defects conferred by temperature-sensitive chromosomal *rpo*

alleles (Weilbaecher et al., 1994)or to confer resistance to an antibiotic (Yuzenkova et al., 2002).

3. Materials

All solutions are prepared using ultrapure water (resistivity of 18 MΩ, e.g. MilliQ) and analytical/molecular biology grade reagents and filtered through bottle-top 0.2 μm PES filter to remove particulate contaminations (unless indicated otherwise). Bacterial growth media are made using purified (by deionization or reverse osmosis to resistivity of >5 MΩ, e.g. Elix) water and biotechnology grade media components and sterilized by autoclaving. All sterilized media can be stored at room temperature (unless indicated otherwise), all protein purification and chromatographic solutions are stored at 4 °C (unless indicated otherwise). We list the manufacturers for supplies and reagents that we currently use; alternative sources can be tried but we cannot guarantee that they will work identically.

3.1. Bacterial growth media and supplements

1. LB growth medium for liquid culture: weigh 125 g of Difco LB Miller (Luria-Bertani) dry pre-mix, dissolve in 4.5 L of water, add water to 5 L total volume, and mix. Dispense 600 mL of prepared LB per 2 L Erlenmeyer flask (total of 8), cover with heavy duty aluminum foil, secure the foil to the flask with the autoclave tape. Dispense the remaining LB (~200 mL) into an autoclavable media bottle. Autoclave media and store at room temperature.
2. 100 mg/mL stock carbenicillin solution: weigh 0.5 g carbenicillin, dissolve in 4 mL of water, add water to 5 mL total, filter through 0.2 μm PES syringe filter, aliquot 1 mL per 1.5 ml microcentrifuge tube, and store at −20°C (*see* Note 1).
3. LB+carbenicillin agar plates: weigh 40 of Difco LB Agar, place into 1 L bottle, add magnetic stir bar and water to 1 L total, cover with foil, secure the foil to the flask with the autoclave tape. Bring the mix to boiling on the hot plate with heavy duty aluminum stirring, and sterilize by autoclaving. Cool autoclaved medium to 50–60°C while stirring, add 1 mL of 100 mg/mL carbenicillin stock solution, continue stirring for another 5 min, and pour the plates. Store plates at 4°C.
4. 1 M stock solution of IPTG: weigh 1.19 g of IPTG, dissolve in 4 mL of water, add water to 5 mL total, filter through 0.2 μm PES syringe filter, make immediately before use or aliquot 1 mL per 1.5 ml microcentrifuge tube, and store at −20°C.

3.2. Protein purification and chromatographic solutions

1. 1.1 M stock solution of Tris-HCl (pH 6.9): weigh 121.14 g of Tris base (e.g. Trizma base), dissolve in 900 ml of water, adjust pH to 6.9 with concentrated HCl (~80 ml), add water to 1 L total.

[1]Carbenicillin can be substituted by ampicillin, but the former performs better due to its higher stability.

2. Cell lysis buffer (50 mM Tris-HCl (pH 6.9), 500 mM NaCl, 5% glycerol): weigh 29.22 g of NaCl, dissolve in 500 mL of water, add 50 mL of 1 M Tris-HCl (pH 6.9), add 50 mL of glycerol, add water to 1 L total.

3. 4 M stock solution of imidazole: weigh 68.08 g of imidazole, dissolve in 200 mL of water, adjust pH to 7.4 with HCl, add water to 250 mL total.

4. 0.5 M stock solution of EDTA (pH 8.0): weigh 186.12 g of $Na_2EDTA{\cdot}2H_2O$, dissolve in 800 mL of water, adjust pH to 8.0 by adding NaOH (~20 g of pellets), add water to 1 L total.

5. 1 M stock solution of DTT: weigh 1.54 g of DL-DTT, dissolve in 8 mL of water, add water to 10 mL total, filter through 0.2 um PES syringe filter, aliquot 1 mL per 1.5 ml microcentrifuge tube, and store at −20°C.

6. 50 mg/mL stock solution of lysozyme: weigh 0.5 g of lysozyme (e.g. human recombinant lysozyme, Sigma-Aldrich), dissolve in 4 ml of water, add water to 5 mL total. Make immediately before use, do not store.

7. Buffer A (50 mM Tris-HCl (pH 6.9), 5% glycerol, 0.5 mM EDTA, 1 mM DTT): combine 500 mL water, 50 mL of 1 M Tris-HCl (pH 6.9), 50 mL of glycerol, 1 mL of 0.5 M EDTA (pH 8.0), 1 mL of 1 M DTT, 1 tablet of complete EDTA-free protease inhibitors cocktail (Roche Applied Science). Add water to 1 L total.

8. Buffer B (50 mM Tris-HCl (pH 6.9), 1.5 M NaCl, 5% glycerol, 0.5 mM EDTA, 1 mM DTT): weigh 87.66 g of NaCl, dissolve in 500 mL water, add 50 mL of 1 M Tris-HCl (pH 6.9), 50 mL of glycerol, 1 mL of 0.5 M EDTA (pH 8.0), 1 mL of 1 M DTT, 1 tablet of complete EDTA-free protease inhibitors cocktail (Roche Applied Science). Add water to 1 L total.

9. 1 M stock solution of Tris-HCl (pH 7.5): weigh 121.14 g of Tris base (e.g. Trizma base), dissolve in 900 ml of water, adjust pH to 7.5 with concentrated HCl (~65 ml), add water to 1 L total.

10. Ni binding buffer (cell lysis buffer + 20 mM imidazole): combine 0.25 mL of 4 M imidazole solution and 49.75 mL of cell lysis buffer in a 50 mL conical tube (e.g. BD Falcon), mix by vortexing and keep on ice. Make immediately prior to use, do not store.

11. Ni elution buffer (cell lysis buffer + 250 mM imidazole): combine 0.625 mL of 4 M imidazole solution and 9.375 mL of cell lysis buffer, mix by vortexing and keep on ice. Make immediately prior to use, do not store.

12. Dialysis buffer AB5 (50 mM Tris-HCl (pH 6.9), 75 mM NaCl, 5% glycerol, 0.5 mM EDTA, 1 mM DTT): weigh 4.38 g of NaCl, dissolve in 500 mL water, add 50 mL of 1 M Tris-HCl (pH 6.9), 50 mL of glycerol, 1 mL of 0.5 M EDTA (pH 8.0), 1 mL of 1 M DTT. Add water to 1 L total.

13. RNAP storage buffer (10 mM Tris-HCl (pH 7.5), 50% glycerol, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT): weigh 5.85 g of NaCl, dissolve in 300 mL of water,

add 10 mL 1 M Tris-HCl (pH 7.5), 0.2 mL 500 mM EDTA (pH 8.0), 0.1 mL 1 M DTT, 500 mL glycerol. Add water to 1 L total.

14. Commercial ready-made materials for running and staining gels. We use NuPAGE MES SDS Running Buffer, NuPAGE LDS Loading Buffer, NuPAGE 4–12% Bis-Tris Gel (all – Life Technologies), Precision Plus Protein Dual Color Standards (Bio-Rad), and GelCode Blue Stain Reagent (Thermo).

4. Methods

All protein purification procedures are performed with refrigeration (on ice, in cold room or in a chromatographic refrigerator, as indicated). All time estimates are given only as a general guidance.

4.1. Over-expression of E. coli RNAP core

1. Transform T7-based over-expression strain of *E. coli* (*e.g.*, BL21 (λDE3))with pVS10 plasmid by electroporation or using chemically competent cells, plate on LB+carbenicillin agar, incubate overnight at 37°C. Remove plate to 4°C to suppress growth of satellite colonies.
2. Inoculate single colony of pVS10-transformed over-expression strain into 100 mL of LB supplemented with 0.1 mL 100 mg/mL carbenicillin solution (0.1 mg/L final concentration) in 250 mL Erlenmeyer flask. Incubate with agitation (250 rpm) overnight at 37°C.
3. Inoculate each of the 2 L Erlenmeyer flasks (600 ml LB) with overnight culture (1:100, v:v), supplement with 0.6 mL carbenicilin stock solution, and incubate with agitation (250 rpm) at 37°C. Monitor culture growth periodically by measuring OD_{600}.
4. Induce RNAP core expression by adding 0.6 mL of 1 M IPTG (to final concentration of 1 mM) once OD600 reaches 0.75 (~4 hours after inoculation). Continue incubation for another 3 hours (*see* Note 2).
5. Harvest cells by centrifugation (6000 g, 10 min at 4 °C, *e.g.* using 1 L bottles in F10S-4X1000 LEX rotor and Sorvall RC6+ centrifuge). Decant and discard the supernatant. Proceed to RNAP core purification or store cell pellets at −80°C (thaw pellets on ice before proceeding with protein purification).

4.2. Cell lysis and clearing of the cell lysate

1. Place centrifuge bottles with cell pellets on ice.

[2]Wild-type *E. coli* RNAP core expresses well when induced from pVS10 plasmid by IPTG at 37 °C, whereas some “mutant” forms (such as large indels) express better at lower temperatures (*e.g.*, 30 °C). Use longer induction times if the cultures are grown at temperatures below 37 °C (*e.g.*, induce for 5 hours at 30 °C as opposed to 3 hours at 37 °C). As an alternative to improve yield and solubility of expressed proteins, use auto-induction protocol, which utilizes expression induction in the stationary phase (Studier, 2005). We have used it extensively with pVS10 and derivative plasmids and a variety of expression strains with great success.

2. Dispense 100 mL of cell lysis buffer into 2 50 mL conical tubes, add 1 tablet of complete EDTA-free protease inhibitors cocktail (Roche Applied Science) into each tube, dissolve by vortexing. Incubate tubes on ice for 15 min.

3. Resuspend cell pellets in cell lysis buffer on ice, using wide tip 10 mL pipette. Estimate final volume (*e.g.*, using graduations on the tubes) and supplement cell suspension with freshly made 50 mg/mL solution of lysozyme to final concentration of 1 mg/mL. Incubate suspension on ice for 30 min (*see* Note 3).

4. Disrupt cells by ultrasonication on ice; *e.g.*, using Tapped Bio Horn and Branson Sonifier Digital 450 at 60% power output, 8×30 sec continuous applications with 2 min cooling intermissions (*see* Note 4).

5. Remove cell debris by centrifugation (29000 g, 30 min at 40°C, *e.g.* using 50 mL Oak Ridge Tubes (Nalgene) in F21S-8X50y BioSEAL rotor and Sorvall RC6+ centrifuge). Transfer supernatants into clean tubes (*e.g.*, Oak Ridge) by aspiration, discard the pellets, and repeat centrifugation (29000 g, 45 min at 4°C; *see* Note 5). Discard the pelleted debris, transfer supernatants into 50 mL conical tubes, keep on ice.

4.3. Ni-affinity chromatography of His_6-tagged RNAP core

All steps take place in the cold room or in a chromatographic refrigerator.

1. Estimate volume of cleared cell lysate using volumetric graduation of conical tubes. Add 4 M imidazole stock solution to each tube to final concentration of 20 mM.

2. Set a His GraviTrap column (GE Healthcare Life Science) into a vertical rack (*e.g.*, Bio-Rad Poly Column Rack) with flow-through collection reservoir for each 50 ml of lysate (*see* Note 6). Attach Labmate (GE Healthcare Life Science) buffer extension reservoir to each column. The rest of the instructions apply to each column.

3. Wash column by gravity flow with 10 mL of ultrapure water.

4. Wash column with 10 mL of Ni binding buffer.

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[3]Lysozyme activity varies greatly with source and preparation. We find human recombinant lysozyme presently to be the best value for large scale preparations, based on price and specific activity.

[4]As alternative for cell disruption use French press. We would not recommend any of the commercially available cell lysis formulations as they didn't perform on par with sonication or the French press. Cell disruptors such as EmulsiFlex (Avestin) used in lieu of sonication tend to produce less active preparations of RNAP core.

[5]Clearing extract by high-speed centrifugation is essential for efficient gravity flow chromatography, as cell debris when not removed clogs the columns filters and bed, interfering with the flow rate and uniformity, even if when column is advertised as capable of handling "uncleared" cell extracts. If you do not have access to centrifuge capable of delivering 29000 g acceleration, perform batch-binding of His_6-tagged RNAP core to Ni Sepharose Fast Flow and pack it into PD-10 gravity column (both GE Healthcare Life Science) per manufacturer's instructions.

[6]His Gravitrap column is packed with Ni Sepharose Fast Flow beads (GE Healthcare Life Science), a medium density (15 μM of Ni^{++} per ml of beads) affinity resin for purification of His_6-tagged proteins. As alternative for purification of His_6-tagged RNAP core a number of Ni^{++} or Co^{++} resins can be used without significant changes in protocol or drastic drop in efficiency; note that Co^{++} affinity media usually have lower protein-binding capacity than Ni^{++} ones. Of interest is high density Ni Agarose from Gold Biotechnology, which features 20–40 μM of Ni^{++} per ml of beads.

5. Apply 50 mL of cleared extract (supplemented with 20 mM imidazole) to the column, drain completely.
6. Wash column with 40 mL of Ni binding buffer.
7. Replace collection reservoir with microcentrifuge tube rack. Label 8 1.5 mL microcentrifuge tubes (*e.g.*, Eppendorf Protein LoBind) 1 through 8 for fraction collection, and place them under the column.
8. Apply 0.5 mL of Ni Elution buffer to the column. Collect flow-through into tube 1.
9. Sequentially apply 7 1 mL aliquots of Ni Elution buffer to the column. Collect flow-through into tubes 2–8. Place tubes on ice.
10. Confirm the presence of RNAP core in fractions 2–8 by SDS-PAGE; use 10–20 μL of each fraction.
11. Combine all RNAP core-containing fractions and dialyze them (*e.g.*, using 3.500 MWCO Slide-A-Lyzer Dialysis Cassette, Thermo) overnight against 100 volumes of dialysis buffer AB5.

4.4. Heparin-affinity chromatography of RNAP core

All the following steps can be implemented in instrument-specific fashion on any medium-pressure liquid chromatography system, the method as written is realized on AKTA FPLC (GE Healthcare Life Science); *see* Note 7.

1. Place pump inlet tubing A and B into buffers A and B, respectively. Run the pump (4 mL/min) without at 100% B until conductivity stabilizes at ~95 mS/cm (~10–15 min). Switch the pump to 0% B and continue running until conductivity stabilizes at ~4 mS/cm (~10–15 min). Lower the rate to 1 mL/min and connect HiPrep Heparin FF 16/10 column (GE Healthcare Life Science); *see* Note 8.
2. To equilibrate the column set the pump at 2 mL/min and 5% B, run until conductivity stabilizes at ~9.5 mS/cm.
3. Load dialyzed sample onto the column at 1 mL/min, wash the column by 20 mL of 5% B at the same rate.
4. Apply gradient 5% B to 100% B over 200 mL at 1 mL/min, monitor protein elution from the column by UV absorbance at 280 or 215 nm. Begin collecting 1.5 mL fractions when conductivity reaches 20 mS/cm. RNAP core usually elutes from heparin column at conductivity between 30 and 45 mS/cm. Stop collecting fractions once conductivity reaches 60 mS/cm. At this time switch the pump to

[7]Heparin-affinity chromatography removes most of the nucleic acid contaminations present in RNAP preparation after the Ni-affinity step. Performing it prior to ion-exchange chromatography is thus very important since nucleic acids have higher affinity to the MonoQ column than does RNAP and if not removed they will interfere with core binding.

[8]Pre-packed HiTrap Heparin FF 16/10 is the preferred column for preparative scale heparin-affinity chromatography, both in terms of capacity and reproducibility. It can be substituted with similar size column packed in-house with Heparin Sepharose 6 Fast Flow (GE Healthcare Life Science); another pre-packed heparin-affinity column from GE, 5 mL HiTrap Heparin HP has much lower capacity and reduced resolution. Connecting several HiTrap columns head-to-tail may approach 16/10 in protein-binding capacity but compromises resolution even further.

100% B and 2 mL/min, continue running for 20 min, switch the pump to 5% B, run for another 20 min and end the run.

5. Based on UV absorbance and conductivity profiles identify RNAP core peak between 30 and 45 mS/cm. Select fractions with UV absorbance within 20% of the maximum value, confirm presence of RNAP core there by SDS-PAGE (use 30–45 μL of each fraction).

6. Combine selected fractions and dialyze them overnight against 100 volumes of dialysis buffer AB5.

4.5. Ion-exchange chromatography of RNAP core

1. Start the pump at 1 mL/min and 5% B, replace heparin column with MonoQ 10/100 GL (GE Healthcare Life Science); *see* Note 9. Continue running till conductivity stabilizes at ~9.5 mS/cm (~15 min).

2. Load dialyzed sample onto the column at 1 mL/min, wash the column by 20 mL of 5% B at the same rate.

3. Apply gradient 5% B to 100% B over 200 mL at 1 mL/min, monitor protein elution from the column by UV absorbance at 280 or 215 nm. Begin collecting 0.8 mL fractions when conductivity reaches 15 mS/cm. RNAP core usually elutes from MonoQ column at conductivity between 20 and 30 mS/cm. Stop collecting fractions once conductivity reaches 50 mS/cm. At this time switch the pump to 100% B, continue running for 20 min, switch the pump to 5% B, run for another 20 min and end the run.

4. Based on UV absorbance and conductivity profiles identify RNAP core peak between 20 and 30 mS/cm. Select fractions with UV absorbance within 20% of the maximum value, confirm presence of RNAP core there in and their purity by SDS-PAGE (use 20 μL of each fraction); *see* Figure 3 for an example.

5. Combine the fractions with highest concentration and purity of RNAP core, change the buffer as per downstream application (*see* Note 10). For storage, dialyze overnight against 500 volumes of RNAP storage buffer, aliquot as needed into screw-top gasketed microcentrifuge tubes, flash-freeze aliquots in liquid nitrogen, and store at −80°C.

Acknowledgments

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[9]MonoQ columns provide the best resolution for RNAP core purification. Q Sepharose is not a recommended substitute due to its significantly reduced resolution. With some success, SOURCE 15Q 4.6/100 PE (GE Healthcare Life Science) can replace MonoQ 10/100 GL, albeit with lower resolution which may require additional polishing steps.

[10]*E. coli* RNAP core purified according to the preceding streamlined protocol is as a rule 95+% pure and 80+% active in *in vitro* transcription applications. For additional polishing steps, repeat ion-exchange chromatographic step or perform size-exclusion chromatography (*e.g.*, using Superdex 200 10/300 GL (GE Healthcare Life Science) in 50% buffer B).

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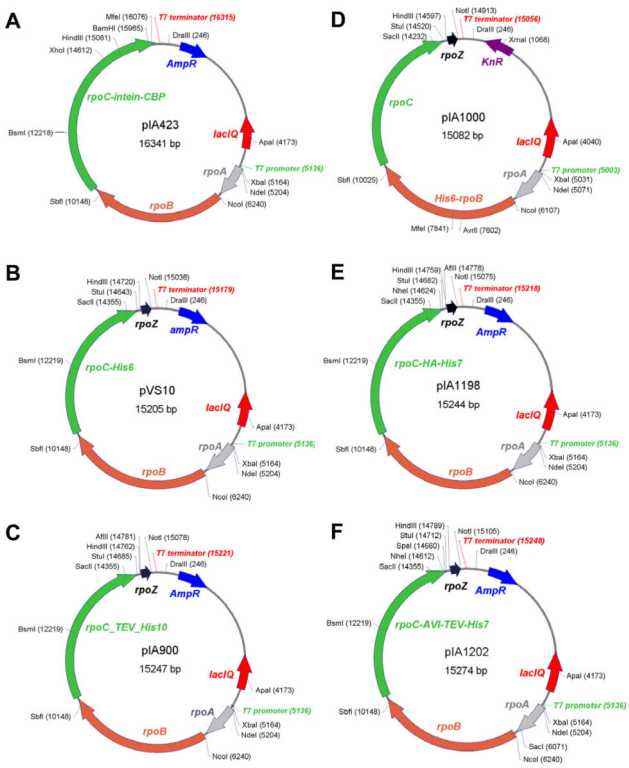

Fig. 1. Polycistronic vectors for expression of *E. coli* core RNAP

Representative examples of plasmids used most often are shown, other variants may be more suitable for a particular application.

A. pIA423 contains *rpoA*, *B*and *C* genes. An intein-chitin binding protein tag (IMPACT-Ni$^{tride;}$, NEB) allows for easy, one-step purification of the core RNAP on chitin matrix.

B. pVS10 (*rpoA*, *B*, *C* and *Z*) encodes a C-terminally His-tagged β′.

C. pIA900 (*rpoA*, *B*, *C* and *Z*) encodes a C-terminally His-tagged β′ which can be removed using TEV protease.

D. pIA1000 (*rpoA*, *B*, *C* and *Z*) encodes an N-terminally His-tagged β.

E. pIA1198 (*rpoA*, *B*, *C* and *Z*) encodes a β′ with C-terminal HA and His tags.

F. pIA1202 (*rpoA*, *B*, *C* and *Z*) encodes a β′ with a C-terminal minimal biotin tag (AVI tag) followed by a His tag ; the latter can be removed using TEV protease, leaving the AVI tag intact.

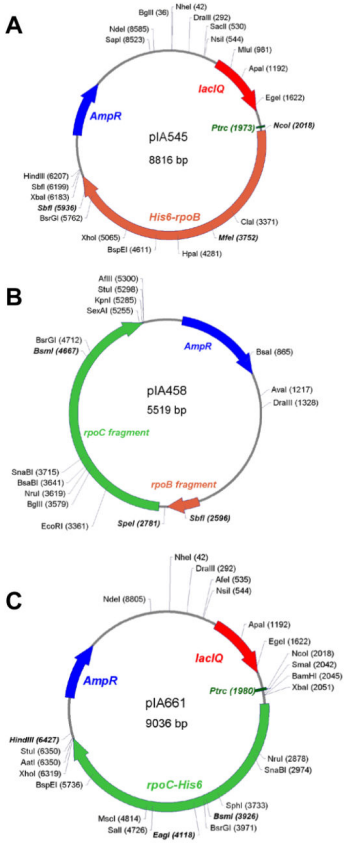

Fig. 2. Plasmids for *rpoB* and *rpoC* mutagenesis

mutant versions of shorter *rpoA* and *rpoZ* can be obtained synthetically from commercial sources. We introduce a desired substitution by site-directed mutagenesis (for example, QuikChange), adding a silent restriction site (if possible) for easy screening. We sequence the mutant fragment between two closest convenient restriction sites and then clone this fragment back into the original plasmid (to make sure there are no mutations elsewhere). Finally, we reclone a fragment flanked by two sites that are unique in **pVS10** (or another similar vector).

A. Mutagenesis of the β subunit. We use three vectors that encode the N-terminally His_6-tagged β subunit under control of P_{trc} promoter most frequently. The wild-type plasmid **pIA545** (shown)encodes the wild-type *rpoB* except a silent *Mfe*I site at β residue 555 ; it complements a temperature sensitive defect in the *rpoB* gene. Two other plasmids have the same structure but lack the *Mfe*I site; instead, they encode rifampicin-resistant *rpoB* alleles: β^{D516V} in **pIA223** and β^{S531F} in **pIA178**. The activity of altered RNAPs can be monitored in the presence of rifampicin, to eliminate concerns of contamination with the wild-type core. Following mutagenesis, we reclone the *Nco*I-*Sbf*I fragment, which encompasses almost entire *rpoB*)into a desired vector (*e.g.*, **pIA821**).

B. Mutagenesis of the C-terminus of β and the N-terminal half of β′. **pIA458** is a Litmus-based plasmid that does not encode any complete RNAP subunit. Mutagenized fragments can be transferred into a vector of choice (*e.g.*, pIA900 for β′ mutants) on the *Sbf*I-*Bsm*I

fragment. pIA458 has as an *Spe*I site in the intergenic region separating the *rpoB* and *rpoC* genes that can be used for screening .

C. Mutagenesis of the C-terminal half of the β subunit. We use **pIA661**, which encodes the wild-type *rpoC* except for a silent *Eag*I site at β′ residue 675, most frequently. Two variants carrying antibiotic-resistant alleles, **pIA388** and **pIA635** (*see* Table 2) can be used instead. These plasmids encode the C-terminally His_6 tagged β′ subunit under control of P_{trc} promoter. The wild-type plasmid complements a temperature-sensitive defect in the *rpoC* gene. Following mutagenesis, we reclone the *Bsm*I-Hin*dIII* fragment into a desired vector (*e.g*., **pVS10**).

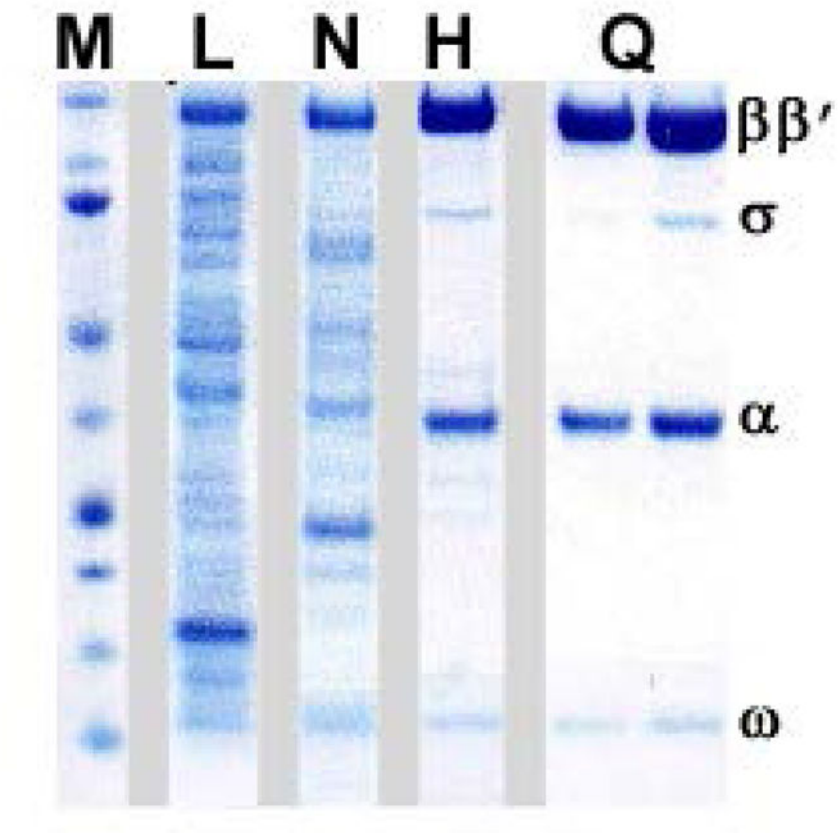

Fig. 3. Sample purification of the *E. coli* RNAP

A variant with a deletion in the β′ jaw (β′ residues 1149–1190) was used. Samples of the cell lysate (L), Ni-NTA eluate (N; *see* section **4.3**), heparin peak fractions (H; *see* section **4.4**), and MonoQ peak fractions (H; *see* section **4.5**) were analyzed by SDS-PAGE using a 4–12% NuPAGE® Bis-Tris pre-cast polyacrylamide gel (Life Technologies). Chromatography through the MonoQ column separates the core enzyme (left) from the holoenzyme (right). The lane marked M was loaded with the molecular weight markers; the positions of the RNAP subunits are indicated on the right.

Table 1

Polycistronic *E. coli* RNAP expression vectors.

Plasmid	Tag and Ab^R allele	Features	Origin & Resistance
Vectors without the *rpoZ* gene			
pIA581	His_6:β	T7P—α—His_6:β—β′; the His_6 tag at the N-terminus of *rpoB*	pMB1 Ampicillin
pIA299	β′:His_6	T7P—α—β—β′:His_6; the His_6 tag at the C-terminus of *rpoC*	pMB1 Ampicillin
pIA423	β′:CBP	T7P—α—β—β′:CBP; a chitin-binding protein/intein tag (CBP; Impact-Ni$^{tride;}$, NEB) fused to the C-terminus of *rpoC*	pMB1 Ampicillin
pIA468	β′:BCCP	T7P—α—β—β′:BCCP; residues 71–156 of the biotin carboxyl carrier protein (BCCP) fused to the C-terminus of *rpoC*	pMB1 Ampicillin
pIA439	His_6:β Rif^R β′:CBP	T7P—α—His_6:β^{S531F}—β′:CBP; His_6 tag at the N-terminus of *rpoB*; Rif^R S531F allele; CBP tag at the C-terminus of *rpoC*	pMB1 Ampicillin
pIA358	α:His_6 β′:CBP	T7P—α:His_6—β—β′:CBP; the His_6 tag at the C-terminus of *rpoA;* CBP tag at the C-terminus of *rpoC*	pMB1 Ampicillin
pIA477	His_6:β Rif^R β′:BCCP	T7P—α—His_6:β^{H526Y}—β′:BCCP: His_6 tag at the N-terminus of *rpoB*; Rif^R H526Y allele; BCCP tag at the C-terminus of *rpoC*	pMB1 Ampicillin
pIA570	His_6:β Rif^R	T7P—α—His_6:β^{S531F}—β′; the His_6 tag at the N-terminus of *rpoB*; Rif^R S531F allele	pMB1 Ampicillin
pIA639	β′:His_6 Stl^R	T7P—α—β—β′^{S793F}:His_6; the His_6 tag at the C-terminus of *rpoC;* Stl^R S793F allele	pMB1 Ampicillin
Vectors with the *rpoZ* gene			
pIA1158	α:His_6	T7P—α:His_6—β—β′—ω; the His_6 tag at the C-terminus of *rpoA*	pMB1 Kanamycin
pIA1000	His_6:β	T7P—α—His_6:β—β′—ω; the His_6 tag at the *rpoB* N-terminus; unique silent *Avr*II and *Mfe*I sites at β residues 477 and 555	pMB1 Kanamycin
pIA1070	His_6:β Rif^R	T7P—α—His_6:β^{D516V}—β′—ω; the His_6 tag at the N-terminus of *rpoB;* Rif^R D516V allele	pMB1 Ampicillin
pVS10	β′:His_6	T7P—α—β—β′:His_6—ω; the His_6 tag at the C-terminus of *rpoC*	pMB1 Ampicillin
pIA787	β′:His_6 β′:PKA	T7P—α—β—β′:RRASV:His_6—ω; the His_6 tag and a protein kinase A (PKA) site at the C-terminus of *rpoC*	pMB1 Ampicillin
pIA821	His_6:β PKA:β Rif^R	T7P—α—His_6:RRASV:β^{S531F} —β′—ω; His_6 tag and PKA site at the N-terminus of *rpoB;* Rif^R S531F allele eliminates an internal kinase site	pMB1 Kanamycin
pIA900	β′:TEV:His_{10}	T7P—α—β—β′:TEV:His_{10}—ω; a removable via TEV protease cleavage His_{10} tag at the C-terminus of *rpoC*	pMB1 Ampicillin
pIA999	β′:biotin	T7P—α—β—β′:GLNDIFEAQKIEWH—ω; a minimal biotin (AVI) substrate at the C-terminus of *rpoC*	pMB1 Ampicillin
pIA1198	β′:HA:His_7	T7P—α—β—β′:YPYDVPDYA:His_7—ω; the HA and His_7 tags at the C-terminus of *rpoC*	pMB1 Ampicillin
pIA1202	β′:biotin: TEV:His_7	T7P—α—β—β′:GLNDIFEAQKIEWH:TEV:His_7—ω; an AVI tag followed by a removable via TEV protease cleavage His_7 tag at the C-terminus of rpoC at the C-terminus of *rpoC*	pMB1 Ampicillin

All vectors have the *rpoA*, *rpoB* and *rpoC* genes expressed under the control of the phage T7 gene 10 promoter. A subset of vectors also have the *rpoZ* gene; for the rest, the ω subunit can be co-expressed from a compatible pIA839 plasmid.

Table 2

Vectors for site-directed mutagenesis or expression of single *E. coli* RNAP subunits

Plasmid	Subunit & Ab^R	Features	Origin & Resistance
pIA281	α^{WT} —	T7P—*rpoA*; α subunit under control of the bacteriophage T7 gene 10 promoter; *Hin*dIII site in *rpoA* is removed	pMB1 Ampicillin
pIA545	β^{WT} —	P_{trc}—His$_6$-*rpoB* ^{WT}; N-terminally tagged β under control of IPTG-inducible P_{trc} promoter; a silent *Mfe*I @ 555	pMB1 Ampicillin
pIA178	β^{S531F} Rif^R	P_{trc}—His$_6$-*rpoB* ^{S531F}; a rifampicin-resistant (Rif^R) S531F allele in the P_{trc}—*rpoB* construct	pMB1 Ampicillin
pIA223	β^{D516V} Rif^R	P_{trc}—His$_6$-*rpoB* ^{D516V}; a Rif^R D516V (marked with a silent *Bsg*I site) allele in the P_{trc}—*rpoB* construct	pMB1 Ampicillin
pIA661	β'^{WT} —	P_{trc}—*rpoC* ^{WT}-His$_6$; C-terminally tagged β′ under control of an IPTG-inducible P_{trc} promoter; a silent *Eag*I @ 675	pMB1 Ampicillin
pIA635	β'^{S793F} Stl^R	P_{trc}—*rpoC* ^{S793F}-His$_6$; a streptolydigin-resistant S793F (marked with a silent *Eco*RV site) allele in the P_{trc}—*rpoC* construct	pMB1 Ampicillin
pIA388	β'^{I774S} CBR^R	P_{trc}—*rpoC* ^{I774S}-His$_6$; a CBR-resistant I774S (marked with a silent *Ear*I site) allele in the P_{trc}—*rpoC* construct	pMB1 Ampicillin
pIA839	ω^{WT} —	P_{BAD}—*rpoZ*; ω subunit under control of arabinose-inducible P_{BAD} promoter	P15A Chloramphenicol
pIA586	σ^{WT} —	T7P—His$_6$-*rpoD;* N-terminally tagged σ^{70} subunit under control of the T7 gene 10 promoter	pMB1 Kanamycin
pIA1127	σ^{WT} —	T7P—His$_6$-*rpoD;* N-terminally tagged σ^{70} under control of the T7 gene 10 promoter; removable His$_6$ tag	pMB1 Kanamycin
pIA458	β^C & β'^N —	A fragment encompassing the C-terminal part of *rpoB*, the linker with an *Spe*I site, and the N-terminal part of *rpoC*	pUC Ampicillin