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Recombinant bacterial RNA polymerase: Preparation and applications

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ABSTRACT

Availability of DNA-dependent RNA polymerase from various bacteria is a key for setting up specific *in vitro* transcription systems necessary for understanding species-specific transcription regulation. We describe here two main strategies for recombinant RNA polymerase preparation—through *in vitro* reconstitution and heterologous co-overproduction in *Escherichia coli*. Both strategies can be used for preparation of large amounts of RNA polymerases from any bacteria for which sequences of *rpo* (RNA polymerase) genes are known.

1. Introduction

DNA-dependent RNA polymerase (RNAP) is the key enzyme of gene expression. RNAP is a multisubunit, multifunctional molecular machine, whose functions are regulated by various cellular factors in response to environmental cues. Despite the very high degree of evolutionary conservation at and around the catalytic center, RNAP from different organisms, including different eubacteria, exhibit species-specific properties such as specificity of promoter recognition or ability to interact with and respond to transcription factors. Thus, understanding transcription regulation in a particular bacterium often means that an *in vitro* transcription system with cognate RNAP needs to be set up.

Bacterial RNAP consists of a catalytically proficient core enzyme (subunit composition $\alpha_2\beta\beta'\omega$, molecular weight ~300–400 kDa) and a specificity subunit σ. A complex of core with σ is called the holoenzyme and is able to specifically recognize promoters. Different σ factors direct the core to different groups of promoters. The smallest RNAP subunit, ω (~10 kDa) is the only subunit that is dispensable for most *in vivo* and *in vitro* functions of the enzyme. While robust procedures suitable for RNAP purification from most bacteria have been developed, their application is hampered by difficulties associated with cultivating, let alone growing large volumes of bacterial cultures needed for biochemical fractionation. Bacterial RNAP is unique in that it can be efficiently reconstituted *in vitro* from isolated recombinant subunits, or prepared by heterologous co-overexpression of subunits in surrogate hosts, opening way for functional and structural analyses of transcription mechanism and regulation in hard-to-cultivate microorganisms. In addition, both the *in vitro* assembly and co-overexpression approaches allow preparing RNAP mutants lacking essential functions. This is very important for reverse genetics analysis of RNAP, which is an essential enzyme, meaning that classical genetic approaches are limited to analysis of viable mutations. Below, we present detailed protocols for preparation of recombinant RNAP by *in vitro* assembly and co-overexpression.

2. In vitro reconstitution of recombinant bacterial RNAP

A general scheme of *in vitro* reconstitution of bacterial RNAP is presented in Fig. 1 and is described in detail in the following sections.

2.1. Expression of recombinant RNAP subunits in Escherichia coli

Plasmids of the pET series (Novagen) have been successfully used as vectors for expression of *rpo* genes from various bacterial sources. Plasmids with *rpo* genes are transformed into the *E. coli* BL21(DE3) cells and transformants are plated on solid medium containing appropriate antibiotics. In PET-based plasmids, the target protein overproduction is induced by the addition of IPTG (usually 1 mM). It is very important to find optimal conditions for overproduction for each RNAP subunit. In our experience, varying growth temperature, IPTG concentration, culture OD_{600} at the time of induction, and cultivation time after the induction allows high- to medium levels of expression of RNAP subunits from various sources, including Gram-negative bacteria (*E. coli*, *Xanthomonas oryzae*, and *Francisella tularensis*), Gram-positive bacteria (*Bacillus subtilis*, *Bacillus cereus*), and several thermophilic

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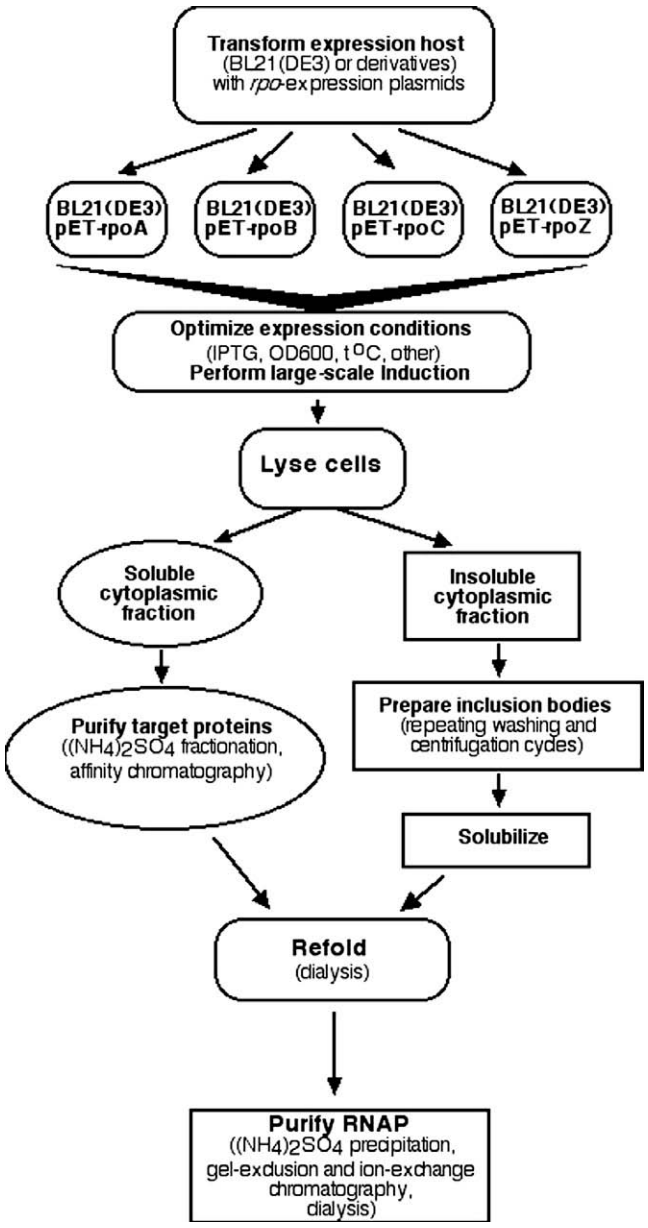

Fig. 1. Preparation of bacterial RNAP core enzyme by *in vitro* reconstitution. The sequence of steps involved in RNAP core enzyme reconstitution is schematically presented. The ω subunit can be omitted from reconstitution reaction with minimal effects on most RNAP functions. See text for details.

organisms (*Thermus aquaticus* and *Aquifex aeolicus*). Should problems with expression be encountered, various procedures such as altering the ratio of isoaccepting tRNAs in the expression host can be used to attempt to increase the yield. Useful information about optimization of induction can be found in the PET system manual from Novagen (http://www.emdbiosciences.com/docs/docs/PROT/TB055.pdf).

Once the induction conditions have been optimized in pilot experiments, large-scale induction is performed. Only freshly transformed cells (grown at 37 °C for no more than 12 h) should be used. Cells are collected from plates by scraping with a microbiological loop and are carefully resuspended in ~1 ml of liquid LB medium in a sterile Eppendorf tube. The resulting suspension is used to inoculate liquid cultures (a plate containing several hundred ~1 mm colonies is sufficient for inoculation of 1 l of LB medium). The medium should contain appropriate antibiotics and should be preheated to 37 °C. The culture is grown at 37 °C with vigorous agitation until OD_{600} reaches 0.4–0.8 (usually 2–3 h), induced with 1 mM IPTG and allowed to grow further (usually for additional 2–5 h; the optimal time of induction time could be determined during the pilot experiments). A 0.1–0.5 ml aliquot of the culture is withdrawn before the induction and stored at room temperature in an Eppendorf tube. When the induction is complete, another aliquot of the culture is withdrawn. Cells from both culture aliquots are collected by centrifugation in a microcentrifuge, the supernatant is removed, and the pellet is resuspended in 20 μl H_2O and equal volume of Laemmli loading buffer is added. Samples are boiled for 5 min and analyzed by SDS–PAGE to determine recombinant RNAP subunits expression levels. If desired levels of overproduction are detected, cells from induced cultures are collected by centrifugation (4000*g*, 10 min, 4 °C), the medium is removed and cell pellet drained and stored at −80 °C until further use.

2.2. Inclusion bodies preparation and purification of His-tagged RNAP subunits from cell extracts

Escherichia coli BL21(DE3) cells transformed with *rpo* gene expression plasmids usually overproduce individual core RNAP subunits at high-level upon induction and these RNAP subunits often form inclusion bodies. Nevertheless, it is very important to determine the localization (cytoplasmic versus inclusion bodies) of recombinant protein when overproducing an RNAP subunit from a new source. This is best done by performing a small-scale trial induction in ~10 ml of LB medium. It is safer to perform localization trials by growing cell cultures in tiny flasks rather than tubes, since induction conditions often do not scale properly from tubes to flasks. Induced cells are collected in an Eppendorf tube, resuspended in 400–500 μl of lysis buffer (see Table 1) and lysed by several 5- to 10-s sonication bursts using a microtip with 1-min rests between the bursts. The tube shall be kept in a water-ice bath during the sonication. An aliquot of lysed cells is next removed and cell debris and inclusion bodies are collected by a 2–5 min centrifugation in a refrigerated microcentrifuge. An aliquot of the cleared cell lysate is removed, the rest of the supernatant discarded and the pellet is resuspended in an initial volume of the lysis buffer

Table 1
Solutions and buffers.

Solution name	Components	Comments
Lysis buffer	40 mM Tris–HCl, pH 7.9; 300 mM KCl; 10 mM EDTA	Before use supplemented with 15 mM 2-ME and 0.1 mM PMSF
Grinding buffer	40 mM Tris–HCl, pH 7.9; 100 mM NaCl; 10 mM EDTA	Before use supplemented with 15 mM 2-ME and 0.1 mM PMSF
Storage buffer	40 mM Tris–HCl, pH 7.9; 200 mM KCl; 50% glycerol; 1 mM EDTA; 1 mM 2-ME	Used for dialysis
Denaturation buffer	6 M guanidine–HCl; 50 mM Tris–HCl, pH 7.9; 10 mM $MgCl_2$; 10 μM $ZnCl_2$; 10% glycerol; 1 mM EDTA	Before use supplemented with 10 mM DTT
Reconstitution buffer	50 mM Tris–HCl pH 7.9; 200 mM KCl; 10 mM $MgCl_2$; 10 μM $ZnCl_2$; 10% glycerol; 1 mM EDTA	Before use supplemented with 1 mM DTT or 2-ME (see text)
TGE buffer	40 mM Tris–HCl, pH 7.9; 5% glycerol; 1 mM EDTA	Before use supplemented with 1 mM 2-ME
Start buffer	20 mM Tris–HCl, pH 7.9; (or Hepes); 500 mM NaCl; 5% glycerol	For metal chelate affinity chromatography
Transcription buffer	40 mM Tris–HCl, pH 8.4; 40 mM KCl; 10 mM $MgCl_2$	