T7 RNA polymerase exhibits a highly active DNA-dependent phage RNA polymerase and is one of the most widely used enzymes in molecular biology (6). Recent developments in the investigation of RNA structures, aptamer and ribozyme evolution and the production of antisense RNAs as well as the application of RNA-based iso-thermal amplification methods, such as the self-sustained sequence replication (3SR) reaction (4,5,8) or nucleic acid sequence-based amplification (2), require large amounts of highly active T7 RNA polymerase with a constant quality. For these applications, T7 RNA polymerase is often purified using purification protocols with several purification steps (3,9).

By N-terminal fusion of a 6-histidine residue tag to the T7 RNA polymerase gene, we have engineered a highly active fusion protein that can be purified very rapidly in a single step using the nickel nitrilotriacetic acid (Ni-NTA) technology (Qiagen GmbH, Hilden, Germany).

To fuse the T7 gene to the 6-histidine residue tag, a polymerase chain reaction (PCR) fragment comprising the N-terminal portion of the T7 RNA polymerase gene fused to a BamHI site was produced using two primers (5'-CGCGATCTCAAGTAAACATCGCTTA-3' and 5'-GATAGTCTAGAGTCTCGAC-3' and pAR1219 (4) as template. This PCR fragment was cut with BamHI and AlwNI. A second fragment encompassing the C-terminal portion of the T7 RNA polymerase gene was obtained by cutting pAR1219 with AlwNI and HinDIII. Both fragments were ligated to the HindIII/BamHI-linearized vector pQE9 (Qiagen GmbH), leading to an in-frame translational fusion of the 6-histidine tag coding sequence of the vector to the T7 RNA polymerase gene. The resulting plasmid, pQE9T7, allows overexpression of the 6-histidine-tagged T7 RNA polymerase in E. coli BL21 (pREP4) lacking the ompT and lon proteases that would cause proteolysis of the protein (7).

For the purpose of purification, a pre-culture in 60 mL TY broth containing 100 µg/mL ampicillin and 25 µg/mL kanamycin was incubated at 37°C overnight on a rotary shaker. The cells were harvested by centrifugation, washed twice and inoculated into 3 L of fresh medium. Expression of T7 RNA polymerase was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at an optical density (OD) of 1.0. After induction, the cells were allowed to grow for an additional three hours and harvested by centrifugation at 3000×g. The cell pellet was resuspended in 40 mL of sonication buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 0.5 mM dithiothreitol [DTT]). Cell disruption was achieved by adding 6 mg/mL lysozyme, 0.5 mg/mL phenylmethylsulfonyl fluoride (PMSF), 0.25 mg/mL bacitracin and 2.5 mM benzamidine and ultrasonication after a 30-min incubation period at 4°C. After centrifugation at 15,000×g for 30 min, the crude extract was pressed twice through a narrow syringe needle and applied immediately with a flow rate of 0.3 mL/min to a 6-mL Ni-NTA Sepharose® column that had been freshly nickel-chelated again and equilibrated with sonication buffer. The column was then washed with 10 vol 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10% glycerol, 0.5 mM DTT, 20 mM imidazole and 20 µg/mL PMSF with a flow rate of 0.5 mL/min. T7 RNA polymerase was eluted with the same flow rate by a 60-mL linear imidazole gradient spanning from 0.02 to 0.5 M in 50 mM sodium phosphate, pH 8.0. 300 mM NaCl and 0.5 mM DTT. Every fraction within the single elution peak detected spectrophotometrically at 280 nm was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing T7 RNA polymerase were pooled and dialyzed overnight against 500 mL 20 mM sodium phosphate, pH 7.7, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 50% glycerol. The volume was then reduced by ultrafiltration. The analysis of the initial and final stages of the purification by SDS-PAGE (Figure 1) indicated that the single purification step efficiently removes the major part of the other proteins from the crude extract.

The protein concentration in the final preparation was determined according to the method of Bradford (1) with 16.31 µg/µL. The specific promoter-dependent transcription activity of 6-histidine-tagged T7 RNA polymerase was measured by trichloroacetic acid (TCA) precipitation. In a 100-µL reaction mixture consisting of 40 mM Tris-HCl, pH 8.0, 6 mM MgCl2, 10 mM DTT, 2 mM spermidine, 0.5 mM each NTP, 1 µCi [α-32P]UTP (5000

**Figure 1. Analysis of crude extract and single-step purified T7 RNA polymerase by 8% SDS-PAGE.** Lanes 1 and 3 are molecular weight markers (broad range standard; Bio-Rad, Hercules, CA, USA); lane 2 represents the crude extract (30 µg) after lysis, sonication and centrifugation steps; lanes 4–7 are 4 different concentrations (50, 30, 20 and 10 µg, respectively) of the purified, pooled, dialyzed and concentrated eluate from the Ni-NTA metal chelate chromatography column. The gel is stained with Coomassie Brilliant Blue® R-250 and shows the high efficiency of Ni-NTA metal chelate chromatography with 6-histidine-tagged proteins.
of the preparation over more than one month allows the long-term storage of RNase activities which occur (4, 5). The addition of DTT disturbed nuclease activities and could result in the same product pattern as the enzyme was virtually free of the 6-histidine tag did not influence the activity of the enzyme. The total amount of pure RNA polymerase isolated from a 3-L culture was 613 U/mg, 1 U corresponding to the amount of enzyme that incorporates 1 nmol [α-32P]UTP in TCA-precipitable form in 1 h. The specific activity of the 6-histidine tagged T7 RNA polymerase was 1.7 times higher than the specific activity of commercially available T7 RNA polymerase (MBI Fermentas, Vilnius, Lithuania), which had been tested in the same assay. Additionally, in vitro run-off transcription experiments using the fusion protein resulted in the same product pattern as with the commercially available polymerase, indicating that the 6-histidine tag did not influence the activity of the enzyme. The total amount of pure RNA polymerase isolated from a 3-L culture was 32.62 mg or 2 × 10⁷ U.

As established by incubation of labeled double-stranded and single-stranded DNA with 3 U/mL (final concentration) of the polymerase preparation for one hour at 37°C in transcription buffer and by the specific product pattern of in vitro transcription reactions, the enzyme was virtually free of disturbing nuclease activities and could serve as RNA polymerase in 3SR reactions in which RNA and DNA intermediates occur (4, 5). The addition of DTT to a final concentration of 1 mM every 6 months allows the long-term storage of the preparation over more than one year with no detectable loss of activity.

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Differential mRNA Display at the Single-Cell Level

The differential mRNA display technique represents a powerful tool for the identification of genes that are over- or underexpressed in one cell population relative to another (5). To perform differential mRNA display, total RNA is isolated from the two cell populations to be compared, and first-strand copies of both RNAs are made by reverse transcription polymerase chain reaction (RT-PCR) amplification. Not only known differentially expressed genes are detected by this approach, but also previously unknown genes can be detected using short, random PCR primers. Differential mRNA display has already been successfully applied for the identification of genes that are differentially expressed in patients with malignancies (e.g., brain tumors), heart disease and diabetes (6). In the mammary gland, several cDNA fragments have been characterized that are up-regulated in tumor tissue in comparison to normal tissue, with the role of the respective genes in tumorigenesis remaining undetermined so far (4). A disadvantage of the differential display technique is the fact that whole-tissue material obtained from malignant tissues does not consist exclusively of tumor cells but is always contaminated by a variety of nonmalignant cells, such as fibroblasts and peripheral blood cells. Therefore, the use of this technique by itself does not allow for any conclusions regarding the cellular origin of differentially expressed genes because they can not be attributed to a defined cellular subpopulation of the tissue from which the amplified cDNA had been made. To circumvent these limitations, we modified the mRNA display technique for single cells. Single cells can be picked under defined conditions (3) and serve as a distinct source for the isolation and PCR amplification of mRNA. Single-cell PCR has facilitated the characterization of tumor cells, which make up a minority of involved tissue; e.g., single Hodgkin and