T7 RNA Polymerase Purification

Prepare buffers and solutions:

Buffer A (Binding Buffer):	50 mM Tris pH8 100 mM NaCl 5 mM b-mercaptoethanol 1 mM PMSF, 5% glycerol
Buffer B:	50 mM Tris pH8 100 mM NaCl 5 mM b-mercaptoethanol 30 mM Imidazole 5% glycerol
Buffer C (Elution Buffer):	50 mM Tris pH8 100 mM NaCl 5 mM b-mercaptoethanol 500 mM Imidazole 5% glycerol
Buffer D (High-Salt Buffer):	50 mM Tris pH8 1.0 M NaCl 5 mM b-mercaptoethanol 5% glycerol
Buffer E (2x Storage Buffer - glycerol):	40 mM K_2HPO_4 pH7.5 2 mM DTT 2 mM EDTA (2X buffer)

PMSF and b-Mercaptoethanol should be added only on the day where you are using the buffer.
Set pH at temperature at which you will use the buffer, i.e. cool it to 4ºC before setting the pH!
All buffer have to be filtered.

Overexpress T7-RNA polymerase

- § follow protocol for over expression of histidine-tagged proteins in BL21(DE3)
- § the pT7pol is ampicillin resistant
- § molecular weight of T7-RNA polymerase is 98.86 kDa

Cell opening by sonication

1. Add PMSF and b-mercaptoethanol to buffer A.
2. Resuspend the cell pellet in Buffer A [approx. 5ml/g(cell)] in a small (100 mL) beaker and thaw cells by stirring the mixture slowly on ice.
3. Add lysozyme to a final concentration of 1mg/ml and incubate the cell suspension on ice for 30 minutes (resuspend the lysozyme in ~ 1 mL buffer A before addition).
4. Add sodium desoxycholate [12.5mg/g(cell)]
5. Stir the mixture slowly on ice for 10 -15 min.
6. Open cells using a sonicator while sample on ice: at least 5 times for 1 min, short pause in between, shake beaker, intensity level 6, duty cycle 60%
7. Centrifuge cell lysate for 45 minutes at 30000xg, 4°C in JA-20
8. Determine the A_{280} of your lysate and record it for future reference (with a similar extend of cell opening, you should always obtain a similar protein concentration in the lysate, i.e. a similar A_{280} value)

Preparation of the Ni Sepharose slurry

1. Spin the Ni-NTA sepharose resin (about 3mL 50% slurry, i.e. 1.5 mL resin) at 500xg for 2min. Remove the supernatant.
2. Wash the column with 3 column volumes of sterile water. (Gently mix the solid phase with the liquid to create slurry. Spin at 500xg for 2min)
3. Wash the column with 6 column volumes of Buffer A, leave 3 ml Buffer A on the resin to obtain a 50% slurry and divide into 4 (or less) falcon tubes.

Purification by Ni-Sepharose slurry (Batch procedure)

1. Remove 50 mL of cell lysate and store in microfuge tube. Apply the cleared cell lysate to the 4 columns evenly and gently mix thoroughly. Incubate on ice for 60 minutes inverting periodically to bind protein to the resin.
2. ALL FURTHER STEPS ARE PERFORMED AT 4°C
3. Transfer all the column material into 1 falcon tube by spinning 4 columns at 500xg for 2 min removing the supernatant and filling a falcon tube with cleared lysate+resin from other tubes. Remove and store supernatant at 4 °C. Take 50mL sample and store at 4 °C

4. Wash the column 3 times with a full falcon tube volume of Buffer A. Pool the washes, stores at 4 ºC and take 50 mL sample.
5. Add b-mercaptoethanol to Buffer B.
6. Wash the column 4 times with a full falcon tube volume of Buffer B. Pool the washes, store at 4 ºC and take a 50 mL sample.
7. Elute the protein 10 times using 90% column volume of Buffer C. Incubate elution buffer for 5-10 minutes. Save each elutant at a 15mL falcon tube, store in 4 ºC and take a 50 mL sample from each elution step.
8. The column material can be stored with 40 ml of Buffer C for 2 days until you wash it properly as described below.

Purification by Affi-gel Blue-gel resin

1. Check the elution fractions on a 10% SDS-PAGE gel and pool the samples shown to have T7 present (DO NOT concentrate sample or it may precipitate)
2. Prepare Affi-gel Blue-gel resin (about 5mL resin)
   a) wash 2x times with full falcon volume of high-salt Buffer D (centrifuge 500xg for 5 min, remove supernatant)
   b) wash 3x times with full falcon volume of low-salt Buffer (e.g. A or B)
3. Batch bind the pooled elutions to a 1:1 (v/v) ratio of Affi-gel Blue-gel resin by incubating for 1h at 4ºC while slightly shaking
4. Centrifuge at 500xg for 5 min
   - **RETAIN SUPERNATANT**. The T7 DOES NOT bind to the resin and remains in the supernatant
   - Use the T7 containing supernatant for all subsequent steps
5. Wash Affi-gel Blue-gel by rinsing twice with 1x full falcon tube volume of Buffer A (or B), keep for analysis on SDS-PAGE and for RNase test.
6. Do a A_{280} assay to determine the concentration of T7-RNA polymerase from step 4(e=140260$M^{-1}cm^{-1}$), then concentrate T7-RNA polymerase containing supernatant in Vivaspin (MWCO 30,000) at 4000xg, don't concentrate too much (max. concentration is 20 mM)
7. Re-buffer in storage buffer E
   (a) Dilute the solution from step 6 with proper volume of Buffer E to a final 1:10 dilution. Then concentrate this solution in Vivaspin (MWCO 30,000) at 4000xg (do an A_{280} assay to make sure the final concentration is not over 20 mM).
   (b) Repeat step 24(a). This will concentrate T7-RNA polymerase in buffer E with a 1:100 dilution compared to the elution Buffer (Buffer C used in binding to Affi-gel Blue-gel resin)

8. Add 1 volume of 100% glycerol to T7-RNA polymerase from step 4 of purification, this will dilute the storage buffer to 1x in 50% glycerol.
9. Aliquot (500 mL, 250 mL and 50 mL) and place into -20°C freezer (DO NOT shock freeze with liquid nitrogen).
10. Regenerate Affi-gel Blue-gel by eluting RNases using twice 0.5x full falcon tube volume of buffer D (High-Salt Buffer), discard.
11. Wash Affi-gel Blue-gel twice with full falcon tube volume of ddH_2O
12. Store it as 50% slurry in ddH_2O in 4°C fridge.

Test T7-RNA polymerase for any remaining RNases (Use MS2 RNA)

For details see protocol RNase Test

1. mix 0.5 mL of 0.8ug/uL MS2 RNA with 2 mL T7 polymerase
2. Add 10 mL ddH_2O
   Negative control: add 12 mL ddH_2O to 0.5 mL of 0.8 mg/mL MS2 RNA
   Positive control: add 2 mL of human saliva to the 10 mL ddH_2O and 2 mL MS2 RNA
3. Incubate reactions for 60 minutes at 37°C

Run samples on a 15% RNA-Urea PAGE

For details see protocol RNA-Urea PAGE

1. Add 5 mL RNA loading dye (8M urea, 0.025% bromophenol blue, 0.025% xylene cyanol) to 5 mL of each reaction sample
2. Heat gel samples at 90°C-100°C for 3 minutes
3. Preheat the TBE running buffer (1L) for 2x 30seconds in the microwave
4. Pre-run the urea gel for 10 minutes at 300V to warm it up
5. Load gel samples on the 15% TBE-urea gel
6. Run Electrophoresis box for about 30 minutes at 300V (1x TBE). (Note: if the electrophoresis stop for some unknown reasons, or if it can't run up to 300V. then run it at 250V at around 40 minutes or longer)
7. Stain gel using ethidium bromide and take a picture in Brant's Lab

There should be no degradation bands in any but the positive control lane.

15% RNA-Urea PAGE

15% Acryl/bisacrylamide 19:1

1x TBE

4 mM EDTA

Add APS and TEMED for polymerization when ready to pour the gel