Purification of T7 RNA polymerase

### Hsuan-Chun Lin August 11, 2017

Protocol and plasmids are adapted from Luo, Le, Blanton Tolbert’s lab, Case Western Reserve University

Protocol is adapted from Donald C. Rio (Rio, 2013).

T7 RNA polymerase 20x reaction buffer is adapted from Wei Huang, Derek Taylor’s lab, Case Western Reserve University.

## Day 1

1. Autoclave 1L LB medium and two 1000ml flask.
2. Check the Ni-NTA column is clean and regenerated.

## Day 2

### Material check list:

RNA polymerase BL21 stable clone (1 ml/tube concentrated from 5ml bacterial incubation) or 5ml starting culture.

LB medium 1L

Autoclaved 1000ml glass buttle x2

1M IPTG 500ml

Leupeptin (5 mg/mL)

Dissolve leupeptin in **100% DMSO** and store in a polypropylene tube at −20°C.

Ampicillin (sodium salt) (50 mg/mL)

Take 0.5 g ampicillin sodium salt (Sigma-Aldrich, A9518), add ddH2O to 10 mL. and store at −20°C.

Phenylmethysulfonyl fluoride (PMSF, 20 mg/mL)

Dissolve PMSF in **isopropanol** and store in a dark glass bottle at −20°C.

Sodium deoxycholate (8%, w/v)

Prepare fresh for each purification by dissolving 0.8 g of deoxycholic acid, sodium salt in ddH2O.

### Lysis buffer:

Ni-NTA Lysis Buffer

|  |  |  |
| --- | --- | --- |
| Reagent | Quantity (for 200 mL) | Final concentration |
| β-Mercaptoethanol (14.1 M) | 71 μL add fresh | 5 mM |
| Imidazole (1 M, pH 8.0) | 200 μL | 1 mM (pH 8.0) |
| ddH2O | to 200 mL |  |

Usually we prepare **500ml** for assurance. Because microfludizer also need the buffer to wash.

Ni-NTA Lysis Buffer 5X

|  |  |  |
| --- | --- | --- |
| Reagent | Quantity (for 200 mL) | Final concentration |
| Tris-HCl (1 M, pH 8.0) | 50ml | 250 mM |
| NaCl (5 M) | 20ml | 500mM |
| Glycerol (100%) | 50ml | 25% (v/v) |
| ddH2O | to 200 mL |  |

### Protocol

1. Inoculate 1 tube 5ml bacterial stock into the medium.
2. Shake for 4-5 hr on 250rpm, 37oC
3. Check the OD600 around 0.5-0.6
4. Induce the cells in the flasks by adding 0.5ml of IM IPTG per 1L cells.
5. Incubate the cells for 3h at 37oC with shaking at 250rpm.
6. Harvest the cells by 7000rpm, 10min.
7. Resuspend the cells in 25ml of lysis buffer per pellet from 1L culture.
8. Transfer the sample to 50ml conical tube.
9. Add glycerol 1.25ml, final concentration is 5% (v/v)
10. <you can stop here and freeze your *E. coli* at -20oC>
11. Microfludizer
12. Add 50l of 20mg/ml PMSF, and 20l of 5mg/ml leupeptin.
13. Add 0.25ml of 8% (w/v) sodium deoxycholate and incubate with rocking for 20min at 4oC.
14. Make a column of Ni-NTA agarose, using 10 mL of 50% slurry for each liter of starting cell culture. For example, for 2 L of cell culture, use 20 mL of 50% slurry and keep it in 4oC for tomorrow.

## Day3

### Material check list

Ni-NTA Elution Buffer

|  |  |  |
| --- | --- | --- |
| Reagent | Quantity (for 200 mL) | Final concentration |
| Ni-NTA lysis buffer (5×) | 40ml | 1x |
| β-Mercaptoethanol (14.1 M) | 71 μL add fresh | 5mM |
| Imidazole (1 M, pH 8.0) | 100 mL | 500 mM |
| ddH2O | to 200 mL |  |

Ni-NTA Wash Buffer

|  |  |  |
| --- | --- | --- |
| Reagent | Quantity (for 500 mL) | Final concentration |
| Ni-NTA lysis buffer (5×) | 100ml | 1x |
| β-Mercaptoethanol (14.1 M) | 177.5 μL add fresh | 5mM |
| Imidazole (1 M, pH 8.0) | 5 mL | 10 mM |
| ddH2O | to 500 mL |  |

T7-RNAP Dialysis Buffer

|  |  |  |
| --- | --- | --- |
| Reagent | Quantity (for 1L) | Final concentration |
| Potassium phosphate (0.2 M, pH 7.5) | 100 mL | 20mM |
| NaCl (5 M) | 20 mL | 100 mM |
| Dithiothreitol (DTT, 0.5 M) | 20 mL | 10 mM |
| EDTA (0.5 M, pH 8.0) | 0.2 mL | 0.1 mM |
| ddH2O | to 1 L |  |

T7-RNAP Storage Buffer

|  |  |  |
| --- | --- | --- |
| Reagent | Quantity (for 10ml) | Final concentration |
| Potassium phosphate (0.2 M, pH 7.5) | 1mL | 20mM |
| NaCl (5 M) | 0.2 mL | 100 mM |
| Dithiothreitol (DTT, 0.5 M) | 0.2 mL | 10 mM |
| EDTA (0.5 M, pH 8.0) | 2l | 0.1 mM |
| Glycerol | 5ml | 50% |
| ddH2O | to 10ml |  |

### Protocol

Transfer the samples to 50-mL Oak Ridge centrifuge tubes. Centrifuge in a Beckman rotor at 15,500 rpm for 30 min at 4°C.

1. Decant the supernatant into a clean bottle or flask, and keep it on ice.
2. Equilibrate the resin with ≥10 column volumes of lysis buffer. (Here 100ml)
3. Load the protein sample by gravity flow onto the column, and collect the flow through.
4. Wash the resin with three column volumes of lysis buffer (30ml) followed by 10 column volumes of wash (100ml) buffer. Collect the flow through.

Lysis buffer 30ml -> wash buffer (100ml)

To remove all bacterial nuclease contamination from the purified protein, it is critical that you wash the column extensively with ≥10 column volumes of wash buffer before the imidazole elution.

#### Elution

1. Purge the pump with elution buffer. (without linking to your column)

Remember: Once the high concentration of Imidazole enter your column, your protein will immediately eluted.

1. Set flow rate to 0.5ml per minute.
2. Set the fraction collector to time mode, collect 2min per tube. (1ml)
3. Stop the pump, link the column to pump and fraction collector.
4. Start elution and the program of fraction collector. The protein should come out from 5-12mins.

#### *Dialysis, finalize, and functional test*

1. Check 5 μL of each fraction by SDS–PAGE on a 7.5% SDS-polyacrylamide gel.
2. Pool the peak fractions containing T7-RNAP (the protein runs at 100 kDa).
3. Use Millipore concentration kit to concentrate the protein to <1ml.
4. Dialyze the peak fractions against T7-RNAP in 1L dialysis buffer for 24 h at 4°C.
5. Quantitate the purified T7-RNAP by measuring the absorbance at 280 nm (A280). The estimated mg/mL = A280 × (dilution factor/1.3).
6. Dilute the samples to 5ml storage buffer.
7. Aliquot the enzyme in 100- to 200-μL aliquots into 1.5ml Eppendorf.
8. Store the purified T7-RNAP at −20°C.
9. Determine the activity of the purified T7-RNAP. (You can use it in in vitro transcription.

Two Editions of Reaction buffer. Our lab now is using NEB edition.

20X T7 RNAP Reaction buffer(from Taylor lab):

|  |  |  |
| --- | --- | --- |
| Reagent | Quantity (for 5ml) | Final concentration |
| 1 M tris-HCl, pH 8.1 | 4mL |  |
| spermidine | 30 mg (D = 0.925) | Add 32.4ul |
| triton x-100 | 10 L |  |
| DTT | 78mg |  |
| ddH2O | to 5ml |  |

1X Buffer Components (NEB)

1 mM DTT   
2 mM spermidine   
40 mM Tris-HCl   
6 mM MgCl2   
pH 7.9@25°C

## Bibliography

Rio, D. C. (2013). Expression and Purification of Active Recombinant T7 RNA Polymerase from E. coli. *Cold Spring Harbor Protocols*, 1094-1098.