

Sample Preparation of Telomerase Subunits for Crystallization

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[Abstract] Telomerase is a large ribonucleoprotein complex that replicates the linear chromosome ends in most eukaryotes. Large-scale preparation of the telomerase core components *in vitro* has long been a big challenge in this field, hindering the understanding of the catalytic mechanism of telomerase, as well as slowing down the development of telomerase inhibitors for cancer therapy. We have successfully developed a protocol for large-scale preparation of the TRBD-CR4/5 complex of the medaka telomerase *in vitro*, and used this method to study the high-resolution structure of the TRBD-CR4/5 complex by X-ray crystallography. This procedure may be also adapted to purify other protein-RNA complexes for structural studies.

Materials and Reagents

1. pMAL-C2X vector (New England Biolabs, catalog number: N8076S) with an insertion of the protease 3C recognition site between MBP and the fusion protein
2. *Escherichia coli* (*E. coli*) strain ScarabXpress T7lac cells (Scarab Genomics, catalog number: C-1709-05K)
3. LB (Luria-Bertani) medium (BD Bioscience, catalog number: 214906) and LB agar (BD Bioscience, catalog number: 244520)
4. Ampicillin (AMRESCO, catalog number: 0339)
5. Glucose (AMRESCO, catalog number: 0188)
6. Isopropyl β -D-1-thiogalactopyranoside (IPTG) (AMRESCO, catalog number: 0487)
7. Phenylmethylsulfonyl fluoride (PMSF) (Roche Diagnostics, catalog number: 11359061001)
8. Tris(2-carboxyethyl)phosphine (TCEP) (AMRESCO, catalog number: K831)
9. Benzamidine (Sigma-Aldrich, catalog number: 12072)
10. Leupeptin (Sigma-Aldrich, catalog number: 62070)

11. Pepstatin (Sigma-Aldrich, catalog number: 77170)
12. Amylose resin (New England Biolabs, catalog number: E8021S)
13. Ammonium sulfate (Sigma-Aldrich, catalog number: A4418)
14. Sodium chloride (Sigma-Aldrich, catalog number: S3014)
15. Magnesium Chloride (Sigma-Aldrich, catalog number: 63068)
16. Tris-HCl (Amresco, catalog number: 0497)
17. Glycerol (Sigma-Aldrich, catalog number: G5516)
18. Maltose (Sigma-Aldrich, catalog number: 1B1184)
19. HEPES-KOH (Sigma-Aldrich, catalog number: 54457)
20. Spermidine (Sigma-Aldrich, catalog number: 85558)
21. Dithiothreitol (DTT) (Roche Diagnostics, catalog number: 10708984001)
22. Recombinant RNasin Ribonuclease Inhibitor (Promega Corporation, catalog number: N2511)
23. Nuclease-Free Water (Promega Corporation, catalog number: P1193)
24. T7 RNA polymerase (New England Biolabs, catalog number: M0251S)
25. Pyrophosphatase (New England Biolabs, catalog number: M0361S)
26. Glucosamine-6-phosphoate (Sigma-Aldrich, catalog number: G5509)
27. Lysis buffer (see Recipes)
28. Column buffer (see Recipes)
29. RNA buffer (see Recipes)
30. 5x transcription buffer (see Recipes)

Equipment

1. Incubator shaker (Eppendorf, model: New Brunswick™ Innova 44)
2. Centrifuge (Eppendorf, model: 5810R)
3. Ultracentrifuge (Beckman Coulter, model: Optima™)
4. 45Ti rotor (Beckman Coulter)
5. Sonicator (Branson Sonifier)
6. AKTA purifier (GE)

Procedure

A. Preparation of recombinant proteins of the TRBD subunit of telomerase

1. Medaka TRBD subunit (residues 318-579) was inserted into a modified pMAL-2CX vector and the plasmid was transformed into the *E. coli* strain ScarabXpress T7lac cells.
2. Pick a single colony from a freshly streaked plate. Inoculate a starter culture of 5 ml LB medium containing 100 µg/ml ampicillin in a sterile plastic tube. Incubate at 37 °C overnight with shaking at 250 rpm.
3. Dilute the overnight culture into 2 L LB medium containing 0.2% glucose and 100 µg/ml ampicillin, and grow the culture at 37 °C with shaking at 250 rpm until the cell density reaches $OD_{600} = 0.5$.
4. Induce the expression of the recombinant TRBD proteins with 0.1 mM IPTG at 20 °C for 16 h with shaking at 250 rpm.
5. Harvest cells by centrifugation at 4,000 rpm for 15 min at 4 °C (Beckman centrifuge).
6. Resuspend the cell pellet in 50 ml lysis buffer supplemented with 1 mM PMSF, 5 mM benzamidine, 1 µg/ml leupeptin, and 1 µg/ml pepstatin.
7. Sonicate the cell suspension with 6 short burst of 30 sec followed by intervals of 30 sec for cooling (sonication power 100 W).
8. Remove cell debris by ultracentrifuge at 4 °C for 40 min at 46,000 rpm using a 45Ti rotor.
9. Slowly add ammonium sulfate (powder) into cell lysate to 55% saturation (add 16.3 g ammonium sulfate per 50 ml cell lysate). Mix for 30 min at 4 °C with slow stirring.
10. Pellet the precipitated proteins by centrifuge at 4 °C for 15 min at 15,000 rpm using a 45Ti rotor.
11. Solubilize the protein pellet in lysis buffer and mix it with 5 ml pre-equilibrated amylose resin in a 50-ml conical tube. Rock the tube for 2 h at 4 °C.
12. Transfer the bead-protein mixture to a BIO-RAD Econo-Pac column and allow all of the liquid to flow through the column. Then, wash beads with 100 ml lysis buffer.
13. Elute the MBP-TRBD proteins with 15 ml lysis buffer added with 10 mM maltose.
14. Apply the eluted MBP-TRBD proteins to a HiLoad Superdex 200 chromatography column to perform gel filtration purification. The column is pre-equilibrated with 120 ml lysis buffer.
15. Collect the fractions that contain the MBP-TRBD proteins, and treat them with 200 µg protease 3C at 4 °C overnight to cleave the fusion proteins.
16. Slowly pass the protease 3C-digested protein solution through 5 ml pre-equilibrated amylose resin by gravity to remove MBP and the uncleaved MBP-TRBD proteins.

17. Apply the TRBD proteins to a HiLoad Superdex 200 chromatography column to perform gel filtration purification. The column is pre-equilibrated with 120 ml column buffer.
18. Concentrate the purified proteins using Amicon 15 ml centrifugal filter to a concentration of 10 mg/ml (determined by UV₂₈₀ absorbance) and store them at -80 °C in small aliquots.

B. Preparation and purification of the CR4/5 RNAs

1. Prepare the DNA templates for the *in vitro* transcription of the medaka telomerase CR4/5 RNA (nucleotides 170-220), with a glucosamine-6-phosphate-activated ribozyme (GlmS ribozyme) fused at its 3' terminus, by PCR amplification.
2. Set up 5 ml of the following *in vitro* transcription reaction and carry it out at 37 °C overnight.

1 ml	5x transcription buffer
1 ml	NTP mix (25 mM of each NTP)
0.5 ml	DNA template (200 µg/ml)
2.375 ml	Nuclease-free water
50 µl	Recombinant RNasin ribonuclease inhibitor
50 µl	T7 RNA polymerase (3 mg/ml)
25 µl	Pyrophosphatase (100 U/ml)
3. After the *in vitro* transcription, purify the CR4/5-GlmS-ribozyme RNAs from the DNA templates and the excess NTPs by gel-filtration chromatography on a HiLoad Superdex 200 column with the RNA buffer.
4. Pool and concentrate the RNA fractions. Treat them with 1 mM glucosamine-6-phosphate at 25 °C for 30 min to cleave the GlmS ribozyme from the 3' site of CR4/5.
5. Run another gel-filtration chromatography on HiLoad Superdex 200 with the column buffer to purify the CR4/5 RNAs from the GlmS ribozyme.
6. Concentrate the CR4/5 RNAs to 10 mg/ml.

C. Preparation of the TRBD-CR4/5 complex for crystallization

1. Mix the TRBD proteins and the CR4/5 RNAs at a molar ratio of 1:2, and incubate the mixture on ice for 1 h.
2. Purify the TRBD-CR4/5 mixture from the excess CR4/5 RNA by gel-filtration chromatography on a HiLoad Superdex 200 column with the column buffer.
3. Concentrate the TRBD-CR4/5 protein-RNA complexes to 12 mg/ml. Proceed to crystallization trials or store them at -80 °C in small aliquots.

Representative data

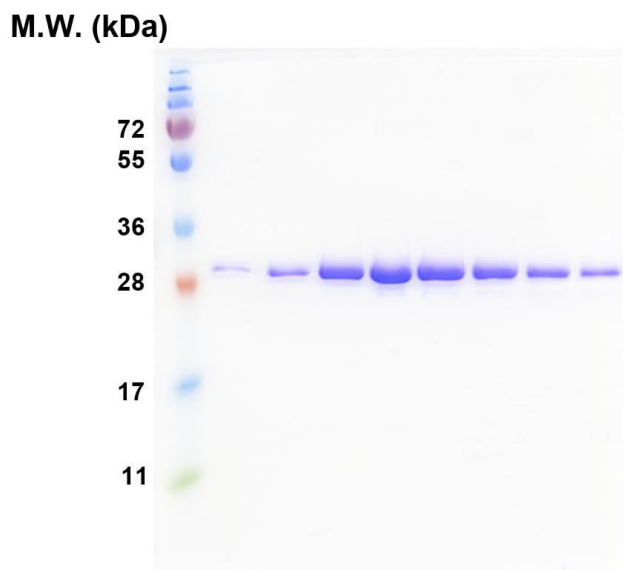


Figure 1. SDS-PAGE of the TRBD proteins after the final-step gel filtration

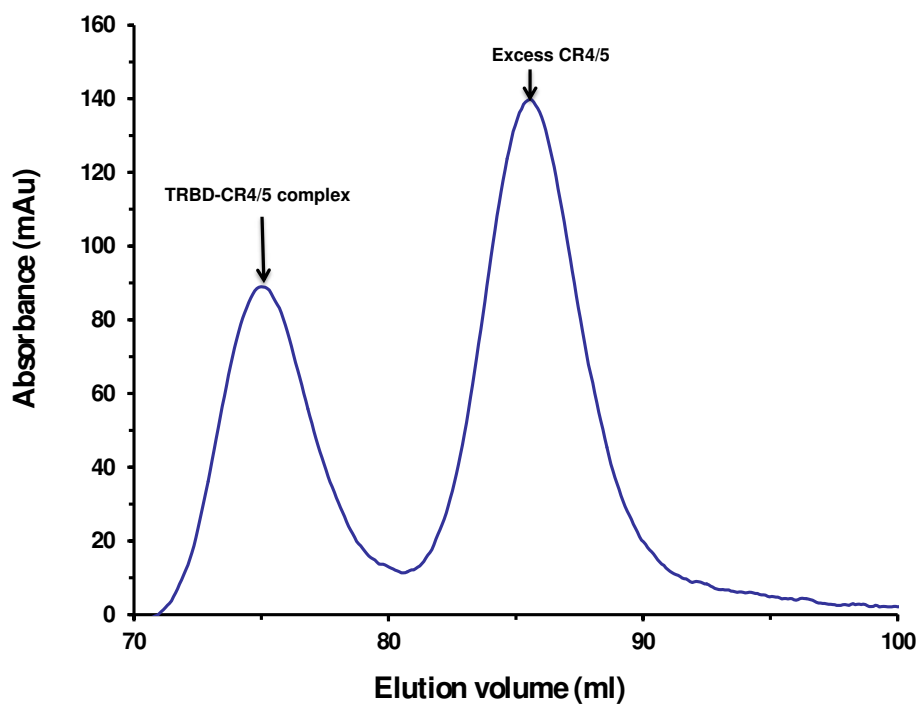


Figure 2. Gel filtration profile of the final-step purification of the TRBD-CR4/5 complex

Recipes

1. Lysis buffer

50 mM	Tris-HCl (pH 7.5)
500 mM	NaCl
1 mM	MgCl ₂
10% (v/v)	Glycerol
1 mM	Tris(2-carboxyethyl)phosphine (TECP)
2. Column buffer

50 mM	Tris-HCl (pH 7.5)
500 mM	NaCl
1 mM	MgCl ₂
1 mM	Tris(2-carboxyethyl)phosphine (TECP)
3. RNA buffer

50 mM	HEPES-KOH (pH 7.5)
150 mM	NaCl
10 mM	MgCl ₂
4. 5x transcription buffer

400 mM	HEPES-KOH (pH 7.5)
120 mM	MgCl ₂
10 mM	spermidine
200 mM	DTT

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