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Isolation of ribosome-associated nascent chains of soluble proteins produced in Escherichia coli

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Protocol status: Working
I (Renuka Kudva) have used this protocol for the paper cited and it has worked. This protocol was developed after discussions with Dr. Andreas Vogt and Dr. Kärt Denks.

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Abstract

Isolation of ribosome-nascent chains of co-translationally folded domains. Detailed description of protocol published in
https://doi.org/10.1073/pnas.1810523115. The arrest sequence used for stalling was TnaC (the leader peptide of the tryptophanase operon).

Guidelines

Protocol can be scaled up to obtain higher yields for biochemical experiments. It can also be adapted for isolating nascent chains of membrane proteins in complex with translocons. Please get in touch with the authors if needed. I recommend performing expression tests to pick clones that give the best yields.

Materials

MATERIALS

- Difco Bacto peptone **Fisher Scientific**
- Magnesium acetate, tetrahydrate **Bio Basic Inc.** **Catalog #**MB0326.SIZE.500g
- BD Bacto™ Yeast Extract **Becton Dickinson (BD)** **Catalog #**212750
- Acetic acid **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**695092
- Sodium chloride **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S3014
- Imidazole **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I5513
- Dodecyl-β-D-maltoside **Carl Roth** **Catalog #**CN26.5
- NuPAGE 4-12% Bis-Tris gel 1.0 mm 10 well **Thermo Fisher Scientific** **Catalog #**NP0321BOX
- TALON Metal affinity resin **Takara Bio Inc.** **Catalog #**635502
- tRNA from E.coli MRE600 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**10109541001
- cOmplete mini EDTA free protease inhibitor cocktail **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**4693159001
- HEPES Sodium salt **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H7006
- potassium acetate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P1190
- sucrose **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**84097
- L-tryptophan **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T0254
- L-arabinose **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A3256

Wash all flasks with milliQ water.

Lysogeny broth (LB)
5 g/l BD Bacto Yeast extract
10 g/l BD Bacto tryptone
10 g/l NaCl

autoclave.

Use flasks without baffels and keep a headspace to culture ratio of 4:1
For example 2 litres distributed into four 2.5 l flasks with 500 ml each for the growth cultures. 100 ml for the overnight culture.

Prepare stock solutions of (Adjust all chemical to room temperatur before opening)
1M HEPES-KOH pH 7.2 (store at room temp) filter through 0.2 or 0.45 µm filter and protect from light.
1M Magnesium acetate (store at room temp) autoclave or filter through 0.2 or 0.45 µm filter
4M Potassium acetate (store at room temp) autoclave or filter through 0.2 or 0.45 µm filter
100 mM Trp (store at -20°C long-term) filter through 0.2 or 0.45 µm filter
10% DDM (store at -20°C long-term)
1M Imidazole pH 8.0 adjust with acetic acid (store at -20°C, store in the dark), filter through 0.2 or 0.45 µm filter
20% Arabinose (prepare prior to use, filter sterilise) (inducer)

Buffer A (pH adjusted to 7.5 with KOH):
50 mM HEPES-KOH
150 mM Potassium acetate
10 mM Magnesium acetate
1 mM Tryptophan
0.025x cOmplete protease inhibitor pill (or 5 mM PMSF)

add PMSF just before use of the buffer.

Buffer B (pH adjusted to 7.5 with KOH):
50 mM HEPES-KOH
150 mM Potassium acetate
10 mM Magnesium acetate
1 mM Tryptophan
0.025X cOmplete protease inhibitor (or 5 mM PMSF)
750 mM sucrose (use a higher concentration to completely separate ribosomes from membranes 1.5 M)
0.1% DDM

Add PMSF directly before use.

Buffer C (pH adjusted to 7.5 with KOH)
20 mM HEPES-KOH
50 mM potassium acetate
5 mM magnesium acetate
125 mM sucrose
2 mM tryptophan
0.03% DDM

Wash Buffer (pH adjusted to 7.5)
50 mM HEPES-KOH
10 mM magnesium acetate
0.025X cOmplete or 5 mM PMSF
250 mM sucrose

Elution buffer (pH adjusted to 7.5)
50 mM HEPES-KOH
150 mM potassium acetate
10 mM magnesium acetate
0.025X cOmplete
150 mM imidazole
250 mM sucrose

Troubleshooting

Safety warnings

Use appropriate personal protective equipement at all times. Assure that you are trained in the handling of all equipment and adhere to local and national waste disposal regulations.

Handle ultra-centrifuges safely.
Wear a face-mask while weighing yeast extract and tryptone for the growth medium, and while weighing DDM. Follow safety instructions during autoclaving.
Take precautions while handling acids for titration.
Handle liquid nitrogen with cryo-protective gear, use eye goggles.
Use safe-seal reaction tubes.

Before start

Use a tuned-expression system for production. This study uses a plasmid under the control of an arabinose promotor, but any expression system (e.g. lac operon) may be used. The inducer needs to be adapted according to the expression vector used.
Engineering of the construct and the vector has not been included in this protocol.
Use the Escherichia coli KC6 strain to efficiently arrest translation of TnaC.

1 Expression tests (perform after transformation of constructs into KC6 cells)

Day 1

Pick 5 single clones from plates and seed into 2 ml LB supplemented with antibiotic of choice. Grow overnight at 37°C by shaking at 200 rpm. Prepare a master plate seeded with each of the clones used.

Day 2

1. Sub-culture the overnight cultures into 50 ml of LB supplemented with antibiotics to an A600 of 0.1. Culture at 37°C till it reaches an A600 of 0.5. (Check A600 every 30 min).
2. After cell growth to an A600 of 0.5, add 0.2% Arabinose (final concentration) to the cultures to induce expression and culture at 37 °C for 01:00:00
3. Cool cultures on ice for 00:15:00
4. Collect cells by centrifugation 3400 x g 00:10:00 at 4 °C
5. Resuspend each cell pellet in 5 mL buffer A.
6. Transfer the 5 mL of cell suspensions into several 2 ml reaction tubes. Supplement with 10mg/ml lysozyme and 10 mg/ml DNAse1.
7. Lyse cells by repeated cycles of freezing and thawing. (Plunge reaction tubes into liquid nitrogen to freeze and transfer tubes to a thermoblock at 42°C to thaw. Repeat 10 times and mix the lysate well between cycles of freezing and thawing.
8. Spin down lysate for 00:30:00 at 15000 x g in a cooled table-top centrifuge at 4 °C .
9. Transfer the lysate to ultracentrifuge tubes (polycarbonate tubes suited for the Beckman TLA 100.3 ultracentrifuge), underlie with Buffer B using a syringe and needle carefully to avoid bubbles. (1 ml of lysate and 1 ml of Buffer B per tube).
10. Centrifuge at 90000 rpm for 01:00:00 in a TLA 100.3 rotor.
11. Discard supernatant after centrifugation the ribosome go into the pellet.
12. Resuspend ribosomal pellet in 30 ul buffer C. Measure concentration on a nanodrop/spectrophotometer at A260.
13. Load different dilutions on SDS-PAGE (A260 of 7 for coomassie and A260 of 14 for Western blotting).
14. Check for differences in expression and make a reserach cell bank (glycerol stocks) of the best expressing clone.

If expression is not sufficient, check more clones or improve expression conditions.

2 Purification of ribosome-associated nascent chains of soluble proteins

Day 1

1. Prepare 2.1 litres of lysogeny broth (use high purity yeast extract and tryptone as described in the Methods section). I divided the medium into four flasks of 500 ml each. Prepare one flask with 100 ml for the seed culture. Autoclave.
2. Prepare buffers.
3. Seed the best expressing clone into 100 ml LB supplemented with antibiotic.
4. Grow overnight at 37°C.

Day 2

1. Dilute the overnight cultures into the flasks containing 500 ml of LB (supplemented with antibiotic for selection) to an A600 of 0.1. Culture at 37°C to an A600 of 0.5. Check for growth every 00:30:00
2. Induce expression of plasmid with 0.2% arabinose for 1 hour.
3. Chill cultures on ice for 30 min.
4. Collect cells by centrifugation 6500 x g for 00:10:00 at 4 °C in a JLA 8.1000 rotor.
5. Measure cell mass (wet weight of the pellet).
6. Resuspend cells in 2 ml BufferA/g cell mass.
7. Lyse cells with an emulsifex (3 passes at 8000 psi).
8. Clarify the cell lysate by centrifugation (30000 x g for 00:30:00 at 4 °C) in a JA25-50 rotor.
9. Distribute the cell lysate into Ti70 ultracentrifuge tubes (Beckman Coulter) (fill to around 12 mL) and underlie with 12 mL of buffer B with a needle and syringe.

10. Centrifuge at 24000 rpm for 20:00:00 at 4°C in a Ti70 rotor.

Day 3

1. Discard supernatant and gently resuspend the ribosome pellet in Buffer A (5 mL). Keep on ice and shake gently till the pellet goes into solution.
2. While the pellet is on ice, prepare the Talon resin. Swirl the bottle to resuspend the resin, pipette out 1 ml in a 15 ml tube and wash thrice with 10 bed volumes of buffer A. Supplement the last wash with 10 ug/ml total E.coli tRNA.
3. Transfer the ribosomal suspension to a dounce homogeniser and use the loose piston to homogenise.
4. Transfer the homogenate to the washed Talon resin and incubate for 01:00:00 for binding (rotate the tube on a roller).
5. Centrifuge at 700 x g for 2 min, discard supernatant.
6. Wash with 5× 10 bed volumes of wash buffer.
7. Elute with 2 bed volumes of elution buffer (incubate for 00:10:00 to 00:15:00 min with elution buffer) before collecting the elution.
8. Transfer eluant to polycarbonate ultracentrifuge tubes suited for the Beckman Coulter TLA 100.3 rotor, and spin at 40000 rpm for 02:00:00 . Discard the supernatant by pipetting out immediately, and resuspend the 70S pellet obtained in 20 µL buffer C. (I recommend a small volume to have a concentrated sample that can be easily diluted for further use. Make small aliquots since it's best to avoid multiple freeze-thaw cycles). Resuspension to be carried out by adding requisite volume to the pellet and letting the pellet go into solution.
9. Plunge freeze in liquid nitrogen and store at -80 °C long-term.