Isolation of homogenous ribosome-nascent-chain complexes from coupled cell-free transcription-translation system

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Method Article

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Abstract

Introduction

Recent studies have made some progress in tracking the conformation of the nascent chains[1-3]; however the earliest steps of folding _in vivo_ \(while chain synthesis is underway) remain unclear. This is in part due to a lack of experimental methods to produce stable ribosome-nascent chain complexes with uniform length of the nascent chains. Translation mixtures usually contain heterogeneous fraction of ribosomes with various lengths of the peptide chains due to re-initiation of the translation; this hinders the precise assessment of the conformation of the nascent chains. Here, we describe a detailed procedure to produce homogeneous fractions of ribosome-nascent-chain complexes with defined length of the stalled peptides.

Reagents

- coupled transcription-translation _E. coli_ cell-free system \(RTS100 _E. coli_ HY kit; Roche Applied Science) - T7 RiboMAX _in vitro_ transcription kit \(Promega) - Total RNA isolation kit \(Promega) - ^{35}S-methionine \(>1000 Ci/mM, Amersham) - Amino acid mix \(1 mM each amino acid, Met-depleted, provided by manufacturer, Roche Applied Science) - 3.75 mM aurintricarboxylic acid \(Sigma) dissolved in methanol - SDS-loading buffer: 3.79g Tris, 1g SDS, 0.5g bromophenol blue, 50ml glycerol, add water to 100ml, pH=6.8

Equipment

Temperature block \(Eppendorf) Ultracentrifuge \(Beckman) with TLA120.2 rotor SDS-PAGE equipment SDS-gel dryer \(Biorad) Phosphoimager \(Fujifilm)

Procedure

1. Dissolve the components of the coupled transcription-translation _E. coli_ cell-free system \(RTS100 _E. coli_ HY kit) as follows: a. The amino acid mixture in 240 µl of reconstitution buffer \(provided by manufacturer). Note that here we used methionine-depleted amino acid mix. b. The _E. coli_ lysate in 360 µl of reconstitution buffer. c. The reaction mixture in 300 µl of reconstitution buffer. 2. Mix the two components \(b and c), aliquot them into 100 µl, and freeze immediately in liquid nitrogen. The 2x master mix can be stored in -80°C for 3 months. Aliquot the amino acid mix in 50 µl and store also at -80 °C. 3. Subclone your gene of interest or truncations of it into pIVEX vector \(Roche Applied Science) or in any T7-promoter based vectors \(e.g., pET-system). The coding sequence of the protein of interest or its truncations should be extended C-terminally by the SecM-sequence \(DYAHFTPQAKFxxxxWIxxxxGIRAGP)[4]in order to stably stall the nascent chains on the ribosomes. The SecM should be linked to the protein or its truncations by a Gly-Ser-rich linker \(preferably 13 amino acids long), which will ensure that the complete full-length nascent chain of interest is outside the ribosomal

tunnel. 4. Add 2.5 ng plasmid DNA to 9.5 µl aliquot of the 2x-master mix \(thawed on ice) and adjust the final volume to 20.5 µl with sterile RNAse-free water. Transcribe for 15 min at 30°C. Perform control reaction under identical conditions using an empty plasmid without an insert. Alternatively, the transcription can be performed separately using _in vitro_ transcription system \(Promega): mRNA is transcribed according to manufacturer instructions with T7 RiboMAX _in vitro_ transcription kit and purified thereafter with total RNA isolation kit. 2 µl purified mRNA from one transcription reaction is added to 9.5 µl aliquot of the 2x-master mix and the final volume is adjusted to 20.5 µl with sterile RNAse-free water. 5. Initiate translation by adding 1 µl 0.6 $mCi.ml^{-1}$ ^{35}S-methionine and 3 µl of the amino acid mix. 6. After 15 seconds add 0.5 µl aurintricarboxylic acid to 75 µM final concentration to synchronize the reaction[5], and incubate further at 30 °C. Alternatively, the translation reaction can be performed from 24 °C- 37 °C if necessary, however the optimal translation temperature for RTS100 _E. coli_ HY \(suggested also by the manufacturer) is 30 °C. 7. Set the time for translation according to the length of the gene of interest, given that the rate of translation at 30 °C is 43-54 amino acid/min, at 37 °C – 130-140 amino acids/min, and at 24 °C - 20-25 amino acids/min. 8. Stop the reaction by immediate chilling on ice[6]; keep an aliquot of 5 µl for further SDS-PAGE analysis. 9. Layer 20 µl of the reaction mixture on a 900 µl of 0.5 M sucrose in 200 mM HEPES buffer pH 7.5, containing 15 mM $MgCl_2$ and 100 mM KOAc and pelleted by ultracentrifugation for 20 min at 4 °C \(TLA120.2 rotor, 120000 rpm). 10. Carefully remove the whole supernatant and gently resuspended the pellet in 20 µl of 200 mM HEPES buffer pH 7.5, containing 15 mM $MgCl_2$ and 100 mM KOAc. 11. Mix 5 µl aliquot with the 5 µl SDS-loading buffer, heat at 95 °C for 3 min and load onto 12% SDS-PAGE. The aliquot from step 8 should be identically processed and loaded on the same gel. 12. Dry the gel using vacuum gel dryer and visualize the translation pattern by phosphoimaging. Note that due to the low concentration of the nascent chains, an overnight exposure at low temperature \(-20 °C) in the lead-shielded cassette is recommended.

Timing

Preparation of buffers and SDS-gel – 4-5 hours \(time is variable depending on the experience) Translation reaction and ultracentrifugation – 1-2 hours SDS-electrophoresis and drying of the gel – 4-5 hours Exposure in the phosphoimager – over night

Critical Steps

Translation \(steps 3-7) is crucial. The yields might be first analyzed on SDS-PAGE before proceeding with the next steps. Perform all the reaction post-translation \(steps 7-9) to decrease the drop-off of the nascent chains.

Troubleshooting

No yields in the transcrition-translation reaction: Use preferably pIVEX vectors for the expression. Note that the RTS100 _E. coli_ HY system is optimized for the pIVEX vectors and with other T7-vectors lower

translational yields might be achieved. Low yields in the transcrition-translation reaction: - increase the time of the reaction by 10-15% as the particular protein might be synthesized with a non-uniform rate of translation through many local attenuations. - increase also the time for transcription to achieve higher starting concentration of mRNA. - perform separate transcription \(step 4) and use higher starting mRNA concentration. - avoid adding any reagents containing glycerol as it might severely reduce the yields of translation reaction.

References

1. Gilbert, R.J. et al. Three-dimensional structures of translating ribosomes by Cryo-EM. _Mol. Cell_ **14**, 57-66 \(2004). 2. Hsu, S.T. et al. Structure and dynamics of a ribosome-bound nascent chain by NMR spectroscopy. _Proc. Natl. Acad. Sci. USA_ **104**, 16516-16521 \(2007). 3. Johnson, A.E. The co-translational folding and interactions of nascent protein chains: a new approach using fluorescence resonance energy transfer. _FEBS Lett._ **579**, 916-920 \(2005). 4. Murakami, A., Nakatogawa, H. & Ito, K. Translation arrest of SecM is essential for the basal and regulated expression of SecA. _Proc. Natl. Acad. Sci. USA_ **101**, 12330-12335 \(2004). 5. Stewart, M.L., Grollman, A.P. & Huang, M.T. Aurintricarboxylic acid: inhibitor of initiation of protein synthesis. _Proc. Natl. Acad. Sci. USA_ **68**, 97-101 \(1971). 6. Woolhead, C.A., Johnson, A.E. & Bernstein, H.D. Translation arrest requires two-way communication between a nascent polypeptide and the ribosome. _Mol. Cell_ **22**, 587-598 \(2006).