Monolith Protocol MO-P-044

Sc-tRNA – Elongator Subcomplex yElp456

tRNA is a class of RNA molecules that plays a role during the translation process – the synthesis of functional proteins. Recently, modifications of RNA molecules were shown to be linked with their biological activity. The elongator complex Elp is formed by two subcomplexes that consist of structurally arranged Elp1, Elp2, Elp3 (Elp123 subunit) and Elp4, ELp5, Elp6 (Elp456 subunit). The Elp456 subunit forms a heterohexameric ring-like structure and is involved in specific interactions with tRNA molecules.

protein – RNA interaction | elongator protein | tRNA binding | tRNA modifications

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A1. Target/Fluorescent Molecule

Sc-tRNA$_{Ala}$

A2. Molecule Class/Organism

RNA
Saccharomyces cerevisiae

A3. Sequence/Formula

5’ GGG CAC AUG GCG CAG UUG GUA GCG CGC UUC CCU UGC AAG GAA GAG GUC AUC GGU UCG AUU CCG GUU GCG UCC A 3’

A4. Purification Strategy/Source

In vitro transcription of tRNA with T7 polymerase:

1. The template for transcription is prepared as double-stranded DNA in a standard PCR approach.[1]
2. Large scale purification of the template is achieved from 2L 2xYT-carbenicilin culture and extraction is performed with QIAfilter® plasmid MEGA or GIGA protocols (Qiagen™) – this process typically yields 3 – 6 mg of DNA.
3. The DNA is dissolved in water and linearized with BbsI or BsaI, using an enzyme concentration of 50 U/ml and overnight incubation at 37°C (BbsI) or 50°C (BsaI).
4. The large scale 10 – 40 mL transcription reaction contains 4 mM rNTPs, 1.3 mM CTP-Cy5, 70 µg/ml linearized DNA template, 1200 U/ml T7 RNA polymerase, 40 mM Tris-HCl (pH 8.1 @37°C), 1 mM spermidine, 5 mM DTT, 0.1% Triton-X, 1 U/ml inorganic phyrophospatase and 40 mM $MgCl_2$. The transcription reaction is incubated for 2 – 4 h at 37°C and stopped by addition of 50 mM EDTA. The transcripts could be stored at –20°C until RNA purification.

[1] Lukavsky and Puglisi, RNA (2004), 10:889–893

5. tRNAs are purified using an ÄKTA-FPLC system, equipped with a 50 mL superloop and three connected in tandem 5 mL HiTrap DEAE columns, using Buffer A (50 mM sodium phosphate, 150 mM sodium chloride and 0.2 mM EDTA, pH 6.5 at room temperature) and Buffer B (50 mM sodium phosphate, 2 M sodium chloride and 0.2 mM EDTA, pH 6.5 at room temperature). Columns are equilibrated with 3 CV of Buffer A. The stopped transcription reaction is loaded into the 50 mL superloop and weak anion-exchange chromatography is performed using the following gradient and collecting 10 mL fractions in sterile 15 mL tubes.
    a. Gradient step 1: 0 – 70 mL, 0% B at 1 mL/min – loading sample on DAEA columns
    b. Gradient step 2: 70 – 100 mL, 0% – 10% buffer B at 2 mL/min – wash free rNTPs
    c. Gradient step 3: 100 – 380 mL, 10% – 30% buffer B at 2 mL/min – separate short abortive transcripts, RNA product, and plasmid DNA
    d. Gradient step 4: 380 – 410 mL, 30% – 100% buffer B at 4 mL/min – columns wash
    e. Gradient step 5: 410 – 455 mL, 100% buffer B at 4 mL/min – columns wash
    f. Gradient step 6: 455 – 485 mL, 100% – 0% buffer B at 4 mL/min – columns re-equilibration
6. 5 µL of each fraction is analyzed by denaturing PAGE (8% acrylamide, 8 M urea)
7. Optional: In case RNA profiles display more than one peak along gradient suggesting alternative, conformations or multimerization of the RNA, each fraction should be analyzed by HPLC-SEC using e.g. a SEC-125 column and Buffer A.
8. Structurally homogeneous RNA is pooled, concentrated and equilibrated into appropriate buffers using 15 mL Centriprep centrifugal devices.

A5. Stock Concentration/Stock Buffer

280 µg/ml | 12 µM
20 mM HEPES pH7.5, 50 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 2 mM DTT

A6. Molecular Weight/Extinction Coefficient

24.9 kDa
940,500 $M^{-1}cm^{-1}$ (ε_{260})

A7. Dilution Buffer

20 mM HEPES, pH 7.5, 50 mM NaCl, 0.05% TWEEN® 20, 2 mM DTT

A8. Labeling Strategy

Labeled during in-vitro transcription by internal covalent attachment of Cy5

A9. Labeling Procedure

Labeling protocol available from New England Biolabs® for RNA synthesis with modified nucleotides (E2050)™. The molar ratio of modified NTP to standard NTP was 1:3.

A10. Labeling Efficiency

Measurement of protein concentration and degree of labeling (DOL) using a NanoDrop™:
nanotempertech.com/dol-calculator

Absorbance A_{260}	17.424	Protein concentration	18.5 µM
Absorbance A_{647}	0.134	Degree-of-labeling (DOL)	0.0289

B1. Ligand/Non-Fluorescent Binding Partner

yElp456 wt

B2. Molecule Class/Organism

tRNA binding protein
Saccharomyces cerevisiae

B3. Sequence/Formula

Elp4

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MSFRKRGEIL NDRGSGLRGP LLRGPPRTSS TPLRTGNRRA PGNVPLSDTT ARLKKLNIAD ESKTKMGLDS SHVGVRPSPA
TSQPTTSTGS ADLDSILGHM GLPLGNSVLV EEQSTTEFHS ILGKLFAAQG IVHNRISDSS ADKTRNGDTH VIVLSLNQMF
AKELPGIYKG SRKQMKKNLI SEEESKVTVQ NLNETQRSTP SRYKDLKIAW KYKLADEKRL GSPDRDDIQQ NSEYKDYNHQ
FDITTRLMPA PIASELTFIA PTQPVSTILS QIEQTIKRND KKLIRIVIPS LLHPAMYPPK MFESSEIIGL MHGVRSLVKK
YYERVVLFAS ISIDIITPPL LVLLRNMFDS VINLEPFNQE MTEFLERVYK SQPGKIQHGL VHILKLPVFT DRGEMRVLKS
EWAFKNGRKK FEIEQWGIPV DDAEGSAASE QSHSHSHSDE ISHNIPAKKT KISLDY
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Elp5

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MASSSHNPVI LLKRILSLTE SSPFILCLDS IAQTSYKLIQ EFVHQSKSKG NEYPIVYISF ETVNKPSYCT QFIDATQMDF
VHLVKQIISY LPAATATQAK KHMVIIDSLN YISTEYITRF LSEIASPHCT MVATYHKDIK DENRTVIPDW NNNYPDKLTL
LQFMATTIVD IDVVLTGTLD TEEVSELLNE FRIPRGLNND IFQLRLVNKR KSGRSLEYDF IVNSNTHEYE LLSTTKQEEE
SSSNGLETPE MLQGLTTFNL GTSNKQKLAK DQVALPFLEA QSFGQGGAIV YEYEKDDDYD EEDPYEDPF
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Elp6

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MGSVQRQDLV LFSDQSVLPA HFFQDSNSHN LFFITHQSCT QPLWMINALV ETHVLGSPSS LNESSSSMLP SSTRSHAVLA
SFIHEQNYFT NSLNKLKIPS NNYNVLDFLS DFIVNNIHNK PRDKILSDVL AKFSAAIQNN PTDTIVIIEQ PELLLSLVSG
LTCSELNNKF ITPLLRQCKV LIIVSNSDIF NIDEYDASVH SSNLQNFYKS SFIKSMINLN LNPLKTGFAK DVTGSLHVCR
GGAPIATSNT SLHVVENEYL YLNEKESTKL FYR
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B4. Purification Strategy/Source

The purification strategy included trial-and-error process of expression and purification of different combinations of full-length Elp4, Elp5, Elp6 (including single 6xHis-tagged protein), followed by mass spectrometry analysis to define stable, interacting and well-expressing hexameric Elp456 protein construct.

The final construct, pBS3576 encodes 6xHis-tag-Elp6$_{1\text{-}273}$, Elp5$_{1\text{-}270}$, Elp4$_{66\text{-}426}$ in tandem for co-expression using pET24d derived vector. Constructs were expressed in E.coli (BL21 Gold (DE3) incubated at 18°C overnight for 12 – 15 h). Bacterial cells were lysed using a French press in 50 mM HEPES (pH 7.5), 300 mM NaCl, 10 mM imidazole, 1 mM DTT, 5% (v/v) glycerol and protease inhibitors. The soluble fraction was cleared by centrifugation (20,000× g for 45 min at 4°C). 6-His-tagged complexes were purified using Ni-NTA affinity chromatography followed by gel filtration with a Superdex 200 column (GE Healthcare™) in 20 mM Hepes-NaOH (pH 7.5), 150 mM NaCl, 2 mM DTT.

B5. Stock Concentration/Stock Buffer

9 mg/mL | 43.7 µM
20 mM HEPES pH 7.5, 50 mM NaCl, 2 mM DTT

B6. Molecular Weight/Extinction Coefficient

206 kDa (theoretical: 136.2 kDa)

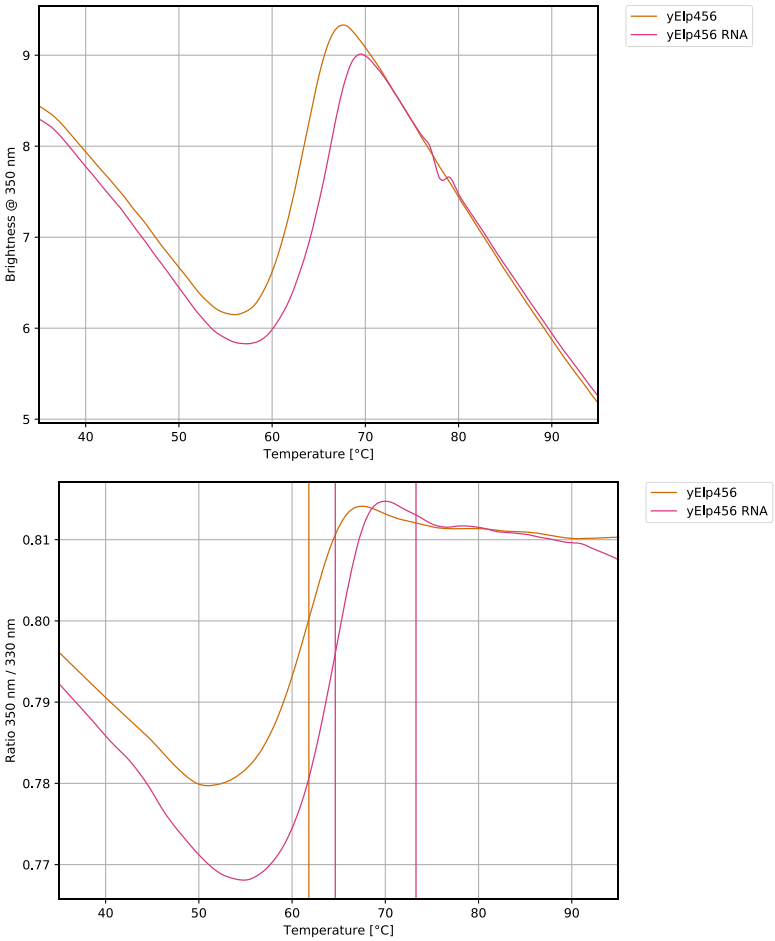
B7. Serial Dilution Preparation

1. Add 193 µL of dilution buffer to 7 µL of labeled Sc-tRNA$_{Ala}$ to obtain 200 µL of a 420 nM solution.
2. Add 16 µL of dilution buffer to 14 µL of 43.7 µM yElp456 to obtain 30 µL of a 20 µM solution.
3. Prepare a PCR-rack with 16 PCR tubes. Transfer 20 µL of the 20 µM yElp456 solution into tube **1**. Then, transfer 10 µL of dilution buffer into tubes **2** to **16**.
4. Prepare a 1:1 serial dilution by transferring 10 µL from tube to tube. Mix carefully by pipetting up and down. Remember to discard 10 µL from tube **16** to get an equal volume of 10 µL for all samples.
5. Add 10 µL of 420 nM labeled Sc-tRNA$_{Ala}$ to each tube from **16** to **1** and mix by pipetting.
6. Incubate for 20 minutes at room temperature in the dark before loading capillaries.

C. Applied Quality Checks

Validation of structural integrity and functionality of yElp456 using Tycho NT.6:
nanotempertech.com/tycho

yElp456	yElp456	T_i = 61.8°C
yElp456 RNA	yElp456 + Sc-tRNA$_{Ala}$	T_i = 64.6°C



D1. MST System/Capillaries

Monolith NT.115 Red (NanoTemper Technologies GmbH)
Premium Capillaries Monolith NT.115 (MO-K025, NanoTemper Technologies GmbH)

D2. MST Software

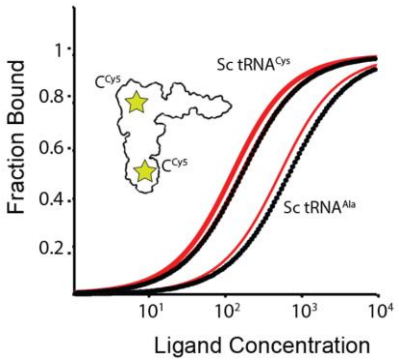
MO.AffinityAnalysis v2.3 (NanoTemper Technologies GmbH)
nanotempertech.com/monolith/#monolith-software

D3. MST Experiment (Assay Buffer/Concentrations/Temperature/MST Power/Excitation Power)

20 mM HEPES pH 7.5, 50 mM NaCl, 0.05% TWEEN® 20, 2 mM DTT
210 nM Sc-tRNA$_{Ala}$ | 10 µM – 31 nM yElp456 | 20°C | low MST power | 20% excitation power

D4. MST Results (Capillary Scan/Time Traces/Dose Response)

K_d = 605.7 ± 17 nM Kojic et al., Nature Communications 9:3195 (2018)



D5. Reference Results/Supporting Results

N/A

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