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Title: Comparative RNA Structure Analysis of Nascent and Mature Transcripts in *Saccharomyces cerevisiae*

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. Filming location: Will the filming need to take place in multiple locations? **Yes**

If **Yes**, how far apart are the locations? **The lab and room (for interviews) are in the same hallway.**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 21

Number of Shots: 51

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

Answers will be slightly edited in accordance with the journal guidelines

INTRODUCTION:

REQUIRED: ~~Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Karla Neugebauer**: We study co-transcriptional RNA processing. This includes understanding nascent RNA folding kinetics while the polymerase is still bound to RNA.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Lokha Boopathy**: CoSTseq was developed to investigate nascent RNA folding in yeast. It is particularly effective for analyzing highly abundant cellular RNAs.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.4. **Leena Sen**: With CoSTseq, researchers can determine in vivo nascent RNA secondary structures and the position of RNA Polymerase along RNA transcripts.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.5. **Lokha Boopathy**: CoSTseq has time-sensitive steps and uses toxic chemicals; it is better to process no more than two samples at a time.

- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.6. **Leena Sen:** From the total RNA generated for CoSTseq, non-biotinylated RNAs can be used for concurrent mature structure probing using traditional DMS-MaPseq.
 - 1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Preparation of Yeast Cells for Co-Transcriptional Structure Tracking Sequencing

Demonstrator: Lokha Boopathy

2.1. To begin, inoculate *Saccharomyces cerevisiae* strain BY4741 (*B-Y-4-7-4-1*) in 50 milliliters of YPAD media [1] and incubate at 30 degrees Celsius with shaking at 200 revolutions per minute until the culture reaches mid-log phase [2].

2.1.1. WIDE: Talent inoculating media with yeast strain BY4741.

2.1.2. Talent placing the culture in a shaker incubator set to 30 degrees Celsius and 200 revolutions per minute.

2.2. Collect 1.8 optical density units, approximately 3 milliliters, of yeast culture and centrifuge at 2,500 *g* for 3 minutes at 4 degrees Celsius using a pre-cooled centrifuge [1]. Discard the supernatant into a waste container [2]. Resuspend the pellet in 10 milliliters of cold PBS [3] and centrifuge again at 2,500 *g* for 3 minutes at 4 degrees Celsius [4]. Remove the supernatant and keep the yeast pellet on ice [5].

2.2.1. Talent loading 3 milliliters of yeast culture taken in a tube into the pre-cooled centrifuge.

2.2.2. Talent pouring the supernatant into a designated waste container.

2.2.3. Talent resuspending the pellet in 10 milliliters of cold phosphate-buffered saline.

2.2.4. Talent placing the tube back into the centrifuge for a second spin.

2.2.5. Talent removing the supernatant and placing the pellet tube on ice.

2.3. Carefully resuspend the yeast pellet in 10 milliliters of cold 0.5 percent sarkosyl without creating bubbles [1]. Incubate the resuspended yeast on ice for 20 minutes to allow permeabilization [2]. Then, pellet the cells at 400 *g* for 5 minutes at 4 degrees Celsius [3] and resuspend the permeabilized yeast cells in 100 microliters of nuclease-free water using a P1000 pipette. [4].

2.3.1. Talent gently resuspending the yeast pellet in 10 milliliters of cold 0.5 percent sarkosyl.

2.3.2. Talent placing the tube on ice for incubation.

2.3.3. Talent centrifuging the sample at 400 *g* for 5 minutes at 4 degrees Celsius.

2.3.4. Talent resuspending the resulting pellet in 100 microliters of nuclease-free

water using a P1000 pipette.

3. Nuclear Run-On and Dimethyl Sulfate (DMS) Probing

Demonstrator: Lokha Boopathy and Leena Sen

3.1. Prepare a working solution of 2.5x transcription buffer with freshly added 5 millimolar dithiothreitol [1] and preheat 2.5x structure probing buffer to 30 degrees Celsius [2].

3.1.1. Talent measuring and mixing 2.5 times transcription buffer with dithiothreitol to create the working solution.

3.1.2. Talent placing the structure probing buffer into the thermomixer set to 30 degrees Celsius.

3.2. In a clean 2-milliliter tube, add all the required reaction components and mix thoroughly [1 and 2].

3.2.1. Talent pipetting 120 microliters of 2.5 times transcription buffer into the tube.

3.2.2. TEXT ON 3.2.1's BACKGROUND:

2.5× Transcription Buffer: 120 µL

ATP (10 mM): 3.75 µL

GTP (10 mM): 3.75 µL

UTP (10 mM): 3.75 µL

Bio-CTP (1 mM): 7.5 µL

Sarkosyl (10%): 15 µL

Nuclease-free Water: 46.25 µL (to 200 µL total)

3.3. Next, add 100 microliters of the prepared yeast cells to the reaction tube [1] and incubate it in the thermomixer set to 30 degrees Celsius with shaking at 500 revolutions per minute for 2 minutes [2].

3.3.1. Talent pipetting 100 microliters of cells into the reaction mixture.

3.3.2. Talent placing the tube in the thermomixer and initiating the 2-minute incubation with shaking. **Author's NOTE: this part was filmed as a separate shot**

3.4. Once the nuclear run-on incubation is complete, quickly add 200 microliters of the

prewarmed 2.5x structure probing buffer and 25 microliters of dimethyl sulfate reagent to the tube simultaneously [1]. Vortex the tube gently in two pulses [2] and place it in an incubator set to 30 degrees Celsius and 500 revolutions per minute [3]. Continue to incubate on the thermomixer for 4 minutes, spacing 30 seconds between samples [3].

3.4.1. Talent adding structure probing buffer and dimethyl sulfate at the same time.

3.4.2. Talent gently vortexing the tube in two short pulses.

3.4.3. Talent placing the tube back into the thermomixer

3.4.4. Talent turning on the timer.

3.5. Prepare the stop and wash buffers ahead of time and place them on ice until use [1]. Stop the dimethyl sulfate methylation by adding 1 milliliter of stop buffer to the reaction tube [2].

3.5.1. Talent retrieving stop buffer from the ice.

3.5.2. Talent adding stop buffer to the DMS-treated sample.

3.6. Now, centrifuge the dimethyl sulfate-labeled samples at 3,500 *g* for 5 minutes in a pre-cooled centrifuge [1]. After the spin, discard the supernatant into an appropriate waste container [2].

3.6.1. Talent placing the tube in the pre-cooled centrifuge.

3.6.2. Talent removing and disposing of the supernatant.

3.7. Add 1 milliliter of chilled wash buffer to the pellet and resuspend [1]. Repeat the centrifugation at 3,500 *g* for 5 minutes [2]. Proceed immediately to RNA extraction and do not freeze the yeast pellet [3].

3.7.1. Talent retrieving wash buffer from the ice and resuspending the pellet in wash buffer.

3.7.2. Talent placing the tube back in the centrifuge for the second spin.

3.7.3. Talent keeping the pellet on ice ready for RNA extraction.

3.8. Resuspend the dimethyl sulfate-labeled yeast pellet in 600 microliters of RNA lysis buffer [1] and then transfer this suspension into a tube containing 40 microliters of 20 percent sodium dodecyl sulfate [2]. Incubate the sample at 65 degrees Celsius for 30 seconds with shaking at 950 revolutions per minute [3].

3.8.1. Talent adding RNA lysis buffer to the yeast pellet.

- 3.8.2. Talent transferring the lysate into tube with sodium dodecyl sulfate.
- 3.8.3. Talent placing tube in the thermomixer at 65 degrees Celsius for the 30-second incubation.

3.9. Next, perform Phenol-Chloroform extraction of RNA following the standard procedure [1] and continue heating it on the thermomixer at 65 degrees Celsius with shaking at 950 revolutions per minute for 2 minutes [2]. Then, allow the sample to sit on ice for 5 minutes [3]. Complete the Phenol-Chloroform extraction of RNA following the standard procedure [4].

- 3.9.1. Talent pipetting pre-warmed acid phenol:chloroform into the lysate tube.
- 3.9.2. Talent incubating the tube in the thermomixer for 2 minutes. **NOTE: Not required to be added in the video**
- 3.9.3. Talent placing the tube on ice.
- 3.9.4. Talent removing the sample from ice and looking at the tube.

4. Biotin Pulldown and RNA Elution

Demonstrator: Leena Sen

4.1. Vortex and add 44 microliters of the streptavidin magnetic beads in a tube per sample containing 80 micrograms of total RNA [1].

- 4.1.1. Talent vortexing a vial of streptavidin magnetic beads and pipetting 44 microliters into labeled sample tubes.

4.2. Place the magnetic beads on a magnetic rack until the beads settle [1]. After removing the storage buffer, resuspend the beads in 1 milliliter of prewash buffer A [2].

- 4.2.1. Talent placing tubes on the magnetic rack.
- 4.2.2. Talent adding prewash buffer A and resuspending the beads.

4.3. Now, incubate the suspension for 2 minutes at room temperature [1]. Then, magnetize and remove the supernatant [2-TXT].

- 4.3.1. Talent allowing the tube to rest on the bench for 2 minutes.
- 4.3.2. Talent removing the tube from the magnetic rack and removing the supernatant. **TXT: Wash again with prewash buffer A and then with prewash**

buffer B

- 4.4. After washing, resuspend the beads in 88 microliters of 2x binding buffer [1]. Transfer 80 microliters of this suspension to a sterile 1.5-milliliter tube containing 80 micrograms of biotinylated RNA sample in 80 microliters [2].
 - 4.4.1. Talent adding 2 times binding buffer to the bead pellet and pipetting to mix.
 - 4.4.2. Talent transferring the bead suspension into tubes containing the biotinylated RNA.
- 4.5. Rotate the bead-sample mixture on a rotator for 20 minutes at room temperature [1]. Then, place the tube on the magnetic rack to collect the flow-through containing mature RNA and save it for precipitation to perform Poly A selection of mature RNAs using DMS-MaPSeq workflow [2]. **Authors: We do not refer to the manuscript in the narration as per our guidelines.**
 - 4.5.1. Talent placing the tube on a rotator and setting a 20-minute timer.
 - 4.5.2. Talent collecting the flow-through and transferring it to a new tube.
- 4.6. Next, rinse the bead-nascent RNA complex twice with 500 microliters of high salt buffer [1]. Then rinse once with 500 microliters of 1x binding buffer followed by a final rinse with 500 microliters of low salt buffer [2].
 - 4.6.1. Talent performing two sequential washes with high salt buffer using a magnetic rack.
 - 4.6.2. Talent rinsing once with 1x binding buffer and removing the supernatant. **Author's NOTE: This part of the step was not filmed - as we have it demonstrated bead washing previously in step 4.3.2. Same footage can be reused here]**
- 4.7. Now, add 300 microliters of RNA reagent to the bead-nascent RNA complex, resuspend thoroughly [1] and incubate on the thermomixer for 5 minutes at 60 degrees Celsius [2].
 - 4.7.1. Talent pipetting RNA reagent into the tube and mixing to resuspend the beads.
 - 4.7.2. Talent placing the tube into the thermomixer for 5-minute incubation.
- 4.8. Next, add 60 microliters of chloroform, vortex [1], and incubate for 3 minutes at room temperature [2]. Centrifuge the sample at 14,000 *g* for 5 minutes in the pre-cooled centrifuge [3].

- 4.8.1. Talent adding chloroform, capping the tube, and vortexing.
- 4.8.2. Talent placing the sample aside on work bench.
- 4.8.3. Talent placing the tube in the centrifuge and initiating the spin.

4.9. Finally, transfer the upper aqueous phase, approximately 180 microliters, to a new collection tube [1]. Discard the remaining organic phase, leaving behind the beads and residual aqueous solution [2-TXT]. After purifying the nascent RNA, perform template-switch reverse transcription, ligate the 5-prime adapter and select libraries for sequencing [3].

- 4.9.1. Talent carefully pipetting the upper aqueous layer into a fresh tube.
- 4.9.2. Talent discarding the lower phase and adding trizol to the beads. **TXT: Repeat the extraction to maximize the nascent RNA recovery; Then, precipitate the RNA using EtOH** **NOTE: Do not show adding trizol to tube, only show removing and discarding the solution.**

4.9.3. *Reuse 3.2.1*

~~TEXT ON 4.9.1's BACKGROUND:~~

- ~~— Quantify the yield of nascent RNA~~
- ~~— Perform template switching reverse transcription~~
- ~~— Ligate 5' adapter and amplify using indexed PCR primers~~
- ~~— Size select and validate libraries for sequencing~~
- ~~- Analyze the sequencing data using the CoSTseq analysis pipeline~~

Results

5. Results

- 5.1. Visualization of the test PCR products on a 1% agarose gel revealed minimal primer dimer formation and a characteristic smear around 300 base pairs for Co-transcriptional Structure Tracking sequencing or CoSTseq (*Cost-Seq*) and DMS-MaPseq (*D-M-S-map-seq*) libraries [1].
 - 5.1.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the smear between 200 and 500 marks in each of the four gel lanes labeled samples 1 and 2.*
- 5.2. The electropherogram for CoSTseq sample 1 confirmed the library size distribution, with a peak around 285 base pairs [1].
 - 5.2.1. LAB MEDIA: Figure 4C. *Video editor: Highlight the peak labelled 285 base pairs in the electropherogram graph.*
- 5.3. Read alignment of *ASC1* mRNA revealed that the CoSTseq library included coverage across the intron confirming that the RNA is nascent[1], whereas the DMS-MaPseq library lacked intron coverage, indicating that the RNA is mature [2].
 - 5.3.1. LAB MEDIA: Figure 4D. *Video editor: Highlight the upper CoSTseq track.*
 - 5.3.2. LAB MEDIA: Figure 4D. *Video editor: Highlight the lower DMS-MaPseq track.*
- 5.4. The co-transcriptional folding matrix of 18S pre-rRNA revealed that the DMS reactivity showed abrupt changes as RNA polymerase progressed from position 790 to 811 along the rDNA locus [1].
 - 5.4.1. LAB MEDIA: Figure 5A. *Video editor: Emphasize the bars near the red arrow on the right.*
- 5.5. DMS reactivity analysis of *ADH1* mRNA showed similar profiles between nascent and mature forms, indicating regions of structural stability [2].
 - 5.5.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the profiles for both nascent and mature ADH1 mRNA.*

5.6. In contrast, SSA2 mRNA exhibited regions of different DMS reactivity between nascent and mature forms, suggesting structural changes during maturation [2].

5.6.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the red arrowheads in both nascent and mature traces for SSA2 mRNA.*

1. Saccharomyces
Pronunciation link: <https://www.howtopronounce.com/saccharomyces>
IPA: /ˌsækəroʊˈmaɪsiːz/
Phonetic Spelling: sak·uh·roh·my·seez
2. cerevisiae
Pronunciation link: <https://www.howtopronounce.com/cerevisiae>
IPA: /ˌserəˈviːsiə/
Phonetic Spelling: seh·ruh·vih·see·eye
3. inoculate
Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculate>
IPA: /ɪˈnoːkjəˌleɪt/
Phonetic Spelling: ih·naa·kyuh·layt
4. incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /ˈɪŋkjəˌbeɪt/
Phonetic Spelling: ing·kyuh·bayt
5. centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
6. supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuːpərˈneɪtənt/
Phonetic Spelling: soo·per·nay·tuhnt
7. resuspend
Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>
IPA: /ˌriːsəˈspend/
Phonetic Spelling: ree·suh·spend
8. pellet
Pronunciation link: <https://www.merriam-webster.com/dictionary/pellet>
IPA: /ˈpɛlɪt/
Phonetic Spelling: pel·it
9. phosphate
Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphate>

IPA: /'fɑ:s,fet/

Phonetic Spelling: faas·fayt

10. saline

Pronunciation link: <https://www.merriam-webster.com/dictionary/saline>

IPA: /'seɪlɪn/

Phonetic Spelling: say·line

11. sarkosyl

Pronunciation link: <https://www.howtopronounce.com/sarkosyl>

IPA: /'sɑ:rkʊs,ɪl/

Phonetic Spelling: saar·koh·sil

12. permeabilization

Pronunciation link: <https://www.howtopronounce.com/permeabilization>

IPA: /,pɜ:miə,bɪlə'zeɪʃən/

Phonetic Spelling: per·mee·uh·bil·uh·zay·shuhn

13. nuclease

Pronunciation link: <https://www.merriam-webster.com/dictionary/nuclease>

IPA: /'nu:kli:,eɪs/

Phonetic Spelling: noo·klee·ays

14. dithiothreitol

Pronunciation link: <https://www.howtopronounce.com/dithiothreitol>

IPA: /,daɪθiʊθ'θraɪtɔ:l/

Phonetic Spelling: dye·thee·oh·thry·ih·tawl

15. thermomixer

Pronunciation link: <https://www.howtopronounce.com/thermomixer>

IPA: /'θɜ:mʊs,mɪksər/

Phonetic Spelling: ther·moh·mik·ser

16. dimethyl

Pronunciation link: <https://www.merriam-webster.com/dictionary/dimethyl>

IPA: /daɪ'mɛθəl/

Phonetic Spelling: dye·meth·uhl

17. sulfate

Pronunciation link: <https://www.merriam-webster.com/dictionary/sulfate>

IPA: /'sʌl,fet/

Phonetic Spelling: sul·fayt

18. methylation

Pronunciation link: <https://www.merriam-webster.com/dictionary/methylation>

IPA: /,mɛθə'leɪʃən/

Phonetic Spelling: meth·uh·lay·shuhn

19. phenol

Pronunciation link: <https://www.merriam-webster.com/dictionary/phenol>

IPA: /'fi:nɔ:l/

Phonetic Spelling: fee·nawl

20. chloroform

Pronunciation link: <https://www.merriam-webster.com/dictionary/chloroform>

IPA: /'klɒ:rəˌfɔːrm/

Phonetic Spelling: klor·uh·form

21. streptavidin

Pronunciation link: <https://www.howtopronounce.com/streptavidin>

IPA: /ˌstreptəˈvɪdɪn/

Phonetic Spelling: strep·tuh·vih·din

22. biotinylated

Pronunciation link: <https://www.howtopronounce.com/biotinylated>

IPA: /ˌbaɪoʊˈtɪnəleɪtɪd/

Phonetic Spelling: bye·oh·tin·uh·lay·tid

23. agarose

Pronunciation link: <https://www.merriam-webster.com/dictionary/agarose>

IPA: /ˈæɡəˌroʊs/

Phonetic Spelling: ag·uh·rohs

24. electropherogram

Pronunciation link: <https://www.howtopronounce.com/electropherogram>

IPA: /ɪˌlektroʊˈfɪrəˌɡræm/

Phonetic Spelling: ih·lek·troh·feer·uh·gram

25. intron

Pronunciation link: <https://www.merriam-webster.com/dictionary/intron>

IPA: /ˈɪnˌtrɔːn/

Phonetic Spelling: in·tron

26. polymerase

Pronunciation link: <https://www.merriam-webster.com/dictionary/polymerase>

IPA: /pəˈlɪməˌreɪs/

Phonetic Spelling: puh·lim·uh·rays

27. locus

Pronunciation link: <https://www.merriam-webster.com/dictionary/locus>

IPA: /ˈloʊkəs/

Phonetic Spelling: loh·kuhs

28. reactivity

Pronunciation link: <https://www.merriam-webster.com/dictionary/reactivity>

IPA: /ˌriːækˈtɪvəti/

Phonetic Spelling: ree·ak·tiv·uh·tee