Native Elongating Transcript Sequencing (NET-seq)

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ABSTRACT

Advances in sequencing technology have led to the development of many high-resolution methodologies that observe genomic activity and gene expression. This unit describes such an approach, native elongating transcript sequencing (NET-seq), which reveals the density of RNA polymerase across the *Saccharomyces cerevisiae* genome with single-nucleotide resolution. A procedure for capturing nascent RNA transcripts directly from live cells through their association with the DNA-RNA-RNAP ternary complex is described. A protocol to create DNA libraries from the nascent RNA, allowing the identity and abundance of the 3′ end of purified transcripts to be revealed by next generation sequencing, is also provided. By deep sequencing this DNA library, a quantitative measure of RNAP density with single-nucleotide precision is obtained. The quantitative nature of the NET-seq dataset relies on the high efficiency of many steps in the protocol. The steps that are most critical are described with suggestions for monitoring their success. *Curr. Protoc. Mol. Biol.* 98:4.14.1-4.14.17. © 2012 by John Wiley & Sons, Inc.

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INTRODUCTION

This unit presents a protocol for native elongating transcript sequencing (NET-seq), which reveals the global density of RNA polymerase at nucleotide resolution (Churchman and Weissman, 2011). In this method (see Basic Protocol 1), the genomic position of the RNAP active site is determined by identifying the 3′ ends of the nascent RNA. Using *Saccharomyces cervisiae* as an example, discussion is provided on how to exploit the intrinsic stability of the RNA polymerase elongation complex to perform a quantitative immunoprecipitation of RNAP without crosslinking (Fig. 4.14.1). The immunoprecipitation is facilitated through the addition of a 3× FLAG epitope tag added endogenously to the C-terminus of the third subunit of RNAP II (Rpb3). A native antibody-based precipitation of RNAP has not been tested, but in theory would allow different isoforms of polymerase (e.g., different phosphorylation states) to be specifically purified. Use of antibodies against the endogenous RNAP would also enable NET-seq to be performed in other organisms without the requirement that they express a modified polymerase.

To quantify the 3′ ends of the nascent RNA, next generation sequencing approaches, such as the Illumina HiSeq, are employed. A DNA library must be created from the purified nascent RNA, which accurately reflects the RNA population. The approach described here (see Basic Protocol 2) uses enzymatic reactions that have been optimized so that each step is highly efficient (>90%). In this manner, the number of bottlenecks through which the RNA must pass through are reduced, lessening the biases that occur during library generation. A useful control is to create and sequence a library from lightly fragmented mature mRNA. By converting the large majority of recovered nascent transcripts to DNA, the biases will be lessened but not necessarily eliminated. To observe the types of biases

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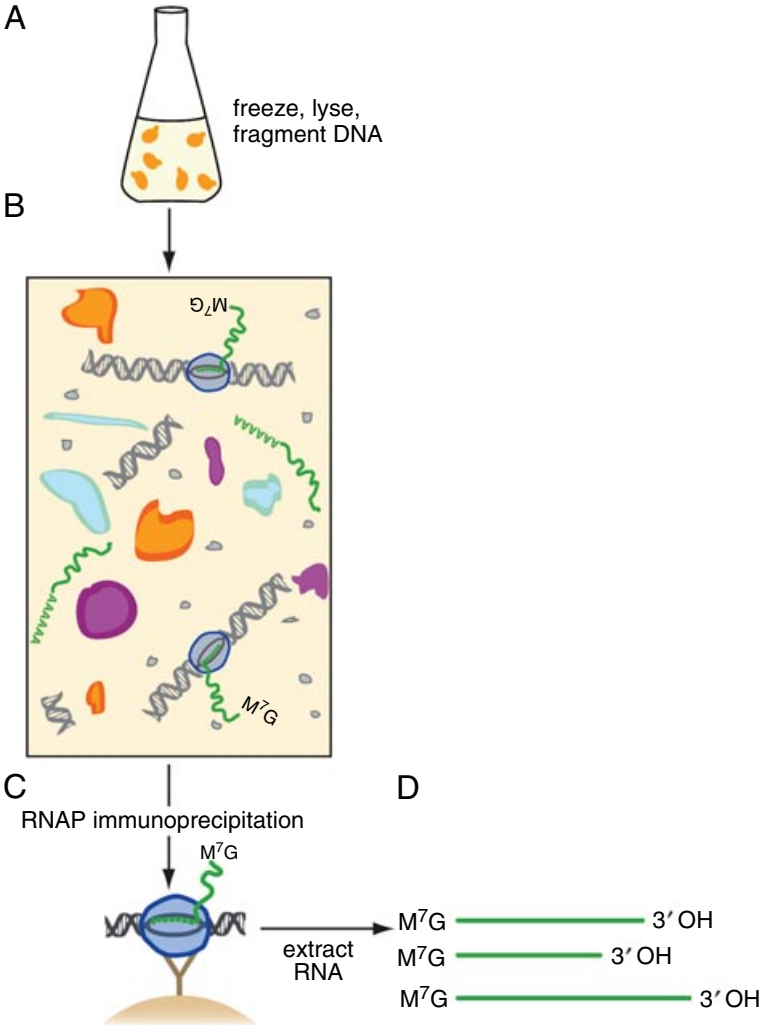

Figure 4.14.1 Schematic of the purification of nascent RNA by immunoprecipitation. (**A**) A yeast culture is grown to mid-log phase in rich medium. (**B**) Lysed yeast with DNase I–digested DNA is used for immunoprecipitation of RNAP (**C**). Nascent RNA is extracted from the purified RNAP (**D**).

that occur, a mature mRNA sample that has been lightly fragmented (at 95°C by alkaline hydrolysis) can be made into a DNA library and sequenced. The variation of the number of reads along gene bodies provides an upper bound to the level of bias.

The Support Protocol details how to rapidly concentrate, flash freeze, and cryogenically lyse a culture of yeast. Basic Protocol 1 describes how nascent RNA is purified through an immunoprecipitation of RNA polymerase. Finally, Basic Protocol 2 is used to create a DNA library for sequencing the 3′ ends of the purified nascent RNA.

BASIC PROTOCOL 1

PURIFICATION OF NASCENT RNA BY IMMUNOPRECIPITATION OF RNA POLYMERASE

Here the immunoprecipitation of 3× FLAG-labeled RNAP is described (Fig. 4.14.1B-D). Frozen yeast grindate is thawed and resuspended in ice-cold lysis buffer. The DNA is then enzymatically fragmented followed by centrifugation to clarify the lysate. Agarose beads associated with FLAG antibodies are added to the lysate to begin the immunoprecipitation. After incubating the beads in the lysate and washing them, elution is stimulated by the addition of 3× FLAG peptide.

Materials

Agarose slurry conjugated with FLAG antibody (Sigma)
Lysis buffer (see recipe), ice cold
Yeast grindate (see Support Protocol)
1 U/μl DNase I (RQ1 RNase-free DNase, Promega)
2× SDS buffer (see recipe)
Wash buffer (see recipe)
2 mg/ml 3× FLAG peptide (Sigma)
Qiagen miRNeasy mini kit
Membrane
FLAG antibody

15-ml conical tubes
Refrigerated centrifuge
Nutator, 4°C
1.5-ml microcentrifuge tubes

Wash agarose slurry

1. To 0.5 ml of agarose slurry in a 15-ml conical tube, add 10 ml of 1× lysis buffer. Centrifuge mixture 2 min at 1000 × *g*, 4°C. Remove and discard supernatant.
2. Repeat step 1. Leave washed beads on ice until ready to use.

Resuspend grindate, digest DNA, and clarify

3. Resuspend 1 g of yeast grindate in 5 ml of ice-cold 1× lysis buffer by pipetting up and down.

   Grindate should be transported to a scale in liquid nitrogen and delivered to the lysis buffer using spatulas chilled in liquid N_2.

4. Add 660 μl (660 U) of RNase-free DNase I and incubate 20 min on ice.
5. Centrifuge digested lysate 10 min at 20,000 × *g*, 4°C. Generate input sample for immunoblot by adding 20 μl of supernatant to 20 μl of 2× SDS buffer and set aside.
6. Add remainder of supernatant to washed beads (from step 2) and incubate 2.5 hr at 4°C on nutator.

Wash beads and elute

7. Centrifuge agarose mixture 2 min at 1000 × *g*, 4°C. Generate unbound sample for immunoblot analysis by adding 20 μl of supernatant to 20 μl of 2× SDS buffer and set aside.
8. Remove and dispose remaining supernatant.
9. Wash beads four times with 10 ml of wash buffer, resuspending beads thoroughly each time and placing on a nutator 2 min at 4°C. Follow by centrifuging supernatant 2 min at 1000 × *g*, 4°C.
10. After last wash, resuspend agarose beads in 1 ml wash buffer and transfer to 1.5-ml microcentrifuge tube.
11. Centrifuge agarose mixture 2 min at 1000 × *g*, 4°C and remove the supernatant.
12. Elute 3×-FLAG-tagged RNAP from beads by addition of an equal bead volume (300 μl) of 2 mg/ml 3× FLAG peptide in 1× lysis buffer.
13. Elute 30 min on ice. Mix gently by flicking bottom of tube every 5 min.
14. Centrifuge agarose mixture 2 min at 1000 × *g*, 4°C and reserve supernatant.

15. Repeat steps 12 to 14 and combine all elutions.

16. To remove any accidental transfer of agarose beads, centrifuge combined elutions 2 min at 1000 × *g*, 4°C and reserve supernatant. Generate elution sample by adding 20 µl of eluted sample to 20 µl of 2× SDS and set aside.

17. Purify RNA from eluted sample using the Qiagen miRNeasy mini kit according to manufacturer's instructions and elute in 30 µl of RNase-free water provided with the kit.

    RNA purification on a column is essential. The FLAG peptide can not be efficiently separated from the RNA using standard phenol/chloroform purification. The miRNeasy kit is used to ensure all lengths of RNA are purified.

18. Quantify RNA using absorption (*APPENDIX 3D* or *APPENDIX 3J* for microvolume methods).

    Typical yield is 1.5 to 4 µg.

    Store RNA for 3 months at −80°C.

Check quality of immunoprecipitation by immunoblot

19. Perform immunoblot on input, unbound, and elution samples reserved in 2× SDS buffer during immunoprecipitation (Negritto and Manthey, 2008).

20. Probe membrane using a FLAG antibody.

    Typically, 90% of RNA polymerase is immunoprecipitated.

21. Prepare DNA sequencing library using the purified RNA following Basic Protocol 2.

BASIC PROTOCOL 2

CONSTRUCTING A DNA SEQUENCING LIBRARY FROM THE 3′ ENDS OF NASCENT RNA

One of the technical challenges in the development of the NET-seq protocol was creating a library generation method that would allow RNA to be converted to DNA in an unbiased manner. First a pre-adenylated DNA linker (linker-1) is ligated onto the purified nascent RNA (Fig. 4.14.2A; Unrau and Bartel, 1998). Fragmentation of the RNA then creates manageable and uniform pieces of RNA. The reverse transcription reaction is performed with a long DNA primer that is flexible due to the addition of carbon spacers in the middle of the oligonucleotide (Fig. 4.14.2B). The resulting DNA product can be readily circularized, using DNA CircLigase, creating a template for PCR (Figure 4.14.2C). Finally, after minimal cycles of PCR, the double-stranded product is ready for sequencing on the Illumina HiSeq (Fig. 4.14.2D).

Materials

1 to 3 µg purified RNA (see Basic Protocol 1)
10 mM Tris·Cl, pH 7.0 and 8.0 (RNase-free; *APPENDIX 2*)
Oligonucleotides (see Table 4.14.1)
Ligation reaction mix (see recipe)
200,000 U/ml truncated T4 RNA ligase 2 (NEB)
0.5 M EDTA (RNase-free; *APPENDIX 2*)
2× alkaline fragmentation buffer (see recipe)
RNA precipitation solution (see recipe)
Isopropanol
80% ethanol, ice cold
2× TBU denaturing loading buffer (Invitrogen)
10-bp DNA ladder

DEPC-treated water
15% TBE-urea polyacrylamide gel (Invitrogen)
SYBR gold (Invitrogen)
1× TBE (Ambion)
Costar Spin-X centrifuge tube filter (0.45-μm cellulose acetate in 2-ml tube; Corning)
15 mg/ml GlycoBlue (Ambion)
3 M sodium acetate, pH 5.5 (RNase-free; *APPENDIX 2*)
RT reaction mix (see recipe)
Superaise.In/DTT mix (see recipe)
200 U/μl Superscript III (Invitrogen)
1 M NaOH (*APPENDIX 2*)
1 M HCl (*APPENDIX 2*)
10% TBE-urea polyacrylamide gel (Invitrogen)
3 M NaCl (*APPENDIX 2*)
Circularization mix (see recipe)
100 U/μl CircLigase (Epicentre)
PCR master mix (see recipe)
2000 U/μl Phusion DNA polymerase (NEB)
6× DNA loading dye (see recipe)
8% TBE polyacrylamide gel (Invitrogen)
DNA soaking buffer (see recipe)

0.5- and 1.5-ml RNase-free, non-stick tubes (Ambion)
37°, 48°, 60°, 70°, 80°, 95°, and 98°C heating blocks
Refrigerated centrifuge
Gel electrophoresis apparatus
20-G needles
Vortex
0.2-ml thin-walled PCR tubes
Thermal cycler
Bioanalyzer DNA high sensitivity chip and reagents (Agilent)

Ligate linker and fragment RNA

1. Prepare three 1.5-ml non-stick, RNase-free tubes each with 1 μg of purified RNA (diluted in 10 mM Tris·Cl, pH 7.0).

   If the immunoprecipitation in Basic Protocol 1 did not yield 3 μg of RNA, prepare fewer reactions. However, it is not recommended to proceed with <1 μg.

2. Prepare a separate control tube with 0.5 μl of 20 μM oGAB11 oligonucleotide and 9.5 μl of 10 mM Tris·Cl, pH 7.0.

3. Denature samples from steps 1 and 2 for 2 min at 80°C and then immediately place on ice.

   Denaturing the RNA prior to ligation lessens the potential of secondary structure to contribute to ligation biases.

4. Pulse-spin sample tubes and add 9 μl ligation reaction mix to each sample. Gently mix well.

5. Add 1 μl (200 U) truncated T4 RNA ligase 2 to each tube and mix well. Incubate 3 hr at 37°C.

6. Place tubes on ice and add 0.7 μl of 0.5 M EDTA.

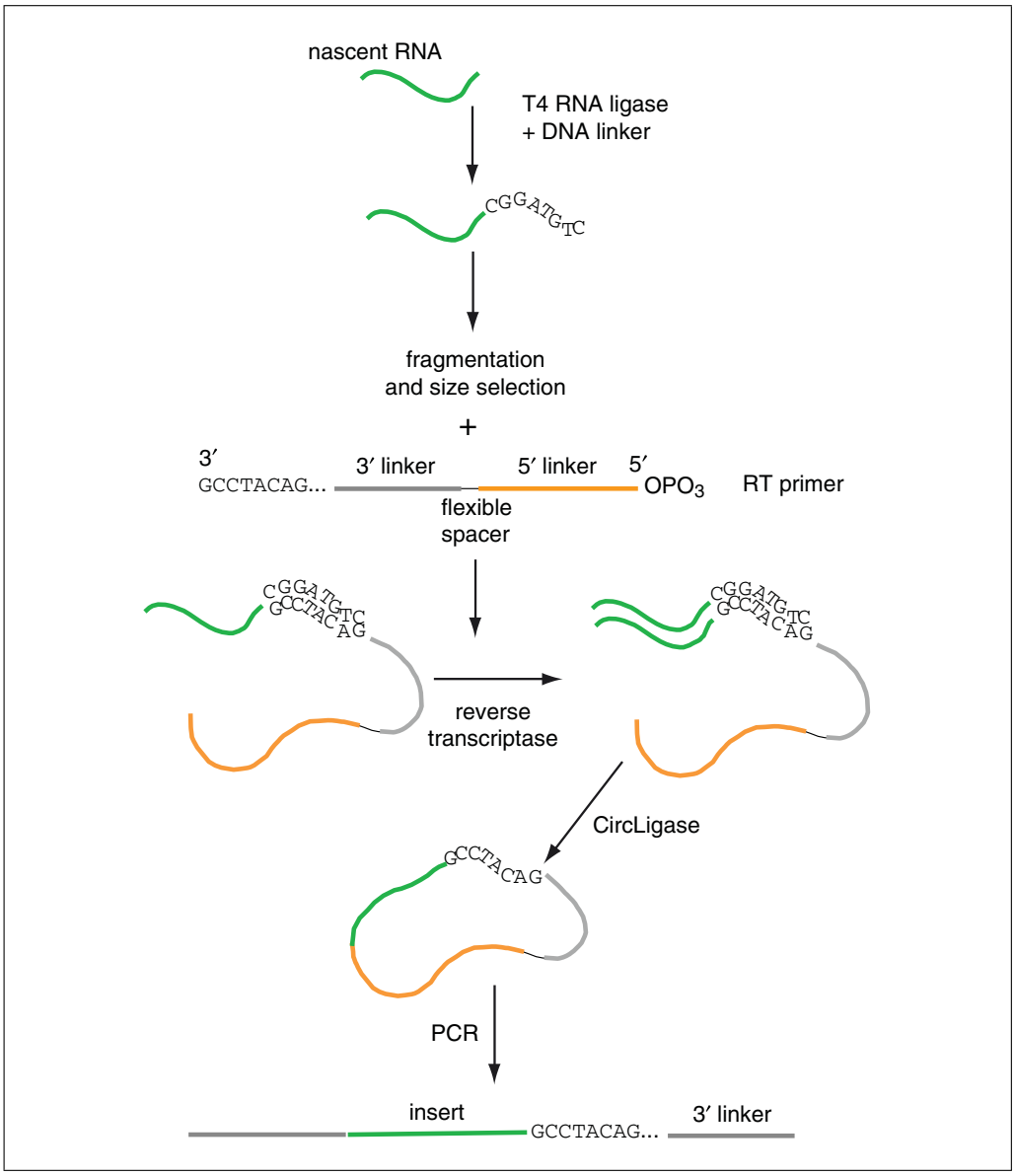

Figure 4.14.2 Schematic of the DNA library construction from nascent RNA. (**A**) A DNA linker is ligated onto the library of nascent RNA. The ligated product is fragmented using alkaline hydrolysis and size selected to be between 35- and 85-nt long. (**B**) Reverse transcription converts the RNA into DNA. (**C**) Circularization of the RT product is performed by CircLigase (Epicentre). (**D**) The circularized product is used for PCR.

7. Add 20 µl of 2× alkaline fragmentation solution to ligation reaction of immunoprecipitated RNA. Do not add solution to control oligonucleotide reaction.
8. Incubate for the calibrated time at 95°C. Place immediately on ice.

   Fragmentation time must be determined empirically for each batch of alkaline fragmentation solution. Incubation times from 15 to 45 min should be tested using a total RNA sample or other easily obtained RNA sample. Most fragments should lie in the range of 40 to 90 nt.

9. Add 0.56 ml RNA precipitation solution to fragmented RNA and to control oligonucleotide sample. Mix well. Add 0.75 ml isopropanol, mix well, and incubate at least 30 min at −20°C.
10. Centrifuge 30 min at 20,000 × *g*, 4°C, to pellet nucleic acids.

Table 4.14.1 DNA and RNA Oligonucleotide Sequences

DNA oligonucleotides	
Linker-1[a]	5′ AppCTGTAGGCACCATCAAT/3ddC 3′
oLSC003	5′Phos/TCGTATGCCGTCTTCTGCTTG/iSp18/ CACTCA/iSp18/AATGATACGGCGACCACCGA TCCGACGATCATTGATGGTGCCTACAG 3′[b]
oNTI230	5′ AATGATACGGCGACCACCGA 3′
oNTI231	5′ CAAGCAGAAGACGGCATACGA 3′
oMHL001[c]	5′AATGATACGGCGACCACCGAGATCGGAAGAGC ACACGTCTGAACTCCAGTCACTGCATC TCCGACGATCATTGATGG 3′
oMHL002[c]	5′ AATGATACGGCGACCACCGAGATCGGAA GAGCACACGTCTGAACTCCAGTCACATGCCA TCCGACGATCATTGATGG 3′
oLSC006	5′-TCCGACGATCATTGATGGTGCCTACAG 3′
RNA oligonucleotide	
oGAB11	5′agu cac uua gcg aug uac acu gac ugu g3′

[a]Available commercially from Integrated DNA Technologies and NEB.
[b]iSp18 are 18-carbon spacers.
[c]Note that oMHL001 and oMHL002 are used when barcoding on the Illumina platform is desired. The underlined sequence is the barcode that is read during sequencing. If more than two samples need barcodes, other sequences can be inserted in place of the underlined sequences. Barcodes should be GC balanced and resistant to single base indel conversion.

11. Remove supernatant and wash pellet in 0.75 ml of 80% ice-cold ethanol. Pulse-spin tube to collect residual ethanol wash at the bottom and carefully remove all liquid.
12. Air-dry pellet for 10 min. Resuspend in 10 µl of 10 mM Tris·Cl, pH 7.0.

All three RNA pellets from the separate ligation reactions should be pooled by sequentially resuspending in the same 10 µl volume.

Store ligated RNA for up to 3 months at −80°C.

Size select fragmented RNA

13. Add 10 µl of 2× TBU denaturing loading buffer to both samples.
14. Prepare a 10-bp ladder by adding 1 µl ladder stock with 9 µl DEPC-treated water and 10 µl of 2× TBU loading buffer.
15. Denature sample, control sample, and ladder for 2 min at 80°C. Place immediately on ice.

It is critical to denature the samples prior to loading them on the gel.

16. Pre-run a 15% TBE-urea polyacrylamide gel for 15 min at 200 V.
17. Load gel and run at 200 V for 65 min.

Perform rapid gel extraction

18. Pierce a 0.5-ml RNase-free, non-stick microcentrifuge tube with a 20-G needle and place it inside a 1.5-ml RNase-free, non-stick tube. Prepare three tubes for the nascent RNA sample and one tube for the oligonucleotide control sample.
19. Stain gel with SYBR gold (6 µl in 60 ml TBE) for 5 min with shaking at room temperature. Image with UV light and excise gel piece. Also excise ligated oligonucleotide.

Place each gel slice into a pierced 0.5-ml RNase-free, non-stick microcentrifuge tube of the nested tubes from step 18.

20. Centrifuge nested tubes 3 min at 20,000 × *g*, room temperature, to force the gel through the needle hole. Shake any residual gel from the small tube into the larger tube.

21. Add 200 µl DEPC-treated water to gel pieces and incubate 10 min at 70°C.

22. Vortex gel slurry for 30 sec and transfer gel mixture to a Costar Spin-X column.

23. Centrifuge 3 min at 20,000 × *g*, room temperature, to recover the elution mixture free of gel debris.

24. Combine eluates for the nascent RNA sample in a new 1.5-ml RNase-free, non-stick tube. Add 2 µl of 15 mg/ml GlycoBlue, and 50 µl of 3 M sodium acetate, pH 5.5, to eluate and mix. Add 0.75 ml isopropanol and mix well. Incubate at least 30 min at −20°C to precipitate nucleic acids.

25. Centrifuge 30 min at 20,000 × *g*, 4°C, to pellet nucleic acids.

26. Remove supernatant, wash pellet in 0.75 ml of 80% ice-cold ethanol, and air-dry for 10 min.

27. Resuspend pellet in 10 µl of 10 mM Tris·Cl, pH 7.0.

    Store up to 3 months at −80°C.

Perform reverse transcription

28. Add 4.6 µl RT reaction mix to RNA sample and control sample and mix well.

29. Denature 2 min at 80°C and place on ice.

30. Add 1.32 µl Superaise.In/DTT mix and 0.82 µl (164 U) Superscript III. Mix well.

31. Incubate 30 min at 48°C.

32. To degrade RNA, add 1.8 µl of 1 M NaOH and mix well. Incubate 20 min at 98°C.

33. Pulse-spin reactions. Neutralize reactions by adding 1.8 µl of 1 M HCl and mix well. Place tubes on ice.

Size select cDNA

34. Add 20 µl of 2× TBU denaturing loading buffer to each sample.

35. Also prepare a 10-bp ladder by adding 1 µl ladder stock to 9 µl DEPC-treated water and 10 µl of 2× TBU loading buffer. Prepare an oLSC003 sample for reference by adding 0.5 µl of 100 µM oLSC003 stock to 9.5 µl DEPC-treated water and 10 µl of 2× TBU loading buffer.

36. Denature samples for 3 min at 95°C prior to loading, pulse-spin, and then place on ice.

    Each RT sample will be split between two lanes (20 µl each).

37. Load a 10% TBE-urea polyacrylamide gel and run for 65 min at 200 V.

Excise bands and extract cDNA

38. Pierce a 0.5-ml non-stick, RNase-free tube with a 20-G needle and place it inside a 1.5-ml non-stick, RNase-free tube. Prepare two tubes for nascent RNA sample and one tube for the oligonucleotide control sample.

Lane number	1	2	3	4	5	6	7	8	9	10
RT primer	X	10-bp DNA ladder	X	X	X	X	X	X	X	X
Fragmented nascent RNA			X	X	X	X	X	X	X	X
Control RNA oligo (28-mer)									X	X

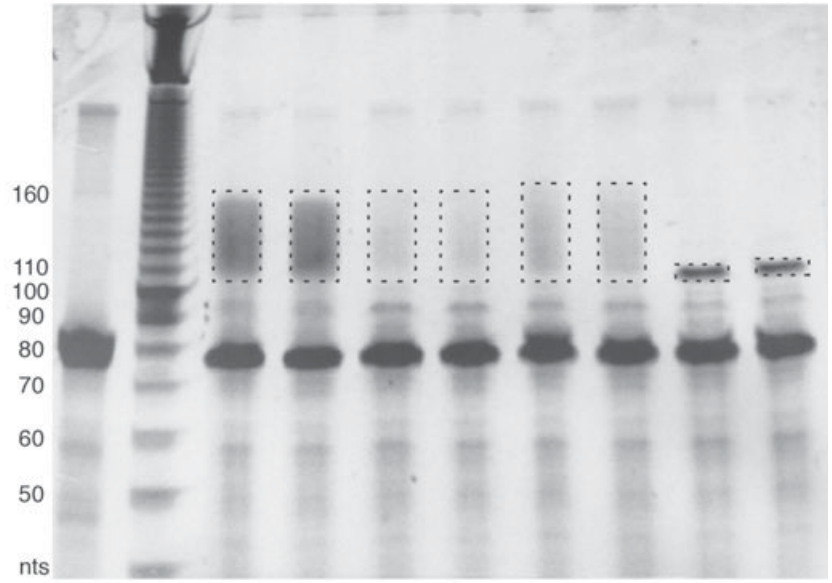

Figure 4.14.3 Representative results of reverse transcription reactions. Lane 1 shows the RT primer. Lane 2 shows a denatured 10-bp DNA ladder. Lanes 3 to 8 show the reverse transcription product and remaining RT primer from three different nascent transcript samples. The dashed boxes show the product to be excised. Lanes 9 and 10 show the reverse transcription product and remaining RT primer from the oGAB11 control RNA oligo sample. The dashed boxes show the product.

39. Stain gel with SYBR gold (6 µl in 60 ml TBE) for 5 min with shaking at room temperature. Image gel with UV light and excise gel piece containing RT product with a disposable scalpel for each sample.

    In the lanes with samples, the remaining primer will be visible at ~80 nt. About 30 nt above the primer, the reverse transcribed product should be visible and extend for ~50 nt. A representative gel is shown in Figure 4.14.3.

40. Excise the product. If the product is not visible, cut band out using the oligonucleotide product as a guide. Also cut out ligated oligonucleotide product. Place each band from the nascent RNA sample into a separate pierced 0.5-ml tube from step 38. Place both oligonucleotide bands into one pierced 0.5-ml tube from step 38.

41. Centrifuge nested tubes 3 min at 20,000 × *g*, room temperature, to force the gel through the needle hole. Shake any residual gel from the small tube into the larger tube.

42. Add 200 µl DEPC-treated water to gel pieces and incubate 10 min at 70°C.

43. Vortex gel slurry 30 sec and transfer gel mixture to a Costar Spin-X column.

44. Centrifuge 3 min at 20,000 × *g*, room temperature, to recover the elution mixture free of gel debris.

45. Transfer eluate to a new 1.5-ml non-stick tube, add 2 µl GlycoBlue and 25 µl of 3 M NaCl, and mix well. Add 0.75 ml isopropanol and mix well. Incubate at least 30 min at −20°C to precipitate the nucleic acids.

46. Centrifuge 30 min at 20,000 × *g*, 4°C, to pellet nucleic acids.

47. Remove supernatant, wash pellet in 0.75 ml of 80% ice-cold ethanol, and air-dry for 10 min.

48. Resuspend pellet in 15 µl of 10 mM Tris·Cl, pH 8.0.

Circularize DNA

49. Add 4 µl circularization mix to each sample and mix well.

50. Add 1 µl (100 U) CircLigase to each sample, mix well.

51. Incubate 60 min at 60°C.

52. Incubate 10 min at 80°C to heat-inactivate enzyme. Place tube on ice if proceeding directly to product amplification or store indefinitely at −20°C.

Perform PCR amplification

53. Add 79.2 µl PCR master mix to 5 µl of circularized DNA sample and mix well. Do this for the oGAB11 control sample as well.

    The new mixture creates the sample master mix.

54. Prepare four sets of 0.2-ml thin-walled PCR tubes with *n* tubes in each set, where *n* is the number of independent samples to be amplified ($n = 2$, if there is only one nascent RNA sample and one oligonucleotide sample).

    Each set of tubes will undergo different numbers of PCR cycles (e.g., 6, 8, 10, 12 cycles).

55. For each tube set, place 16.7 µl sample master mix of each sample into a different tube.

56. Perform PCR as follows:

1 cycle:	30 sec	98°C
11 cycles:	10 sec	98°C
	10 sec	60°C
	5 sec	72°C
1 cycle:	Hold	4°C.

    From the 11 cycles, take the appropriate set of tubes out at end of cycles 6, 8, and 10 and place those tubes on ice.

57. Add 3.4 µl of 6× DNA loading dye to each PCR tube.

58. Load an 8% TBE gel with 20 µl per lane. Run 40 min at 180 V.

59. Stain gel with SYBR gold (6 µl in 60 ml TBE) for 5 min with shaking at room temperature. Image gel with UV light and cut out bands containing PCR product with a disposable new scalpel for each sample. Choose bands from PCR reactions that have not saturated nor produced any high-molecular weight products. Note that any primer carryover from the RT step will appear as an ∼70-bp product. Use caution to avoid inclusion of primer carryover. Use observation of oGAB11 control to confirm proper product size.

Purify DNA from gel

Here the rapid purification cannot be used as it would denature the double-stranded DNA.

60. Place excised gel pieces in a 0.5-ml tube that was pierced with a 20-G needle and place it inside a 1.5-ml non-stick tube.
61. Centrifuge nested tubes 3 min at 20,000 × *g*, room temperature, to force the gel through the needle hole. Shake any residual gel from the small tube into the larger tube.
62. Soak gel in 0.67 ml DNA soaking buffer overnight with agitation at room temperature.
63. Cut the tip off of a 1000-µl pipet tip and transfer gel and soaking mixture to the top of a Spin-X column.
64. Centrifuge 3 min at 20,000 × *g*, room temperature, to recover the soaking mixture free of gel debris.
65. Transfer eluate to a new 1.5-ml non-stick tube. Add 2.0 µl GlycoBlue (15 mg/ml) to the elution mixture, then add 0.68 ml isopropanol, mix well, and precipitate at least 30 min at −30°C.
66. Centrifuge 30 min at 20,000 × *g*, 4°C, to pellet nucleic acids.
67. Remove supernatant, wash pellet in 0.75 ml of 80% ice-cold ethanol, and air-dry.
68. Resuspend pellet in 5 µl of 10 mM Tris·Cl, pH 8.
69. Using a Bioanalyzer DNA high-sensitivity chip according to manufacturer's instructions, determine the concentration of DNA.

    Typical concentrations are 20 nM.

    Sequencing can now proceed on an Illumina sequencer using oLSC006 as a sequencing primer.

    These DNA sequencing libraries are only compatible with single-end sequencing kits for the Illumina HiSeq.

YEAST CELL HARVESTING BY FILTRATION AND CRYOGENIC LYSIS

SUPPORT PROTOCOL

This protocol describes how to rapidly harvest yeast culture and perform lysis under cryogenic conditions to preserve protein complexes (Fig. 4.14.1A). A yeast strain expressing a C-terminal epitope tagged (3× FLAG) Rpb3 is grown to mid-log phase in rich YPD medium. The culture is rapidly filtered to minimize transcriptional activity in response to change of temperature and concentration. Yeast are then scraped from the filter and plunged rapidly into liquid nitrogen (Fig. 4.14.4). To lyse yeast, a mixer mill is employed as the canisters can be periodically cooled during lysis, allowing the cell wall to be disrupted at liquid nitrogen temperature. As the resulting ground cells (or grindate) can be stored long term at −80°C, 2 liters per strain are prepared at a time. Grindate from ∼1 liter is used for the subsequent immunoprecipitation.

Materials

Liquid nitrogen
Yeast strain expressing a C-terminal epitope tagged (3× FLAG) Rpb3 grown to mid-log phase in rich YPD medium
Microfiltration assembly (90-mm, ULTRA-WARE):
  4-liter side-arm flask with a fritted glass support base
  Glass funnel

Anodized aluminum clamp
No. 8 silicone stopper
Nitrocellulose (0.45-μm, 90-mm diameter membranes; Whatman)
Vacuum source
Deep Styrofoam container (dry-ice shipping container) containing tube rack
Forceps
50-ml conical tubes
20-G needles
Metal spatulas with one flat end and one curved end, pre-chilled
Tongs
Mixer mill, 50-ml chambers and 25-mm stainless steel ball (Retsch) or TissueLyserII (Qiagen)
Cryo-gloves

Filter yeast culture and flash freeze

1. Set up microfiltration apparatus and connect it to the house vacuum (Fig. 4.14.4A).
2. Fill a Styrofoam container with a tube rack with liquid nitrogen. Use forceps to fill labeled 50-ml conical tube with liquid nitrogen and set into the tube rack in the Styrofoam container.

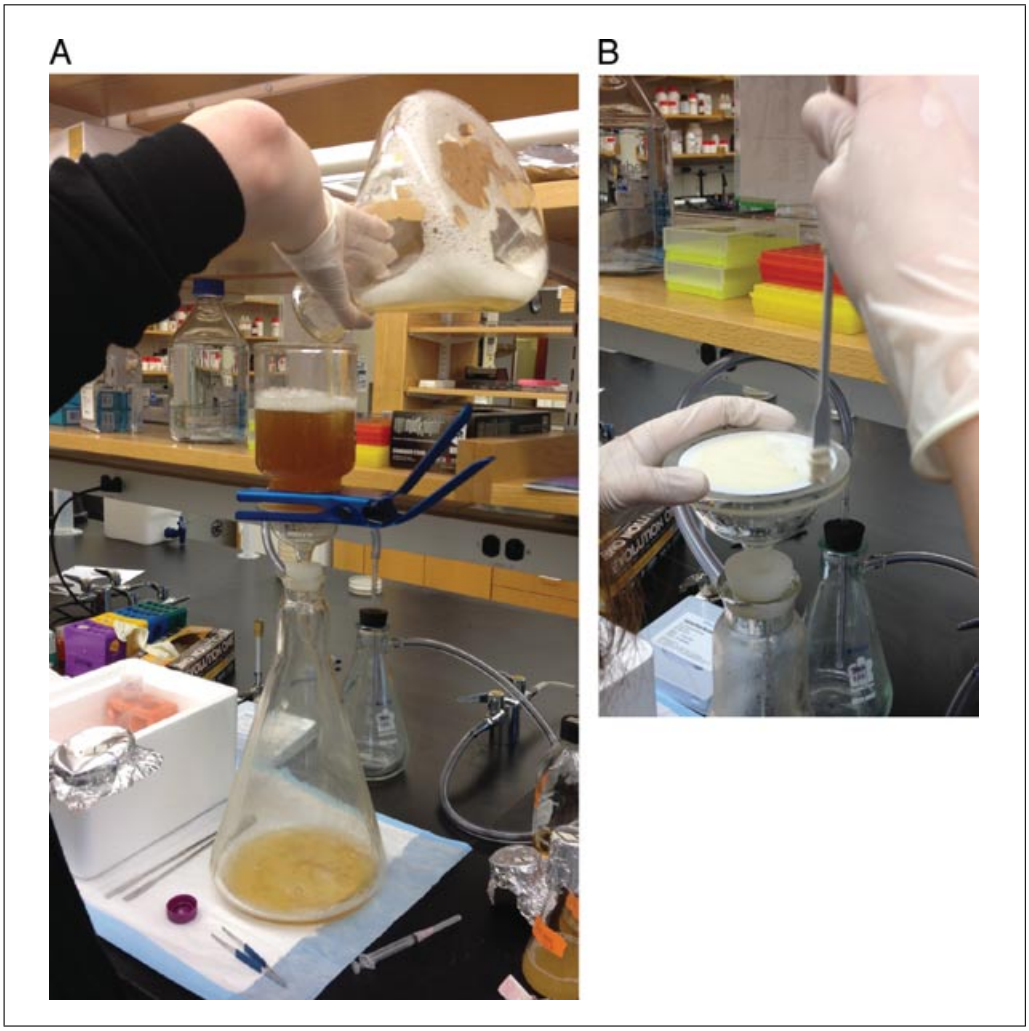

Figure 4.14.4 Images of the filtration and recovery of yeast culture. (**A**) One liter of yeast culture at mid-log phase is filtered onto a 0.45-μm nitrocellulose membrane. (**B**) The paste of yeast is scraped off using a spatula that has been pre-chilled in liquid nitrogen.

The container should be deep enough so that 2/3 of the conical tube is immersed in liquid nitrogen.

3. Using a 20-G needle, puncture holes into the caps of the conical tubes and set aside.
4. Turn on vacuum. Pour 1 liter at a time of the yeast culture into filter apparatus with a fresh membrane. When all the liquid has gone through, immediately remove clamp and expose the membrane coated with a paste of yeast cells.
5. Acting quickly, use a gloved finger to carefully remove foam from top of cells.
6. Use the flat end of pre-chilled metal spatula to scrape cells without tearing the membrane (Fig. 4.14.4b). Transfer cells into a 50-ml conical that is at least half full with liquid nitrogen.

   Use a pre-chilled spatula (in liquid nitrogen), which will decrease the freezing time due to reduction of the Leidenfrost effect.
7. Once the cells start to freeze solid (~3 sec), knock them off the spatula with another prechilled spatula.

   This can take a few tries as the pellet can become attached to the spatula.
8. Cap 50-ml conical tube and tilt to remove most of the liquid nitrogen through the holes of the cap.

   Frozen cells can be stored for 6 months at −80°C.

Lyse by mixer mill

9. Using tongs, place an opened mixer mill chamber and stainless steel ball into a Styrofoam box mostly full of liquid nitrogen. Wait until the boiling of the liquid nitrogen stops.

   Simmering will proceed for some time. It is not necessary to wait until all bubbling stops.
10. Remove chamber and place frozen yeast sample and ball inside. Screw chamber together and place into the liquid nitrogen.

    The sample can be kept, until ready to place in chambers, in a tube rack immersed in the liquid nitrogen. It is important to maintain the sample at a very cold temperature.
11. Take chamber out of liquid nitrogen with tongs. Place chamber into holder on mixer mill and tighten into place.
12. Allow mixer mill to run 3 min at 15 Hz.
13. Remove chamber from mixer mill and place back into the liquid nitrogen. Immerse until aggressive boiling stops.

    It is not necessary to wait until all simmering stops.
14. Repeat steps 11 to 13 five additional times.
15. Place a new labeled 50-ml conical tube into tube rack in liquid nitrogen.
16. Remove chamber from liquid nitrogen with tongs and unscrew. With liquid nitrogen–chilled spatulas, scrape and remove as much ground cells (or "grindate") as possible from chambers and place into conical tube sitting in liquid nitrogen.

    It is easiest to use curved tipped spatulas to obtain all of the grindate off the walls of the chambers.

    The grindate can be stored for 6 months at −80°C.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Alkaline fragmentation buffer, 2×

100 mM $NaCO_3$, pH 9.2 (for 1.25 ml: 0.15 ml of 0.1 M Na_2CO_3 and 1.10 ml of 0.1 M $NaHCO_3$)

2 mM EDTA (*APPENDIX 2*)

Store in 125-µl aliquots in air-tight, screw-top tubes indefinitely at room temperature. It is important that a given aliquot be used only a few times, as air exchange alters the pH of the mixture. Calibration of fragmentation is needed with each new batch of solution aliquoted.

Circularization mix

For 5 µl (one reaction), add:
2 µl 10× Circ buffer (Epicentre, supplied with Circligase enzyme)
1 µl 1 mM ATP (0.2 mM final)
1 µl 50 mM $MnCl_2$ (10 mM final; *APPENDIX 2*)
Store up to 5 hr on ice

DNA loading dye, 6×

For 10 ml, add:
1.5 g Ficoll 400 (15% w/v)
25 mg Orange G
25 mg xylene cyanol
Bring up to 10 ml with water
Store indefinitely at room temperature

DNA soaking buffer

For 668 µl (one reaction), add:
40 µl 5 M NaCl (0.3 M final; *APPENDIX 2*)
6.7 µl 1 M Tris·Cl, pH 8.0 (10 mM final; *APPENDIX 2*)
1.3 µl 0.5 M EDTA (0.97 mM final; *APPENDIX 2*)
620 µl DEPC-treated or RNase-free water
Store indefinitely at room temperature

Ligation reaction mix

For 9 µl (one reaction), add:
4.8 µl 50% PEG (mol. wt. 8.000; 26.6% final)
2 µl 10× RNA ligase buffer (with T4 truncated ligase; NEB)
1 µl 1 µg/µl linker-1 (0.11 µg final)
1.2 µl DEPC-treated or RNase-free water
Store up to 5 hr on ice

Lysis buffer, 1×

1× 10× lysis buffer stock (see recipe)
10 mM $MnCl_2$ (*APPENDIX 2*)
1× proteinase inhibitors (complete, EDTA-free; Roche)
50 U/ml SUPERase.In
Store up to 5 hr on ice

Lysis buffer, 10×

200 mM HEPES, pH 7.4 (*APPENDIX 2*)
1100 mM KOAc (*APPENDIX 2*)
5% Triton X-100
1% Tween 20
Store for 1 year

PCR master mix

For 79.2 µl (one reaction), add:
16.7 µl 5× Phusion HF buffer (supplied with Phusion polymerase; NEB)
1.7 µl dNTPs, 10 mM each (0.21 mM each final)
0.4 µl 100 µM oNTI230 (0.51 µM final)
0.4 µl 100 µM oNTI231 (0.51 µM final)
59.2 µl DEPC-treated or RNase-free water
0.8 µl 2000 U/ml Phusion polymerase (20.2 U/ml final; NEB)
Store up to 5 hr on ice

Note that for sequencing applications where barcoding is used, oMHL001 or oMHL002 is used in this mix instead of oNTI230 at the same concentration.

RNA precipitation solution

For 562 µl (one reaction), add:
60 µl 3 M sodium acetate, pH 5.5 (0.32 M final; *APPENDIX 2*)
2 µl 15 mg/ml Glycoblue (0.053 mg/ml final)
500 µl DEPC-treated or RNase-free water
Store up to 5 hr at room temperature

RT reaction mix

For 4.6 µl (one reaction), add:
3.28 µl 5× FS buffer (supplied with Superscript III enzyme; Invitrogen)
0.82 µl dNTPs, 10 mM each (1.78 mM each final)
0.5 µl 100 µM oLSC003 (10.9 µM final)
Store up to 5 hr on ice

SDS buffer, 2×

For 1 ml, add:
500 µl 4× SDS buffer (*APPENDIX 2*)
200 µl 1 M DTT (200 mM final; *APPENDIX 2*)
300 µl water
Store up to 5 hr at room temperature

SDS buffer, 4×

200 mM Tris·Cl, pH 7.0 (*APPENDIX 2*)
8% SDS (*APPENDIX 2*)
0.4% bromophenol blue
40% glycerol
Store up to 1 year at room temperature

Superaise.In/DTT mix

For 1.32 µl (one reaction), add:
0.5 µl 20 U/µl Superase.In (7.6 U/µl final; Ambion)
0.82 µl 0.1 M DTT (62 µM final; *APPENDIX 2*)
Store up to 5 hr on ice

Wash buffer

1× 10× lysis buffer stock (see recipe)
50 U/ml SUPERase.In
1 mM EDTA (*APPENDIX 2*)
Store up to 5 hr on ice

COMMENTARY

Background Information

The final nucleotide of a nascent RNA reports where the active site of RNA polymerase lies along the genome. NET-seq exploits this fact to obtain nucleotide resolution of RNAP density through sequencing the 3′ ends of nascent transcripts. Two other strategies exist to measure RNAP density across the genome but have lower resolution (∼50 bp). One strategy is RNAP chromatin immunoprecipitation (ChIP), where the DNA bound to RNA polymerase is captured and identified by hybridization to a microarray or by sequencing (Mayer et al., 2010). A drawback to ChIP is that it is not strand-specific and does not distinguish between active and inactive complexes. A second approach, global run-on sequencing (GRO-seq), where metazoan nascent RNA is labeled in vitro enabling its purification and subsequent identification, does identify active RNAP in a strand-specific manner (Core et al., 2008). While GRO-seq is limited to 50-bp resolution, it does provide a method for the investigation of mammalian transcription. NET-seq has yet to be developed for metazoan cells.

The key to the quantitative nature of the NET-seq protocol is the accurate conversion of RNA to DNA such that the sequencing data reflects the distribution of purified RNA. To reduce the number of bottlenecks that could create a bias, each step in the NET-seq protocol has been optimized so that the steps are at least 90% efficient. Additionally, NET-seq does not use crosslinking to freeze the elongation complex. By removing this step, the protocol has fewer steps and is protected from biases that may arise from crosslinking.

Critical Parameters and Troubleshooting

Each step of the NET-seq protocol must be as efficient as possible so that the data are quantitative. The following steps are the most critical and should be monitored to ensure that the reagents are working as expected.

1. Immunoprecipitation: The large majority of RNAP should be purified by immunoprecipitation, which can be monitored by immunoblotting. It is typical to recover 90% of the solubilized RNAP.

2. Ligation: Ligation efficiency can change with the lot of truncated T4 RNA ligase 2; it is critical to monitor this continually. By including the RNA oligonucleotide, oGAB11, as a control and sizing marker, the ligation efficiency is determined internally. Ligation should occur on >90% of input RNA. It is also possible to purify ligase from an expression vector in *E. coli.* The resulting ligase typically shows high efficiency.

3. Circularization: As in the first ligation step, the efficiency of the circularization ligation can change with the lot of the enzyme. The CircLigase enzyme can be tested by circularizing the reverse transcription primer, oLSC003. As the circularized product will migrate more slowly than the linear primer, the efficiency of the reaction can be monitored by electrophoresis.

In general, by passing oGAB11 through the library generation protocol for each library preparation, one can quickly isolate the problem if there is a failure. Additionally, it is useful to note the lot number of each enzyme used during library preparation, since failures are typically due to a faulty enzyme.

Anticipated Results

The immunoprecipitation should yield 1.5 to 4 μg of RNA. The result of the sequencing run typically has the following characteristics for wild-type yeast (for the s288c strain, BY4741): 38% reads align uniquely to the genome, 55% reads aligning to rRNA, and 2% reads aligning to tRNA. Finally, it is expected that read density will be comparable across introns and 5′ exons. Since polymerase density in yeast is highest at the 5′ ends of genes, it is not as informative to compare intronic read densities to 3′ exon read densities.

Time Considerations

The full NET-seq protocol can be performed over 5 days. Bringing a yeast culture to log phase, flash freezing, and cryogenic lysis can be done on the first day. The

immunoprecipitation takes the majority of a second day. Finally, the library generation protocol takes 2 to 3 days. However, there are many stopping points so the protocol can be performed in a very flexible manner. Only the immunoprecipitation needs almost 1 full day to be devoted to NET-seq.

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