Chapter 3

Illuminating Enhancer Transcription at Nucleotide Resolution with Native Elongating Transcript Sequencing (NET-Seq)

Olga Jasnovidova, Mirjam Arnold, and Andreas Mayer

1 Introduction

Transcription by RNA polymerase II (Pol II) is not restricted to genes and also occurs in intergenic regions including enhancers [1–3]. Transcription at enhancer regions is usually bidirectional where transcription in one direction is accompanied by a transcriptional activity in the opposite orientation [4, 5]. Both transcriptional activities originate in close proximity to each other. Transcripts that arise from enhancer regions, the so-called enhancer RNAs (eRNAs), are unstable with half-lives significantly lower as compared to those of messenger RNAs [6]. Due to these features enhancer transcription and eRNAs mostly escape detection by standard RNA-Seq methods that typically measure steady-state levels of stable and mainly cytoplasmic mature RNAs [7].

To study the regulation and functional roles of enhancer transcription methods are required that ideally provide a DNA strand-specific and quantitative measure for transcription genome-wide with high spatial resolution. One genome-wide approach, which combines all these features and can be used to study enhancer transcription is native elongating transcript sequencing (NET-Seq). NET-Seq was originally developed for budding yeast [8]. In the following years, NET-Seq protocols became available for other species including mammals [9, 10], plants [11, 12], fission yeast [13, 14], and for bacteria [15, 16]. Although NET-Seq has so far

Olga Jasnovidova and Mirjam Arnold contributed equally to this work.

Tilman Borggrefe and Benedetto Daniele Giaimo (eds.), *Enhancers and Promoters: Methods and Protocols*, Methods in Molecular Biology, vol. 2351, https://doi.org/10.1007/978-1-0716-1597-3_3,

mainly been used to study nascent transcription by Pol II, the yeast NET-Seq protocol has been adapted for the analysis of Pol I transcription [17]. New applications that originated from the NET-Seq approach have also become recently available [18–22].

The NET-Seq approach consists of four major modules: (a) enrichment of transcribing RNA polymerase along with the nascent RNA, (b) efficient conversion of 3′-nascent RNA ends into a sequencing library, (c) next-generation sequencing, and (d) computational data analysis. NET-Seq exploits the high stability of the ternary complex formed by transcribing Pol II, the DNA template, and the nascent RNA [23]. Following cell lysis, transcribing Pol II along with the nascent RNA is purified. In the original NET-Seq protocol for budding yeast, this was accomplished by immunoprecipitation (IP) of Pol II elongation complexes [8]. Our NET-Seq approach for mammalian cells uses a simple and efficient chromatin fractionation method to enrich transcribing RNA polymerase together with the nascent RNA (Fig. 1a) [9, 24, 25]. The chromatin fractionation approach relies on a protocol originally developed by the Schibler and Black laboratories [26–28] and has been extensively optimized to ensure that nearly all Pol II elongation complexes are captured in the chromatin fraction [9]. This fractionation method simplifies the protocol due to fewer experimental steps and most importantly avoids artifacts that typically arise from IP mainly due to cross-reactivity of antibodies, epitope masking, and low IP efficiencies.

To avoid run-on transcription during sample processing, cell lysis and chromatin preparation are performed in the presence of α-amanitin, a potent Pol II inhibitor [29, 30]. The high stability of the Pol II elongation complex, even in the presence of high amounts of urea, salts, or detergents [26, 31, 32], allows stringent washes of the chromatin and results in an almost complete enrichment of transcribing Pol II (>95%) along with the nascent RNA (Fig. 1a). Next, nascent RNA is prepared from the chromatin and a DNA linker that contains a unique molecular identifier (UMI) is ligated to the 3′-ends of the nascent RNA (Fig. 1b). The UMI serves as a molecular barcode and allows PCR duplicates to be identified and computationally removed. After RNA fragmentation, 3′-ends of the nascent RNA are strand-specifically converted into cDNA and circularized (Fig. 1b). cDNA that originated from mature chromatin-associated RNAs are removed by subtractive hybridization (Fig. 1c). Before the NET-Seq library can be submitted for next-generation sequencing, it is amplified using a limited and optimized number of PCR amplification cycles.

Following deep sequencing from the 3′-end, the NET-Seq data is computationally analyzed. By mapping NET-Seq reads to the reference genome and by recording the 3′-most nucleotide corresponding to the last nucleotide that was incorporated into the nascent RNA chain, the genome-wide and DNA strand-specific

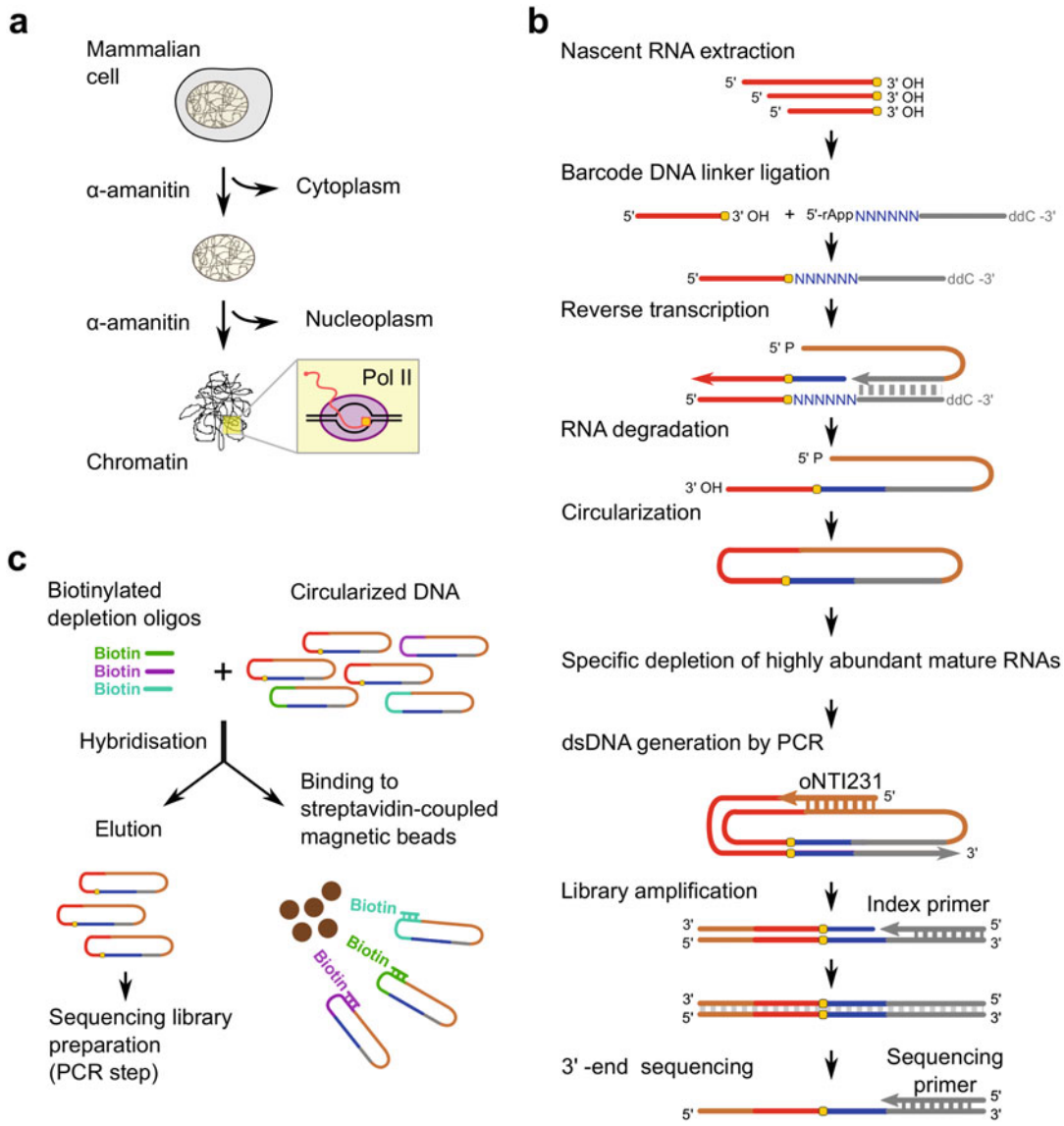

Fig. 1 Schematic overview of the main steps of the NET-Seq protocol. (**a**) Chromatin preparation (*see* 3.2). Purification of transcriptionally engaged RNA polymerases by chromatin isolation in the presence of α-amanitin. (**b**) Key experimental steps of the NET-Seq library preparation and deep sequencing (*see* 3.4 and 3.5). 3′-ddC, 3′-dideoxycytosine; 5′-rApp, 5′-riboadenylate; dsDNA, double-stranded DNA. (**c**) Specific depletion of highly abundant mature RNAs (*see* 3.4.6). Schematic view of specific depletion of highly abundant mature RNAs is accomplished by subtractive hybridization with biotinylated DNA oligos that are complementary to the target cDNA

density of transcriptionally engaged Pol II is revealed with single-nucleotide resolution.

Here, we provide step-by-step guidance for performing NET-Seq in mammalian cells. This protocol includes an updated list of enzymes, reagents, and kits. All experimental steps starting from

Table 1
Overview of experimental steps and time considerations

Day	Subheading	Procedure	Time	Can I stop after?	How can I stop? (°C)
1	2.2.1	Preparation of buffers for subcellular fractionation	1 h		
	3.2	Subcellular fractionation	1 h	Yes	−80
	3.3	RNA purification with miRNeasy kit, DNase I cleavage on the column	1.5–2 h	Yes	−80
2	3.4.1	Ligation with DNA barcode linker	4 h		
	3.4.1	RNA fragmentation	20 min		
	3.4.2	Purification of the ligated and fragmented RNA	30 min	Yes	−80
		15% TBE-urea gel	100 min		
		RNA size selection	30 min		
		RNA extraction from the **15% TBE-urea** gel	80 min	Yes	−80
3	3.4.3	cDNA synthesis by Superscript IV	1 h	Yes	−20
	3.4.4	**10% TBE-urea** gel	95 min		
		cDNA size selection	30 min		
		ss cDNA extraction from PAGE	1 h	Yes	−20
	3.4.5	cDNA circularization	1.5 h	Yes	−20
	3.4.6	Depletion of abundant RNA species	2 h		
		Precipitation of depleted circular cDNA	3 h–overnight	Yes	−20
4		Recovery of precipitated circular cDNA	1.5 h	Yes	−20
	3.4.7	Test PCR	30 min		
		8% TBE gel	80 min		
	3.4.8	Optimized PCR	30 min	Yes	−20
		8% TBE gel	80 min		
5		Library extraction from the PAGE gel	3 h–overnight		
		DNA precipitation	4 h	Yes	−20

the cell lysis to the submission of NET-Seq libraries for next-generation sequencing can be completed within 5 days. Table 1 outlines the time requirements of each experimental module.

2 Materials

2.1 Equipment and Supplies

1. UV-Vis Spectrophotometer, e.g., Nanodrop2000 (Thermo-Fisher Scientific).
2. RNase/DNase-free PCR tubes, 0.2 mL.
3. RNase/DNase-free microcentrifuge tubes, 0.5 mL.
4. DNA/RNA low-binding RNase/DNase-free microcentrifuge tubes, 1.5 and 2 mL.

5. Microcentrifuge tube filter, e.g., Costar Spin-X centrifuge tube filters (Sigma-Aldrich).
6. RNase/DNase-free centrifuge tubes, 15 mL.
7. Syringe needle, 20G.
8. RNase/DNase-free scalpels.
9. Cell counter.
10. Refrigerated centrifuge.
11. Refrigerated microcentrifuge.
12. TBE-urea gels, 15% (w/v) (Life Technologies).
13. TBE-urea gels, 10% (w/v) (Life Technologies).
14. TBE gels, 8% (w/v) (Life Technologies).
15. Mini-Cell polyacrylamide gel box, XCell SureLock (Life Technologies).
16. Electrophoresis power supply.
17. Gel staining box.
18. Microcentrifuge tube rotator.
19. Vortexer.
20. Magnetic rack for 1.5 mL tubes.
21. PCR Thermal cycler.
22. Thermomixer for 1.5 and 2 mL tubes.
23. 2100 Bioanalyzer or Tapestation (Agilent Technologies).
24. Qubit™ Fluorometer, Assay Tubes, Quant-iT™ Qubit RNA BR Assay-kit, and Qubit™ dsDNA HS Assay-Kit (ThermoFisher Scientific).
25. Low retention filter tips.
26. 1 mL wide orifice pipette tips.
27. 5PRIME Phase Lock Gel, 2 mL tubes, heavy (QuantaBio).

2.2 Reagents and Buffers

All buffers need to be freshly prepared using RNase-free reagents and should be kept on ice till use.

2.2.1 Chromatin Preparation

1. 50× Protease inhibitor mix: dissolve one tablet of Protease inhibitor mix cOmplete™, EDTA-free (Roche) in 1 mL of pre-cooled RNase-free H_2O. Use immediately or store aliquots for up to 1 year at −20 °C.
2. 1 mM α-Amanitin stock solution: dissolve 1 mg of α-amanitin (Sigma-Aldrich) in 1 mL of RNase-free H_2O. Use immediately or store aliquots for up to 1 year at −20 °C.
3. 5 M urea stock solution: dissolve 1.5 g of urea, molecular biology grade, in 4 mL of RNase-free H_2O. Urea solution is not stable, therefore use on the same day.

4. 50% (w/v) sucrose stock solution: dissolve 25 g of sucrose, molecular biology grade, in 50 mL of RNase-free H_2O. Filter-sterilize, make 10 mL aliquots, and store at 4 °C for up to several months.
5. Cytoplasmic lysis buffer: 0.15% (v/v) NP-40, 10 mM Tris–HCl (pH 7.0), 150 mM NaCl, 25 μM α-amanitin, 10 U SUPERase.In™, and 1× protease inhibitor mix. For one reaction, prepare 250 μL of the buffer: mix 3.8 μL of 10% (v/v) NP-40, 2.5 μL of 1 M Tris–HCl (pH 7.0), 7.5 μL of 5 M NaCl, 5 μL of 50× protease inhibitor mix, 6.2 μL of 1 mM α-amanitin, 0.6 μL of SUPERase. In™ (20 U/μL), and 224.4 μL of RNase-free H_2O.
6. Sucrose buffer: 10 mM Tris–HCl (pH 7.0), 150 mM NaCl, 25% (w/v) sucrose, 25 μM α-amanitin, 20 U SUPERase.In™, and 1× protease inhibitor mix. For one reaction, prepare 500 μL of the buffer: mix 5 μL of 1 M Tris–HCl (pH 7.0), 15 μL of 5 M NaCl and 250 μL of 50% (w/v) filter-sterilized sucrose, 10 μL of 50× protease inhibitor mix, 12.5 μL of 1 mM α-amanitin, 1.2 μL of SUPERase.In™ (20 U/μL), and 206.3 μL of RNase-free H_2O.
7. Nuclei wash buffer: 0.1% (v/v) Triton X-100, 1 mM EDTA, 25 μM α-amanitin, 40 U SUPERase.In™, and 1× protease inhibitor mix in 1× PBS. For one reaction, prepare 600 μL of the buffer: mix 1.2 μL of 0.5 M EDTA solution, 6 μL of 10% (v/v) Triton X-100, 12 μL of 50× protease inhibitor mix, 15 μL of 1 mM α-amanitin, 1.5 μL of SUPERase.In™ (20 U/μL), and 564.3 μL of 1× PBS.
8. Glycerol buffer: 20 mM Tris–HCl (pH 8.0), 75 mM NaCl, 0.5 mM EDTA, 50% (v/v) glycerol, 0.85 mM DTT, 25 μM α-amanitin, 10 U SUPERase.In™, and 1× protease inhibitor mix. For one reaction, prepare 250 μL of the buffer: mix 5 μL of 1 M Tris–HCl (pH 8.0), 3.8 μL of 5 M NaCl, 0.5 μL of 0.25 M EDTA, 125 μL of 100% (v/v) filter-sterilized glycerol, 2.1 μL of 0.1 M filter- sterilized DTT, 5 μL of 1× protease inhibitor mix (50×), 6.2 μL of 1 mM α-amanitin, 0.6 μL of SUPERase.In™ (20 U/μL), and 101.8 μL of RNase-free H_2O.
9. Nuclei lysis buffer: 1% (v/v) NP-40, 20 mM HEPES (pH 7.5), 300 mM NaCl, 1 M urea, 0.2 mM EDTA, 1 mM DTT, 25 μM α-amanitin, 10 U SUPERase.In™, and 1× protease inhibitor mix. For one reaction, prepare 280 μL of the buffer: mix 25 μL of 10% (v/v) NP-40, 5 μL of 1 M HEPES (pH 7.5), 0.5 μL of 0.1 M EDTA, 15 μL of 5 M NaCl, 25 μL of 10 M filter-sterilized urea, 2.5 μL of 0.1 M filter-sterilized DTT, 5 μL of 50× protease inhibitor mix, 6.2 μL of 1 mM α-amanitin, 0.6 μL of SUPERase (20 U/μL), and 165.2 μL of RNase-free H_2O.

10. Chromatin resuspension solution: 25 μM α-amanitin, 20 U SUPERase.In™ and 1× protease inhibitor mix in 1× PBS. For one reaction, prepare 120 μL of the buffer: mix 3 μL of 1 mM α-amanitin, 2.4 μL of 50× protease inhibitor mix, 0.3 μL of SUPERase.In™ (20 U/μL), and 114.3 μL of 1× PBS.

2.2.2 Nascent RNA Extraction

Work under the chemical hood! Phenol and chloroform are toxic.

1. miRNeasy mini kit (50; Qiagen) (*see* **Note 1**), RNase-free DNase set (50; Qiagen).
2. Chloroform, molecular biology grade.
3. Ethanol, molecular biology grade.

2.2.3 Library Preparation

1. DNA soaking buffer: for one reaction (668 μL), mix 6.7 μL of 1 M Tris–HCl (pH 8.0), 40 μL of 5 M NaCl, 1.3 μL of 0.5 M EDTA, and 620 μL of RNase-free H_2O. Prepare buffer immediately before the experiment and keep at room temperature.
2. 1 N HCl: dilute 821 μL of 37% hydrochloric acid concentrate by adding DNase-free H_2O to a final volume of 10 mL. Store 1 N HCl for up to 1 year at room temperature.

Name	Function	Sequence	Length (nt)
oGAB11	Positive control	AGUCACUUAGCGAUGUACACUGACUGUG	28
Hexamer barcode DNA linker	Ligation	5rApp/NNNNNNCTGTAGGCACCATCAAT/3ddC	25
oLSC007	Reverse transcription	5Phos/ATCTCGTATGCCGTCTTCTGCTTG/iSp18/CACTCA/iSp18/TCCGACGATCATTGATGGTGCCTACAG	57
universal reverse primer oNTI23	PCR amplification	CAAGCAGAAGACGGCATACGA	22
indexed forward primer	PCR amplification, sample barcoding	AATGATACGGCGACCACCGAGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCAC (Illumina TruSeq index) TCCGACGATCATTGATGG	86
oLSC006	Custom sequencing primer	TCCGACGATCATTGATGGTGCCTACAG	27

N: random nucleotides to distinguish unique sequences from PCR duplicates
5rApp: 5′-riboadenylate, necessary for the RNA 3′-OH ligation
3ddC: 3′-dideoxycytidine to avoid self-ligation
iSp18: internal 18-atom hexa-ethylenglycol spacer
5Phos: 5′-phosphate
Illumina index: 6-nucleotide TruSeq index of choice

3. RNA and DNA oligos for NET-Seq library construction:
4. Depletion oligo mix for abundant mature RNA species: resuspend each of the 20 biotinylated DNA oligos (*see* below, **Note 2**; every oligo contains a 5′ biotin-tetraethylene glycol group) with 10 mM Tris–HCl (pH 8.0) to the final concentration of 200 μM. Next, combine 5 μL of each depletion oligo solution and mix. The final concentration of each DNA oligo in the depletion oligo mix will be 10 μM. The final volume of the depletion oligo mix is 100 μL. Prepare the mix before use and store indefinitely at −20 °C.

Gene	Transcript	DNA sequence
RNA5S1	rRNA	GTACTTGGATGGGAGACCGCCTGGGAATACCGGGTG
RNA28S5	rRNA	TGCGATCTATTGAAAGTCAGCCCTCGACACAAGGGTTTGT
RNVU1-1	snRNA	GGTAGTGGGGGACTGCGTTCGCGCTTTCCCCTG
SNORD3D	snoRNA	TTGGGGAGTGAGAGGGAGAGAACGCGGTCTGAGTGG
RNU2-1	snRNA	CATCGACCTGGTATTGCAGTACCTCCAGGAACGGTGCA
RNA5-8S5	rRNA	CCTCCCGGGGCTACGCCTGTCTGAGCGTCGCT
RNU5B-1	snRNA	GTCTTAAGCTAATTTTTTGAGGCCTTGTTCCGACAAGGCT
SNORD80	snoRNA	CGCTGATGAGCAATATTAAGTCTTTCGCTCCTATCTGATG
SNORD31	snoRNA	ATACCGCCCCAGTCTGATCAATGTGTGACTGAAAGGTA
SNORD12C	snoRNA	CATCAGATCGACAATGCTGACGTCTTATATTTTGCCAGTTAG
SNORD29	snoRNA	GCTCACTATGACCGACAGTGAAAATACATGAACACCTGAG
RNU5E-1	snRNA	CTTAACCCAATTTTTTGAGGCCTTGCTTTGGCAAGGCT
SNORD81	snoRNA	CCAACTTGAACTCTCTCACTGATTACTTGATGACAA
RNVU1-7	snRNA	AGTGGGGGACTGCGTCCGCGCTTTCCCCTG
SNORD27	snoRNA	GCATATGGCTGAACTTTCAAGTGATGTCATCTTACTACTGAG
SNORD12B	snoRNA	GATCGACTATGTTGATCTAACTTTTCTAAGCCAGTTTCTGTCTG
RNU4-1	snRNA	TGGCAATTTTTGACAGTCTCTACGGAGACTGCTGTAGGC
SNORD118	snoRNA	CTCTGCGTAATCAGGTCTTGCAACACCCTGATTGCTCC
MT-TM	mitochondrial tRNA	CCCATACCCCGAAAATGTTGGTTATACCCTTCCCGTAC
MT-TV	mitochondrial tRNA	CACTTAGGAGATTTCAACTTAACTTGACCGCTCTGACCA

5. 2× Magnetic bead equilibration buffer: 5 mM Tris–HCl (pH 7.0), 2 M NaCl, 1 mM EDTA and 0.2% (v/v) Triton X-100. To prepare 1 mL for up to five reactions, mix 5 μL of 1 M Tris–HCl (pH 7.0), 400 μL of 5 M NaCl, 2 μL of 0.5 M EDTA, 20 μL of 10% Triton X-100, and 573 μL of RNase-free H_2O. Store it at room temperature for up to several months.
6. Circular cDNA precipitation mix: for one reaction (326 μL), mix 2 μL of GlycoBlue, 24 μL of 5 M NaCl, and 300 μL of RNase-free H_2O.
7. Gel soaking buffer: for one gel piece, prepare 668 μL of buffer. Mix 6.7 μL 1 M Tris–HCl (pH 8.0), 40 μL 5 M NaCl, 1.3 μL 0.5 M EDTA, 620 μL nuclease-free H_2O to each tube.
8. 6× DNA loading buffer: dissolve 6 g of sucrose and 30 mg of Orange G (Sigma-Aldrich) in 20 mL of RNase-free H_2O. Adjust to a final volume of 25 mL using RNase-free H_2O. Store protected from light at room temperature for up to 1 year.
9. Gel staining solution: to stain one TBE or TBE-urea gel, add 4 μL of SYBR Gold nucleic acid gel stain to 40 mL of 1× TBE buffer and mix. Prepare this solution immediately before use and keep it at room temperature protected from light.
10. NEBNext® Magnesium RNA Fragmentation Module: NEBNext® RNA Fragmentation Buffer, NEBNext® RNA Fragmentation Stop Solution.
11. RNA Clean and Concentrator-5 (ZYMO Research).
12. ZR small-RNA PAGE Recovery Kit (ZYMO Research).
13. T4 RNA ligase 2 truncated module: polyethylene glycol (PEG) 8000, molecular biology grade, 10× T4 RNA ligase buffer, T4 RNA ligase 2 truncated (NEB).
14. SYBR Gold nucleic acid gel stain (10,000× concentrate; Life Technologies).
15. DNA ladder (20 bp; Takara Bio).
16. 2× TBE-urea (TBU) denaturing sample buffer (Life Technologies).
17. SuperScript™ IV Reverse Transcriptase, 5× RT buffer, 0.1 M DTT (ThermoFisher Scientific).
18. 10× TBE buffer.
19. dNTP mix (10 mM; Life Technologies).
20. CircLigase ssDNA ligase (100 U/μL), 10× CircLigase reaction buffer, 1 mM ATP, 50 mM $MnCl_2$ (Epicentre).
21. Dynabeads MyOne streptavidin C1 (Life Technologies).
22. 20× SSC buffer.

23. Phusion high-fidelity (HF) DNA polymerase (2000 U/mL) and 5× Phusion HF buffer (NEB).
24. DMSO, molecular biology grade.

3 Methods

3.1 Cell Culture

Grow adherent or suspension cells in a suitable growth medium and at an appropriate density or concentration, as recommended by the ENCODE guidelines. For isolation of the chromatin-associated RNA, the cell population should have a viability of at least 90%. The method presented here can be combined with various treatments such as addition of transcription inhibitors (e.g., flavopiridol [9]).

3.2 Chromatin Preparation

The chromatin preparation method works successfully for an input of 1–15 million cells. Up to six samples can typically be processed in parallel.

Use RNA/DNA low-binding tubes and low-binding pipette tips throughout the procedure.

It is essential to prepare all buffers with RNase-free reagents immediately before the experiment and precool buffers on ice till use. The 5 M urea stock solution needs to be freshly prepared before each experiment. The preparation of the buffers requires 45–60 min.

Unless indicated otherwise, all steps are performed on ice.

1. Count cells and collect 1×10^7 cells in a 15 mL falcon tube. Pellet cells for 4 min at 200 × *g* at 4 °C. Discard the supernatant.
2. To wash out media traces, gently resuspend cells in 5 mL of ice-cold PBS. Pellet cells for 4 min at 200 × *g* at 4 °C. Discard the supernatant.
3. Carefully resuspend cells in 0.5 mL of ice-cold PBS. Transfer the cells into 1.5 mL tubes, spin 3 min at 60 × *g*, 4 °C. Discard the supernatant.
4. Resuspend the cell pellet in 200 μL of cytoplasmic lysis buffer by gently pipetting up and down several times using a wide-bore P1000 tip. Incubate the cells on ice for 5 min (*see* **Note 3**).
5. During the incubation prepare tubes with sucrose cushion. Pipette 400 μL of sucrose buffer into a new 1.5 mL tube. Briefly spin tubes.
6. Using a wide bore tip carefully layer the lysed cells onto the sucrose cushion. The cell lysate will form a layer on the top of the cushion. Centrifuge for 10 min at 16,000 × *g*, 4 °C. The nuclei will form a white pellet.

7. Discard the supernatant (cytoplasm) or transfer into a new 1.5 mL tube. Avoid lifting the nuclei pellet (*see* **Note 4**).
8. Gently wash nuclei pellet with 500 µL nuclei wash buffer. Centrifuge for 2 min at 1150 × *g*, 4 °C. Discard the supernatant.
9. Carefully resuspend the nuclei pellet in 200 µL glycerol buffer by pipetting up and down several times using a wide-bore P1000 tip. Ensure that the buffer was mixed well before adding to the sample. The nuclei suspension should be homogenous.
10. Add 200 µL nuclei lysis buffer. Pipette up and down with a cut tip. Invert the tube a few times, until the "precipitating" chromatin becomes visible.
11. Pulse-vortex three times for 5 s. Incubate on ice for 2 min. Centrifuge for 2 min at 18,500 × *g*, 4 °C.
12. Discard the supernatant (nucleoplasm) or transfer to a new 1.5 mL tube (*see* **Note 4**).
13. Briefly spin and discard the supernatant.
14. Wash the chromatin pellet with 0.5 mL of PBS. This will help to remove nucleoplasmic remnants.
15. Add 100 µL of chromatin resuspension buffer to the chromatin pellet.
16. Add 700 µL Qiazol. Place the tube on a rotator for 10 min. Vortex for 5 min. Repeat until chromatin is completely dissolved (*see* **Note 5**).

Potential pause point: store the sample at −80 °C.

3.3 Purification of Nascent RNA

Phenol and chloroform are toxic. Perform all steps under the chemical hood.

1. Prepare the phase lock tubes by spinning them for 30 s at 12,000 × *g* at room temperature.
2. Transfer the sample dissolved in Qiazol into the phase lock tube.
3. Add 140 µL chloroform (1 volume of Qiazol: 0.2 volume of chloroform).
4. Thoroughly mix the sample by gently inverting the tube for 15 s. Do not vortex! Incubate for 2 min at room temperature.
5. Centrifuge for 5 min at 13,000 × *g* at room temperature.
6. Transfer the aqueous phase to a 1.5 mL tube. Measure volume of the solution by pipetting. Add 1.5 sample volumes of 100% (v/v) ethanol. Mix by inversion (*see* **Note 6**).
7. Continue RNA extraction according to manufacturer's instructions. Critical: perform on-column DNase I cleavage according to manufacturer's instructions.

8. Elute RNA with 35 μL nuclease-free H_2O. Add H_2O directly onto the membrane, incubate for 2 min at room temperature. Centrifuge for 2 min at 10,000 × *g*, room temperature. Discard the column and keep the eluate on ice.
9. Quantify the RNA using Nanodrop or Qubit fluorometer according to the manufacturer's instructions. In case human cancer cell lines are used, the expected RNA yield is 1–2 μg per one million of cells (*see* **Note 7**).
10. Keep samples on ice and proceed.

Potential pause point: store the sample at −80 °C for months.

3.4 Preparation of Sequencing Library

3.4.1 DNA Barcode Linker Ligation, RNA Fragmentation, and RNA Clean Up

Next, 3′-hydroxyl group (-OH) of nascent RNAs are ligated to the DNA linker, which contains a random hexamer sequence at its 5′-end. In a typical NET-Seq library preparation, 3 μg of the RNA are used as an input. As a control of the ligation efficiency, we recommend to also process the custom control RNA oligo oGAB11 (*see* 2.2.3).

1. Denature 3.5 μg of RNA using a heating block or PCR cycler for 2 min at 80 °C. Cool the RNA on ice for 2 min. Also, denature 3 μL of oGAB11 (*see* **Note 8**).
2. Prepare the DNA linker ligation master mix at room temperature as described below. Mix well. Add the enzyme at the end and mix.

Reagent	RNA sample (triplicate!) (μL)	Master mix for 3.5 reactions (μL)	oGAB11 control (μL)
RNase/DNase-free H_2O	Count	Count	5.2
PEG 8000 [50% (v/v)]	8	28	8
DMSO	2	7	2
10× T4 RNA ligase buffer	2	7	2
Hexamer barcode DNA linker (100 μM)	1	3.5	1
RNA sample (1 μg/reaction)	Count	Count	–
oGAB11 (10 μM)	–	–	1
Truncated T4 RNA ligase 2	1	3.5	1
Total volume	20	20/tube	20

3. Incubate the reaction at 37 °C for 3 h in a PCR cycler.
4. Transfer ligation reactions on ice. Keep the oGAB11 control sample on ice (no fragmentation). For the oGAB11 control, proceed with **step 8**. For the other samples, proceed with **step 5**.
5. Add 2.0 μL NEBNext® RNA Fragmentation Buffer to each tube (*see* **Note 9**). Mix by pipetting.
6. Incubate at 95 °C for 10 min. Preheat the PCR cycler before adding the samples (*see* **Note 10**).
7. Stop fragmentation immediately by adding 2 μL of NEBNext® RNA Fragmentation Stop Solution to each sample. Briefly vortex samples, spin and mix by pipetting up and down several times. Keep samples on ice.
8. Pool the three reactions corresponding to the same original sample. Purify the ligated and fragmented RNA using the ZYMO RNA clean and concentrator-5 according to manufacturer's protocol. Purify the ligated oGAB11 control.
9. Elute ligated RNA with 17 μL RNase-free H_2O. Repeat the elution with additional 17 μL RNase-free H_2O into the same tube. Elute oGAB11 with 12 μL of RNase-free H_2O.
10. Keep the purified ligated and fragmented RNA on ice.

Potential pause point: store the sample at −80 °C.

3.4.2 RNA Size Selection and Gel Extraction

1. For the samples, add 30 μL 2× TBU sample buffer to 30 μL eluate; for the oGAB11 control, add 10 μL 2× TBU sample buffer to 10 μL of the oGAB11 eluate as well as to 10 μL of the unligated oGAB11. Denature the samples at 80 °C for 2 min in a heating block. Prepare the Takara 20 bp DNA ladder by mixing 9.5 μL H_2O and 0.5 μL of the ladder with 10 μL TBU sample buffer. Denature the DNA ladder at 80 °C for 5 min. Place samples and DNA ladder on ice for 3 min.
2. Pre-run a 15% (w/v) TBE-urea acrylamide gel for 15 min at 200 V.
3. After pre-cleaning the wells by pipetting the running buffer, load 10 μL of the ladder, and 20 μL sample into different wells. Also, load 20 μL of each of the oGAB11 controls.
4. Run the gel for 65 min at 200 V at room temperature.
5. Stain the gel for 5 min in 1× SYBR Gold in 1× TBE running buffer on a shaker.
6. Visualize the RNA on a blue light table. Wear suitable protective glasses! If the linker ligation worked efficiently, >90% of oGAB11 should be shifted by 25 nt to the higher molecular weight as compared to unligated oGAB11.

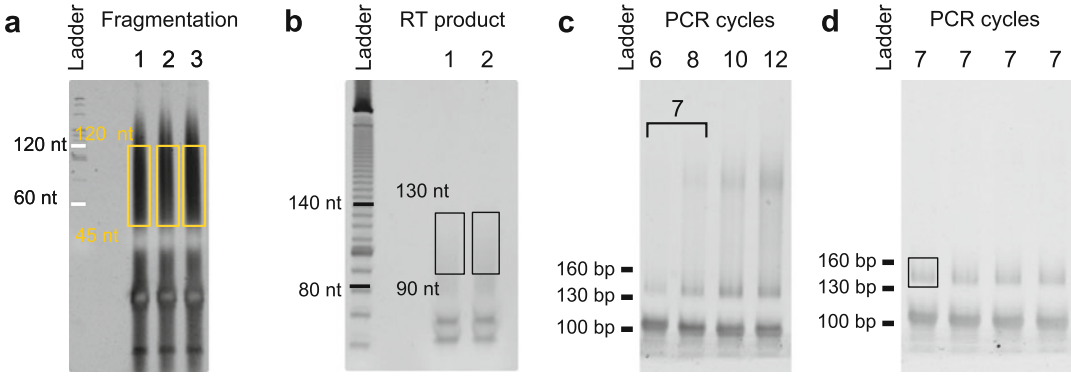

Fig. 2 Gallery of size selections performed during NET-Seq library preparation. (**a**) Size selection of fragmented RNA (*see* 3.4.2). The fragmented RNA was separated on a 15% (w/v) TBE-urea gel. 1 μg of fragmented RNA was loaded per lane. The size-selected region is shown by yellow frames. (**b**) Size selection of cDNA following reverse transcription (RT) (*see* 3.4.4). The cDNA was separated on a 10% (w/v) TBE-urea gel and size-selected, as indicated by black frames. RT, reverse transcription. (**c**) PCR amplifications to determine the lowest number of amplification cycles that is required (*see* 3.4.7). The PCR was stopped after 6, 8, 10, and 12 amplification cycles. The PCR products were separated on an 8% (w/v) TBE gel. The NET-Seq library runs at ~150 bp. The PCR product that arises from empty circles runs at ~120 bp. PCR products in the higher molecular range are indicative for overamplification. Seven amplification cycles were optimal in this NET-Seq library preparation. (**d**) Final NET-Seq libraries were separated on an 8% (w/v) TBE gel (Subheading 3.4.8). The excised band is indicated by a black box. NET-Seq library was generated for human K562 cells (A–D)

7. Cut out the smear between 45 and 120 nt (*see* Fig. 2a).
8. Homogenize gel slices. Pierce the bottom of a 0.5 mL low-bind tube with a 20G needle and place into a 2 mL low-bind tube. Transfer the gel slice of each lane into the pierced tube. Centrifuge for 4 min at 20,000 × *g* at room temperature. Repeat centrifugation, if pieces of the gel remain in the inner tube. Discard the pierced tube.
9. Purify the ligated RNA using the ZR small-RNA PAGE Recovery Kit. Add 400 μL RNA recovery buffer.
10. Transfer the homogenized gel to a Zymo-Spin III-F filter using a wide bore P1000 tip.
11. Incubate the sample on the column for 15 min at 65 °C and 1000 rpm in a thermomixer.
12. Freeze the sample for 5 min on dry ice or at a −80 °C.
13. Transfer the sample to 65 °C for 5 min.
14. Centrifuge the Zymo-Spin III-F filter at 5000 × *g* for 2 min.
15. Transfer the flow-through to a Zymo-Spin IIICG column. Centrifuge for 30 s at 2000 × *g*.

16. Collect the flow-through and measure its volume. Add two volumes of RNA MAX buffer to the flow-through. Mix by pipetting.
17. Transfer up to 700 µL to a white Zymo-Spin IC column. Transfer the material from the three gel pieces of the same sample that were processed separately onto the same column.
18. Centrifuge for 30 s at 13,000 × *g*. Repeat to load the remaining sample volume. Discard the flow-through. Place the column back into the collection tube.
19. Add 400 µL RNA Prep buffer to the column and centrifuge for 1 min at 13,000 × *g*. Discard the flow-through.
20. Add 800 µL RNA Wash buffer and centrifuge for 30 s at 13,000 × *g*. Discard the flow-through. Repeat this step with 400 µL RNA Wash buffer.
21. Place the column into a fresh collection tube and centrifuge for 2 min at 13,000 × *g* to remove the residual wash buffer. Place the column into a fresh 1.5 mL tube.
22. Add 11 µL nuclease-free H_2O onto the membrane. Let the column stand on the benchtop for 2 min, then centrifuge for 1 min at 10,000 × *g* to elute the RNA.
23. Reapply the eluate to the column. Let the column stand on the benchtop for 2 min before centrifugation, centrifuge for 1 min at 10,000 × *g*.
24. Keep the RNA on ice.

Potential pause point: store the sample at −80 °C for months.

3.4.3 Reverse Transcription

1. If only one sample is further processed, add the required amount of oLS007 oligo (*see* 2.2.3) and dNTPs directly to 10 µL of RNA. Keep on ice. If multiple samples are further processed, prepare a master mix and add 1.3 µL per RNA sample. Mix several times by pipetting.

Reagent	For one reaction (µL)
oLS007 oligo (10 µM)	0.5
dNTP (10 mM)	0.8
ligated and fragmented RNA	10
Total volume	11.3

2. Denature the RNA and allow oligo annealing using a PCR cycler: 2 min at 80 °C, 5 min at 65 °C, 2 min at 4 °C. Transfer tubes to ice.

3. Prepare the reverse transcription reaction mix as described below:

Reagent	For one reaction (μL)
5× SSIV buffer	3.5
0.1 M DTT	0.85
RNase OUT	0.85
Superscript™ IV	0.85
Total volume	6

4. If more than one sample is processed, prepare a master mix without Superscript™ IV and aliquot. Add Superscript™ IV and mix by pipetting several times. Proceed sample by sample.
5. Perform reverse transcription for 20 min at 55 °C in a PCR cycler.
6. Degrade the RNA by alkaline RNA hydrolysis. Add 1.8 μL of 1 M NaOH to each tube and mix by pipetting several times. Incubate the reaction for 20 min at 98 °C in a PCR cycler.
7. Stop and neutralize the reaction by adding 1.8 μL of 1 N HCl.

Potential pause point: store the sample at −20 °C overnight.

3.4.4 ssDNA Size Selection and Gel Extraction

1. Add 19 μL of 2× TBU sample buffer to each sample. Prepare the DNA ladder as described in **step 1**, Subheading 3.4.2. Denature the samples and the ladder at 95 °C for 3 min in the PCR cycler, then place samples and ladder on ice for 3 min.
2. Pre-run a 10% (w/v) TBE-urea acrylamide gel for 15 min at 200 V in 1× TBE.
3. Load 10 μL of the ladder or 20 μL sample per lane.
4. Run the gel for 65 min at 200 V at room temperature.
5. Transfer the gel into the staining solution and stain for 5 min on a shaking platform.
6. Visualize the DNA on a blue light table. Wear suitable protective glasses!
7. Cut out the part between 90 nt and 170 nt (*see* Fig. 2b).
8. Homogenize the gel pieces as described in **step 8**, Subheading 3.4.2. Prepare one tube per lane to be extracted.
9. Use the ZR small-RNA PAGE Recovery Kit for purification as described in **steps 9–21**, Subheading 3.4.2. Pool the material of the same sample that was processed separately upon loading to the Zymo-Spin IC column (*see* 3.4.2, **step 17**).

10. Add 16 μL nuclease-free H_2O onto the column. Let the column stand on the benchtop for 2 min. Centrifuge for 1 min at 10,000 × *g* to elute the ssDNA.
11. Reapply the eluate to the column as described in the previous step. Keep the ssDNA on ice.

Potential pause point: store the sample at −20 °C indefinitely.

3.4.5 Circularization

1. Add 2.0 μL 10× CircLigase buffer, 1.0 μL 1 mM ATP, 0.5 μL 50 mM $MnCl_2$, and 1.0 μL CircLigase to 15.5 μL cDNA from **step 11**, Subheading 3.4.4. Mix by pipetting several times (*see* **Note 11**).

Reagent	For one reaction (μL)
10× CircLigase buffer	2
1 mM ATP	1
50 mM $MnCl_2$	0.5
eluted ssDNA	15.5
CircLigase	1
Total volume	20

2. In a PCR cycler, incubate the reaction for 60 min at 60 °C, followed by 10 min at 80 °C to stop the reaction.
3. Keep the sample on ice.

Potential pause point: store the sample indefinitely at −20 °C.

3.4.6 Depletion of Abundant Mature RNAs

1. Prepare a circular cDNA precipitation mix, aliquot the amount needed for one library (326 μL) into a new 1.5 mL tube. Keep on ice.
2. Prepare a 1× magnetic bead equilibration buffer by diluting the 2× magnetic bead equilibration buffer. One reaction requires 330 μL.
3. Resuspend the streptavidin-coated magnetic beads by gentle vortexing. For one reaction, transfer 110 μL of beads into a new 1.5 mL tube. In case of multiple reactions beads can be combined in one tube (e.g., for N reactions, take N.2 × volume).
4. Place the tube on a magnetic rack for 2 min. Remove the clarified liquid.
5. Remove the tube from the magnetic rack. Resuspend beads in 110 μL of 1× magnetic bead equilibration buffer. Place the tube on a magnetic rack for 2 min. Remove the clarified liquid. Repeat for a total of three washes.

6. Following the final wash, resuspend the beads for one reaction in 40 μL of 2× magnetic bead equilibration buffer. If multiple reactions are prepared, scale up the volume accordingly. Pipette slowly to avoid foaming. Prepare 40 μL aliquots of washed and resuspended beads.
7. Equilibrate the beads at 37 °C in a thermomixer for 15–30 min.
8. Meanwhile, proceed with the hybridization reaction. Add the components listed below directly to the circularized cDNA obtained in Subheading 3.4.5.

Reagent	For one reaction (μL)
circularized cDNA	20
Depletion oligo mix	4
20× SSC buffer	4
RNase/DNase-free H_2O	12
Total volume	**40**

9. In a PCR cycler, perform the hybridization of depletion oligos and the complementary cDNA using the following program.

Step	Temperature	Time
Denature	99 °C	90 s
Annealing	99 °C → 37 °C in 0.1 °C steps; 1 s per 0.1 °C	
Final annealing	37 °C	15 min

10. Add the hybridization mix (40 μL) to 40 μL of the equilibrated magnetic beads and mix well by pipetting. Incubate for 15 min at 37 °C and 1000 rpm in a thermomixer. This needs to be done quickly to prevent premature cooling of the samples.
11. Transfer tubes to a magnetic rack, incubate for 1 min, and then transfer the entire supernatant to a new 1.5 mL tube containing 326 μL of the circular cDNA precipitation mix. It is critical to avoid transferring any magnetic beads. Mix well and keep on ice.
12. Add 600 μL of 100% isopropanol, mix well and put on ice for 15 min. Precipitate at −20 °C overnight.
13. Pellet the ssDNA precipitate by centrifugation for at least 1 h at 20,000 × *g*, 4 °C. Carefully remove the supernatant and avoid touching the blue pellet.
14. Wash the pellet with 750 μL of freshly prepared ice-cold 80% (v/v) ethanol. Spin at 20,000 × *g* and 4 °C for 2 min. Discard

the supernatant. Repeat for a total of two washes. After the final wash, briefly spin the tube and carefully remove the remaining liquid. Dry the pellets at room temperature until all visible liquid has evaporated and the pellet appears transparent, i.e. for 8–10 min. Resuspend the pellet in 16 μL nuclease-free H_2O.

Potential pause point: store the sample indefinitely at −20 °C.

3.4.7 PCR Cycle Optimization

In order to minimize the amount of PCR duplicates and the formation of concatemers, it is crucial to limit the number of amplification cycles. To determine the minimal number of required PCR cycles, we usually test 6, 8, 10 and 12 cycles. Different index primers can behave differently.

1. For each condition, prepare one reaction as shown below (*see* **Note 12**). To test all four conditions, prepare a master mix. Mix the primers by pipetting up and down several times before adding to the master mix. Add the enzyme and gently mix. Aliquot 20 μL into new PCR tubes. Keep the samples on ice.

Reagent	For one reaction (μL)	Master mix for 4.2 reactions (μL)
5× Phusion HF buffer	3.8	16
10 mM dNTP	0.375	1.6
indexed forward primer (100 μM)	0.1	0.4
universal reverse primer oNTI231 (100 μM)	0.1	0.4
RNase-free H_2O	14.4	60.5
Phusion DNA polymerase	0.225	0.9
DNA template	1	4.2
Total volume	20	20/tube

2. Perform PCR as follows:

Step	Temperature (°C)	Time (s)	Cycles
Denature	98	30	1
Denature Annealing Extension	98 60 72	10 10 5	6–12
	4	Pause	1

3. Stop the PCR after completion of the respective amplification cycle by placing the sample on ice. Do not stop or pause the PCR program.
4. Add 4 μL 6× DNA loading buffer per PCR sample. Prepare the DNA ladder and add an appropriate volume of 6× DNA loading buffer. Load 24 μL of each sample and an appropriate volume of the ladder on an 8% (w/v) TBE gel.
5. Run the gel at 180 V for 60 min.
6. Stain the gel in 1× TBE containing 1× SYBR Gold for 5 min on a shaker at room temperature.
7. Visualize the RNA on a blue light table or UV light. Wear suitable protective glasses!

3.4.8 NET-Seq Library Amplification and Final Quality Checks

For the final library amplification, choose the number of PCR amplification cycles that result in a clearly visible PCR product at around 150 bp but does not yet lead to products of higher molecular weight (Fig. 2c, d, *see* **Note 12**).

1. Prepare a master mix for 6–8 reactions as shown in Subheading 3.4.7.
2. Perform PCR as shown in Subheading 3.4.7 using the optimized number of PCR cycles.
3. Perform the gel electrophoresis as described in Subheading 3.4.7.
4. Visualize the PCR product on a blue light table. Wear suitable protective glasses! Excise the broad band at around 150 nt, corresponding to the NET-Seq library.
5. PCR products of two lanes are extracted together. Pierce the bottom of a 0.5 mL low-bind tube with a 20 G needle and place into a 2 mL low-bind tube as described in **step 8**, Subheading 3.4.2. Transfer the gel slices (from two lanes) into the pierced 0.5 mL tube. Centrifuge for 4 min at 20,000 × *g*, room temperature. Repeat, if gel pieces remain in the inner tube. Discard the inner tube.
6. Add 668 μL of fresh DNA soaking buffer. Put the tubes on a shaker at room temperature, 1400 rpm overnight.
7. Transfer the gel slurry from two lanes onto one filter tube and centrifuge for 3 min at 20,000 × *g*. Repeat the centrifugation step to increase the recovery of the NET-Seq library.
8. Add 2 μL of GlycoBlue and 680 μL 100% isopropanol to each tube. Mix by inverting the tubes several times. Put the sample to −20 °C for at least 1 h.

Potential pause point: store the sample overnight at −20 °C.

9. Centrifuge the sample for at least 1 h at 20,000 × *g*, 4 °C to collect the precipitated library. A blue compact pellet is visible.
10. Remove the supernatant. Wash the pellets by adding 750 μL freshly prepared ice-cold 80% (v/v) ethanol. Immediately remove most of the ethanol without disturbing the pellet. Spin 2 min at 12,000 × *g*, 4 °C. Carefully remove any remaining supernatant. Air-dry the pellets until they become transparent (<10 min). Carefully resuspend the pellets of the same sample in 6 μL ice-cold 10 mM Tris–HCl, pH 8.0.
11. For concentration measurements and quality check, prepare 1:5 and 1:10 library dilutions. For 1:5 dilution, mix 1 μL of the library and 4 μL of 10 mM Tris–HCl, pH 8.0. Mix well by pipetting. For 1:10 dilution, mix 2 μL of 1:5 dilution and 2 μL of 10 mM Tris–HCl, pH 8.0.
12. Measure the concentration of 1:5 and 1:10 dilutions using Qubit HS dsDNA assay following the manufacturer's instructions. Verify library size distribution by BioAnalyzer measurements using the HS DNA kit. Follow the manufacturer's instructions. Store the NET-Seq library and the dilutions at −20 °C.

3.5 Next-Generation Sequencing

The obtained NET-Seq libraries are sequenced from the 3′-end using the custom sequencing primer oLSC006 (Subheading 2.2.3). NET-Seq libraries are usually sequenced on HiSeq, NextSeq, and NovaSeq Illumina platforms. For monitoring transcription at protein-coding and non-coding genes in human cells, a reasonable coverage is obtained with >100 million reads. In case NET-Seq is mainly used for studying enhancer transcription, we recommend to sequence NET-Seq libraries to greater depth due to overall lower transcription levels at enhancer regions.

3.6 Computational Analysis

Finally, the obtained mammalian NET-Seq data sets are computationally analyzed. An updated version of the computational NET-Seq analysis pipeline is provided at our GitHub site that can be accessed here: https://github.molgen.mpg.de/MayerGroup/net-seq-pipeline. Briefly, NET-Seq data processing begins with the extraction of the hexamer barcode, followed by trimming of adapter sequences and aligning the sequencing reads to the reference genome. The 5′-nucleotide of the reads corresponding to the 3′-most nucleotide of the original nascent RNA is extracted and recorded. PCR duplicates are identified as reads with identical sequences and the same barcodes and are removed. Our analysis pipeline also identifies artifacts that can arise during reverse transcription due to mispriming as reads for which the hexamer barcode is identical to the adjacent sequence in the reference genome. The computational analysis of NET-Seq data results in DNA strand-specific occupancy profiles of transcriptionally engaged Pol II (Fig. 3).

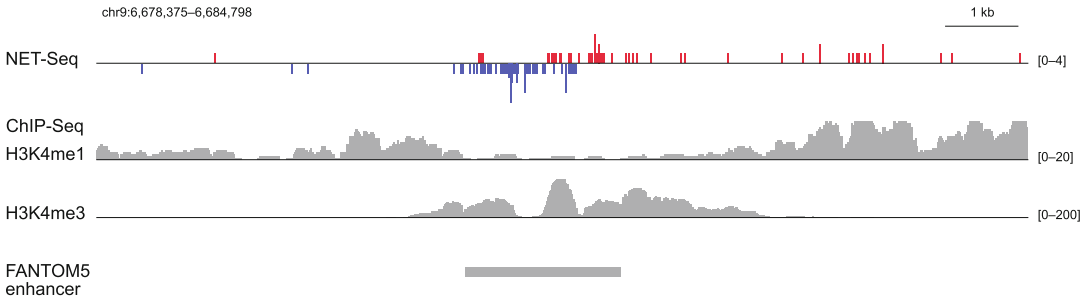

Fig. 3 Exemplary NET-Seq result for a transcribed enhancer. NET-Seq reads for Watson and Crick strands are displayed in red and blue, respectively. ChIP signal for H3K4 mono- and tri-methylation [33], and Fantom5 enhancer annotation are displayed as reference. Results are shown for human K562 cells

4 Notes

1. In order to capture short RNA species (<200 nt), such as nascent RNAs associated with promoter-proximally paused Pol II, it is critical to use the miRNeasy mini kit designed to capture both short and long RNAs (>17 nt).
2. The set of depletion oligos listed here was originally designed for HeLa cells but also functions efficiently for K562 and HEK293 cells, and potentially also for other human cell lines. You can easily replace or add depletion oligos if necessary to increase the fraction of informative reads.
3. Efficiency of cell lysis can be monitored using light microscopy. On a glass slide, mix 1 μL of Trypan blue dye with 1 μL of lysed cells. Under the microscope, nuclei of lysed cells will appear as small and dark blue colored dots as compared to non-lysed cells, which are larger in size and less colored. Always use a sample of non-lysed cells as a control.
4. For each new cell line, we recommend to monitor the success of the cell fractionation by Western blot analysis probing at least for the cytoplasmic marker GAPDH, the chromatin marker histone H2B and for p-Ser2 RPB1 corresponding to the elongating form of Pol II. For Western blot analysis, perform the fractionation as described in Subheading 3.2. Omit α-amanitin and RNase inhibitors in subcellular fractionation buffers. Solubilize the chromatin pellet by benzonase nuclease treatment: add 1 μL of benzonase to the chromatin fraction, incubate sample with gentle agitation at room temperature until the pellet is solubilized. Adjust volumes of the cytoplasmic, nucleoplasmic, and chromatin samples to allow comparisons of Western blot signals between the subcellular fractions. >95% of p-Ser2 RPB1 should be detected in the chromatin

fraction. A lower percentage indicates harsh handling of cells or nuclei. For additional information, please consult our protocol on subcellular RNA-Seq [24].

5. Pipetting with a regular P1000 helps to dissolve vortex-resistant chromatin clumps. Do not use a syringe. Incomplete resuspension will decrease the RNA yield.
6. If the organic and aqueous phases are not well separated after centrifugation, this could be due to a high density of the aqueous phase. To reduce the density, add 50–100 μL of either RNase-free H_2O or PBS to the sample prior to centrifugation.
7. The number of cells that are required as an input for the NET-Seq library preparation depends on the cell type. Stable cancer cell lines have overall more expression as compared to primary cells. Therefore, less cells are required as an input as compared to primary cells.
8. Avoid presence of free divalent ions in the RNA samples as they will cause degradation of RNA upon heating.
9. RNA fragmentation is based on the reactivity of Mg^{2+} ions. While other buffer formulations are also able to fragment RNA, we found NEBNext® RNA Fragmentation Buffer to give the most reproducible results.
10. The RNA fragmentation time should be optimized for each new lot of fragmentation buffer. To determine the optimal fragmentation time, prepare a ligation reaction mix for four reactions. Add reactions to four 0.2 mL tubes. To save reagents, the DNA linker as well as the ligase can be replaced by nuclease-free H_2O. Do not replace other buffer components. Add 2 μL NEBNext® RNA Fragmentation Buffer to each tube and proceed as explained in **step 6** of 3.4.1, but stop fragmentation after 6, 8, 10, and 12 min. Purify the samples as described in **steps 8 to 9** of 3.4.1 and monitor the size distribution with both gel electrophoresis using a 15% TBE-urea gel and a BioAnalyzer or TapeStation.
11. The concentration of Mn^{2+} ions in the circularization reaction is critical. An excess of Mn^{2+} ions can result in a brown precipitate and a failure of the circularization reaction. Another possibility for inefficient circularization is the degradation of ATP over time. Therefore, we recommend to store ATP in aliquots to limit the number of freeze-thaw cycles. We also recommend to replace unused ATP solutions every 4 months.
12. If none of the tested PCR conditions seem suitable, you can increase the sample volume to 1.5 or 2.0 μL per reaction. Using more than 1 μL of template will reduce the number of PCR cycles that are required to obtain sufficient amounts of the NET-Seq library.

Acknowledgments

We thank all members of the Mayer group for critical comments on the manuscript. This work was funded by the Max Planck Society (to A.M.) and the Deutsche Forschungsgemeinschaft (DFG, grant no. 418415292 to A.M. and the International Research Training Group (IRTG) 2403 to A.M. and M.A.). O.J. was supported by a 2017 FEBS Long-Term Fellowship.

References

1. Kim T-K, Hemberg M, Gray JM et al (2010) Widespread transcription at neuronal activity-regulated enhancers. Nature 465:182–187
2. Li W, Notani D, Rosenfeld MG (2016) Enhancers as non-coding RNA transcription units: recent insights and future perspectives. Nat Rev Genet 17:207–223
3. Andersson R, Gebhard C, Miguel-Escalada I et al (2014) An atlas of active enhancers across human cell types and tissues. Nature 507:455–461
4. Lam MTY, Li W, Rosenfeld MG et al (2014) Enhancer RNAs and regulated transcriptional programs. Trends Biochem Sci 39:170–182
5. Chen H, Du G, Song X et al (2017) Non-coding transcripts from enhancers: new insights into enhancer activity and gene expression regulation. Genom Proteom Bioinformat 15:201–207
6. Schwalb B, Michel M, Zacher B et al (2016) TT-seq maps the human transient transcriptome. Science 352:1225–1228
7. Wissink EM, Vihervaara A, Tippens ND et al (2019) Nascent RNA analyses: tracking transcription and its regulation. Nat Rev Genet 20:705–723
8. Churchman LS, Weissman JS (2011) Nascent transcript sequencing visualizes transcription at nucleotide resolution. Nature 469:368–373
9. Mayer A, di Iulio J, Maleri S et al (2015) Native elongating transcript sequencing reveals human transcriptional activity at nucleotide resolution. Cell 161:541–554
10. Nojima T, Gomes T, Grosso ARF et al (2015) Mammalian NET-Seq reveals genome-wide nascent transcription coupled to RNA processing. Cell 161:526–540
11. Zhu J, Liu M, Liu X et al (2018) RNA polymerase II activity revealed by GRO-seq and pNET-seq in Arabidopsis. Nat Plants 4:1112–1123
12. Kindgren P, Ivanov M, Marquardt S (2020) Native elongation transcript sequencing reveals temperature dependent dynamics of nascent RNAPII transcription in Arabidopsis. Nucleic Acids Res 48:2332–2347
13. Shetty A, Kallgren SP, Demel C et al (2017) Spt5 plays vital roles in the control of sense and antisense transcription elongation. Mol Cell 66:77–88.e5
14. Wery M, Gautier C, Descrimes M et al (2018) Native elongating transcript sequencing reveals global anti-correlation between sense and antisense nascent transcription in fission yeast. RNA 24:196–208
15. Larson MH, Mooney RA, Peters JM et al (2014) A pause sequence enriched at translation start sites drives transcription dynamics in vivo. Science 344:1042–1047
16. Vvedenskaya IO, Vahedian-Movahed H, Bird JG et al (2014) Interactions between RNA polymerase and the "core recognition element" counteract pausing. Science 344:1285–1289
17. Clarke AM, Engel KL, Giles KE et al (2018) NETSeq reveals heterogeneous nucleotide incorporation by RNA polymerase I. Proc Natl Acad Sci 115:E11633–E11641
18. Drexler HL, Choquet K, Churchman LS (2020) Splicing kinetics and coordination revealed by direct nascent RNA sequencing through nanopores. Mol Cell 77:985–998.e8
19. Mylonas C, Tessarz P (2019) NET-prism enables RNA polymerase-dedicated transcriptional interrogation at nucleotide resolution. RNA Biol 16:1156–1165
20. Hirabayashi S, Bhagat S, Matsuki Y et al (2019) NET-CAGE characterizes the dynamics and topology of human transcribed cis-regulatory elements. Nat Genet 51:1369–1379
21. Fischl H, Howe FS, Furger A, Mellor J (2017) Paf1 has distinct roles in transcription elongation and differential transcript fate. Mol Cell 65:685–698.e8
22. Imashimizu M, Takahashi H, Oshima T et al (2015) Visualizing translocation dynamics and

nascent transcript errors in paused RNA polymerases in vivo. Genome Biol 16:98

23. Bernecky C, Herzog F, Baumeister W et al (2016) Structure of transcribing mammalian RNA polymerase II. Nature 529:551–554
24. Mayer A, Churchman LS (2017) A detailed protocol for subcellular RNA sequencing (subRNA-seq). Curr Protoc Mol Biol 120:4.29.1–4.29.18
25. Mayer A, Churchman LS (2016) Genome-wide profiling of RNA polymerase transcription at nucleotide resolution in human cells with native elongating transcript sequencing. Nat Protoc 11:813–833
26. Wuarin J, Schibler U (1994) Physical isolation of nascent RNA chains transcribed by RNA polymerase II: evidence for cotranscriptional splicing. Mol Cell Biol 14:7219–7225
27. Pandya-Jones A (2011) Pre-mRNA splicing during transcription in the mammalian system. Wiley Interdiscip Rev RNA 2:700–717
28. Bhatt DM, Pandya-Jones A, Tong A-J et al (2012) Transcript dynamics of proinflammatory genes revealed by sequence analysis of subcellular RNA fractions. Cell 150:279–290
29. Brueckner F, Cramer P (2008) Structural basis of transcription inhibition by α-amanitin and implications for RNA polymerase II translocation. Nat Struct Mol Biol 15:811–818
30. Lindell TJ, Weinberg F, Morris PW et al (1970) Specific inhibition of nuclear RNA polymerase II by alpha-amanitin. Science 170:447–449
31. Cai H, Luse DS (1987) Transcription initiation by RNA polymerase II in vitro. Properties of preinitiation, initiation, and elongation complexes. J Biol Chem 262:298–304
32. Kireeva ML, Komissarova N, Waugh DS et al (2000) The 8-nucleotide-long RNA:DNA hybrid is a primary stability determinant of the RNA polymerase II elongation complex. J Biol Chem 275:6530–6536
33. Shah RN, Grzybowski AT, Cornett EM et al (2018) Examining the roles of H3K4 methylation states with systematically characterized antibodies. Mol Cell 72:162–177.e7