# Tissue NET-seq (native elongating transcript sequencing)

Forked from Profiling metazoan transcription genome-wide with nucleotide resolution using NET-seq (native elongating transcript sequencing)

Sep 02, 2022

Mihaela Bozukova[1], Peter Tessarz[1]

[1]Max Planck Institute for Biology of Ageing



dx.doi.org/10.17504/protocols.io.kxygxzj3dv8j/v1

churchmanlab

Peter Tessarz

ABSTRACT

Quantifying crucial steps in gene regulation during transcription elongation, such as promoter-proximal pausing, requires high resolution methods to map the transcription machinery across the genome. Native Elongating Transcript sequencing (NET-seq) interrogates the 3' ends of nascent RNA through sequencing, providing a direct visualization of RNA Polymerase II (Pol II) positions genome-wide with strand specificity and single nucleotide resolution. As NET-seq does not require previous labeling of mRNA, it is ideally-suited for measuring nascent transcription in tissues. In brief, actively transcribing Pol II together with the nascent RNA is quantitatively purified from cells by cellular fractionation. To prevent run-on transcription, fractionation is performed in the presence of the potent Pol II inhibitor α-amanitin, which prevents NTP recognition and catalysis by the Pol II trigger loop. Then, the isolated nascent RNA is fragmented, converted into cDNA and processed into a sequencing library employing a minimal number of PCR cycles. The library preparation method is designed in a way that upon random fragmentation of the nascent transcripts, only the 3’ ends of nascent transcripts carrying a free hydroxy-group are sequenced. This reveals the position of transcriptionally engaged Pol II at nucleotide resolution.

We modified the original NET-seq protocol (Mayer & Churchman, 2016) for application in murine liver tissue and named this new adaptation of the protocol tissue NET-seq (tNET-seq). The main change in the protocol pertains to the nuclei isolation. Fresh liver tissue were placed in ice-cold PBS, cut into small pieces and homogenized in nuclei isolation buffer (Sigma, NUC101) using a Dounce tissue homogenizer.

A published analysis pipeline for NET-seq can be found at the Churchman lab Github.

DOI

dx.doi.org/10.17504/protocols.io.kxygxzj3dv8j/v1

PROTOCOL CITATION

Mihaela Bozukova, Peter Tessarz 2022. Tissue NET-seq (native elongating transcript sequencing). **protocols.io** https://protocols.io/view/tissue-net-seq-native-elongating-transcript-sequen-cescteaw

FUNDERS ACKNOWLEDGEMENT

Max Planck Society

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Mayer, Andreas, and L. Stirling Churchman. 2016. “Genome-Wide Profiling of RNA Polymerase Transcription at Nucleotide Resolution in Human Cells with Native Elongating Transcript Sequencing.” Nature Protocols 11 (4): 813–33

FORK NOTE

FORK FROM

Forked from Profiling metazoan transcription genome-wide with nucleotide resolution using NET-seq (native elongating transcript sequencing), Robert ietswaart

KEYWORDS

Transcription, NET-seq, RNA polymerase, RNA, Methods, Nascent transcript, Gene expression, Elongation, tissue



CREATED

Aug 03, 2022

LAST MODIFIED

Sep 02, 2022

PROTOCOL INTEGER ID

68132

GUIDELINES

**Notes**

1. Irritant, handle with care and dispose of the waste according to institutional regulations.
2. Toxic, handle with care and dispose of the waste according to institutional regulations.
3. Irritant, harmful, and hazardous to the environment: handle with care and dispose of the waste according to institutional regulations.
4. Toxic, corrosive, and an irritant: handle with care and dispose of waste according to institutional regulations.
5. When the fractionation protocol is being tested on a new cell type it is recommended that the cellular fractions are checked for subcellular marker proteins in order to confirm that the fractionation is working. To do this, probe the WB membrane with antibodies raised against GAPDH (cytoplasm), the U1 snRNP70 (nucleoplasm) and histone 2B (chromatin).
6. The CTD specificities of these Pol II–specific antibodies have been extensively characterized, so we recommend using these specific antibodies when testing the fractionation protocol.
7. Benzonase (250 U) can be used to resuspend the sticky chromatin pellet in solution (for the WB control, Box 1).
8. Flammable, corrosive, and an irritant: handle with care and dispose of waste according to institutional regulations
9. Volatile, toxic, and an irritant: handle with care and dispose of waste according to institutional regulations.
10. It is important to use the miRNeasy mini kit (or miRNeasy micro kit for small cell numbers) instead of the RNeasy mini kit to avoid RNA length biases. Use personal protective equipment when handling reagents in the kits; the RWT buffer is corrosive and an irritant, and the QIAzol lysis reagent contains phenol and is toxic and corrosive. Dispose of waste according to institutional regulations.
11. Handle sharps with care and dispose according to institutional regulations.
12. Keep track of the enzyme lot; ligase activity can vary between different batches.
13. Using an N10 linker increases the dynamic range of the NET-seq library and decreases PCR duplicates (compared to the N6 linker used in (Mayer and Churchman 2016)).
14. Use personal protective equipment when handling reagents in the kit; buffers are irritants. Dispose of waste according to institutional regulations.
15. Nucleic acid stains are usually mutagenic; use personal protective equipment when handling. SYBR Gold stain is flammable; dispose of waste according to institutional regulations.
16. Corrosive and an irritant: handle solutions with care and dispose of waste according to institutional



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regulations. Limit the freeze-thaw cycles for this reagent.
17. Toxic and hazardous to the environment: handle with care and dispose of waste according to institutional regulations.
18. Highly flammable, volatile, and an irritant: handle solutions with care and dispose of waste according to institutional regulations.
19. NET-seq has been successfully carried out on several human cell lines (e.g. K562, HeLa S3, HEK293T, and MOLT-4 cells), including challenging cell types such as neurons. The NET-seq protocol has also been successfully applied to other cell types such as Drosophila cells (S2 and S2R+ cells) and mouse cells (NIH 3T3 cells) and others.
20. Perform a western blot control in parallel with the sample, or prior to performing fractionation on the sample to ensure the cellular fractionation protocol is optimized for the specific tissue being used. The western blot control is a separate sample from the sample being prepared to make a sequencing library.
21. We used roughly 1/2 of a liver per sample.
22. If there is a small amount of supernatant remaining in the tube after the greater quantity has been removed, it is helpful to use a small pipette tip to get rid of the residual supernatant.
23. For more effective layering, tilt the tube at a 45 degree angle and pipette lysate onto the side of the tube.
24. Some chromatin pellets can be difficult to resuspend. If you find this to be the case, you can either increase the volume of the CRS (up to 300 µl) and try to solubilize the chromatin pellet again or you can proceed directly to the RNA extraction (Steps section 1.3.3) by adding Qiazol to your chromatin pellet (which is in 50 µl of CRS).
25. Alternatively, the chromatin pellet can also be solubilized by gentle vortexing.
26. The expected absorbance at (A260/A280) ratio is ~2.0, indicative of highly purified RNA.
27. The fragmentation time needs to be adjusted whenever a new batch of alkaline fragmentation solution is applied (or when the solution has not been used for long periods of time -i.e. months). An over fragmentation or under fragmentation of the nascent RNA pool can lead to systematic biases. In a typical experiment RNA is fragmented between 10 and 40 min at 95 °C.
28. The un-ligated oGAB11 control is only used for visualizing the efficiency of the ligation reaction by comparing un-ligated oGAB11 to ligated oGAB11. After visualization of the gel, un-ligated oGAB11 control is not subjected to any further steps in the protocol.
29. Use a needle and syringe to flush out the excess urea that may settle in the gel wells prior to loading samples.
30. The efficiency of the linker ligation is usually >95%, the efficiency of the ligation is monitored using the oGAB11 control by PAGE (see Figure 2).
31. If no cDNA smear is observed continue to the PCR step; sometimes PCR product will be visible even when the RT product is not visible by eye. Other possible reasons for not observing the RT product are that the linker ligation was inefficient or the reverse transcriptase enzyme lost activity. One way to prevent these problems is to replace the ligation reaction and RT reaction reagents every 4 months.
32. Empty circles are circularized DNA molecules that arise from un-extended RT primers, and hence they do not contain any information about the original nascent RNA.
33. Do not dilute the NET-seq library for the quantification and characterization step if less than 1 µg of starting material was used to construct the sequencing library.

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MATERIALS TEXT

**1.1** Preparation of buffers and working area for cellular fractionation.
RNase free reagents and solutions should be used for RNA work only and no other laboratory techniques
1. RNase Away Reagent (Thermo Scientific) (see Note 1 in the Guidelines section)
2. α-Amanitin solution (1 mM): Dissolve 1 mg of α-amanitin (Sigma-Aldrich) in 1 ml of RNase-free H2O. The α-amanitin solution can be stored in aliquots for up to 1 year at −20 °C (see Note 2 re α-amanitin).
3. Halt protease inhibitor cocktail (Thermo Scientific, # 87786)
4. Nuclear Isolation Buffer (Sigma, NUC101) was supplemented with 1x Halt protease inhibitor cocktail, 40U RNAsin (Promega, # N2511) and 25 µM α-amanitin.
5. Nuclei wash buffer (NWB): For one reaction, mix the reagents below. Freshly prepare the NWB before use with RNase-free reagents and keep on ice (see Note 3 in the Guidelines section re Triton X-100 and Note 1 re EDTA).

| A | B | C |
|---|---|---|
| **Nuclei wash buffer (NWB)** | | |
| Reagents | Volume (µl) | Final concentration |
| 10% (vol/vol) Triton X-100 | 105 | 0.1% (vol/vol) |
| 5 M NaCl | 15 | 150 mM |
| 0.5 M EDTA (cat. no.) | 2 | 1 mM |
| 1mM α-amanitin solution | 25 | 25 µM |
| RNAsin (20 U/µl) | 2 | 40 U |
| protease inhibitor mix (50×) | 20 | 1× |
| PBS | 941 | 1× |



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7. Glycerol buffer (GB): For one reaction, mix the reagents below. Prepare a 0.25 M EDTA solution (from 0.5 M stock solution) in 1× PBS. Freshly prepare the GB before use with RNase-free reagents and keep on ice.

| A | B | C |
|---|---|---|
| **Glycerol buffer (GB)** | | |
| Reagents | Volume (µl) | Final concentration |
| 1 M Tris-HCl (pH 8.0) | 5 | 20 mM |
| 5 M NaCl | 3.8 | 75 mM |
| 100% (vol/vol) filter-sterilized glycerol | 125 | 50% (vol/vol) |
| 0.25 M EDTA | 0.5 | 0.5 mM |
| 0.1 M filter-sterilized DTT | 2.1 | 0.85 mM |
| 1mM α-amanitin solution | 6.2 | 25 µM |
| SUPERase.In (20 U/µl) | 0.6 | 10 U |
| protease inhibitor mix (50×) | 5 | 1× |
| RNase-free H2O | 101.8 | – |

8. Nuclei lysis buffer (NLB): For one reaction, mix the reagents below. Prepare a 0.1 M EDTA solution (from 0.5 M stock solution) in 1× PBS. Freshly prepare the NLB before use with RNase-free reagents and keep on ice.

| A | B | C |
|---|---|---|
| **Nuclei lysis buffer (NLB)** | | |
| Reagents | Volume (µl) | Final concentration |
| 1 M HEPES (pH 7.5) | 5 | 20 mM |
| 5 M NaCl | 15 | 300 mM |
| 10% (vol/vol) NP-40 | 25 | 1% (vol/vol) |
| 0.1 M EDTA | 0.5 | 0.2 mM |
| 0.1 M filter-sterilized DTT | 2.5 | 1 mM |
| 10 M filter-sterilized urea | 25 | 1 M |
| 1mM α-amanitin solution | 6.2 | 25 µM |
| SUPERase.In (20 U/µl) | 0.6 | 10 U |
| protease inhibitor mix (50×) | 5 | 1× |
| RNase-free H2O | 165.2 | – |

9. Chromatin resuspension solution (CRS): For three reactions, mix the reagents below. Freshly prepare the NWB before use with RNase-free reagents and keep on ice.

| A | B | C |
|---|---|---|
| **Chromatin resuspension solution (CRS)** | | |
| Reagents | Volume (µl) | Final concentration |
| 1mM α-amanitin solution | 5 | 25 µM |
| SUPERase.In (20 U/µl) | 4 | 20 U |
| protease inhibitor mix (50×) | 10 | 1× |
| PBS | 190.5 | 1× |

10. Oligonucleotide sequences used for tNET-seq library preparation. Illumina barcode sequences are underlined. rApp:



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5’-adenylated ribonucleotide. $(N)_6$: random hexameric sequence. 3ddC: 3’dideoxycytosine. 5Phos: 5’-phosphate. iSp18: internal 18-atom hexa-ethylenglycol spacers.

| A | B |
|---|---|
| **Name** | **Sequence (5’ to 3’)** |
| DNA linker | rApp(N)6CTGTAGGCACCATCAAT3ddC |
| oGAB11 (RNA control oligo) | AGUCACUUAGCGAUGUACACUGACUGUG-OH |
| RT primer oLSC007 | 5Phos/ATCTCGTATGCCGTCTTCTGCTTG/iSp18/CACTCA/iSp18/TCCGACGATCATTGATGGTGCCTACAG |
| PCR forward primer MHL001 | AATGATACGGCGACCACCGAGATCGGAAGAGCACACGTCTGAACTCCAGTCACTGCATCTCCGACGATCATTGATGG |
| PCR forward primer MHL002 | AATGATACGGCGACCACCGAGATCGGAAGAGCACACGTCTGAACTCCAGTCACATGCCATCCGACGATCATTGATGG |
| PCR reverse primer (oNTI231) | CAAGCAGAAGACGGCATACGA |
| Sequencing primer (oLSC006) | TCCGACGATCATTGATGGTGCCTACAG |

Linker: (N10), random decameric sequence or UMI (handmixed by Integrated DNA Technologies); 3ddC, 3'-dideoxycytidine; iSp18, internal 18-atom hexa-ethylenglycol spacer; 5rApp, 5'-riboadenylate (5'-adenine nucleotide of the pre-adenylated Linker is a ribonucleotide, rA). oLSC007: 5Phos, 5'-phosphate. Barcode primer: lowercase region represents the barcode index for one library which allows for multiplexing with other libraries on the same instrument (use separate index sequences for each library, a list of other index sequences can be found on the Illumina website). The primer also contains a region for the sequencing primer (oLSC006) to anneal, and the p5 sequence for binding to the flow cell.

11. Depletion oligonucleotides for the 20 most abundant chromatin-associated mature RNAs captured in NET-seq libraries from mESCs cells. Biotinylated oligonucleotides were purchased from Sigma Aldrich in HPLC quality.



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| A | B | C |
|---|---|---|
| **Gene** | **Transcript type** | **DNA sequence (5' to 3')** |
| Snord49a | snoRNA | AGTCAGCCAGGAGCAGTTATCGTCAGTTATCGAC |
| Rn45s | rRNA | GAGAGCCGCCCGAACGACCGACTTCCCTACGGGCCC |
| Snord65 | snoRNA | CTTCAGAAAACCATAGGCTCACCACTACCAATCT |
| Snord82 | snoRNA | GAACCATGGGGTTGAAATGAAATATGCTGATGTGCT |
| Snord49b | snoRNA | GTCAGCTAACTAGGGATGTCGTCAGTTGTCGCAT |
| Snord2 | snoRNA | AGTGATCAGCAAGAGTATTCTCTTCATTTCAGGTCA |
| Snord99 | snoRNA | TCTCAGTCCCATATCCGCATTTCTCATCCATAGA |
| Snord95 | snoRNA | CAGCTCAGAAACAGCCTCTGGATTTCAGCAAAGCAA |
| Snord55 | snoRNA | CGTGGGGAAGCCAACCTTGGAGAGCTGAGCGTGC |
| Snord68 | snoRNA | CATCAGATGGAAAAGGGTTCAAAAGTACTTTCAT |
| Snord32a | snoRNA | GACTGTGAGATCAACCCATGCACCGCTCTGAGACTC |
| Snord87 | snoRNA | GTTTCTTTGAAGAGAGAATCTTAAAAGACTGAGA |
| Rmrp | ncRNA | CGCACCAACCACACGGGGCTCATTCTCAGCGCGGCTAC |
| Snord100 | snoRNA | CTCGCTGAGGAAACTGCACGTCACCCTCCTGAAA |
| Snora68 | snoRNA | GTGCAGTGCCCCCCAGAGTGAATCAGTAGGCTCTACAGAA |
| Rnu3a | snRNA | AACCACTCAGACTGTGTCCTCTCCCTCTCAACCCTCAA |
| Snord42b | snoRNA | GAGACCTGTGATGTCTTCAAAGGAACCACTGATG |
| Snord83b | snoRNA | TGAGGAATTATTCCCTGTTGCCTTCCTTCTGAGA |
| Snord110 | snoRNA | TTGCTCAGACACATGGAGTCGTCAGTGATCTCTCAGGG |
| Snord47 | snoRNA | CCTCAGAAATAAAATGGAACGGTTTAAAGGTGAT |



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BEFORE STARTING

The following steps are based on the protocol described in (Mayer and Churchman 2016), but modified to work in tissues. The main change in the protocol pertains to the nuclei isolation.

## Tissue homogenization and cell fractionation

1 Place the fresh liver tissue in ice-cold PBS, cut into smaller pieces and homogenize in 3 ml of nuclei isolation buffer using a Dounce tissue homogenizer (Wheaton). After complete homogenization, transfer the sample to a Falcon tube. Add 3 ml of nuclei isolation buffer to the remaining pieces in the tissue homogenizer, homogenize further and transfer the remnants also to the tube. Following an incubation on ice for 5 min, pass the samples through a 70-µm cell strainer and collect the nuclei by centrifuging at 500 x g for 20 min.
Resuspend the nuclei pellet in 4 ml of nuclei isolation buffer. After incubation on ice for 5 min, collect the nuclei by centrifuging at 500 x g for 5 min. To remove cytoplasmic remnants, wash the nuclei pellet with 1600 µl nuclei wash buffer and centrifuge at 1,150 x g for 5 min. Repeat the washing with 800 µl nuclei wash buffer. Then, gently resuspend the pellet in 200 µl of glycerol buffer using a cut 1,000-µl tip and transfer the suspension to a new 1.5-ml RNase-free microcentrifuge tube. To lyse nuclei, add 400 µl of nuclei lysis buffer, mix samples by pulsed vortexing and incubate on ice for 20 min. After assessing nuclei lysis of a small aliquot under a light microscope, centrifuge the samples at 18,500 x g for 2 min. Completely remove the supernatant (nucleoplasmic fraction) and resuspend the chromatin pellet in 50 µl chromatin resuspension solution.

## Preparation of nascent RNA

2 **1.** Add 700 µl of QIAzol lysis reagent (part of miRNeasy mini kit, Qiagen) to the resuspended chromatin
**2.** Mix thoroughly by slowly pipetting up and down using a 1-ml syringe with a 22G needle. Alternatively, the chromatin pellet can also be solubilized by gentle vortexing. Mix very carefully until the solution is homogeneous.



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Mix it slowly to avoid spilling the sample. (at this point the sample can be stored at −80 °C for months).
**3.** Prepare RNA using the miRNeasy mini kit according to the manufacturer's instructions, including the optional on-column DNase treatment using the RNase-free DNase set (Qiagen).
**4.** Elute the nascent RNA in 25 µl RNase-free $H_2O$. Assess the quantity and quality of the prepared RNA using NanoDrop 2000 (Thermo Scientific). RNA yield from one whole liver typically ranged from 10 to 56 µg. The absorbance A260/A280 ratios were around 2.1.

## Barcode DNA linker ligation

3 1. Spray down all work surfaces, pipettes, etc. with RNase away
2. Denature the RNA sample (from the cellular fractionation, section 1.3.3) and 5 µl of the oGAB11 control (10 µM; Table 1 in the Materials section) for 2 min at 80 °C in a Thermomixer. Place on ice.
3. Prepare linker ligation mix for each RNA sample and for oGAB11 in 0.2-ml RNase-free PCR tubes (see Materials section 1.4.1, Linker ligation, step 4). Mix until reaction is homogeneous, poor mixing will negatively affect the ligation efficiency.
4. Add 1 µl Truncated T4 RNA ligase 2 (200U) to each RNA sample as well as the oGAB11 control.
5. Incubate the ligation samples overnight for 10 - 16 hours at 16 °C in a thermal cycler (Note 31 in the Guidelines section).
6. Place on ice and add 0.7 µl of EDTA (0.5 M) to each ligation sample to stop the ligation reaction.

| A | B | C | D |
|---|---|---|---|
| **Component** | **Amount per reaction (µl)** | **Final** | |
| **RNA sample** | **oGAB11 control** | | |
| PEG8000 (50% v/v) | 8.0 | 8.0 | 20% (v/v) |
| DMSO | 2.0 | 2.0 | 10 % (v/v) |
| T4 RNA ligase buffer (10x) | 2.0 | 2.0 | 1x |
| Barcode DNA linker (1 µg) | 1.0 | 1.0 | |
| RNA sample (1 µg) | 6.0 | - | |
| oGAB11 (10 µM) | - | 1.0 | 0.5 µM |
| RNase-free H2O | - | 5.0 | |
| Truncated T4 RNA ligase 2 | 1.0 | 1.0 | 200 U |

## RNA fragmentation

4 Prior to RNA fragmentation, precipitate the RNA to remove PEG, which can affect the fragmentation reaction.
1. To each sample, add 60 µl of 3 M sodium acetate, 2 µl GlycoBlue (15 mg/ml) and 600 µl of 100 % ethanol.
2. ncubate the samples at -80°C for 45 min. (precipitates can be stored at -80 °C overnight
1. Centrifuge the samples for 30 min at full speed.
2. Wash the samples twice with 500 µl of each 100 % ethanol and 70 % ethanol.
3. Air-dry the pellets for 5 min and resuspend in 20 µl sterile water (10 µl for oGAB11 ligation control).

5 Following precipitation, add 20 µl of 2× alkaline fragmentation solution to each sample and mix. Please note that Steps 4.3 and 5.1 are not performed for the oGAB11 ligation control.
1. Fragment the RNA at 95 °C in a thermal cycler for 20 min. The fragmentation time needs to be adjusted whenever a new batch of alkaline fragmentation solution is applied. An over-fragmentation or under-fragmentation of the nascent RNA pool can lead to systematic biases. In a typical experiment RNA is fragmented between 10 and 40 min at 95 °C. The optimal fragmentation time is when most RNA molecules are in the required size range, usually between 35 and 100 nt.
2. Following fragmentation, precipitate the RNA and the oGAB11 ligation control as described above (steps 4.1-4.3).



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3. Add 20 µl of 2× TBE-urea denaturing sample buffer to each RNA sample and mix it.
4. Prepare the RNA control ladder and the oGAB11 control. Add 1.0 µl 0.5 µl of RNA control ladder or 1 µl oGAB11 to 9 µl of RNase-free $H_2O$. Add 10 µl of 2× TBU denaturing sample buffer to each control sample and mix it.
5. Denature the RNA sample and RNA control samples (oGAB11 ligation control, ladder and oGAB11) for 2 min at 80°C. Cool the samples on ice for 3 min.
6. Pre-run a 15% (wt/vol) polyacrylamide TBE-urea gel at 200 V for 15 min in 1× TBE.
7. Separate the fragmented RNA samples and the RNA control samples (including the oGAB11 ligation control) by PAGE at 200 V for 65 min.
8. Stain the gel in 50 ml of gel staining solution for 5 min at room temperature on a shaker. Protect the gel from light during staining by the use of a black gel box.
9. Visualize the fragmented RNA and the oGAB11 ligation control under blue light. For the fragmented RNA, excise the region between 35 (in the middle of the dye front) and 100 nt. For the oGAB11 ligation control, excise the narrow band at ~55 nt.
10. Extract the RNA from the gel slice by rapid gel extraction:
a. Pierce the bottom of a 0.5-ml RNase-free microcentrifuge tube with a 23G needle.
b. Put the pierced 0.5-ml tube in a 1.5-ml RNase-free microcentrifuge tube.
c. Combine and place the gel slices for each sample into the inner pierced 0.5-ml tube.
d. Centrifuge the mixture at 20,000g for 4 min at room temperature.
e. Add 200 µl RNA recovery buffer (Zymo Research, R1070-1-10) to the excised gel slices.
f. Incubate the sample for 15 min at 70°C in a Thermomixer (1500 rpm).
g. Vortex the mixture for 30 s at a medium intensity setting.
h. Cut the tip off of a 1,000-µl pipette tip and transfer the gel slurry into a Zymo-Spin IV Column (Zymo Research, C1007-50).
i. Freeze for 2 min at -80°C and subsequently thaw by placing at 70°C for 1 min.
j. Centrifuge the mixture at 20,000g for 30 secat room temperature.
k. The expected eluate volume is ~200 µl.
11. Precipitate the RNA by adding 90 µl of 3 M sodium acetate, 2 µl GlycoBlue (15 mg/ml) and 900 µl of 100 % ethanol. Proceed as described above (step 28).
12. Re-suspend the size-selected RNA and the oGAB11 ligation control in 10 µl of pre-cooled RNase-free $H_2O$.

cDNA synthesis by reverse transcription

6 1. Synthesize cDNA using the SuperScript™ III First-Strand Synthesis System (Thermo Fischer) following the manufacturer's instructions. In brief, add 0.8 µl dNTPs (10 mM) and 0.5 µl of the reverse primer oLSC007 (10 µM, Table 2) to 10 µl of RNA sample and to the oGAB11 control. After incubating at 80°C in a thermal cycler for 2 min and cooling on ice for 3 min, add 10 µl of cDNA synthesis mix consisting of 2 µl of 10x RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT™ (40 U/µl) and 1 µl of SuperScript® III RT (200 U/µl) to each sample.
2. Incubate the mixture for 30 min at 48°C in a thermal cycler.
3. Add 1.8 µl of 1 N NaOH, mix well and incubate the reaction for 20 min at 98°C.
4. Neutralize the reaction by adding 1.8 µl of 1 N HCl; mix well and put the reaction on ice.
5. Add 20 µl of 2× TBU denaturing sample buffer to each cDNA sample and the oGAB11 cDNA control, and then mix.
6. Prepare the DNA control ladder. Add 1.0 µl of DNA control ladder to 9 µl of RNase-free $H_2O$. Add 10 µl of 2× TBU denaturing sample buffer to the ladder and mix.
7. Denature the cDNA sample, oGAB11 cDNA control and DNA control ladder for 3 min at 95°C in a Thermomixer. Cool the samples on ice for 3 min.
8. Pre-run a 10% (wt/vol) polyacrylamide TBE-urea gel at 200 V for 15 min in 1× TBE.
9. Separate the cDNA sample, the oGAB11 cDNA control and the DNA ladder by PAGE at 200 V for 65 min.
10. Stain the gel in 50 ml of gel staining solution for 5 min at room temperature on a shaker. Protect the gel from light during staining by the use of a black gel box.
11. Visualize the gel under blue/UV light and excise the cDNA between 85 and 160 nt.
12. Extract the cDNA from the gel slice by rapid gel extraction (see step 5.10). To 200 µl eluate, add 25 µl of 5 M NaCl and mix.
13. Precipitate the cDNA by adding 2 µl GlycoBlue (15 mg/ml) and 750 µl of 100 % ethanol. Proceed as described above (step 28). Resuspend cDNA and oGAB11 control cDNA in 15 µl of pre-cooled RNase-free $H_2O$ each. The cDNA can be stored indefinitely at −20°C.



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Circularization of cDNA

7 1. Prepare circularization mix (Table 4) and store it on ice.
2. Add 4 µl of circularization mix to 15 µl of cDNA sample and the oGAB11 cDNA control sample in a 0.2-ml RNase-free PCR tube, and then mix well.
3. Add 1 µl of CircLigase (100 U/µl) and mix.

| Component | Amount per reaction (µl) | Final |
|---|---|---|
| CircLigase reaction buffer (10x) | 2.0 | 1x |
| ATP (1 mM) | 1.0 | 50 µM |
| MnCl2 (50 mM) | 1.0 | 2.5 mM |

4. Incubate the CircLigase reaction for 60 min at 60°C and for 10 min at 80°C in a thermal cycler. Circularized cDNA can be stored indefinitely at −20°C.

Specific depletion of highly abundant mature RNAs

8 1. For depletion of the 20 most abundant chromatin-associated mature RNAs (see Materials, step 11), prepare one specific depletion reaction per sample in 0.2-ml DNase-free PCR tubes. Please note that the oGAB11 control sample is not subjected to the depletion procedure.
2. Perform subtractive hybridization in a thermal cycler (Table 7).
3. Prepare Dynabeads MyOne streptavidin C1 (10 mg/ml) at room temperature.
a. Re-suspend the beads by gentle vortexing.
b.Transfer 100 µl of beads per depletion reaction to a DNase-free 1.5-ml microcentrifuge tube.
c.Place the tube on a magnetic rack for 1 min and withdraw all of the supernatant from the tube.
d.Remove the tube from the magnetic rack and re-suspend beads in 100 µl of bind/wash buffer.
e.Repeat this washing procedure (steps 3 and 4) two more times.
f.Place the tube on a magnetic rack for 1 min and withdraw the supernatant from the tube.
g.Remove the tube from the magnetic rack and re-suspend the beads in 30 µl of bind/wash buffer.
h.Transfer 25 µl of the re-suspended and washed beads to a new tube.
i.Place the tube in a Thermomixer at 37°C to equilibrate for 15–30 min.
4. Transfer 40 µl of depletion reaction directly from the 0.2-ml PCR tube in the thermal cycler (from Step 71) to the washed and equilibrated beads in the Thermomixer. Immediately mix by pipetting.

Composition of the depletion reactions.

| A | B | C |
|---|---|---|
| **Component** | **Amount per reaction (µl)** | **Final** |
| Circularization reaction (from step 7.4) | 20 | |
| Depletion DNA oligo pool | 4.0 | 1 µM |
| SSC, 20x | 4.0 | 2x |
| DNase-free H2O | 12.0 | |

Parameters for depletion of highly abundant mature RNAs.

| | Temperature | Time |
|---|---|---|
| **Denature** | 99°C | 90 s |
| **Annealing** | 99-37°C in 0.1°C steps | 1s (per 0.1°C step) |
| **Final annealing** | 37°C | 15 min |

5. Incubate the mixture in the Thermomixer for 15 min at 37°C with mixing at 1,000 rpm.



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6. Transfer the tubes from the Thermomixer into a magnetic rack and leave them for 1 min. Transfer the supernatant into a new 1.5-ml microcentrifuge tube. The supernatant needs to be transferred carefully. Any remaining magnetic beads in the supernatant will have a negative impact on subsequent steps.
7. Precipitate the oligo-depleted, circularized cDNA by adding 6 µl of 5 M sodium chloride, 2 µl GlycoBlue (15 mg/ml) and 250 µl of 100 % ethanol. Proceed as described above (step 4.1-4.3). Resuspend the cDNA in 10 µl sterile water. DNA can be stored indefinitely at −20°C.

## PCR amplification of the cDNA sequencing library

9 1. Prepare PCR mix for four pilot PCR amplification reactions for both the cDNA sample and the oGAB11 control cDNA sample (Table 8). Mix well and store it on ice.
2. For each PCR, put 19 µl of PCR master mix in a 0.2-ml RNase-free PCR tube.
3. Add 1 µl of circularized cDNA and mix it well.
4. Perform PCR pilot amplifications (Table 9). Remove one PCR tube for each sample at the end of the extension step after 6, 8, 10 and 12 cycles.

Composition of PCR amplification reactions.

| Component | Amount for 4 reactions (µl) | Final |
|---|---|---|
| Phusion HF buffer (5x) | 15.2 | 1x |
| dNTPs (10 mM) | 1.5 | 0.2 mM |
| Forward primer (Illumina index primer, 100 µM) | 0.4 | 0.5 µM |
| oNTI231 (reverse primer, 100 µM) | 0.4 | 0.5 µM |
| DNase-free H2O | 57.6 | |
| Phusion DNA polymerase (2 U/µl) | 0.9 | 1.8 U |

Parameters for PCR amplification.

| Cycle number | Denature | Anneal | Extend |
|---|---|---|---|
| 1 | 98°C, 30s | | |
| 2-14 | 98°C, 10 s | 60°C, 10 s | 72°C, 5 s |

5. Add 3.4 µl of 6× DNA loading dye to each tube and mix well.
6. Prepare DNA control ladder. Add 1.0 µl of DNA control ladder to 9 µl of DNase-free $H_2O$. Add 2 µl of 6× DNA loading dye and mix well.
7. Separate the PCR products and the DNA control ladder by TBE gel electrophoresis on an 8% (wt/vol) TBE gel at 180 V for 55 min.
8. Stain the gel in 50 ml of gel staining solution for 5 min at room temperature on a shaker. Protect the gel from light during staining by the use of a black gel box.
9. Visualize the gel under blue/UV light and identify the optimal PCR amplification cycle for each cDNA sequencing library. The NET-seq library runs at ~150 nt. The optimal PCR amplification cycle is characterized by a clear band at ~150 nt and the absence of PCR products at the higher-molecular-weight range.
10. Perform four PCR amplification reactions per sample with the optimal amplification cycle. The oGAB11 control sample is not subjected to amplification. Add 6 µl of 6× DNA loading dye to each sample and mix well. Separate the PCR products by TBE gel electrophoresis on a 4% (wt/vol) low melt agarose gel at 80 V for 2 h.
11. For each sample, excise the band that contains the PCR product from the gel. Excise the broad band at ~150 nt. Avoid contamination from the lower band that runs at ~120 nt, representing PCR product from empty circles. Empty circles are circularized cDNA molecules that arise from unextended RT primers, and hence they do not contain any information about the original nascent RNA.
12. Purify the final NET-seq library using the Nucleospin™ Gel and PCR Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions. Elute in 10 µl of Tris-HCl (10 mM, pH 8.0). The DNA sequencing library can be stored indefinitely at −20°C.



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Quantification and characterization of the NET-seq library

10 1. Prepare a 1:5 dilution of the NET-seq library by adding 1 µl of the NET-seq library to 4 µl of Tris-HCl (10 mM, pH 8.0); mix well.
2. Use 1 µl of the diluted NET-seq library for quantification with the Qubit fluorometer using the Qubit dsDNA HS assay kit. Prepare the sample and perform the measurement according to the manufacturer's protocol.
3. Use 1 µl of the diluted NET-seq library for characterization on the Agilent Bioanalyzer; use the high-sensitivity DNA analysis kit according to the manufacturer's instructions.
4. Sequence the human NET-seq library from the 3' end on the Illumina platform using oLSC006 (Table 2) as a custom sequencing primer.



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