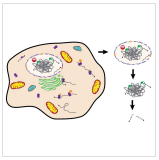

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Profiling metazoan transcription genome-wide with nucleotide resolution using NET-seq (native elongating transcript sequencing)

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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. NET-seq applied to human cells has uncovered regions of Pol II pausing at the boundaries of retained exons and convergent antisense transcription near transcription start sites (Mayer et al. 2015). It has also been used to investigate regulators of productive elongation (Winter et al. 2017), and the directionality of promoter regions (Jin et al. 2017). Here, we describe the experimental protocol for metazoan cells that includes a spike-in control enabling normalization across samples. We also report on an improved bioinformatics pipeline for NET-seq. Together, the protocol yields a fast and non-perturbative method to map Pol II transcription genome-wide, revealing complex and global transcriptional events.

1. Introduction

Transcription regulation has many layers of complexity. Canonically, we understand that transcription is controlled by promoters and distal regulatory regions, or enhancers, that are proposed to be the primary determinant of cell type–specific gene expression. Additionally, transcription elongation and other post-initiation events, such as promoter-proximal pausing, are emerging as crucial regulatory steps in controlling gene expression (Adelman and Lis 2012; Margaritis and Holstege 2008). Non-coding RNA transcripts, such as long intergenic non-coding and antisense RNAs, are involved in gene regulation of specific genes or of larger regions, such as in X chromosome inactivation (Rinn and Chang 2012; Ietswaart, Wu, and Dean 2012).

The deep analysis of steady-state pools of RNA have yielded useful insights into nascent transcription (Boswell et al. 2017), but they fail to capture unstable RNA species, such as enhancer RNAs, and transcriptional pausing. Traditional transcription run-on techniques only observe a few genes at a time. As transcription elongation has emerged as a crucial regulatory step in controlling gene expression, it is critical to directly monitor the elongation process to identify all layers of gene regulation. Native elongating transcript sequencing (NET-seq) is a non-perturbative method which detects actively elongating RNA polymerase II (Pol II) genome-wide --with strand-specific nucleotide resolution, in vivo. This chapter provides a step-by-step protocol for NET-seq and a bioinformatics pipeline for sequence analysis.

The NET-seq protocol begins with purifying nascent RNA, which is done by cellular fractionation in metazoan cells (Figure 1a). Briefly, cells are lysed, and the nuclei are isolated from the cytoplasm by centrifugation through a sucrose cushion buffer. The nuclei are then washed to eliminate any remaining cytoplasmic matter. The chromatin fraction is isolated from the nucleoplasm using urea, salt, and mild detergents. While urea removes most chromatin-bound proteins, it does not remove histones or elongating RNA polymerase from DNA. The RNA polymerase-RNA-DNA ternary complex is stable and capable of withstanding high concentrations of salt, urea, and detergents (Wuarin and Schibler 1994; Cai and Luse 1987). Due to this stability, the isolated chromatin fraction is enriched for nascent RNAs that arise from transcriptionally engaged RNA polymerase complexes. As the histone proteins also remain on DNA after urea treatment, the chromatin fraction can be isolated through low-speed centrifugation. Isolating the chromatin fraction completes the process of purifying the nascent RNA. An advantage of this purification approach is that a restart of transcription in vitro is not required and can therefore isolate nascent RNA from Pol II in multiple transcriptional states. Furthermore, possible biases arising from antisera-based purification of Pol II from epitope masking and cross-reactivity are avoided.

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After the nascent RNA has been purified, the sequencing library preparation can proceed (Figure 1b). This begins with the ligation of a linker to the 3' end of the RNA, which preserves the information on the 3' end of RNAs and allows for nucleotide precision of the location of Pol II. The linker contains a random sequence at the 5' end that serves as a unique molecular identifier (UMI), enabling the bioinformatic detection of multiple library generation biases, including PCR duplicates and reverse transcription mispriming events. After 3' ligation of the linker, the RNA is fragmented and size selected. Fragmenting the RNA helps avoid length biases in any downstream enzymatic reactions. After fragmentation, the RNA is reverse transcribed to create single-stranded cDNA using a primer with a long overhang. The cDNA, containing a 3' adaptor, is then circularized. Sequence elements introduced in the RT primer can then serve as a 5' adaptor to allow for PCR amplification (see Figure 1b). The PCR product is then sequenced using a next generation sequencing platform, typically Illumina. The resulting sequencing reads are aligned, and data analysis is then performed. The NET-seq bioinformatics pipeline for sequence alignment has improved compared to the original pipeline in Mayer et al. 2015; the RT mispriming and PCR duplicate removal scripts now perform a more stringent comparison of read alignment (reducing the number of reads filtered out at this stage) which generates a 2-3 fold higher coverage (scripts are available at Churchman lab GitHub).

NET-seq surveys transcriptional processes and Pol II occupancy with DNA strand specificity and nucleotide resolution. It does not require any genetic modification or any metabolic labeling of nascent RNA. NET-seq also works on a number of different cell types including various human cell lines (Winter et al. 2017; Mayer et al. 2015), as well as mouse and Drosophila cells (see Note 19). It should be noted that RNA processing intermediates and some mature chromatin-associated RNAs may be included in the library, although these can be computationally removed through comparison with gene annotations. Furthermore, NET-seq does not provide information about the positions of pre-initiation complexes and transcription start sites. In sum, NET-seq is a straightforward, easy to use, in vivo, high resolution methodology that captures complex and global transcriptional events by directly monitoring nascent coding, non-coding, antisense, intergenic, and enhancer RNAs.

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KEYWORDS

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

IMAGE ATTRIBUTION

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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 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. To optimize the cellular fractionation for different cell types the cytoplasmic and/or nuclei lysis buffers can be adjusted. The volumes, salt concentration, and/or detergent concentrations can be adjusted in both lysis buffers. In the nuclei lysis buffer, the urea concentration can also be adjusted. Run the western blot control (see Box 1) to determine if the cellular fractionation is working on a specific cell type. A western blot signal of GAPDH found in the chromatin fraction could indicate reduced nuclei lysis efficiency and the nuclei lysis buffer should be optimized by adjusting the above-mentioned components. A western blot signal of Pol II (CTD Ser5-P and/or Ser2-P) found in the cytoplasmic or nucleoplasmic fractions could indicate that the cytoplasmic lysis buffer is too harsh for the specific cell type; the cytoplasmic lysis buffer should be optimized by adjusting the above-mentioned components.
21. It is important to regularly check cell lines to ensure that they are authentic and are not infected with mycoplasma; use sterile equipment and wear gloves to minimize the risk of contamination.
22. Wash cells twice with 10 ml of PBS buffer (pre-chilled on ice). Scrape cells into 1 ml PBS. Count cells (fractionation was optimized for 1×10^7 cells, if fractionating fewer cells see Note 24) then wash cells with 10 ml of PBS (prechilled). Collect cells by centrifugation at 500g for 2 min at 4 °C. Remove supernatant by aspiration (it is critical to remove all supernatant to avoid diluting the lysis buffer). Proceed to cellular fractionation (Steps section 1.3).

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23. Perform a western blot control (described in Box 1) in parallel with the sample, or prior to performing fractionation on the sample to ensure the cellular fractionation protocol is optimized for the specific experimental conditions being used (e.g. cell type, cell number, etc.) and to ensure that the protocol can be performed successfully in your hands. The western blot control is a separate sample from the sample being prepared to make a sequencing library.
24. For samples where cell numbers are limited, cellular fractionation can be performed on as few as 1×10^6 cells. For the cellular fractionation (Steps section 1.3), reduce the volumes of the buffers used by half. It is important to note that though fractionation can be performed on 1×10^6 cells, for consistent results in the construction of the sequencing library, at least 300 ng of chromatin RNA need to be obtained from the fractionation.
25. A spike-in control can be added for normalization. This is recommended when anticipating widespread changes in gene expression profiles between samples. Spike-in control cells are counted and 4% are added to the sample cells that were collected by centrifugation (see Steps section 1.2.1 step 9). The spike-in cells are mixed with the sample cells and the remainder of the protocol is performed on the mixed cells (starting at Steps section 1.2.1 step 11). The Drosophila and human reads can be distinguished bioinformatically (see Steps section 1.5).
26. 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.
27. For more effective layering, tilt the tube at a 45 degree angle and pipette lysate onto the side of the tube.
28. 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).
29. Alternatively, the chromatin pellet can also be solubilized by gentle vortexing.
30. The expected absorbance at (A260/A280) ratio is ~2.0, indicative of highly purified RNA.
31. The original NET-seq protocol (Mayer and Churchman 2016) incubated the ligation reaction for 3 h at 37 °C in a thermal cycler. Our lab has since found that less RNA degradation occurs when the ligation reaction is incubated for 10 - 16 hours at 16 °C.
32. 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.
33. A precipitation protocol can be found in **(Mayer and Churchman 2016)**.
34. 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.
35. Use a needle and syringe to flush out the excess urea that may settle in the gel wells prior to loading samples.
36. 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).
37. 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.
38. The protocol for depletion can be found in **(Mayer and Churchman 2016)**.
39. 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.
40. 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.
41. When starting with 1 µg of RNA (from HeLa S3 cells), the total yield of a typical NET-seq library (determined by Qubit) is usually 10–20 ng. This will vary depending on the amount of RNA used to construct the sequencing library.
42. Compared to our previous alignment pipeline **(Mayer et al. 2015)**, RT mispriming and PCR duplicate removal scripts now perform a more stringent comparison of read alignment to reduce the number of reads filtered out at this stage. Combined with an increased random barcode length of 10nt, this results in a threefold increase in RNA polymerase coverage.
43. It is expected that 40-55% of the reads without barcode are mapped uniquely with STAR. This fraction reduces to only 10-20% for the reads with random barcode, as their sequences mostly do not align to the reference genome.

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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. Protease inhibitor mix (50x): Dissolve one tablet of Protease inhibitor mix cOmplete (EDTA-free, Roche) in 1 ml of chilled RNase/DNase-free H2O. The protease inhibitor mix can be stored in aliquots for up to 1 year at −20 °C (see Note 1 in the Guidelines section re Protease inhibitor mix).
4. Cytoplasmic lysis buffer (CLB): For one reaction, mix the reagents below. Freshly prepare the CLB before use with RNase-free reagents and keep on ice (see Note 1 in the Guidelines section re NP-40).

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A	B	C
Cytoplasmic lysis buffer (CLB)		
Reagents	Volume (µl)	Final concentration
1 M Tris-HCl (pH 7.0)	2.5	10 mM
5 M NaCl	7.5	150 mM
10% (vol/vol) NP-40	3.8	0.15% (vol/vol)
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	224.4	–

5. Sucrose buffer (SB): For one reaction, mix the reagents below. Freshly prepare the SB before use with RNase-free reagents and keep on ice.

A	B	C
Sucrose buffer (SB)		
Reagents	Volume (µl)	Final concentration
1 M Tris-HCl (pH 7.0)	5	10 mM
5 M NaCl	15	150 mM
50% (wt/vol) filter-sterilized sucrose	250	25% (wt/vol)
1mM α-amanitin solution	12.5	25 µM
SUPERase.In (20 U/µl)	1.2	20 U
protease inhibitor mix (50×)	10	1×
RNase-free H2O	206.3	–

6. 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
SUPERase.In (20 U/µl)	2.5	40 U
protease inhibitor mix (50×)	20	1×
PBS	940.5	1×

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.

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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×

1.2 Preparation of cells for fractionation

1.2.1 Washing and counting cells

1. 1×10^7 cells grown according to the supplier's guidelines (if cell numbers are limited, see Note 24 in the Guidelines section).
2. PBS, 1×
3. Tissue culture dish, 15 × 2.5 cm
4. Cell scrapers

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1.3 Cellular Fractionation

1.3.1 Isolation of nuclei fraction

1. CLB, SB, and NWB (prepared as described in section 1.1)
2. RNase/DNase-free microcentrifuge tubes, 1.5 ml
3. P1000 tips with the bottom (~1cm) cut off with a clean razor blade
4. GAPDH antibody (6C5; Thermo Scientific), for WB control (see Note 5 in the Guidelines section)

1.3.2 Isolation of chromatin fraction

1. GB, NLB, and CRS (prepared as described in section 1.1)
2. RNase/DNase-free microcentrifuge tubes, 1.5 ml
3. Pol II CTD Ser2-P antibody (3E10; Active Motif), for WB control (see Note 6 in the Guidelines section)
4. Pol II CTD Ser5-P antibody (3E8; Active Motif), for WB control (see Note 6 in the Guidelines section)
5. Histone 2B antibody (FL-126; Santa Cruz Biotechnology), for WB control (see Note 5 in the Guidelines section)
6. Benzonase nuclease, for WB control (see Note 7 in the Guidelines section)
7. SDS buffer (2×), for WB control: Mix the reagents below. Makes enough buffer to process at least 50 subcellular fractions. Prepare SDS before use and store at room temperature for up to several months (see Note 8 in the Guidelines section re SDS).

A	B	C
SDS buffer (2×)		
Reagents	Volume (ml)	Final concentration
1 M Tris-HCl (pH 7.0)	5	100 mM
20% (wt/vol) SDS	10	4% (wt/vol)
100% (vol/vol) filter-sterilized glycerol	10	20% (vol/vol)
filter-sterilized and deionized H2O	25	–

1.3.3 Preparation of nascent RNA

1. Chloroform, molecular biology grade (see Note 9 in the Guidelines section)
2. miRNeasy Mini Kit (50; Qiagen). For fractionations on small amounts of cells (less than 2×10^6 cells) use the miRNeasy Micro Kit (50; Qiagen) (see Note 10 in the Guidelines section)
3. RNase-free DNase set (50; Qiagen)
4. RNase/DNase-free microcentrifuge tubes, 1.5 ml
5. Needle, 20G (see Note 11 in the Guidelines section)
6. Syringe, 1 ml

1.4 Constructing DNA sequencing library from isolated nascent RNA

1.4.1 Linker ligation

1. T4 RNA ligase 2, truncated (NEB) (see Note 12 in the Guidelines section)
2. Linker (Integrated DNA Technologies), see Table 1 and Note 13
3. oGAB11 (Integrated DNA Technologies), see Table 1
4. Ligation mix: mix the reagents below (for one reaction). Freshly prepare the ligation mix before use (with RNase-free reagents) and keep on ice.

A	B	C	D
Ligation mix			
Reagents	Amount per reaction (ul)		Final concentration
	RNA sample	oGAB11 control	
PEG8000 (50% (vol/vol))	8.0	8.0	20% (vol/vol)
T4 RNA ligase buffer (10×)	2.0	2.0	1×
DMSO	2.0	2.0	10% (vol/vol)
Linker (1 µg)	1.0	1.0	
RNA sample	1.0–6.0*	–	1 µg
oGAB11 (10 µM)	–	1.0	0.5 µM
RNase-free H20	0–5.0*	5.0	
Truncated T4 RNA ligase 2	1.0	1.0	200 U

T4 RNA ligase buffer and PEG8000 are supplied as reagents with T4 RNA ligase 2, truncated. *Final total volume of ligation mix is 20 µl.

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5. EDTA, RNase-free (0.5 M) (see Note 1 in the Guidelines section)
6. RNase/DNase-free PCR tubes, 0.2 ml

1.4.2 RNA fragmentation
1. Alkaline fragmentation solution (2×): For a 5-ml stock solution, mix 0.6 ml of 0.1 M Na2CO3 (sodium carbonate, proteomics grade) and 4.4 ml of 0.1 M NaHCO3 (sodium bicarbonate). Store 500 µl aliquots in air-tight screw-cap tubes at room temperature (22 °C) for up to 4 months (see Note 1 in the Guidelines section re Na2CO3).
2. RNA Clean & Concentrator-5 kit (Zymo) (see Note 14 in the Guidelines section)
3. 10 mM Tris-HCl (pH 7.0)

1.4.3 Size select Fragmented RNA and perform gel extraction
1. TBE buffer, 10×
2. RNA control ladder (the ladder should span from 10 bp to at least 150 bp)
3. TBE-urea (TBU) denaturing sample buffer, 2× (Thermo Scientific)
4. TBE-urea gels, 15% (wt/vol)
5. Gel staining solution (for staining one TBE or TBE-urea gel): Mix 5 µl of SYBR Gold nucleic acid gel stain (10,000× concentrate) and 50 ml of 1× TBE buffer. Prepare this solution immediately before use; keep at room temperature and protected from light (see Note 15 in the Guidelines section).
6. RNase-free H2O
7. RNase/DNase-free microcentrifuge tubes, 0.5 ml
8. RNase/DNase-free microcentrifuge tubes, 1.5 ml
9. Microcentrifuge tube filter: Costar Spin-X centrifuge tube filters (Sigma-Aldrich), for rapid gel extraction
10. Scalpels (see Note 11 in the Guidelines section)
11. Needle, 22G (see Note 11 in the Guidelines section)
12. RNA Clean & Concentrator-5 kit (Zymo) (see Note 14 in the Guidelines section)
13. 10 mM Tris-HCl (pH 7.0)

1.4.4 Reverse Transcription (RT)
1. SuperScript III first-strand synthesis system (Thermo Scientific)
2. oLSC007, RT primer (Integrated DNA Technologies), see Table 1
3. RT reaction mix: mix the reagents below (for one reaction). Freshly prepare the RT reaction mix before use (with RNase-free reagents) and keep on ice.

A	B	C
RT reaction mix		
Reagents	Amount per reaction (µl)	Final concentration
5× First-strand buffer	3.3	1×
dNTPs (10 mM)	0.8	0.5 mM
oLSC007 (10 µM)	0.5	0.3 µM

First-strand buffer is supplied as a reagent with SuperScript III first-strand synthesis system.

4. SUPERase.In/DTT mix: For one reaction (1.3 µl), mix 0.5 µl of SUPERase.In (20 U/µl, Thermo Scientific) and 0.8 µl of 0.1 M DTT (part of the SuperScript III first-strand synthesis system). Freshly prepare the solution before use and store on ice (see Note 4 in the Guidelines section re DTT).

1.4.5 Degradation of RNA after cDNA production
1. NaOH solution (1.0 N) (see Note 16 in the Guidelines section)
2. HCl, hydrochloric acid concentrate (see Note 16 in the Guidelines section)
3. RNase/DNase-free PCR tubes, 0.2 ml

1.4.6 Size select cDNA and gel extraction
1. DNA control ladder (the ladder should span from 50 bp to at least 250 bp)
2. TBE-urea (TBU) denaturing sample buffer, 2× (Thermo Scientific)
3. TBE-urea gels, 10% (wt/vol) (Life Technologies)
4. Gel staining solution (see section 1.4.3 step number 5)
5. Oligo Clean & Concentrator kit (Zymo) (see Note 14 in the Guidelines section)
6. 10 mM Tris-HCl (pH 8.0)
7. RNase-free H2O
8. RNase/DNase-free microcentrifuge tubes, 0.5 ml
9. RNase/DNase-free microcentrifuge tubes, 1.5 ml

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10. Microcentrifuge tube filter: Costar Spin-X centrifuge tube filters (Sigma-Aldrich), for rapid gel extraction
11. Scalpels (see Note 11 in the Guidelines section)
12. Needle, 22G (see Note 11 in the Guidelines section)
13. Mini-Cell polyacrylamide gel box, XCell SureLock

1.4.7 Circularization of cDNA
1. CircLigase ssDNA ligase (100 U/µl; Epicentre)
2. Circularization mix: mix the reagents below (for one reaction). Freshly prepare the circularization mix before use and keep on ice.

A	B	C
Circularization mix		
Reagents	Amount per reaction (µl)	Final concentration
CircLigase 10× reaction buffer	2.0	1×
ATP (1 mM)	1.0	50 µM
MnCl2 (50 mM)	1.0	2.5 µM

ATP, MnCl2 and CircLigase reaction buffer are supplied as reagents with CircLigase ssDNA ligase (see Note 17 in the Guidelines section re MnCl2).

3. RNase/DNase-free PCR tubes, 0.2 ml

1.4.8 PCR amplification
1. Phusion high-fidelity (HF) DNA polymerase (2,000 U/ml; NEB)
2. oNTI231, reverse primer (Integrated DNA Technologies), see Table 1
3. Barcode primer (Integrated DNA Technologies), see Table 1
4. PCR mix: mix the reagents below (for four reactions). Freshly prepare the PCR mix before use and keep on ice.

A	B	C
PCR mix		
Reagents	Amount for 4 reactions (uL)	Final concentration
DNase-free H2O	57.6	
Phusion HF buffer, 5×	15.2	1×
dNTPs (10 mM)	1.5	0.2 mM
Barcode primer (100 µM)	0.4	0.5 µM
oNTI231 (100 µM)	0.4	0.5 µM
Phusion DNA polymerase (2 U/µl)	0.9	1.8 U

Phusion HF buffer is supplied as a reagent with Phusion HF DNA polymerase.

1.4.9 PCR size selection and gel extraction
1. DNA Gel Loading Dye (6x)
2. DNA control ladder (the ladder should span from 50 bp to at least 250 bp)
3. TBE gels, 8% (wt/vol) (Life Technologies)
4. Gel staining solution (see section 1.4.3 step number 5)
5. RNase/DNase-free PCR tubes, 0.2 ml
6. Scalpels (see Note 11 in the Guidelines section)

1.4.10 PCR product cleanup
1. DNA soaking buffer: For one reaction (668 µl total volume), mix 620 µl of RNase-free H2O, 40 µl of 5 M NaCl, 6.7 µl of 1 M Tris-HCl (pH 8.0), and 1.3 µl of 0.5 M EDTA. Freshly prepare the buffer before use and keep it at room temperature (see Note 1 re EDTA).
2. GlycoBlue (15 mg/ml)
3. Isopropanol, molecular biology grade (see Note 18 in the Guidelines section)
4. 80% Ethanol, molecular biology grade (see Note 18 in the Guidelines section)
5. 10 mM Tris-HCl (pH 8.0)
6. RNase/DNase-free microcentrifuge tubes, 0.5 ml

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7. RNase/DNase-free microcentrifuge tubes, 1.5 ml
8. Microcentrifuge tube filter: Costar Spin-X centrifuge tube filters (Sigma-Aldrich), for rapid gel extraction
9. Needle, 22G (see Note 11 in the Guidelines section)

1.4.11 Quantification and characterization of NET-seq library
1. Qubit dsDNA high-sensitivity (HS) assay kit (Life Technologies)
2. High sensitivity DNA analysis kit (Agilent Technologies)
3. Qubit fluorometer
4. Agilent Bioanalyzer
5. oLSC006, sequencing primer (Integrated DNA Technologies), see Table 1

TABLE 1 | DNA and RNA oligos for NET-seq library preparation (5'–3')

A	B
DNA oligo	
Linker	/5rApp/(N10)CTGTAGGCACCATCAAT/3ddC
oLSC007 (RT primer)	/5Phos/ATCTCGTATGCCGTCTTCTGCTTG/iSp18/CACTCA/iSp18/TCCGACGATCATTGATGGTGCCTACAG
oNTI231 (reverse primer)	CAAGCAGAAGACGGCATACGA
Barcode primer (forward primer)	AATGATACGGCGACCACCGAGATCTACACGATCGGAAGAGCACACGTCTGAACTCCAGTCACcttgtaTCCGACGATCATTGATGG
oLSC006 (sequencing primer)	TCCGACGATCATTGATGGTGCCTACAG
RNA oligo	
oGAB11	agucacuuagcgauguacacugacugug

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.

BEFORE STARTING

The following steps are based on the protocol described in (Mayer and Churchman 2016), which contains a general overview and explanation of NET-seq with comparisons to other methodologies. Several improvements have been made to the NET-seq protocol since the published protocol, including an increase in the dynamic range of the NET-seq library (see Note 13 in the Guidelines section), the addition of a spike-in control for normalization that enables analysis of widespread changes in gene expression profiles between samples, streamlined RNA and cDNA purification, and improved sequence alignment generating a 2-3 fold higher coverage compared to the original bioinformatics pipeline.

1.1 Preparation of buffers and working area for cellular fractionation. Prepare all buffers on the day of fractionation before collecting cells.
1h

1 Spray down all worksurfaces, pipettes, etc. with RNase away.

2 Prepare CLB, SB, NWB, GB, NLB, and CRS buffers (see Materials section 1.1 for buffer components) and store all solutions on ice.

3 Make sure tabletop centrifuge is at 4 °C.

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1.2 Preparation of cells for fractionation

4 Cellular fractionation has been successfully performed on several different cell types (see Note 19 in the Guidelines section). The protocol was originally optimized for HeLa S3 or HEK293T cells (Mayer and Churchman 2016). When fractionating other cell types optimization may be required (see Note 20). Cells are ideally grown according to internationally recognized guidelines (such as those suggested by ENCODE) and handled according to the supplier's instructions (see Note 21 in the Guidelines section).

1.2.1 Washing and counting cells 15m

5 The following steps are for suspension cells, if working with adherent cells see Note 22 in the Guidelines section for steps on how to prepare the cells.

6 Count out 1×10^7 cells as input for each fractionation, including a western blot control sample (see Note 23 in the Guidelines section). If cell numbers are limited, fractionation can be performed on fewer cells (see Note 24 in the Guidelines section).

7 Collect cells by centrifugation at 500g for 2 min at room temperature.

8 Wash with 10 ml of PBS (room temperature).

9 Collect cells by centrifugation at 500g for 2 min at room temperature.

10 Add spike-in cells (Drosophila S2, used for normalization) to the sample cells collected by centrifugation (the addition of a spike-in is optional, see Note 25 in the Guidelines section).

11 Repeat wash (step 8) and centrifugation (step 9).

12 Remove supernatant by aspiration. It is important to remove the supernatant completely to avoid diluting the lysis buffer (see Note 26 in the Guidelines section).

1.3 Cellular Fractionation (Figure 1a) 1h 45m

Citation: Danya J Martell, Robert Ietswaart, Brendan M. Smalec, Stirling Churchman (06/23/2021). Profiling metazoan transcription genome-wide with nucleotide resolution using NET-seq (native elongating transcript sequencing). https://dx.doi.org/10.17504/protocols.io.bpymmpu6

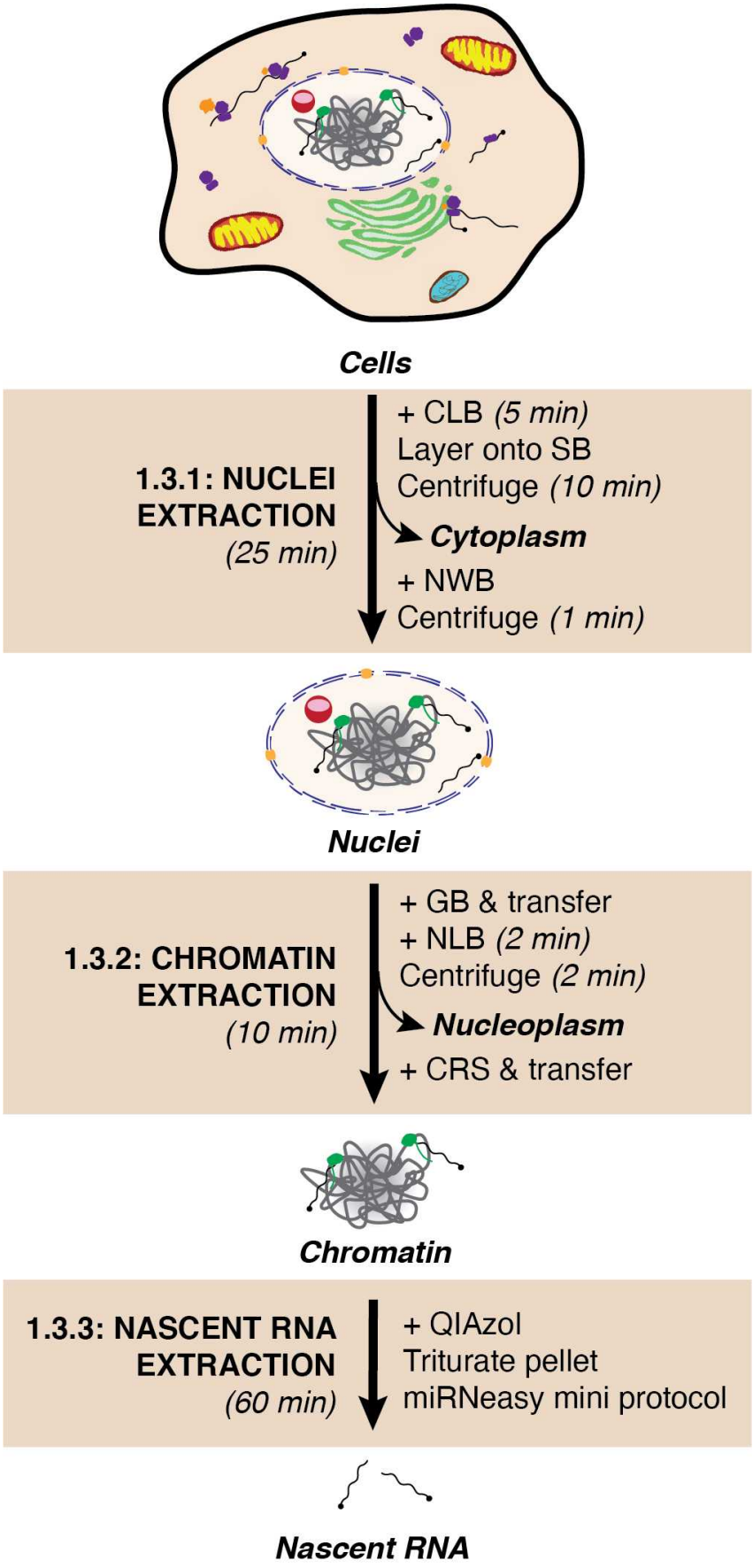

Figure 1| (a) Schematic overview of the cellular fractionation protocol (1.3). CLB, cytoplasmic lysis buffer; SB, sucrose buffer; NWB, nuclei wash buffer; GB, glycerol buffer; NLB, nuclei lysis buffer; CRS, cytoplasmic resuspension solution.

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nuclei wash buffer, GB, glycerol buffer, NLB, nuclei lysis buffer, CRS, cytoplasmic resuspension solution.

1.3.1 Isolation of nuclei fraction

14 Using a cut P1000 pipette tip, add 200 µl of CLB to cells and mix by pipetting the sample up and down ten times. Transfer sample to an RNase-free 1.5 ml microcentrifuge tube using a cut 1,000 µl pipette tip.

15 Incubate the cell lysate on ice for 5 min.

16 During incubation period, pipette 500 µl of SB to an RNase-free 1.5 ml microcentrifuge tube.

17 After incubation period, use a cut 1,000 µl pipette tip to layer the cell lysate sample onto the SB (see Note 27 in the Guidelines section).

18 Centrifuge layered lysate at 16,000g for 10 min at 4 °C to collect nuclei.

19 Pipette off supernatant completely from the nuclei pellet (supernatant contains the cytoplasmic fraction, retain for western blot, described in Box 1)

20 Wash nuclei pellet with 800 µl of NWB (either by inverting the tube or pipetting up and down using a cut pipette tip).

21 Collect cell nuclei by centrifugation at 1,150g for 1 min at 4 °C.

22 Pipette off supernatant from the nuclei pellet. It is important to remove the supernatant completely in order to remove cytoplasmic mature RNAs (see Note 26 in the Guidelines section).

1.3.2 Isolation of chromatin fraction

23 Add 200 µl of GB to the nuclei. Use a cut 1,000 µl tip to resuspend the washed nuclei pellet by pipetting up and down.

24 Transfer the nuclei suspension to a new 1.5 ml RNase-free microcentrifuge tube.

25 Layer 200 µl of NLB onto nuclei suspension and briefly mix by pulsed vortexing.

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26 Incubate the mixture on ice for 2 min.

27 Centrifuge at 18,500g for 2 min at 4 °C to collect the chromatin pellet.

28 Pipette off supernatant from the chromatin pellet. It is important to completely remove the supernatant in order to remove nucleoplasmic RNAs (retain supernatant for the nucleoplasm fraction of western blot, described in Box 1).

29 Add 50 µl CRS to the chromatin pellet, transfer pellet in CRS to a new 1.5 ml tube and then resuspend pellet in CRS (see Note 28 in the Guidelines section). Perform this step on the experimental sample (for the preparation of nascent RNA, section 1.3.3) and the control sample (for the western blot analysis, as described in Box 1).

1.3.3 Preparation of nascent RNA

30 Add 700 µl of QIAzol (lysis reagent in miRNeasy mini kit, Qiagen) to the resuspended chromatin (see Note 10 in the Guidelines section re QIAzol).

31 Solubilize chromatin pellet by slowly pipetting up and down using a 1-ml syringe with a 22G needle (see Note 29 in the Guidelines section). Mix very carefully (to avoid spilling the sample) until the solution is homogeneous (at this point the sample can be stored at −80 °C for months).

32 Prepare RNA using the miRNeasy mini kit (or miRNeasy micro kit for small cell numbers, see Materials section 1.3.3, Preparation of nascent RNA, step 2) according to the manufacturer's instructions, including optional DNase treatment.

33 Measure the prepared RNA quantity and quality using a NanoDrop (see Note 30 in the Guidelines section). The RNA yield is usually in the range of 20–30 µg (this can vary quite a lot depending on the type of cells used). If fractionation was performed on less than 1×10^7 cells (or RNA yield is less than 20 µg) see Note 24 in the Guidelines section re RNA amount needed to construct sequencing library. Store the isolated RNA at −80 °C (can be stored for months) until ready to construct sequencing library.

1.4 Constructing DNA sequencing library from isolated nascent RNA (Figure 1b)

Citation: Danya J Martell, Robert Ietswaart, Brendan M. Smalec, Stirling Churchman (06/23/2021). Profiling metazoan transcription genome-wide with nucleotide resolution using NET-seq (native elongating transcript sequencing). https://dx.doi.org/10.17504/protocols.io.bpymmpu6

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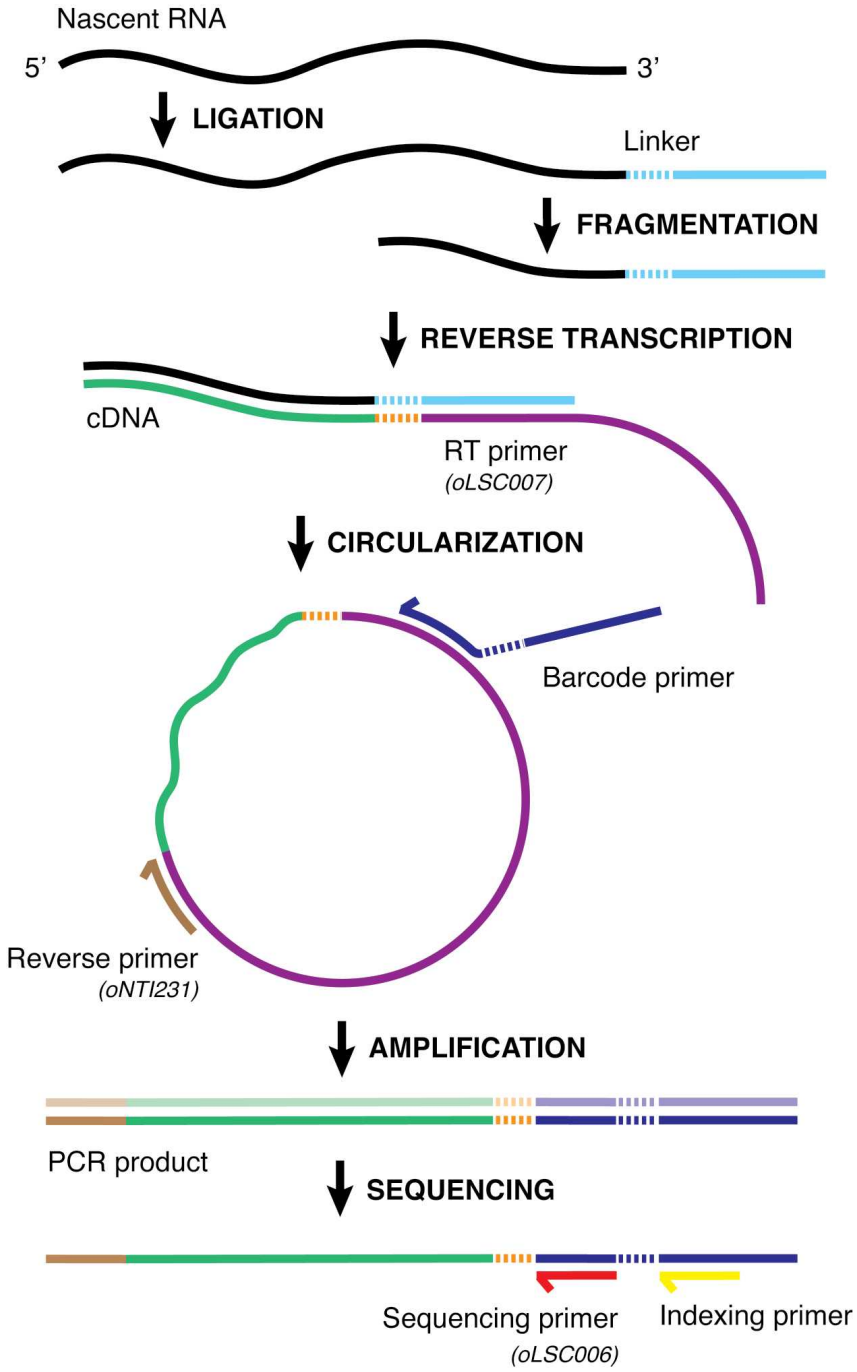

Figure 1 | (b) Schematic overview of the DNA sequencing library preparation (1.4).

1.4.1 Linker ligation (Figure 2) 12h

35 Spray down all work surfaces, pipettes, etc. with RNase away

36 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.

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37 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.

38 Add 1 µl Truncated T4 RNA ligase 2 (200U) to each RNA sample as well as the oGAB11 control.

39 Incubate the ligation samples overnight for 10 - 16 hours at 16 °C in a thermal cycler (Note 31 in the Guidelines section).

40 Place on ice and add 0.7 µl of EDTA (0.5 M) to each ligation sample to stop the ligation reaction.

41

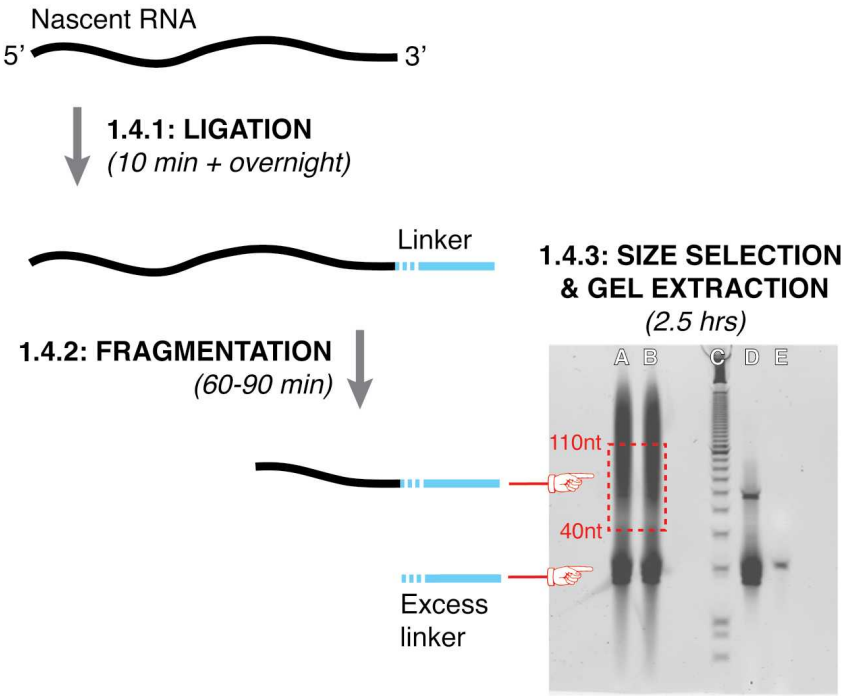

Figure 2| RNA ligation, fragmentation and size selection. RNA is ligated to the Linker overnight and then fragmented for an optimal period of time (Box 2). The fragmented RNA (lanes A and B) is separated on a 15% (wt/vol) TBE-urea gel and excised from the gel between 35 to 110 nt (excised area represented by the red dotted box). Lane C, DNA ladder; lane D, ligated oGAB11 control; lane E, un-ligated oGAB11. The RNA was obtained from K562 cells and ~1 µg of RNA was used per fragmentation reaction.

1.4.2 RNA fragmentation (Figure 2) 2h 30m

42 Perform fragmentation calibration test (see Box 2)

43 Add 20 µl of 2× alkaline fragmentation solution (see Materials section 1.4.2, RNA fragmentation, step 1) to each sample (from the ligation reaction) and mix. Do not perform fragmentation steps for the oGAB11 ligation control.

44 Incubate the sample at 95 °C in a thermal cycler for the calibrated time (see Box 2) to fragment RNA (see Note 32 in the Guidelines section).

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45 To purify the fragmented RNA, use the RNA Clean & Concentrator-5 kit (Zymo) and elute RNA from column with 10 uL of 10 mM Tris-HCl (pH 7.0). Alternatively, RNA can be prepared by RNA precipitation (see Note 33 in the Guidelines section).

1.4.3 Size select Fragmented RNA and perform gel extraction (Figure 2) 2h

46 Prepare RNA ladder and un-ligated oGAB11 control by adding 1.0 µl of RNA control ladder or oGAB11 to 9 µl of RNase-free H2O. Add 10 µl of 2× TBU denaturing sample to the RNA ladder and oGAB11 control, mix well (see Note 34 in the Guidelines section).

47 Prepare all RNA samples (including the ligated oGAB11 control) by adding 10 µl of 2× TBU denaturing sample buffer to each sample, mix well.

48 Denature the RNA sample, RNA control samples (ligated oGAB11 and un-ligated oGAB11 controls), and RNA ladder for 2 min at 80 °C. Place the samples on ice.

49 Pre-run a 15% (wt/vol) polyacrylamide TBE-urea gel at 200 V for 15 min in 1× TBE. Thoroughly flush out gel wells prior to pre-running the gel (see Note 35 in the Guidelines section).

50 Load gel with the RNA ladder, RNA control samples (ligated oGAB11 and un-ligated oGAB11), and the fragmented RNA sample. Separate by PAGE at 200 V for 65 min.

51 Stain the gel in 50 ml of gel staining solution for 5 min at room temperature on a shaker (use a black gel box to protect gel from light while staining).

52 Visualize the gel under blue/UV light.

53 For the fragmented RNA sample, excise region between 40 and 110 nt. For the ligated oGAB11, excise a narrow band ~55 nt (see Note 36 in the Guidelines section).

54 Perform rapid gel extraction on the excised gel (see Box 3).

55 After rapid gel extraction is performed, use the RNA Clean & Concentrator-5 kit (Zymo) and elute RNA from column with 10 uL of 10 mM Tris-HCl (pH 7.0) (at this point, RNA can be stored up to 3 months at -80 °C). Alternatively, RNA can be prepared by RNA precipitation (see Note 33 in the Guidelines section).

1.4.4 Reverse transcription (Figure 3) 1h

Citation: Danya J Martell, Robert Ietswaart, Brendan M. Smalec, Stirling Churchman (06/23/2021). Profiling metazoan transcription genome-wide with nucleotide resolution using NET-seq (native elongating transcript sequencing). https://dx.doi.org/10.17504/protocols.io.bpymmpu6

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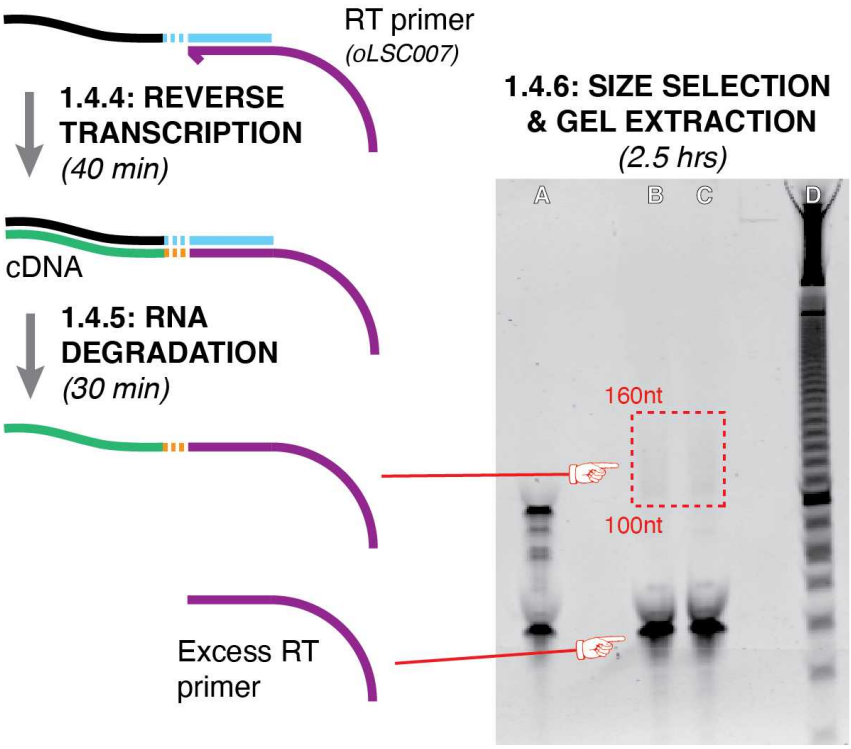

Figure 3| Reverse transcription of RNA to cDNA, RNA degradation, and size selection of the cDNA.The RT product (lanes B and C) is separated on two lanes of a 10% (wt/vol) TBE-urea gel. The red dotted box shows the region where the cDNA is excised from the gel (in the range of 100–160 nt). Lane A, oGAB11 control; lane D, DNA ladder.

57 Prepare RT reaction mix in a 0.2-ml RNase-free PCR tube (see Materials section 1.4.4**,** Reverse Transcription, step 3), store on ice.

58 Add 4.6 µl of RT reaction mix to 10 µl of the RNA sample and the oGAB11 control sample.

59 Denature the sample mixtures (including the oGAB11 control) by incubating for 2 min at 80 °C in a thermal cycler. Place on ice.

60 Add 1.3 µl of SUPERase.In/DTT mix (see Materials section 1.4.4**,** Reverse Transcription, step 4) and mix well.

61 Add 0.8 µl of SuperScript III (200 U/µl) and mix.

62 Incubate for 30 min at 48 °C in a thermal cycler.

1.4.5 Degradation of RNA after cDNA production (Figure 3) 30m

63 Add 1.8 µl of 1 N NaOH, mix well and incubate 20 min at 98 °C. This step degrades the RNA after cDNA was produced

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by RT.

64 Neutralize the reaction by adding 1.8 µl of 1 N HCl; mix well and place on ice.

1.4.6 Size select cDNA and gel extraction (Figure 3) 2h

65 Prepare the DNA control ladder. Add 1.0 µl of DNA control ladder to 9 µl of RNase-free H2O. Add 10 µl of 2× TBU denaturing sample buffer and mix.

66 Add 20 µl of 2× TBU denaturing sample buffer to the cDNA sample and the oGAB11 cDNA control, mix well.

67 Denature the cDNA sample, oGAB11 cDNA control and DNA control ladder for 3 min at 95 °C in a Thermomixer. Place the samples on ice.

68 Prerun a 10% (wt/vol) polyacrylamide TBE-urea gel at 200 V for 15 min in 1× TBE. Thoroughly flush out gel wells prior to pre-running the gel (see Note 35 in the Guidelines section).

69 Load gel (20 uL per lane, each sample will be split into two lanes) with the cDNA sample, the oGAB11 cDNA control and the DNA ladder. Separate by PAGE at 200 V for 65 min.

70 Stain the gel in 50 ml of gel staining solution for 5 min at room temperature on a shaker (use a black gel box to protect gel from light while staining).

71 Visualize the gel under blue/UV light.

72 For the cDNA sample, excise region between ~85 and 160 nt (use oGAB11 as baseline and cut up ~70 nt). For the cDNA oGAB11 control, excise a narrow band ~85 nt. If no cDNA smear is observed see Note 37 in the Guidelines section.

73 Perform rapid gel extraction on the excised gel (see Box 3). There will be two gels for one sample since each sample was split into two lanes, combine the two gels from one sample into a single tube.

74 After rapid gel extraction is performed, use the Oligo kit (Zymo) and elute cDNA from column with 15 uL of 10 mM Tris-HCl (pH 8.0) (at this point, the cDNA can be stored indefinitely at −20 °C). Alternatively, cDNA can be prepared by precipitation (see Note 33 in the Guidelines section).

1.4.7 cDNA Circularization (Figure 4) 1h 30m

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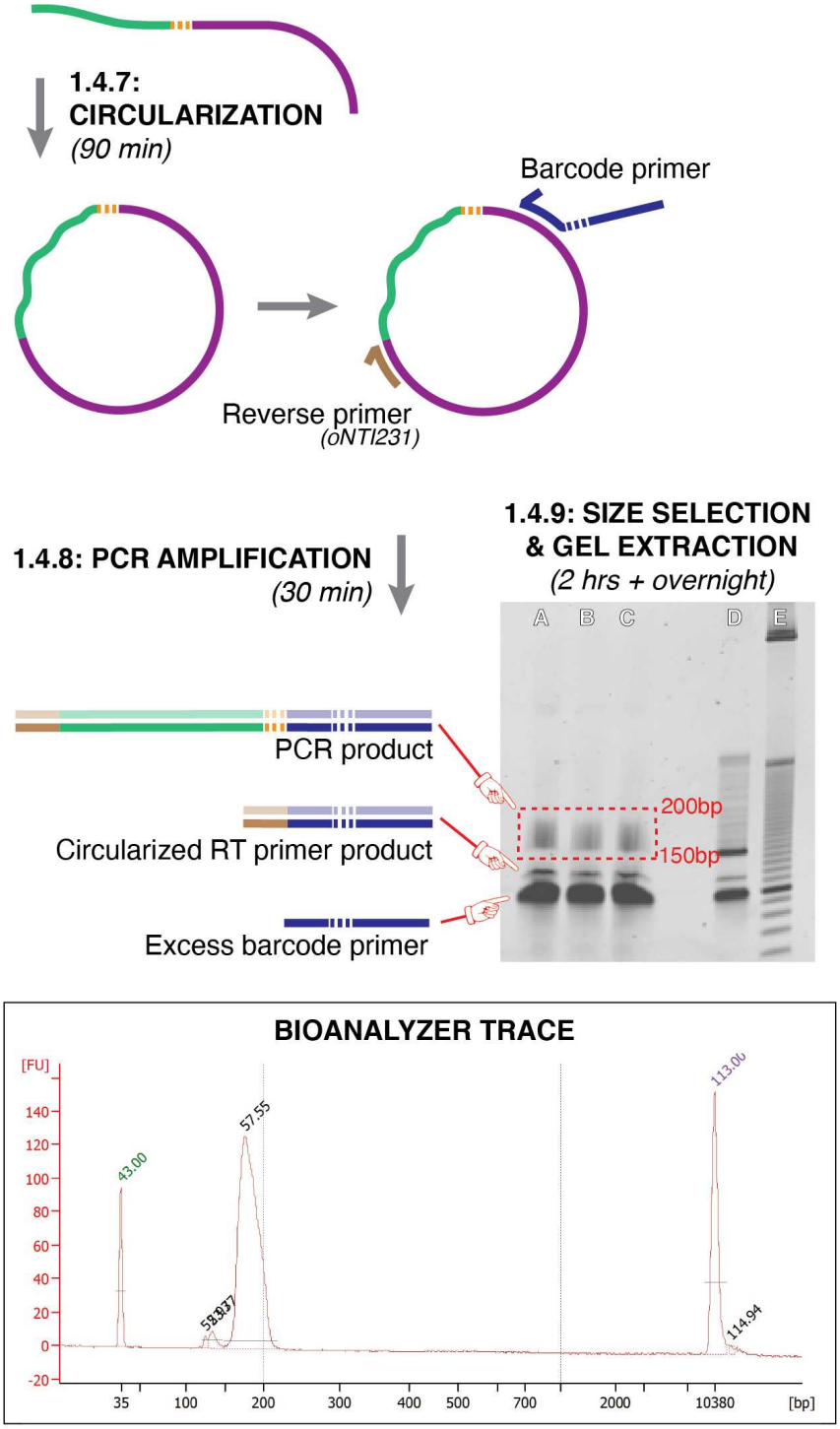

Figure 4| Circularization followed by PCR amplification of cDNA. Size selection and quantification (bioanalyzer trace) of the PCR product. The PCR product (lanes A, B, and C) is separated on an 8% (wt/vol) TBE gel and excised from the gel between 150 to 200 nt (excised area represented by the red dotted box). The PCR product that arises from circularized RT primer and the excess barcode primer are labeled on the gel image. Lane D, oGAB11 control; lane E, DNA ladder. The Bioanalyzer trace shows a human NET-seq library generated from 500 ng of RNA. The trace shows a major peak around 150 nt and a smaller peak around 120 nt (corresponding to the circularized RT primer product). The sequencing reads that result from this smaller peak are computationally removed. The peaks at 35 and 10380 nt are the manufacturer's internal markers. FU, fluorescence units.

76 Prepare circularization mix (see Materials section 1.4.7, Circularization of cDNA, step 2) and store it on ice.

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77 In a 0.2-ml RNase-free PCR tube, add 4 µl of circularization mix to 15 µl of the cDNA sample and the oGAB11 cDNA control sample, mix well.

78 Add 1 µl of CircLigase (100 U/µl) and mix.

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

80 Optional: at this point a specific depletion of highly abundant mature RNAs can be performed (see Note 38 in the Guidelines section)

1.4.8 PCR amplification (Figure 4) 1h

81 Perform PCR amplification test (Box 4)

82 Prepare PCR mix for four PCR amplification reactions for both the cDNA sample and the oGAB11 control cDNA sample (see Materials section 1.4.8**,** PCR amplification, step 4). Mix well and store on ice.

83 For each PCR, put 19 µl of PCR master mix in a 0.2-ml RNase-free PCR tube.

84 Add 1 µl of circularized cDNA and mix it well.

85 Perform PCR amplifications (as shown in Table 2) with determined optimal number of PCR cycles (see Box 4).

TABLE 2 | PCR amplification

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

1.4.9 PCR size selection and gel extraction (Figure 4) 2h

86 Prepare DNA control ladder. Add 1.0 µl of DNA control ladder to 9 µl of DNase-free H2O. Add 2 µl of 6× DNA loading dye and mix well.

87 Add 3.4 µl of 6× DNA loading dye to each PCR sample tube and mix well.

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88 Load gel with the PCR products and DNA control ladder. Separate by TBE gel electrophoresis on an 8% (wt/vol) TBE gel at 180 V for 55 min.

89 Stain the gel in 50 ml of gel staining solution for 5 min at room temperature on a shaker (use a black gel box to protect gel from light while staining).

90 Visualize the gel under blue/UV light.

91 For each sample, excise the band from the gel that contains the PCR product. The NET-seq library is a broad band that runs at ~150 nt (use oGAB as baseline and cut up ~50 nt). Avoid contamination from the lower band that runs at ~120 nt, representing PCR product from empty circles (see Note 39). The oGAB11 control sample is not subjected to this step, or any further steps.

1.4.10 PCR product cleanup 16h

92 Perform the first four steps of rapid gel extraction (see Box 3)

93 After centrifugation, add 670 µl of DNA soaking buffer and mix.

94 Incubate the samples overnight at room temperature in a Thermomixer (set at 1,500 r.p.m.).

95 Use a cut 1,000 µl pipette tip to transfer the gel slurry into a microcentrifuge tube filter.

96 Centrifuge at 20,000g for 3 min at room temperature. Transfer the eluate to a new 1.5 ml microcentrifuge tube.

97 Precipitate the NET-seq library by adding 2 µl of 15 mg/ml GlycoBlue and 680 µl of isopropanol. Mix well then incubate the precipitations at −20 °C for ≥1 h (the DNA precipitations can be stored at −20 °C overnight).

98 Centrifuge at 20,000g for 30 min at 4 °C to pellet the RNA.

99 Remove the supernatant and wash the pellet with 750 µl of 80% (vol/vol) ice-cold ethanol.

100 Spin the sample at 20,000g for 2 min at 4 °C, and then remove the supernatant. Air-dry the RNA pellet for 10 min at room temperature.

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101 Resuspend the NET-seq library in 7 - 10 µl of Tris-HCl (10 mM, pH 8.0). The DNA sequencing library can be stored indefinitely at −20 °C.

1.4.11 Quantification and characterization of NET-seq library (Figure 4) 1h

102 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 (see Note 40 in the Guidelines section).

103 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 (see Note 41 in the Guidelines section).

104 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.

105 Sequence using oLSC006. Sequence the human NET-seq library from the 3' end (75 bp single-end sequencing) on the Illumina platform using oLSC006 (Table 1 in the Materials section) as a custom sequencing primer. Perform sequencing according to the manufacturer's instructions. NET-seq libraries are typically sequenced on MiSeq, HiSeq or NextSeq next-generation sequencing platforms. A reasonable coverage is obtained with 100–200 million reads.

1.5 Bioinformatics analysis (Figure 5)

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106

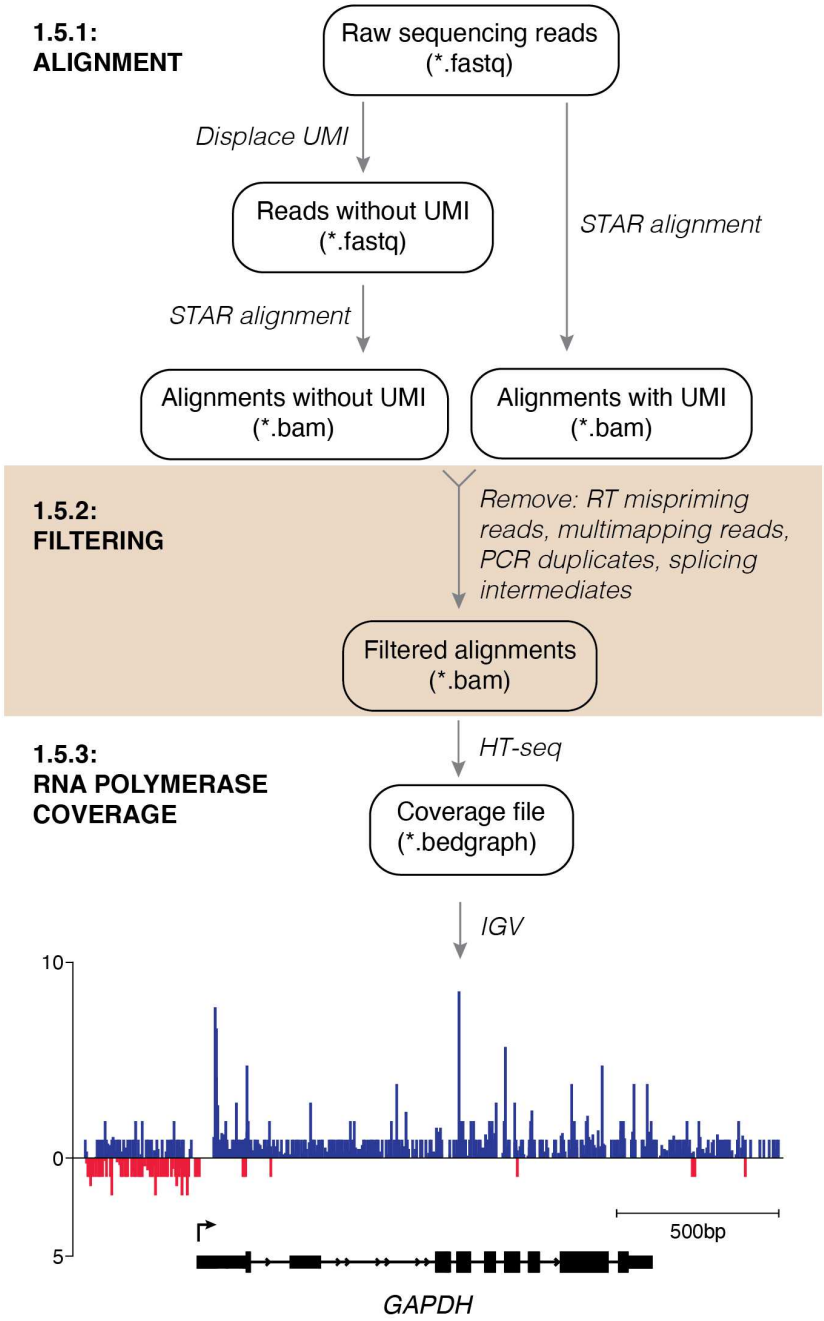

Figure 5| Schematic overview of the bioinformatics pipeline and DNA strand–specific RNA polymerase II coverage at a representative gene, GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). The Pol II density for HeLa S3 cells (Mayer et al. 2015) at the positive (+) and negative (−) DNA strand is shown in blue and red, respectively. The transcription start site as well as the direction of transcription are marked by a black arrow; and exonic regions are portrayed as blue boxes while intronic regions are portrayed as blue lines. Total number of raw reads in library: 169953553. Number of alignments after filtering steps: 37398108 (22% of total library).

107 Below we describe in brief the custom bioinformatics pipeline (see Figure 5) that is used to generate NET-seq coverage files. The scripts are available at Churchman lab GitHub . See Note 42 in the Guidelines section for a description of the improvements relative to our previous pipeline (Mayer et al. 2015).

1.5.1 Alignment of sequencing reads

108 Assess read quality control with FastQC.

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109 Displace 5’ end random barcode sequence from sequence to read identifier line and save as a separate fastq file. This barcode serves as a unique molecular identifier (UMI) and originates from the linker (see Table 1 in the Materials section).

110 Align fastq files with and without UMI sequences separately to a human reference genome using STAR (See Note 43 in the Guidelines section). In case a Drosophila spike-in was used, concatenate the human and Drosophila fasta files to generate a combined reference genome.

1.5.2 Filter out read alignments that do not reflect RNA polymerase position.

111 Remove alignments resulting from RT mispriming events, i.e. cases where RT priming did not occur at the complementary sequence site on the ligated linker but instead at a position within the RNA fragment. The resulting reads can be identified as they lack the random UMI sequence encoded on the linker and therefore align well to the reference genome both with and without UMI displacement: when the STAR alignment score of a read with the UMI sequence equals the UMI length (10nt) plus the alignment score from that read without UMI.

112 Remove alignments that map to multiple genomic locations by filtering for their MAPQ value (< 50) using samtools.

113 Remove alignments resulting from PCR duplication events, i.e. cases of multiple amplicons that originate from the same RNA fragment. The resulting multiple reads are bioinformatically indistinguishable as their alignments share the same position, strand, chromosome, UMI sequence and CIGAR score. To filter out these PCR duplicates, we maintain only one of the alignments.

114 Remove alignments that could arise from splicing intermediates, i.e. 3’ ends of annotated exons and introns.

1.5.3 Determine RNA polymerase coverage genome-wide from read alignments.

115 Use HTseq to generate a coverage (bedgraph) file containing the read counts at a base-pair resolution over the reference genome as determined from the 5’ end of the read alignments (without UMI) on the opposite strand. The resulting positions correspond to the 3’ ends of nascent RNA and thereby map RNA polymerase positions. The coverage files will be used for a variety of downstream bioinformatics analyses such as visual inspection (e.g. using IGV), metagene profiling (e.g. Bedtools), differential expression (e.g. DEseq2).

Box 1 | Western blot control: checking cell fractionation

116 The western blot control is used to determine whether or not the cellular fractionation protocol has been executed successfully. The efficiency of the fractionation can be quantified by measuring the amount of elongating Pol II captured in the chromatin fraction (ideally ≥95%). When the fractionation protocol is being performed for the first time or being tested on a new cell type, the cytoplasm, nucleoplasm, and chromatin fractions should be checked for subcellular marker proteins (see Note 5 in the Guidelines section) to confirm the fractionation is working (see Note 20 in the Guidelines section).

117 Adjust the nucleoplasmic (from steps 1.3.2) and chromatin fraction (from steps 1.3.2) volumes to the cytoplasmic fraction (from steps 1.3.1) volume by adding 1× PBS. Mix each fraction well and ensure the chromatin pellet is completely resuspended (see Note 7 in the Guidelines section).

118 Add 50 µl of 2× SDS buffer (see Materials section 1.3.2, Isolation of chromatin fraction, step 7) to 50 µl of each subcellular fraction and boil the samples at 95 °C for 5 min (at this point, samples can be stored for months at −20 °C).

119 Separate samples by standard SDS-PAGE (loading 10 µl of each boiled sample per lane is usually sufficient).

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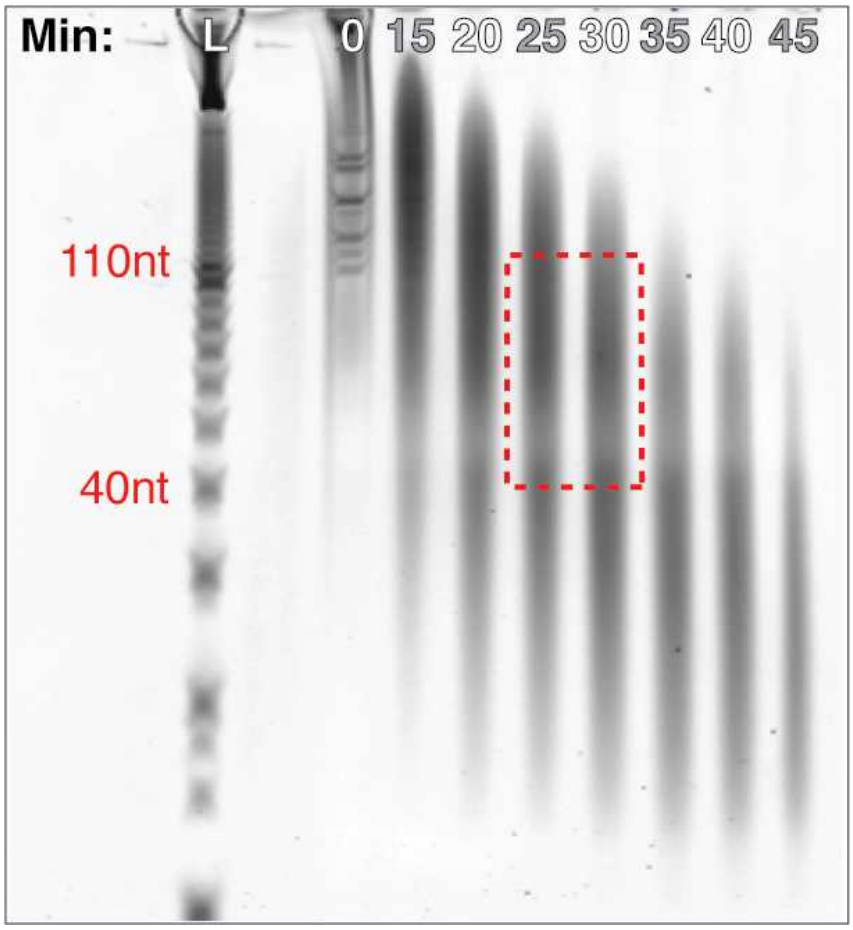
120 Probe the membrane overnight at 4 °C with Pol II CTD Ser2-P antibody (3E10, 1:1,000 dilution) and Pol II CTD Ser5-P antibody (3E8, 1:1,000 dilution). These antibodies target transcribing RNA Pol II (see Note 6 in the Guidelines section).

Box 2 | Fragmentation test

121



122 Performing RNA fragmentation results in a homogeneous RNA pool which helps to avoid length biases in any downstream enzymatic reactions. The fragmentation test can be performed using total RNA (or any easily-obtained RNA sample). Once the appropriate fragmentation time is determined (where most of the RNA molecules range from 40 –110 nt), fragmentation can be performed on the nascent RNA (obtained from cellular fractionation) using the determined time (see directly above); the size range of the nascent RNA and total RNA pools do not vary enough for the fragmentation time to be different.

123 Take 9 samples of RNA (~1ug each) from a total RNA sample (or any other easily obtained RNA sample). Re-suspend each RNA sample in 8uL of RNase-free water and add 12uL of the linker ligation mix, excluding the linker and ligase (see Materials section **1.4.1,** Linker ligation, step 4). Add 20 µl of 2× alkaline fragmentation solution (see Materials section 1.4.2**,** RNA fragmentation, step 1) to each sample and mix well (see Note 32 in the Guidelines section); fragmentation is not performed for the oGAB11 control.

124 Fragment each RNA sample at 95 °C in a thermal cycler for the following times: 0, 15, 20, 25, 30, 35, 40, 45, and 50 minutes.

To purify the fragmented RNA, use the RNA Clean & Concentrator-5 kit (Zymo) and elute RNA from column with 10 uL

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125 of 10 mM Tris-HCl (pH 7.0). Alternatively, RNA can be prepared by RNA precipitation (see Note 33 in the Guidelines section).

126 Prepare RNA ladder (denoted as L in figure) by adding 1.0 µl of RNA control ladder to 9 µl of RNase-free H2O. Add 10 µl of 2× TBU denaturing sample to the RNA ladder and mix well.

127 Prepare all RNA samples by adding 10 µl of 2× TBU denaturing sample buffer to each sample, mix well.

128 Denature the RNA sample and RNA ladder for 2 min at 80 °C. Place the samples on ice.

129 Pre-run a 15% (wt/vol) polyacrylamide TBE-urea gel at 200 V for 15 min in 1× TBE. Thoroughly flush out gel wells prior to pre-running the gel (see Note 35 in the Guidelines section).

130 Load gel with the RNA ladder and the fragmented RNA sample. Separate by PAGE at 200 V for 65 min.

131 Stain the gel in 50 ml of gel staining solution for 5 min at room temperature on a shaker (use a black gel box to protect gel from light while staining).

132 Visualize the gel under blue/UV light. The optimal fragmentation time is when most RNA molecules are in the required size range, between 40 and 110 nt (the optimal fragmentation time in the figure below is between 25 and 30 minutes).

Box 3 | Rapid gel extraction

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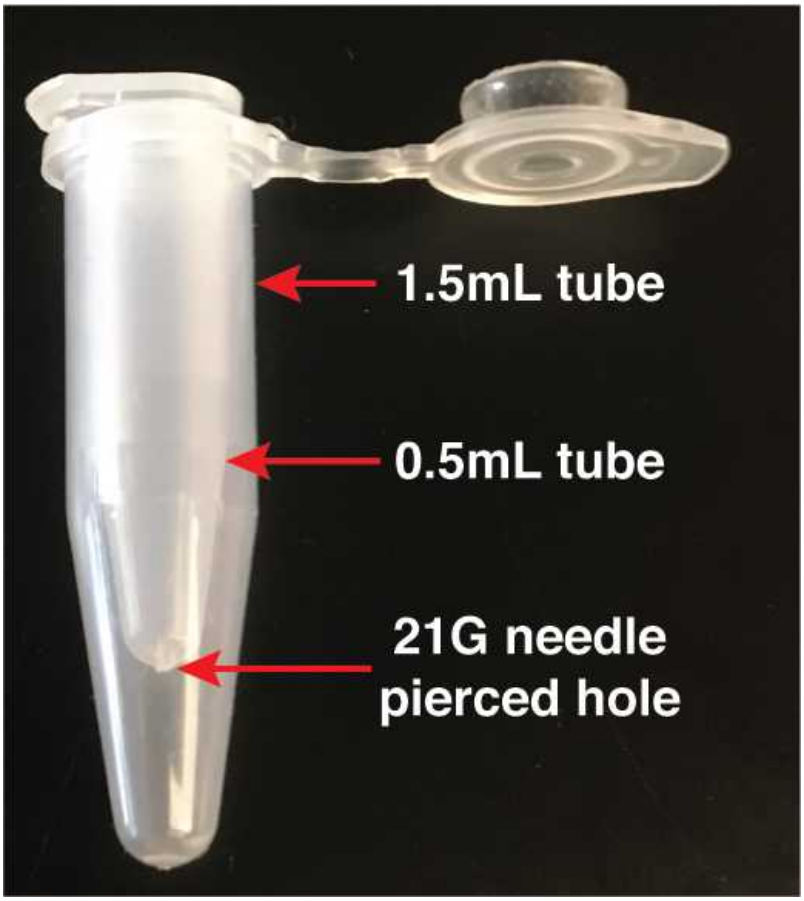
133 

134 This protocol for rapidly extracting RNA or cDNA from a polyacrylamide TBE-urea gel is similar to the gel extraction protocol described by the Weissman laboratory (Ingolia et al. 2012; Churchman and Weissman 2012).

135 Use a 21G needle to pierce the bottom of a 0.5-ml RNase-free microcentrifuge tube.

136 Place the pierced 0.5-ml tube into a 1.5-ml RNase-free microcentrifuge tube.

137 Put the gel slice (from fragmentation, RT, or PCR) into the pierced 0.5-ml tube.

138 Centrifuge the nestled tubes, containing the gel slice, at 20,000g for 4 min at room temperature.

139 Add 200 µl of RNase-free H2O to the chopped-up gel and mix.

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140 Incubate the sample mixture for 10 min at 70 °C in a Thermomixer.

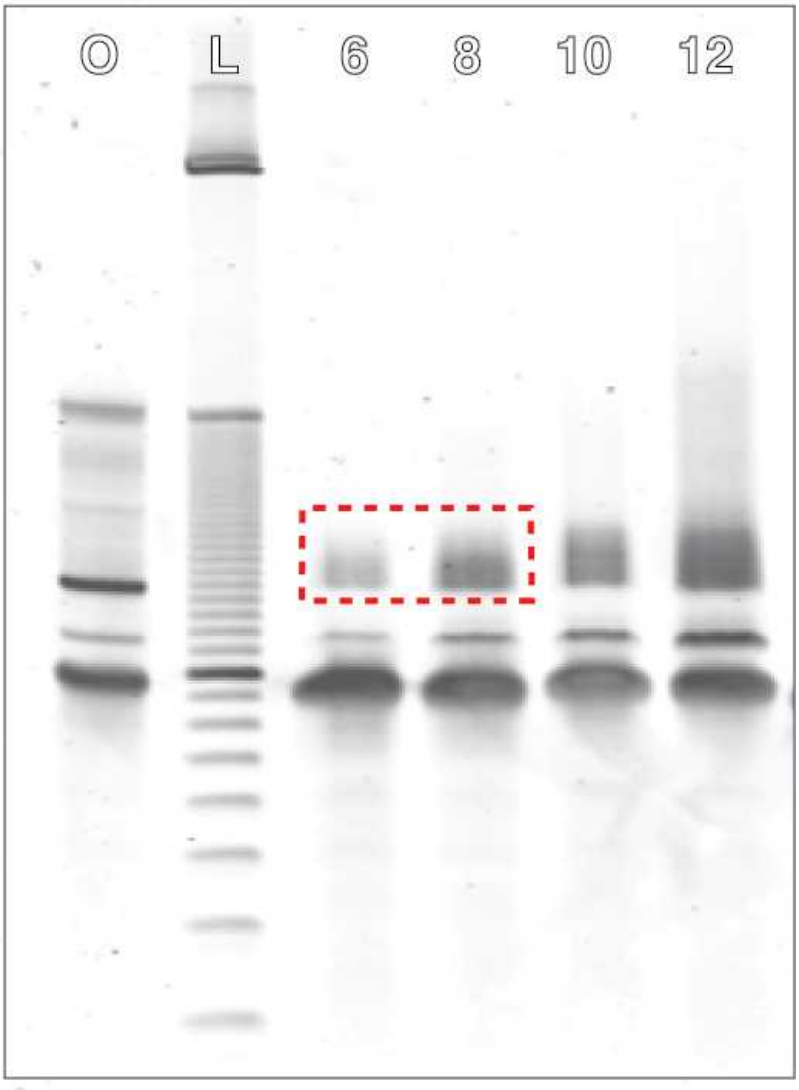
141 Vortex the mixture for 30 s at medium intensity.

142 Cut the tip off of a 1,000-µl pipette tip and transfer the gel mixture into a microcentrifuge tube filter.

143 Centrifuge the mixture at 20,000g for 3 min at room temperature.

Box 4 | PCR amplification test

144



145 The NET-seq library is PCR amplified using an Illumina index forward primer (Barcode primer, see Table 1 in the

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Materials section) and a reverse primer (oNTI231; Table 1 in the Materials section) specific to NET-seq (Figure 4). For each NET-seq library preparation, the minimum number of PCR cycles needed to obtain a sequencing library must to be determined. The NET-seq library is then amplified with the determined minimal amount of PCR cycles; this avoids overamplification which can result in unwanted products such as PCR duplicates.

146 Prepare the PCR master mix (see Materials section 1.4.8, PCR amplification, step 4) for four pilot PCR amplifications for each cDNA sample and the oGAB11 cDNA control sample (denoted as O in figure). Mix well and store it on ice.

147 For each PCR sample, put 19 µl of PCR master mix in a 0.2-ml RNase-free PCR tube.

148 Add 1 µl of circularized cDNA and mix it well.

149 Perform PCR pilot amplifications (described in Table 2). Remove one PCR tube for each sample at the end of the extension step after 6, 8, 10 and 12 amplification cycles.

150 Add 3.4 µl of 6× DNA loading dye to each tube and mix well.

151 Prepare DNA control ladder (denoted as L in figure) by adding 1.0 µl of ladder to 9 µl of DNase-free H2O. Then add 2 µl of 6× DNA loading dye and mix well.

152 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.

153 Stain the gel in 50 ml of gel staining solution (see Materials section 1.4.8, PCR amplification) for 5 min at room temperature on a shaker (use a black gel box to protect gel from light while staining).

154 Visualize the gel under blue/UV light and identify the optimal PCR amplification cycles for each cDNA sequencing library. The optimal PCR amplification cycles for the NET-seq library, which runs at ~150 nt, is determined by the presence of a clear band at ~150 nt and no higher-molecular-weight PCR products (the optimal number of cycles in the figure above is between 6 and 8).

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