# Base-pair-resolution genome-wide mapping of active RNA polymerases using precision nuclear run-on (PRO-seq)

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Published online 21 July 2016; doi:10.1038/nprot.2016.086

**We provide a protocol for precision nuclear run-on sequencing (PRO-seq) and its variant, PRO-cap, which map the location of active RNA polymerases (PRO-seq) or transcription start sites (TSSs) (PRO-cap) genome-wide at high resolution. The density of RNA polymerases at a particular genomic locus directly reflects the level of nascent transcription at that region. Nuclei are isolated from cells and, under nuclear run-on conditions, transcriptionally engaged RNA polymerases incorporate one or, at most, a few biotin-labeled nucleotide triphosphates (biotin-NTPs) into the 3′ end of nascent RNA. The biotin-labeled nascent RNA is used to prepare sequencing libraries, which are sequenced from the 3′ end to provide high-resolution positional information for the RNA polymerases. PRO-seq provides much higher sensitivity than ChIP-seq, and it generates a much larger fraction of usable sequence reads than ChIP-seq or NET-seq (native elongating transcript sequencing). Similarly to NET-seq, PRO-seq maps the RNA polymerase at up to base-pair resolution with strand specificity, but unlike NET-seq it does not require immunoprecipitation. With the protocol provided here, PRO-seq (or PRO-cap) libraries for high-throughput sequencing can be generated in 4–5 working days. The method has been applied to human, mouse, *Drosophila melanogaster* and *Caenorhabditis elegans* cells and, with slight modifications, to yeast.**



## INTRODUCTION

The ability to measure the density of RNA polymerase across the genome provides a comprehensive and quantitative snapshot of transcription[1]. Collecting a series of these snapshots in response to regulatory switches reveals the identity of genes that respond immediately or secondarily to specific signals, and it provides critical insights into the mechanisms of their regulation[2]. Quantification of RNA polymerase density along the genes is also critical for deciphering the regulatory steps involved in transcription.

In addition to protein-coding genes, many other regions in the genome (such as upstream divergent regions, regions downstream of mRNA poly A sites and enhancers) are transcribed to various extents. Enhancers produce short unstable RNAs (eRNAs) that do not encode proteins[3] but delineate major hubs of transcription regulation[4]. Differential regulation of enhancer-mediated transcription is implicated in various diseases[5], and understanding this regulation is important for deciphering the transcriptional changes in response to developmental, nutritional and environmental cues. However, sequencing of total RNA by RNA-seq is inefficient in detecting these unstable RNAs.

A number of methods have been described that enrich and sequence nascent RNAs associated with RNA polymerase. These methods either are based on immunoprecipitation of RNA polymerase[6–8] or are dependent on the purification of insoluble chromatin[9]. Therefore, these methods are highly dependent on antibody specificity or the purity of the chromatin fraction, respectively. We have developed nuclear run-on–based methods to map active RNA polymerases and their start sites genome-wide at up to base-pair resolution[10,11]. In these methods, the endogenous activity of RNA polymerase is used to selectively label nascent RNAs. The ability to affinity-purify nuclear run-on RNA multiple times during the course of library preparation provides an approximate million-fold enrichment of the nascent RNA over other forms of RNA and thereby effectively eliminates background[10]. Furthermore, because the RNA is sequenced, the direction of transcription can be unambiguously identified.

### Development of PRO-seq

PRO-seq is based on global run-on sequencing (GRO-seq), a genome-wide adaptation of nuclear run-on assays that have been used classically to measure transcription of target genes. In GRO-seq, bromouridine-labeled nascent RNAs are affinity-purified and analyzed by high-throughput sequencing to map RNA polymerase positions. Extremely high sensitivity and specificity is achieved through multiple distinct affinity-purification steps[10,12]. GRO-seq uses bromouridine as the substrate for the nuclear run-on reaction, enabling RNA polymerase to add multiple nucleotides to the nascent RNA. Therefore, the resolution of GRO-seq is on the order of tens of bases.

However, to understand the molecular mechanisms of transcriptional elongation and promoter-proximal pausing, RNA polymerase mapping at base-pair resolution is required. Such resolution enables mechanistic modeling of how DNA sequences, nucleosomes or other DNA-binding factors affect RNA polymerase elongation and gene expression[2,13]. To achieve base-pair resolution, we used a modified nuclear run-on that

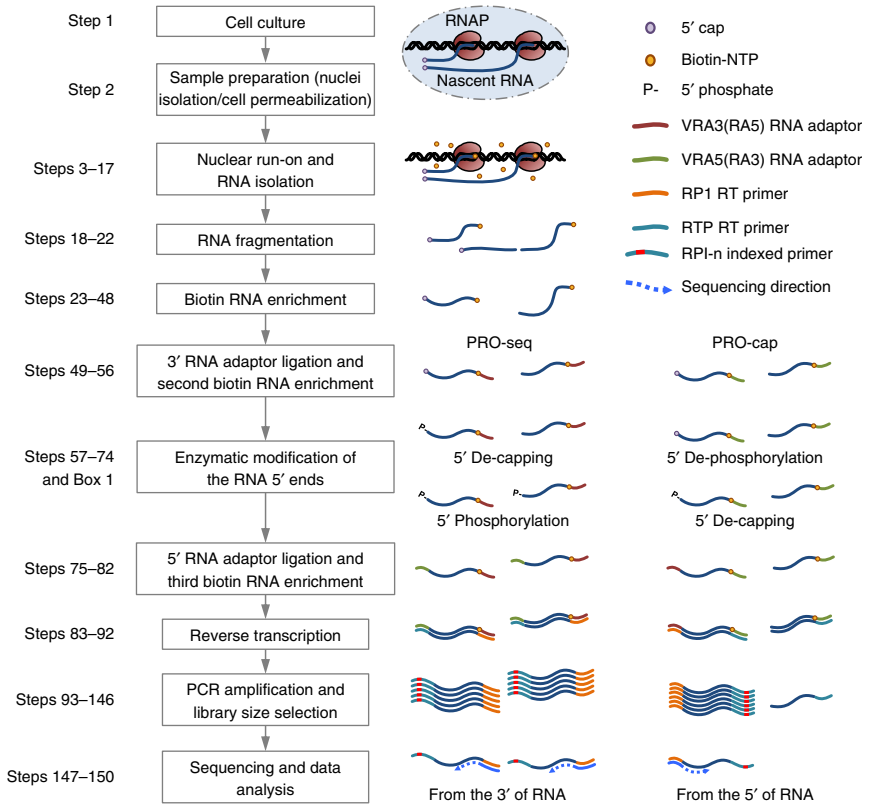


**Figure 1** | Flowchart of the PRO-seq and PRO-cap protocol. Stages of the procedure are outlined, along with schematics indicating the status of the RNA polymerase and the nascent RNA. PRO-seq and PRO-cap share common steps, but the procedures are different at the adaptor ligation, reverse-transcription and 5′ modification stages. In PRO-seq, the 3′ RNA adaptor is modified to have the reverse complement sequence of a standard 5′ RNA adaptor. Similarly, the 5′ RNA adaptor and the reverse-transcription primer are modified for PRO-seq. This allows 3′ sequencing of the RNA on a standard Illumina platform. For PRO-cap, standard small RNA adaptors are used, which allows 5′ sequencing as usual. In the 5′ modification step, PRO-seq RNAs are decapped and rephosphorylated to ensure that all RNA is accessible for 5′ RNA adaptor ligation. PRO-cap RNAs are dephosphorylated first to convert all forms of 5′ ends, except for the 5′ capped ends, to 5′ hydroxyl ends, in order to restrict the ligation of 5′ adaptors to only the cap containing nascent RNAs after cap removal. Then, the 5′ caps are converted to 5′ monophosphates using the tobacco acid pyrophosphatase, and they can be ligated to a 5′ RNA adaptor.



limits the number of labeled nucleotides added to the nascent RNA[10,11,14–16]. In PRO-seq, biotin-labeled NTPs are provided as the substrates for the nuclear run-on reaction. The incorporation of a biotin-NTP by an RNA polymerase inhibits further incorporation of biotin-NTPs into the nascent RNA. Sequencing from the 3′ end of the nascent transcript therefore identifies the last incorporated NTP and reveals the precise location of the active site of the RNA polymerase engaged with its nascent RNA.

Identification of the precise position of TSSs is also important for understanding how DNA elements, general transcription factors and transcription activators recruit RNA polymerase to genes and enhancers. RNA polymerase initiates transcription at a TSS and quickly transcribes a short region before pausing at a promoter-proximal site. However, because the nascent transcripts are sequenced from the 3′ end in PRO-seq, the positional information of where RNA polymerase began transcription is mostly lost. We therefore developed PRO-cap by modifying the sequencing strategy of PRO-seq to sequence the capped nascent RNA from the 5′ end, enabling TSSs to be identified at the RNA synthesis level[11].

## Overview of the procedure

A general overview of the PRO-seq and PRO-cap experimental procedures is shown in **Figure 1**. Nuclei from cells are rapidly isolated, and native nucleotides are washed away to halt transcription. However, RNA polymerases remain engaged on the DNA and retain their enzymatic activity. Incubation of isolated nuclei with biotin-labeled NTPs allows the RNA polymerases to actively elongate and label the nascent RNA. For PRO-seq only, the labeled nascent RNA is hydrolyzed with NaOH to generate RNA fragments suitable for sequencing (~100 bp in length). The RNA containing a biotin nucleotide is then enriched by affinity purification using streptavidin-coated magnetic beads. The biotin–streptavidin interaction is very stable ($K_d \sim 10^{-14}$ mol/l), which allows stringent washing of the magnetic beads to minimize contamination with unlabeled RNA. A 3′ sequencing adaptor is then ligated to the hydroxyl (OH) group at the 3′ end of nascent RNA, followed by another affinity purification to further enrich the nascent RNA and remove unligated adaptor sequences. Preparation for 5′ sequencing adaptor ligation differs for PRO-seq and PRO-cap. For PRO-seq, the 5′ cap is removed from unhydrolyzed short nascent RNA using either tobacco acid pyrophosphatase (TAP) or RNA 5′ pyrophosphohydrolase (RppH). The 5′ OH generated by base hydrolysis is then converted to 5′ phosphate by treatment with T4 polynucleotide kinase (PNK). For PRO-cap, uncapped RNA with a 5′-monophosphate is degraded using 5′-phosphate-dependent exonuclease. 5′-Triphosphates and monophosphates are removed from any remaining contaminating uncapped RNA with alkaline phosphatase. Only then is the 5′ cap of nascent RNA removed with TAP or RppH treatment. After these chemical modifications, a 5′ sequencing adaptor is ligated to the nascent RNA, and a third round of affinity purification is performed to enrich for nascent RNA with sequencing adaptors on both ends. Nascent RNA is then reverse-transcribed and test-PCR-amplified to determine an appropriate number of PCR cycles for final amplification; this latter step is critical to avoid over-amplification. During the final amplification, barcodes can be added so that multiple libraries can be pooled for sequencing. Finally, the PCR-amplified libraries are size-selected for a range of 140–350 bp and sent for high-throughput sequencing. Sequencing depths of 25–50 million for mammalian cells, 10–20 million for organisms with smaller genome size such as *Drosophila* and 5–10 million for yeast cells provide useful information. A relatively short read length such as 40–50 bp

is sufficient. The sequencing data are generated as a text-based list of short nucleotide sequences, and sequencing quality parameters are provided in a 'fastq' format[17]. The sequences may contain varying lengths of adaptor sequences, which are removed before aligning the adaptor-trimmed sequences to the appropriate genome. Finally, the aligned sequences are used to generate coverage files that can be used to visualize and analyze the data.

**Advantages and limitations of PRO-seq**

The key advantages of PRO-seq are as follows:

- It provides base-pair resolution and strand-specific information of global RNA polymerase occupancy.
- Background RNA contamination is hugely reduced because of almost a million-fold purification of biotinylated nascent RNAs.
- It is highly sensitive in detecting rare and common nascent RNAs with a large dynamic range (>$10^5$).
- It can identify short unstable nascent RNAs transcribed from enhancer regions.

PRO-seq also has a number of limitations that should be considered when deciding which genome-wide RNA polymerase mapping strategy to use.

- In principle, PRO-seq results are ensemble profiles of potentially heterogeneous populations of cells—and this is generally true for all multicell, high-throughput sequencing analyses. Unlike mature RNA molecules that are present in multiple copies per cell, RNA polymerase at a specific genomic position can yield at most only two copies of nascent RNA. Therefore, although it may be possible to adapt PRO-seq to measure nascent transcript levels for abundantly expressed genes in single cells, genome-wide mapping of RNA polymerase in single cells using PRO-seq would remain a challenge.
- PRO-seq detects only the active RNA polymerase, and thus RNA polymerases in the preinitiation complex will not be detected. There is also a possibility that other forms of stalled RNA polymerases, such as back-tracked polymerases, may not be detected, although nuclear run-on conditions may allow some of these polymerases to re-align the active site through thermal motion. Generally, the signals seen by ChIP-seq of RNA polymerase II (Pol II) and our genome-wide run-on methods quantitatively agree[18], so the bulk of Pol II is detectable by GRO- and PRO-seq methods.
- Compared with GRO-seq, which adds a longer extension to the 3′ end of nascent RNA[10], PRO-seq adds only one or a few nucleotides in order to provide higher-resolution mapping. However, there is a possibility that RNA polymerases positioned very close to the TSS may not be detected because the nascent RNA may not be long enough to be uniquely mapped to the genome. In this case, GRO-seq may provide more accurate quantification of promoter-proximal RNA polymerases. Similarly, RNA polymerases positioned in a repetitive sequence region of the genome are difficult to unambiguously map to a particular repeat.
- PRO-seq does not distinguish nascent transcription derived from different RNA polymerases (Pol I versus Pol II versus Pol III) unless it is carried out in the presence of inhibitors of specific RNA polymerases. In addition, unlike NET-seq, nascent RNA associated with specific RNA polymerase modifications (e.g., phosphorylations of the C-terminal domain) cannot be selectively detected using PRO-seq[8].

**Applications of PRO-seq and PRO-cap**

The most common use of PRO-seq is for the analysis of genome-wide transcription levels with directional information and with higher resolution and sensitivity than an RNA polymerase ChIP-seq assay. PRO-seq provides an independent layer of gene expression analysis distinct from that of mRNA-seq, revealing the transcriptional stages of regulation before the influence of mRNA processing or stability control. The increased resolution and the directional information become useful in distinguishing upstream divergent (also called upstream antisense) transcription[10,19].

PRO-cap[11] can capture TSSs at the nascent RNA synthesis level, in contrast to other TSS analyses that use mature RNA[20–22]. This becomes an advantage in detecting enhancer transcripts[3,23,24], upstream antisense transcription[3,10] or other types of unstable transcripts, and avoiding post-transcriptional background capping events[25].

**Alternatives to PRO-seq**

RNA polymerase can be mapped genome-wide by a variety of different strategies.

- *ChIP-seq.* In this approach, RNA polymerase proteins are cross-linked to the DNA, and then Pol II is purified by immunoprecipitation. Pol-II-associated DNA is identified and quantified by high-throughput sequencing, thereby providing an estimate of the amount of Pol II at different sites on the genome. The resolution of ChIP is usually limited by the size of the fragmented DNA in the chromatin at the immunoprecipitation step. A variant of this method, called ChIP-exo, overcomes the resolution limitation by additionally treating the DNA fragments from the Pol II ChIP with a DNA exonuclease[26]. The exonuclease digests DNA from the 3′ end of both strands, stopping near the cross-linked polymerase complex. An additional limitation of ChIP-seq is that Pol-II-unbound genomic regions that interact with a Pol-II-bound region through three-dimensional looping can be falsely identified with this method because of the use of cross-linking. Finally, ChIP-seq will map all forms of Pol II, including transcriptionally inactive Pol IIs and Pol IIs in antisense orientation; therefore, the direction of the transcription is not directly disclosed.
- *Permanganate footprinting.* This method can be used to identify the single-stranded DNA created by the transcription bubble formed on DNA by the RNA polymerase. The nontemplate strand of the DNA is exposed to single-strand-specific breakage at T residues through a series of chemical treatments. A method called permanganate–ChIP-seq couples permanganate footprinting to Pol II ChIP and thereby maps the cleaved ends of the DNA from the single-stranded region of transcription bubbles[27]. This directly maps the transcription active site with high resolution. Permanganate mapping depends on the presence of thymine residues in the nontemplated single-stranded DNA of the bubble that are not masked by protein binding. Although the protocol enriches for Pol II in a single chromatin immunoprecipitation, other regions that expose single-stranded thymine—such as in other DNA–RNA hybrids or intrastrand DNA hairpins—could contribute to background.
- *NET-seq.* A number of chromatin-bound nascent-RNA-based methods, including NET-seq and its variants, have been developed for mapping RNA polymerase[6,8,9,28]. In the original

NET-seq protocol[6], the RNA polymerase complex is immunoprecipitated and the co-purified native RNA is sequenced. The 3′ end of the nascent RNA provides base-pair-resolution mapping of RNA polymerase. This method is ideally suited to examining the occupancy of differently modified RNA polymerases. In practice, the efficiency of NET-seq relies on the degree of enrichment provided by the single immunoprecipitation step. Because the method detects the 3′ ends of all RNAs that are associated with Pol II, it also captures the 3′ ends of intermediates of co-transcriptional splicing and micro-RNA production that can complicate mapping of transcriptionally engaged Pol II (ref. 8).

### Experimental design

**Cells.** In our lab, we have successfully generated PRO-seq libraries for cells from plants (G.T.B., unpublished data), yeast (G.T.B., unpublished data), *Drosophila*[11] and mammals[3,29]. In general, the higher the number of cells, the better the PRO-seq read coverage of the genome. However, a minimum of 5–10 million nuclei or permeabilized cells is required for a single PRO-seq library, regardless of cell type. In principle, the application of PRO-seq in yeast, including *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, is very similar to that in other organisms; however, some alterations are required in yeast cell permeabilization[30], run-on reaction and post-run-on RNA extraction procedures[31]; required modifications for yeast are indicated in the appropriate steps of the PROCEDURE.

**Sample preparation.** Isolation of nuclei for nuclear run-on is a critical step in the procedure not only to preserve the enzymatic activity of the RNA polymerase but also to capture the precise position of the RNA polymerase on genes. Starting with 10–20 million cells per library is recommended, considering the efficiency of the nuclei isolation process (~50%). The whole process should take place in a cold room on ice as far as is possible. Isolated nuclei can be resuspended in the glycerol-containing storage buffer, and they can be quickly frozen in liquid nitrogen for long-term storage at −80 °C. We have used permeabilized cells in PRO-seq as an alternative to isolating nuclei, which makes handling easier and reduces loss of sample; cell permeabilization has a much higher efficiency (~90%) than nuclei isolation. Cell permeabilization conditions may differ between cell types and may need to be optimized; we provide a general method for permeabilization in the PROCEDURE, as well as a version optimized for yeast cells.

**Spike-in for library normalization.** Disproportionate loss of RNA and/or cDNA can occur during multiple stages of the PRO-seq library preparation, which spans 4–5 d and involves several handling steps. Even with the use of identical starting material, uneven loss of libraries could affect the genome-wide RNA polymerase density between libraries. To control for handling effects on library yield, a small fraction (1–5%) of cells with a distinct genome can be added during library preparation; adding an identical number of spike-in cells to different libraries enables normalization between different conditions. We have used *S. pombe* to normalize *S. cerevisiae* (and vice versa) and *Drosophila* cells to normalize mammalian cells (and vice versa). When the cell permeabilization approach is used, spike-in cells should be added and permeabilized together with the experimental cells. For the nuclei isolation approach, spike-in cells should be added to the experimental cells before nuclei isolation, and the cells should be dounced together.

**Nuclear run-on.** In PRO-seq, biotin-NTPs are used as the nuclear run-on substrates. The $K_m$ of each of the NTPs as substrates for RNA polymerase lie in the range of 1–20 μM (ref. 32). Therefore, a final substrate concentration greater than a 1–20 μM range (~25 μM) is, in general, sufficient for each biotin-NTP substrate. Depending on the purpose of the experiment, biotin-NTP substrates can be supplied in different combinations: individual-biotin run-on, 4-biotin run-on, 2-biotin run-on or 1-biotin run-on.

- *Individual-biotin run-on*. To obtain the most precise mapping of the RNA polymerase, four separate PRO-seq libraries should be made, each supplied with only one type of biotin-NTP in the nuclear run-on reaction. This ensures that the RNA polymerase adds only one, or at most a few (when the polymerase is positioned at multiple stretches of same nucleotide), biotin-NTPs to the nascent RNA. In this case, four times more sample is required.
- *4-Biotin run-on*. We found that all four biotin-NTPs can be supplied in a single reaction and the Pol II incorporates only one or at most a few bases, giving an equivalent resolution to that of an individual-biotin run-on. The reason for this is unclear, but we speculate that steric hindrance in the active site of the RNA polymerase prevents incorporation of multiple biotinylated nucleotides.
- *2-Biotin run-on*. When the amount of sample or the cost is limited, unlabeled NTPs can be used in combination with biotin-NTPs. The use of biotinylated purine nucleotides (biotin-ATP, biotin-GTP) is more costly than that of the pyrimidine nucleotides (biotin-CTP, biotin-UTP). A combination of biotin-CTP, biotin-UTP, ATP and GTP can be supplied to the nuclear run-on reaction, providing reasonable resolution and cost.
- *1-Biotin run-on*. If a longer run-on RNA is preferred, combinations of biotin-CTP with unlabeled CTP, UTP, ATP and GTP can be used effectively in a biotin-NTP form of GRO-seq. This approach can be useful for increasing sequencing coverage of RNA polymerases that reside near the TSSs. Although most transcriptionally engaged RNA polymerases near the 5′ ends reside between 30 and 60 nucleotides from the TSS[11], RNA polymerases closer to the TSS may fail to map uniquely. In addition, the longer run-on extensions of nascent RNAs may be desired for distinguishing allele-specific nascent transcription.

**PCR amplification of the PRO-seq library.** When the number of cells and/or nuclei is limited for PRO-seq library preparation, a higher number of PCR amplification cycles will be required to generate a sufficient library for reliable quantification and accurate loading into the sequencer. However, a higher number of PCR cycles can result in amplification bias of some sequences. To avoid PCR-induced biases, molecular barcodes[33] can be introduced as part of the 3′ RNA adaptor, which is ligated to the nascent RNAs. Duplicate reads generated by PCR overamplification can be identified by identical barcodes and computationally filtered at the stage of mapping the sequenced reads to the genome.

## MATERIALS

### REAGENTS

▲ CRITICAL Extreme care should be taken to avoid nuclease contamination. Use nuclease-free reagents and change gloves routinely.

- Appropriate cell line(s)—e.g., K562, GM12878, MCF7, HeLa, embryonic stem cells, mouse embryonic fibroblasts, mouse 3T3 cells, *Drosophila* S2 and yeast
  ! CAUTION Before use, cells should be checked for contamination.

**Chemical stocks**

- Diethyl pyrocarbonate (DEPC; Sigma-Aldrich, cat. no. D5758)
  ! CAUTION DEPC is toxic and harmful. Proper eyeshield, faceshield, full-face respirator and gloves are required while handling DEPC.
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S9888)
- Potassium chloride (KCl; Avantor, cat. no. 6858-04)
- Magnesium chloride ($MgCl_2$; Avantor, cat. no. 5958-04)
- Sucrose (Sigma-Aldrich, cat. no. S0389)
- Calcium chloride ($CaCl_2$; Sigma-Aldrich, cat. no. C1016)
- Magnesium acetate ($MgAc_2$; Sigma-Aldrich, cat. no. M5661)
- Ammonium acetate ($NH_4Ac$; Sigma-Aldrich, cat. no. A1542)
- Sodium acetate (NaOAc; Sigma-Aldrich, cat. no. S2889)
- EDTA (Sigma-Aldrich, cat. no. E9884)
- EGTA (Sigma-Aldrich, cat. no. E3889)
- Protease inhibitor cocktail, EDTA-free (Roche, cat. no. 11873580001)
- Sodium hydroxide (NaOH; Avantor, cat. no. 7708-10)
- Triton X-100 (Calbiochem, cat. no. 9410)
- Nonidet P40 (NP40) substitute (Sigma-Aldrich, cat. no. 11332473001)
- Sarkosyl (Sigma-Aldrich, cat. no. L5125)
- Tween-20 (Sigma-Aldrich, cat. no. P9416)
- PBS, pH 7.4 (Gibco, cat. no. 10010031)
- Tris (Avantor, cat. no. 4109-02)
- Hydrochloric acid (HCl; Avantor, cat. no. 4613-05)
- DTT (Sigma-Aldrich, cat. no. D0632)
- Betaine (Sigma-Aldrich, cat. no. B0300)
- Glycerol (Sigma-Aldrich, cat. no. G5516)

**Biotin nuclear run-on and enrichment**

- Biotin-11-ATP (PerkinElmer, cat. no. NEL544001EA)
- Biotin-11-CTP (PerkinElmer, cat. no. NEL542001EA)
- Biotin-11-GTP (PerkinElmer, cat. no. NEL545001EA)
- Biotin-11-UTP (PerkinElmer, cat. no. NEL543001EA)
- ATP, 10 mM (Roche, cat. no. 11277057001)
- GTP, 10 mM (Roche, cat. no. 11277057001)
- UTP, 10 mM (Roche, cat. no. 11277057001)
- P-30 column, RNase-free (Bio-Rad, cat. no. 732-6250)
- Streptavidin M280 beads (Invitrogen, cat. no. 112.06D)

**Reagents for nucleic acid extraction**

- Trizol (Ambion, cat. no. 115596018) ! CAUTION Trizol is harmful, and contact with skin or eyes or inhalation should be avoided. Use it inside a fume hood.
- Trizol LS (Ambion, cat. no. 10296028) ! CAUTION Trizol is harmful, and contact with skin or eyes or inhalation should be avoided. Use it inside a fume hood.
- Chloroform (Calbiochem, cat. no. 3150)
- GlycoBlue (Ambion, cat. no. AM9515)
- Ethanol, 100% (vol/vol) (Pharmco-AAPER, cat. no. 111000200)
- Ethanol, 75% (vol/vol)
- Phenol:chloroform, Tris buffered (Thermo Scientific, cat. no. 17909)
  ! CAUTION Phenol:chloroform is harmful, and contact with skin or eyes or inhalation should be avoided. Use it inside a fume hood.
- Phenol (Ambion, cat. no. 9700) ! CAUTION Phenol is harmful, and contact with skin or eyes or inhalation should be avoided. Use it inside a fume hood.

**Enzymes and recombinant protein reagents**

- RNase inhibitor, 40 units per µl (Ambion, cat. no. AM2696)
- T4 RNA ligase I, 10 units per µl (NEB, cat. no. M0204). Supplied with 10× T4 RNA ligase buffer, 10 mM ATP and PEG, 50% (wt/vol)
- 5′-Phosphate-dependent exonuclease, 1 unit per µl (Epicenter, cat. no. TER51020) (required for PRO-cap only). Supplied with 10× reaction buffer A
- Alkaline phosphatase, 10 units per µl (NEB, cat. no. M0290) (required for PRO-cap only). Supplied with 10× alkaline phosphatase buffer. Alternatively, Antarctic phosphatase, 5 units per µl (NEB, cat. no. M0289), can be used
- Tobacco acid pyrophosphatase (TAP), 10 units per µl (Epicenter, cat. no. T19500). Supplied with 10× TAP buffer. Alternatively, RNA 5′ pyrophosphohydrolase (RppH), 5 units per µl (NEB, cat. no. M0356S), can be used with ThermoPol Reaction Buffer (NEB, cat. no. B9004S)
- T4 polynucleotide kinase (PNK), 10 units per µl (NEB, cat. no. M0201) (required for PRO-seq only). Supplied with 10× PNK buffer
- Superscript III reverse transcriptase (Invitrogen, cat. no. 56575). Supplied with 5× first-strand buffer and 0.1 M DTT
- dNTP mix, 12.5 mM each (Roche, cat. no. 03622614001)
- Phusion polymerase, 2 units per µl (NEB, cat. no. M0530). Supplied with 5× High-Fidelity buffer
- RNA and DNA oligos (custom synthesis from IDT DNA, RNase-free HPLC purified). See **Table 1** and Reagent Setup for details. Further information about barcoding and sequencing indexes can be found at http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/truseqsampleprep/truseq-library-prep-pooling-guide-15042173-01.pdf

**Electrophoresis**

- DNA-grade agarose (Bio-Rad, cat. no. 161-3102EDU)
- Tris/acetic acid/EDTA (TAE), 50× (Bio-Rad, cat. no. 161-0773). Alternatively, a 50× TAE buffer can be made in-house (see Reagent Setup section)
- Glacial acetic acid (Sigma-Aldrich, cat. no. 537020) for in-house preparation of 50× TAE
- Gel-loading dye, Orange G 6× (NEB, cat. no. B7022S)
- 100-bp DNA ladder (Life Technologies, cat. no. 15628-019)
- 10-bp DNA ladder (Life Technologies, cat. no. 10821-015). Alternatively, 25-bp DNA ladders (Thermo Fisher, cat. no. 10597011) can be used
- SYBR Gold nucleic acid gel stain, 6× (Life Technologies, cat. no. S-11494)
- Acrylamide (Protogel), 30% (wt/vol) (National Diagnostics, cat. no. EC-980). 30% (wt/vol) Acrylamide/methylene bisacrylamide solution from other sources is also compatible
- TEMED (Bio-Rad, cat. no. 161-0800)
- Ammonium persulfate (APS), 10% (wt/vol) (Bio-Rad, cat. no. 161-0700).
- Tris/boric acid/EDTA (TBE), 10× (Bio-Rad, cat. no. 161-0770). Alternatively, a 10× TBE buffer can be made in-house (see Reagent Setup section)
- Boric acid (Sigma-Aldrich, cat. no. B6768) for in-house preparation of 10× TBE

**Software for data analysis**

- Adaptor removal software, such as cutadapt[34] (http://code.google.com/p/cutadapt/)
- Mapping or alignment software, such as bwa[35] (https://sourceforge.net/projects/bio-bwa/files/) or bowtie[36] (https://sourceforge.net/projects/bowtie-bio/files/)
- Tools for generation of coverage information, such as SAMtools[37] (https://sourceforge.net/projects/samtools/files/) and BEDTools[38] (https://sourceforge.net/projects/bedtools/files/)

### EQUIPMENT

- 2 Heat blocks, one set at 37 °C and the other set at 65 °C, each filled with water equilibrated at the appropriate temperature
- Dounce homogenizer (Wheaton Scientific, cat. no. 357546)
- Magnetic separator for streptavidin magnetic beads (Invitrogen, cat. no. K1585-01)
- Rotating stand (Thermo Barnstead Labquake Rotator, cat. no. 415110)
- Refrigerated centrifuge (Eppendorf, cat. no. 5417R)
- Microcentrifuge (Eppendorf, cat. no. 5415D)
- SpeedVac dryer (Thermo Scientific, cat. no. 20-548-134)
- Dark Reader transilluminator (Clare Chemical Research, cat. no. DR89X)

### REAGENT SETUP

▲ CRITICAL All reagents, solutions and buffers should be made with DEPC-treated water (DEPC-$H_2O$).

**DEPC-$H_2O$** Add 0.1% (vol/vol) DEPC to $H_2O$. Mix it overnight and then autoclave and filter-sterilize the solution with a 0.22-µm filter. DEPC-$H_2O$ can be prepared in advance and stored at room temperature (25 °C) for up to a year. ! CAUTION DEPC is toxic and harmful. Proper eyeshield, faceshield, full-face respirator and gloves are required while handling DEPC.

**5 M NaCl** Dissolve 14.61 g of NaCl in 50 ml of $H_2O$ with 0.1% (vol/vol) DEPC, mix it overnight and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ CRITICAL 5 M NaCl can be prepared in advance and stored at room temperature for up to a year.

**TABLE 1** | Oligonucleotides required for PRO-seq and PRO-cap.

| | Oligo name | Sequence (5′–3′) | Purpose | Comments |
|---|---|---|---|---|
| Oligos for PRO-seq | VRA3 | GAUCGUCGGACUGUAGAACUCUGAAC-/inverted dT/ | RNA adaptor for ligation to the 3′ end of nascent RNA at Step 49 | The 5′ end is phosphorylated and the 3′ end is protected by an inverted dT |
| | VRA5 | CCUUGGCACCCGAGAAUUCCA | RNA adaptor for ligation to the 5′ end of nascent RNA at Step 75 | The 5′ end is not phosphorylated |
| | RP1 | AATGATACGGCGACCACCGAGATCTACACGT TCAGAGTTCTACAGTCCGA | DNA oligo for reverse transcription of adaptor-ligated nascent RNA at Step 84 | |
| Oligos for PRO-cap | RA3 | UGGAAUUCUCGGGUGCCAAGG-/inverted dT/ | RNA adaptor for ligation to the 3′ end of nascent RNA at Step 49 | The 5′ end is phosphorylated and the 3′ end is protected by an inverted dT |
| | RA5 | GUUCAGAGUUCUACAGUCCGACGAUC | RNA adaptor for ligation to the 5′ end of nascent RNA at Step 75 | The 5′ end is not phosphorylated |
| | RTP | GCCTTGGCACCCGAGAATTCCA | DNA oligo for reverse-transcription of adaptor-ligated nascent RNA at Step 84 | |
| PCR primers for library amplification | RP1 | AATGATACGGCGACCACCGAGATCTACACGT TCAGAGTTCTACAGTCCGA | DNA oligo for PCR amplification of cDNA in both PRO-seq and PRO-cap at Steps 94 and 102 | Same as the RT oligo for PRO-seq |
| | RPI-n | CAAGCAGAAGACGGCATACGAGAT NNNNNN GTGACTGGAGTT CCTTGGCACCCGAGAATTCCA | DNA oligo with barcodes for PCR amplification of cDNA in both PRO-seq and PRO-cap at Steps 94 and 104 | The six Ns represent the barcodes for Illumina TRU-seq multiplexing. For example, the barcode in RPI-1 is CGTGAT |



**4 M KCl** Dissolve 3.73 g of KCl in 50 ml of $H_2O$ with 0.1% (vol/vol) DEPC, mix it overnight and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 4 M KCl can be prepared in advance and stored at room temperature for up to a year.

**1 M $MgCl_2$** Dissolve 4.76 g of $MgCl_2$ in 50 ml of $H_2O$ with 0.1% (vol/vol) DEPC, mix it overnight and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 1 M $MgCl_2$ can be prepared in advance and stored at room temperature for up to a year.

**1 M Sucrose** Dissolve 171.15 g of sucrose in 500 ml of $H_2O$ with 0.1% (vol/vol) DEPC, mix it overnight and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 1 M Sucrose can be prepared in advance and stored at room temperature for up to a year.

**1 M $CaCl_2$** Dissolve 5.55 g of $CaCl_2$ in 50 ml of $H_2O$ with 0.1% (vol/vol) DEPC, mix it overnight and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 1 M $CaCl_2$ can be prepared in advance and stored at room temperature for up to a year.

**1 M $MgAc_2$** Dissolve 7.12 g of $MgAc_2$ in 50 ml of $H_2O$ with 0.1% (vol/vol) DEPC, mix it overnight and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 1 M $MgAc_2$ can be prepared in advance and stored at room temperature for up to a year.

**1 M $NH_4Ac$** Dissolve 3.85 g of $NH_4Ac$ in 50 ml of $H_2O$ with 0.1% (vol/vol) DEPC, mix it overnight and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 1 M $NH_4Ac$ can be prepared in advance and stored at room temperature for up to a year.

**1 M NaOAc, pH 5.3** Dissolve 4.1 g of NaOAc in 50 ml of $H_2O$, and adjust the pH to 5.3; add 0.1% (vol/vol) DEPC, mix it overnight and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 1 M NaOAc can be prepared in advance and stored at room temperature for up to a year.

**0.5 M EDTA** Dissolve 29.22 g of EDTA in 100 ml of DEPC-$H_2O$, and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 0.5 M EDTA can be prepared in advance and stored at room temperature for up to a year.

**0.1 M EGTA** Dissolve 19.02 g of EGTA in 50 ml of DEPC-$H_2O$, and then autoclave and filter-sterilize the solution. ▲ **CRITICAL** 0.1 M EGTA can be prepared in advance and stored at room temperature for up to a year.

**1 N NaOH** Dissolve 2 g of NaOH in 50 ml of DEPC-$H_2O$, and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 1 N NaOH can be prepared in advance and stored in 50-µl aliquots at −80 °C for up to a year. Use a freshly thawed aliquot each time. ! **CAUTION** NaOH is corrosive, and contact with skin or eyes or inhalation should be avoided.

**10% (vol/vol) Triton X-100** Dissolve 5 ml of Triton X-100 in 45 ml of DEPC-$H_2O$ and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 10% (vol/vol) Triton X-100 can be prepared in advance and stored at room temperature for up to a year.

**10% NP40** Dissolve 5 ml of NP40 in 45 ml of DEPC-$H_2O$ and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 10% NP40 can be prepared in advance and stored at room temperature for up to a year.

**2% Sarkosyl** Dissolve 1 g of Sarkosyl in 50 ml of DEPC-$H_2O$ and filter-sterilize the solution with a 0.22-µm filter. ! **CAUTION** Sarkosyl is an irritant, and contact with skin or eyes, or inhalation should be avoided. ▲ **CRITICAL** 2% Sarkosyl can be prepared in advance and stored at room temperature for up to a year.

**1% Tween-20** Dissolve 1 ml of Tween-20 in 49 ml of DEPC-$H_2O$, and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 1% Tween-20 can be prepared in advance and stored at room temperature for up to a year.
**1 M Tris-HCl, pH 6.8** Dissolve 6.06 g of Tris base in 50 ml of DEPC-$H_2O$, adjust the pH to 6.8 with HCl and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** The buffer can be prepared in advance and stored at room temperature for up to a year.
**1 M Tris-HCl, pH 7.4** Dissolve 6.06 g of Tris base in 50 ml of DEPC-$H_2O$, adjust the pH to 7.4 with HCl and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** The buffer can be prepared in advance and stored at room temperature for up to a year.
**1 M Tris-HCl, pH 8.0** Dissolve 6.06 g of Tris base in 50 ml of DEPC-$H_2O$, adjust the pH to 8.0 with HCl and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** The buffer can be prepared in advance and stored at room temperature for up to a year.
**1 M DTT** Dissolve 1.54 g of DTT in 10 ml of DEPC-$H_2O$, and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 1 M DTT can be prepared in advance and stored at −20 °C for up to a year.
**1 mM Biotin-11-CTP** Mix 10 µl of 10 mM stock in 90 µl of DEPC-$H_2O$ to obtain 1 mM dilution. ▲ **CRITICAL** The buffer can be prepared in advance and stored at 4 °C for up to a year.
**1 mM Biotin-11-UTP** Mix 10 µl of 10 mM stock in 90 µl of DEPC-$H_2O$ to obtain a 1 mM dilution. ▲ **CRITICAL** The buffer can be prepared in advance and stored at 4 °C for up to a year.
**50× TAE** Dissolve 121 g of Tris base, 28.55 g of glacial acetic acid and 50 ml of 0.5 M EDTA in DEPC-$H_2O$ to a final volume of 500 ml, and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** The buffer can be prepared in advance and stored at 4 °C for up to a month.
**10× TBE** Dissolve 54 g of Tris base, 27.5 g of boric acid and 20 ml of 0.5 M EDTA in DEPC-$H_2O$ to a final volume of 500 ml, and then autoclave and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** The buffer can be prepared in advance and stored at 4 °C for up to a month.
**Douncing buffer (for nuclei isolation)** The Douncing buffer is 10 mM Tris-HCl, pH 7.4, 300 mM sucrose, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1% (vol/vol) Triton X-100, 0.5 mM DTT, one tablet of protease inhibitors cocktail per 50 ml and 4 units per ml RNase inhibitor. ▲ **CRITICAL** The buffer without DTT, protease inhibitors and RNase inhibitor can be prepared and stored at 4 °C for up to a month. Add fresh DTT, protease inhibitors and RNase inhibitor immediately before use.
**Permeabilization buffer (for nonyeast cells)** The permeabilization buffer is 10 mM Tris-HCl, pH 7.4, 300 mM sucrose, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.05% Tween-20, 0.1% NP40 substitute, 0.5 mM DTT, one tablet of protease inhibitors cocktail per 50 ml and 4 units per ml RNase inhibitor. ▲ **CRITICAL** The buffer without DTT, protease inhibitors and RNase inhibitor can be prepared and stored at 4 °C for up to a month. Add fresh DTT, protease inhibitors and RNase inhibitor immediately before use.
**Permeabilization buffer (for yeast cells)** The permeabilization buffer is 0.5% Sarkosyl, 0.5 mM DTT, one tablet of protease inhibitors cocktail per 50 ml and 4 units per ml RNase inhibitor. ▲ **CRITICAL** The buffer without DTT, protease inhibitors and RNase inhibitor can be prepared and stored at 4 °C for up to a month. Add fresh DTT, protease inhibitors and RNase inhibitor immediately before use.
**Storage buffer** The storage buffer is 10 mM Tris-HCl, pH 8.0, 25% (vol/vol) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA and 5 mM DTT. ▲ **CRITICAL** The buffer without DTT can be prepared and stored at 4 °C for up to a month. Add fresh DTT immediately before use.
**2× Nuclear run-on master mix (for nonyeast cells)** The 2× nuclear run-on (NRO) master mix is 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl and 1 mM DTT. ▲ **CRITICAL** The buffer without DTT can be prepared and stored at 4 °C for up to a month. Add fresh DTT immediately before use.
**2× NRO master mix (for yeast cells)** The 2× NRO master mix is 40 mM Tris-HCl, pH 7.7, 400 mM KCl, 64 mM $MgCl_2$ and 1 mM DTT. ▲ **CRITICAL** The buffer without DTT can be prepared and stored at 4 °C for up to a month. Add fresh DTT immediately before use.
**AES buffer (for yeast cells only)** The AES buffer is 50 mM NaOAc, pH 5.3, 10 mM EDTA and 1% SDS.
**High-salt wash buffer** The high-salt wash buffer is 50 mM Tris-HCl, pH 7.4, 2 M NaCl and 0.5% (vol/vol) Triton X-100 in DEPC $H_2O$. ▲ **CRITICAL** The buffer can be prepared in advance and stored at 4 °C for up to a month.
**Binding buffer** The binding buffer is 10 mM Tris-HCl, pH 7.4, 300 mM NaCl and 0.1% (vol/vol) Triton X-100 in DEPC $H_2O$. ▲ **CRITICAL** The buffer can be prepared in advance and stored at 4 °C for up to a month.
**Low-salt wash buffer** The low-salt wash buffer is 5 mM Tris-HCl, pH 7.4 and 0.1% (vol/vol) Triton X-100 in DEPC $H_2O$. ▲ **CRITICAL** The buffer can be prepared and stored at 4 °C for up to a month.
**Prewashed streptavidin-coated magnetic beads** Take 90 µl of streptavidin M280 beads per library. Place the beads on a magnetic separator for 1 min and discard the supernatant. Prewash by resuspending in 0.1 N NaOH + 50 mM NaCl in DEPC-$H_2O$ for 1 min, place the mixture on the magnetic separator for 1 min and remove the supernatant. Wash the beads twice with 100 mM NaCl in DEPC $H_2O$. After removing the wash buffer, resuspend the beads in 150 µl of the binding buffer and make three aliquots of 50 µl each. Scale up accordingly when processing multiple samples. ▲ **CRITICAL** The washed beads can be prepared in advance and stored at 4 °C for up to a week.
**2.2% Agarose gel** Add 3.3 g of DNA-grade agarose to 150 ml of 1× TAE. Mix by swirling and heat it using a lab microwave until the mixture bubbles and looks clear.
**Gel elution buffer** The gel elution buffer is 10 mM Tris-HCl, pH 8.0, 0.5 mM $NH_4Ac$, 10 mM $MgAc_2$, 1 mM EDTA and 0.1% SDS. ▲ **CRITICAL** The buffer can be prepared in advance and stored at room temperature for up to a month.
**DNA and RNA oligos** Oligos for PRO-seq and PRO-cap should be dissolved in DEPC $H_2O$ at a concentration of 100 mM. PCR primers should be dissolved in DEPC $H_2O$ at a concentration of 25 mM. ▲ **CRITICAL** The DNA and RNA oligos can be stored at −80 °C for up to 10 years.
**Ammonium persulfate** Dissolve 1 g of APS in 10 ml of DEPC-$H_2O$ and filter-sterilize the solution with a 0.22-µm filter. ▲ **CRITICAL** 10% (wt/vol) APS can be prepared in advance and stored at –20 °C for up to a year.

## PROCEDURE

### Cell culture ● TIMING 24 h

**1|** Seed cells at a concentration that will enable them to reach ~80% confluency in 24 h. For a PRO-seq experiment using adherent fibroblasts, 4–6 150-mm cell culture dishes yield sufficient cells (~$10^7$ cells; see the Experimental design section for further details). For yeast cells, plate them to ensure that they are in the exponential phase of growth ($OD_{600}$ = 0.5) at the time of harvest.
**! CAUTION** Check cell lines for *Mycoplasma* contamination before setting up the experiment.

### Sample preparation ● TIMING 1 h

▲ **CRITICAL** Samples should be prepared in a cold room (4 °C) to avoid unsolicited run-on.
**2|** Prepare samples by isolating nuclei (option A) or by cell permeabilization (use option C for yeast cells and option B for other cell types).

All centrifugation steps for sample preparation are performed in a cold centrifuge (4 °C) at 1,000*g* (unless otherwise stated) for 5 min.

**(A) Nuclei isolation**

(i) Harvest adherent cells by scraping and centrifugation and nonadherent cells by centrifugation at 1,000*g* for 5 min at 4 °C.
(ii) Resuspend the cell pellet in 10 ml of ice-cold PBS and centrifuge the mixture at 1,000*g* for 5 min at 4 °C.
(iii) Resuspend the cell pellet in ice-cold douncing buffer (1 × $10^6$ cells per ml).
▲ **CRITICAL STEP**: If you are using spike-in cells, add them at this point to the cells resuspended in the douncing buffer.
(iv) Incubate the cells for 5 min on ice and dounce 25 times using a Dounce homogenizer.
(v) Transfer the dounced nuclei to either a 15- or 50-ml conical tube and centrifuge the nuclei.
(vi) Wash the nuclei twice by resuspending the pellet in 5 ml of douncing buffer, followed by centrifugation.
(vii) Resuspend the pellet in storage buffer (5–10 × $10^6$ nuclei per 100 µl of storage buffer), flash-freeze the solution in liquid nitrogen and store it at –80 °C.
■ **PAUSE POINT** The nuclei in the storage buffer can be stored at –80 °C for up to 5 years.

**(B) Cell permeabilization (nonyeast cells)**

(i) Harvest adherent cells by scraping and centrifugation, and harvest nonadherent cells by centrifugation at 1,000*g* for 5 min at 4 °C.
(ii) Resuspend the cell pellet in 10 ml of ice-cold PBS and centrifuge the mixture at 1,000*g* for 5 min at 4 °C.
(iii) Resuspend the cell pellet in ice-cold permeabilization buffer (1 × $10^6$ cells per ml).
▲ **CRITICAL STEP** Spike-in cells, if used, should be added to the cells resuspended in permeabilization buffer.
(iv) Incubate the cells for 5 min on ice and centrifuge the permeabilized cells.
(v) Wash the cells twice by resuspending them in 5 ml of permeabilization buffer, followed by centrifugation.
(vi) Resuspend the cell pellet in storage buffer (5–10 × $10^6$ permeabilized cells per 100 µl of storage buffer), flash-freeze the solution in liquid nitrogen and store it at –80 °C.
■ **PAUSE POINT** The permeabilized cells in storage buffer can be stored at –80 °C for up to 5 years.

**(C) Cell permeabilization (optimized for yeast)**

(i) Harvest exponentially growing yeast cells by centrifugation at 400*g* for 5 min at 4 °C.
(ii) Resuspend the cell pellet in 10 ml of ice-cold PBS and centrifuge at 400*g* for 5 min at 4 °C.
(iii) Resuspend the cell pellet in ice-cold yeast permeabilization buffer (1 × $10^6$ cells per ml).
▲ **CRITICAL STEP** Spike-in cells, if used, should be added to the cells resuspended in yeast permeabilization buffer.
(iv) Incubate the cells for 20 min on ice and centrifuge them at 400*g*.
(v) Resuspend the cell pellet in storage buffer (25–50 × $10^6$ permeabilized cells per 100 µl of storage buffer), flash-freeze the solution in liquid nitrogen and store it at –80 °C.
■ **PAUSE POINT** The permeabilized cells in storage buffer can be stored at –80 °C for up to 5 years.

**Nuclear run-on ● TIMING 1.5 h**

**3|** Prepare a 2× NRO master mix; for nonyeast cells, prepare the master mix according to the first table, and for yeast cells use the second table.

| Reagents | Volume per reaction (µl) | Final concentration – 1× (in 200-µl reaction) (mM) |
|---|---|---|
| Tris-Cl, pH 8.0 (1 M) | 1 | 5 |
| $MgCl_2$ (1 M) | 0.5 | 2.5 |
| DTT (0.1 M) | 1 | 0.5 |
| KCl (4 M) | 7.5 | 150 |
| DEPC-$H_2O$ | 18 | |

| Reagents | Volume per reaction (µl) | Final concentration – 1× (in 200-µl reaction) (mM) |
|---|---|---|
| Tris-Cl pH 7.7 (1 M) | 4 | 20 |
| $MgCl_2$ (1 M) | 6.4 | 32 |
| DTT (0.1 M) | 1 | 0.5 |
| KCl (4 M) | 10 | 200 |
| DEPC-$H_2O$ | 6.6 | |

**4|** Depending on the type of run-on experiment (see the Experimental design section), prepare a 2× reaction mix according to option A (individual-biotin run-on), option B (4-biotin run-on), option C (2-biotin run-on) or option D (1-biotin run-on). If you are processing multiple libraries at once, scale up accordingly (**Supplementary Table 1**).

**(A) Individual-biotin run-on 2× reaction mix**

(i) Transfer a 28-µl aliquot of NRO master mix to each of four separate microcentrifuge tubes.
(ii) Add 5 µl of biotin-11-ATP (1 mM) to one of the tubes containing NRO master mix. Label this mix 'A'.
(iii) Repeat Step 4A(ii) for the remaining three biotin-11-NTPs (1 mM each) and the three remaining tubes containing NRO master mix and label them 'C', 'G' and 'U' accordingly.
(iv) Add 15 µl of DEPC-$H_2O$ to all four tubes.
(v) Add 2 µl of RNase inhibitor and 50 µl of 2% Sarkosyl to all four tubes. From Step 5, each tube will be processed as a separate sample.

**(B) 4-Biotin run-on 2× reaction mix**

(i) Transfer a 28-µl aliquot of NRO master mix to a microcentrifuge tube.
(ii) Add 5 µl each of all 4 biotin-11-NTPs (1 mM each) to the NRO master mix aliquot.
(iii) Add 2 µl of RNase inhibitor and 50 µl of 2% Sarkosyl.

**(C) 2-Biotin run-on 2× reaction mix**

(i) Transfer a 28-µl aliquot of NRO master mix to a microcentrifuge tube.
(ii) Add 5 µl each of biotin-11-CTP (1 mM) and biotin-11-UTP (1 mM) to the NRO master mix aliquot.
(iii) Add 2.5 µl each of ATP (10 mM) and GTP (10 mM) to the mix.
(iv) Add 5 µl of DEPC-$H_2O$.
(v) Add 2 µl of RNase inhibitor and 50 µl of 2% Sarkosyl.

**(D) 1-Biotin run-on 2× reaction mix**

(i) Transfer a 28-µl aliquot of NRO master mix to a microcentrifuge tube.
(ii) Add 5 µl of biotin-11-CTP (1 mM) to the NRO master mix aliquot.
(iii) Add 1 µl of CTP (0.05 mM) to the mix.
(iv) Add 2.5 µl each of ATP (10 mM), GTP (10 mM) and UTP (10 mM) to the mix.
(v) Add 6.5 µl of DEPC $H_2O$.
(vi) Add 2 µl of RNase inhibitor and pipette up and down several times.
(vii) Add 50 µl of 2% Sarkosyl and pipette up and down 15 times.

**5|** Preheat 100 µl of the appropriate 2× reaction mix prepared in Step 4 to 37 °C for mammalian cells or 30 °C for yeast and insect cells.

**6|** Using a cut-off P200 pipette tip, add 100 µl of nuclei or permeabilized cells (in storage buffer from Step 2) to 100 µl of preheated 2× reaction mix, gently but thoroughly pipette the mixture 15 times and place it in a heat block at the appropriate temperature.

▲ **CRITICAL STEP** Sarkosyl in the 2× reaction mix causes the run-on mixture to become very viscous (except for yeast). When adding the nuclei or permeabilized cells to the reaction mix and when mixing by pipetting up and down, use a wide-bore pipette tip or cut the last centimeter off a normal one with ethanol-wiped clean scissors or a razor blade.

**7|** Incubate the cells for 3 min (5 min for yeast cells), with gentle tapping at the incubation midpoint.

**RNA extraction ● TIMING 1 h**

**8|** Extract RNA using option A for nonyeast nuclei or permeabilized cells or option B for yeast.

**(A) RNA extraction from nonyeast nuclei or permeabilized cells**

(i) Add 500 µl of Trizol LS and mix it well by vortexing to stop the reaction.
(ii) Incubate the homogenized sample for 5 min at room temperature to allow the complete dissociation of nucleoprotein complexes, and add 130 µl of chloroform.
(iii) Vortex the sample vigorously for 15 s, and incubate it at room temperature for 2–3 min.
(iv) Centrifuge the sample at 14,000*g* for 5 min at 4 °C, transfer the aqueous phase to a new tube and add 1 µl of GlycoBlue.

**(B) RNA extraction from yeast cells or nuclei**

(i) Pellet cells or nuclei after the run-on reaction at 400*g* for 5 min at 4 °C, and quickly resuspend them in 500 µl of phenol.
(ii) Add an equal volume of AES buffer, and incubate the mixture at 65 °C for 5 min with periodic vortexing. Let the mixture rest on ice for 5 min, and then add 200 µl of chloroform.
(iii) Vortex the sample vigorously for 15 s, and incubate it at room temperature for 2–3 min.
(iv) Centrifuge the sample at 14,000*g* for 5 min at 4 °C, transfer the aqueous phase to a new tube and add 1 µl of GlycoBlue and NaOAc to a final concentration of 200 mM.

**9|** Add 2.5× volume of 100% room-temperature ethanol (vol/vol) and vortex it for 10 s.

**10|** Incubate the sample at room temperature for 10 min.

**11|** Centrifuge the sample at 14,000*g* for 20 min at 4 °C. The RNA precipitate forms a gel-like pellet on the side and bottom of the tube.

**12|** Remove the supernatant completely.

**13|** Add 750 µl of 75% (vol/vol) ethanol.
■ **PAUSE POINT** The RNA pellet in 75% (vol/vol) ethanol can be stored for up to a week at –80 °C.

**14|** Mix the sample by vortexing, and centrifuge it at 14,000*g* for 5 min at 4 °C.

**15|** Remove all of the supernatant.

**16|** Air-dry the RNA pellet for 5–10 min.
▲ **CRITICAL STEP** It is important not to let the RNA pellet dry completely, as this will greatly decrease its solubility.

**17|** For PRO-seq, re-dissolve the RNA pellet in 20 µl of DEPC-$H_2O$ and proceed to Step 18. For PRO-cap, re-dissolve the RNA pellet in 50 µl of DEPC-$H_2O$ and proceed to Step 23.

**RNA fragmentation by base hydrolysis (PRO-seq only)** ● **TIMING 0.5 h**
**18|** Heat-denature the RNA at 65 °C on a heat block for 40 s, and then place the tubes on ice.

**19|** Add 5 µl of ice-cold 1 N NaOH and incubate the mixture on ice for 10 min.

**20|** Add 25 µl of 1 M Tris-HCl, pH 6.8.

**21|** Perform buffer exchange once by running the 50-µl base-hydrolyzed RNA sample through a P-30 column according to the manufacturer's instructions.

**22|** Add 1 µl of RNase inhibitor.

**Biotin RNA enrichment** ● **TIMING 3 h**
**23|** Mix ~50 µl of the RNA sample from Step 22 for PRO-seq or from Step 17 for PRO-cap with 50 µl of prewashed streptavidin beads.

**24|** Incubate the bead mixture at room temperature on a rotator set at 8 r.p.m. for 20 min.

**25|** Place the mixture on a magnet for 1 min and remove the liquid.

**26|** Resuspend the beads in 500 µl of ice-cold high-salt wash buffer for a 1-min wash using a rotator.

**27|** Place the mixture on a magnet for 1 min, and remove the buffer.

**28|** Repeat Steps 26 and 27.

**29|** Wash the beads two times with 500 µl of binding buffer for a minute and use the magnet to facilitate removal of buffer.

**30|** Wash the beads once with 500 µl of low-salt wash buffer.

**31|** Resuspend the beads in 300 µl of Trizol and vortex them thoroughly.

**32|** Incubate the beads for 3 min at room temperature.

**33|** Add 60 µl of chloroform.
▲ **CRITICAL STEP** Inaccurate pipetting of chloroform leads to incomplete phase separation of the Trizol when transferring a small volume.

**34|** Vortex the beads thoroughly (for more than 20 s) and incubate the tubes for 3 min at room temperature.

**35|** Centrifuge the beads at 14,000*g* for 5 min at 4 °C.

**36|** Transfer ~180 µl of the aqueous layer to a new tube.

**37|** Remove and discard the organic phase, leaving the beads and the unpipetted aqueous phase.

**38|** Extract RNA from the beads once more by repeating Steps 31–35.

**39|** Collect ~180 µl of the aqueous layer and combine it with the sample from Step 36.

**40|** Add 360 µl of chloroform to the pooled aqueous layer and vortex the mixture.

**41|** Centrifuge the mixture at 14,000*g* for 5 min at 4 °C.

**42|** Transfer ~350 µl of the aqueous layer to a clean tube.

**43|** To the collected aqueous layer, add 1 µl of GlycoBlue and 900 µl of 100% (vol/vol) ethanol and vortex.

**44|** Incubate the samples at room temperature for 10 min and centrifuge them at 14,000*g* for 20 min at 4 °C.
**? TROUBLESHOOTING**

**45|** Add 750 µl of 75% (vol/vol) ethanol.
■ **PAUSE POINT** The RNA pellet in 75% (vol/vol) ethanol can be stored for up to a week at –80 °C.

**46|** Mix the samples by vortexing and centrifuge them at 14,000*g* for 5 min at 4 °C.

**47|** Remove all of the residual liquid.

**48|** Air-dry the RNA pellet for 5–10 min.
▲ **CRITICAL STEP** Do not re-dissolve in $H_2O$ without the RNA adaptor. The RNA pellet should be dissolved in a small volume of RNA-adaptor-containing solution to minimize the adaptor ligation reaction volume.

**3′ RNA adaptor ligation ● TIMING 4.5 h**
**49|** Dilute 0.5 µl of 100 µM 3′ RNA adaptor in 3.5 µl of DEPC-$H_2O$. For PRO-seq, use VRA3 RNA adaptor. For PRO-cap, use RA3 RNA adaptor. For processing multiple samples, scale up accordingly.

**50|** Re-dissolve the RNA pellet from Step 48 in 4 µl of the 3′ RNA adaptor dilution.

**51|** Heat-denature the mixture at 65 °C in a heat block for 20 s, and then place it on ice.

**52|** Make the RNA ligation mix as shown below. When processing multiple samples, scale up accordingly (**Supplementary Table 2**).

| Reagents | Volume per reaction (µl) | Final concentration |
|---|---|---|
| T4 RNA ligase buffer (10×) | 1 | 1× |
| ATP (10 mM) | 1 | 1 mM |
| 50% PEG | 2 | 10 % |
| RNase inhibitor | 1 | 4 units per µl |
| T4 RNA ligase I | 1 | 1 unit per µl |

**53|** Add 6 µl of the mix to the 4 µl of RNA (10 µl final).
▲ **CRITICAL STEP** GlycoBlue may form a precipitate in the presence of high PEG concentrations, but this is not reported to affect the ligation efficiency.

**54|** Incubate the mixture at 20 °C for 4 h, and then store it at 4 °C until ready to proceed to the next step.
■ **PAUSE POINT** The ligation reaction can be left at 4 °C overnight.

**Second biotin RNA enrichment ● TIMING 3 h**
**55|** Bring the volume of the adaptor-ligated RNA from Step 54 to 50 µl by adding 40 µl of DEPC-$H_2O$.

**56|** Perform a second biotin enrichment by repeating Steps 23–48 with the 50 µl of ligated RNA sample.
■ **PAUSE POINT** The RNA pellet in 75% (vol/vol) ethanol can be stored for up to a week at –80 °C.

**Enzymatic modification of the RNA 5′ ends ● TIMING 3.5–4 h**
**57|** Re-dissolve the RNA pellet from Step 56 in 5 µl of DEPC-$H_2O$.

**58|** Heat-denature the mixture briefly at 65 °C in a heat block for 20 s, and then place it on ice.

**59|** If you are performing PRO-cap, degrade the uncapped RNA containing 5′-monophosphate, and remove the 5′-triphosphate and monophosphate from uncapped RNA, as described in **Box 1** (**Supplementary Tables 3** and **4**), before proceeding to Step 60. For PRO-seq, continue directly to Step 60.

**60|** Prepare 5′ cap repair enzyme mix, depending on the availability of TAP or RppH. When using TAP, prepare the enzyme mix in the first table. When using RppH, prepare the enzyme mix in the second table. When processing multiple samples, scale up accordingly (**Supplementary Table 5**).

| Reagents | Volume per reaction (µl) | Final concentration |
|---|---|---|
| DEPC-$H_2O$ | 3 | |
| TAP buffer (10×) | 1 | 1× |
| RNase inhibitor | 0.5 | 2 units per µl |
| TAP | 0.5 | 0.5 units per µl |

| Reagents | Volume per reaction (µl) | Final concentration |
|---|---|---|
| DEPC-$H_2O$ | 2.5 | |
| ThermoPol Reaction Buffer (10×) | 1 | 1× |
| RNase inhibitor | 0.5 | 2 units per µl |
| RppH | 1 | 0.5 units per µl |

**61|** Add 5 µl of the appropriate enzyme mix to the 5 µl of RNA from Step 58 for PRO-seq or 5 µl of RNA from Step 24 of **Box 1** for PRO-cap.

**62|** Incubate the mixture at 37 °C for 1 h.

**63|** For PRO-seq, proceed to Step 64 for hydroxyl repair. For PRO-cap, add 90 µl of DEPC $H_2O$ to the 10 µl of RNA from Step 62 and proceed directly to Step 67.

**Hydroxyl repair (PRO-seq only) ● TIMING 2 h**
**64|** Prepare PNK mix as shown below. When processing multiple samples, scale up accordingly (**Supplementary Table 6**).

| Reagents | Volume per reaction (µl) | Final concentration |
|---|---|---|
| DEPC-$H_2O$ | 65 | |
| PNK buffer (10×) | 10 | 1× |
| ATP (10 mM) | 10 | 1 mM |
| RNase inhibitor | 2.5 | 1 unit per µl |
| PNK | 2.5 | 0.25 units per µl |

## Box 1 | Degradation of RNA containing 5′-monophosphate and removal of 5′-triphosphate and -monophosphate from the RNA for PRO-cap ● TIMING 4 h

1. Prepare the 5′-phosphate-dependent exonuclease enzyme mix. When processing multiple samples, scale up accordingly (**Supplementary Table 3**).

| Reagents | Volume (μl) per reaction | Final concentration |
|---|---|---|
| DEPC-$H_2O$ | 2.5 μl | |
| Buffer A (10×) | 1 μl | 1× |
| RNase inhibitor | 0.5 μl | 2 units per μl |
| 5′-Phosphate-dependent exonuclease | 1 μl | 0.1 units per μl |

2. Add 5 μl of the mix to the RNA from Step 58 of the main PROCEDURE (10 μl final).
3. Incubate the mixture at 30 °C for 1 h.
4. Add 300 μl of Trizol and vortex the mixture for 5 s.
5. Add 60 μl of chloroform, vortex the mixture for 15 s and incubate it for 2 min at room temperature.
6. Centrifuge the mixture at 14,000*g* for 5 min at 4 °C.
7. Transfer ~180 μl of the aqueous layer to a clean microcentrifuge tube.
8. Add 180 μl of chloroform to the aqueous layer from step 7 and vortex for 5 s.
9. Centrifuge the mixture at 14,000*g* for 5 min at 4 °C and collect ~180 μl of the aqueous layer.
10. Add 0.5 μl of GlycoBlue and 450 μl of 100% (vol/vol) ethanol to the aqueous layer from step 9, and pellet the RNA by centrifuging at 14,000*g* for 20 min at 4 °C.
11. Wash the RNA pellet in 75% (vol/vol) ethanol by repeating Steps 45–48 of the main PROCEDURE.

■ **PAUSE POINT** The RNA pellet in 75% (vol/vol) ethanol can be stored for up to a week at –80 °C.

12. Re-dissolve the RNA pellet in 5 μl of DEPC-$H_2O$, and heat-denature the mixture briefly in a 65 °C heat block for 20 s and then place it on ice.
13. Prepare the alkaline phosphatase enzyme mix. When processing multiple samples, scale up accordingly (**Supplementary Table 4**).

| Reagents | Volume (μl) per reaction | Final concentration |
|---|---|---|
| DEPC-$H_2O$ | 3 μl | |
| Alkaline phosphatase buffer (10×) | 1 μl | 1× |
| RNase inhibitor | 0.5 μl | 2 units per μl |
| Alkaline phosphatase | 0.5 μl | 0.5 units per μl |

14. Add 5 μl of the mix to the RNA from step 12 (10 μl final).
15. Incubate the mixture at 37 °C for 1 h.
16. Add 300 μl of Trizol and vortex the mixture for 5 s.
17. Add 60 μl of chloroform, vortex the mixture for 15 s and incubate it for 2 min at room temperature.
18. Centrifuge the mixture at 14,000*g* for 5 min at 4 °C.
19. Transfer ~180 μl of the aqueous layer to a clean microcentrifuge tube.
20. Add 180 μl of chloroform to the aqueous layer from step 19 and vortex the mixture for 5 s.
21. Centrifuge the mixture at 14,000*g* for 5 min at 4 °C and collect ~180 μl of the aqueous layer.
22. Add 0.5 μl of GlycoBlue and 450 μl of 100% (vol/vol) ethanol to the aqueous layer from step 21, and pellet the RNA by centrifuging at 14,000*g* for 20 min at 4 °C.
23. Wash the RNA pellet in 75% (vol/vol) ethanol by repeating Steps 45–48 of the main PROCEDURE.

■ **PAUSE POINT** The RNA pellet in 75% (vol/vol) ethanol can be stored for up to a week at –80 °C.

24. Re-dissolve the RNA pellet in 5 μl of DEPC-$H_2O$, heat-denature briefly in a 65 °C heat block for 20 s and then place the mixture on ice. Proceed from Step 60 of the main PROCEDURE.

**65|** Add 90 μl of the mix to the 10 μl of RNA from Step 62.

**66|** Incubate the mixture at 37 °C for 1 h.

**67|** Add 300 μl of Trizol and vortex the mixture for 5 s.

**68|** Add 60 μl of chloroform, vortex for 15 s and incubate the mixture for 2 min at room temperature.

**69|** Centrifuge the mixture at 14,000*g* for 5 min at 4 °C.

**70|** Transfer ~280 μl of the aqueous layer to a clean microcentrifuge tube.

**71|** Add 280 µl of chloroform to the aqueous layer from Step 70 and vortex for 5 s.

**72|** Centrifuge the mixture at 14,000*g* for 5 min at 4 °C, and transfer ~280 µl of the aqueous layer to a clean microcentrifuge tube.

**73|** Add 0.5 µl of GlycoBlue and 700 µl of 100% (vol/vol) ethanol to the aqueous layer from Step 72, and pellet the RNA by centrifuging at 14,000*g* for 20 min at 4 °C.

**74|** Wash the RNA pellet in 75% (vol/vol) ethanol by repeating Steps 45–48.
▲ **CRITICAL STEP** Do not re-dissolve in $H_2O$ without the RNA adaptor. The RNA pellet should be dissolved in a small volume of RNA-adaptor-containing solution to minimize the adaptor ligation reaction volume.
■ **PAUSE POINT** The RNA pellet in 75% (vol/vol) ethanol can be stored for up to a week at –80 °C.

**5′ RNA adaptor ligation ● TIMING 4.5 h**
**75|** Dilute 0.5 µl of 100 µM 5′ RNA adaptor in 3.5 µl of DEPC-$H_2O$. For PRO-seq, use VRA5 RNA adaptor. For PRO-cap, use RA5 RNA adaptor. For processing multiple samples, scale up accordingly.

**76|** Re-dissolve the RNA pellet from Step 74 in 4 µl of the 5′ RNA adaptor dilution.

**77|** Heat-denature the mixture at 65 °C in a heat block for 20 s, and then place it on ice.

**78|** Make the RNA ligation mix, as described in Step 52. When processing multiple samples, scale up accordingly (**Supplementary Table 2**).

**79|** Add 6 µl of the RNA ligation mix to the 4 µl of RNA (10 µl final).
▲ **CRITICAL STEP** GlycoBlue may form a precipitate in the presence of high PEG concentrations, but it is not reported to affect the ligation efficiency.

**80|** Incubate the mixture at 20 °C for 4 h, and then store it at 4 °C until ready to proceed to the next step.
■ **PAUSE POINT** The ligation reaction can be left at 4 °C overnight.

**Third biotin RNA enrichment ● TIMING 3 h**
**81|** Bring the volume of the adaptor-ligated RNA to 50 µl by adding 40 µl of DEPC-$H_2O$.

**82|** Perform a third biotin enrichment by repeating Steps 23–48 with the 50 µl of ligated RNA sample.
■ **PAUSE POINT** The RNA pellet in 75% ethanol (vol/vol) can be stored for up to a week at –80 °C.

**Reverse transcription ● TIMING 2 h**
**83|** Re-dissolve the RNA pellet in 10 µl of DEPC-$H_2O$.

**84|** Make reverse-transcription (RT) primer mix as shown below.

| Component | Amount per reaction (µl) | | Final concentration in 20-µl volume (µM) |
|---|---|---|---|
| | PRO-seq | PRO-cap | |
| RP1 reverse-transcription primer (100 µM) | 0.5 | – | 2.5 |
| RTP reverse-transcription primer (100 µM) | – | 0.5 | 2.5 |
| 12.5 mM dNTP mix | 1 | 1 | 625 |
| DEPC-$H_2O$ | 1 | 1 | |

**85|** Add 2.5 µl of the RT primer mix to the 10 µl of re-dissolved RNA.

**86|** Heat the mixture to 70 °C for 2 min, chill it on ice for 2 min and briefly spin at 500–1,000*g* at 25 °C for 5 s.

**87|** Prepare the RT buffer mix as shown below. When processing multiple samples, scale up accordingly (**Supplementary Table 7**).

| Reagents | Volume per reaction (µl) | Final concentration |
|---|---|---|
| First-strand buffer (5×) | 4 | |
| DTT (0.1 M) | 1 | 5 mM |
| RNase inhibitor | 1 | 2 units per µl |

**88|** Add 6 µl of the RT buffer mix to the 12.5 ìl of RNA-primer mix from Step 86.

**89|** Incubate the mixture for 5 min at 37 °C.

**90|** Add 1.5 µl of Superscript III RT enzyme and mix (total 20 µl).

**91|** Incubate the mixture for 15 min at 45 °C, and then for 40 min at 50 °C, 10 min at 55 °C and 15 min at 70 °C.

**92|** Add 6 µl of DEPC-$H_2O$ to the RT reaction (total 26 µl).
■ **PAUSE POINT** The reverse-transcribed cDNA can be stored for a month at –20 °C.

**Test PCR amplification** ● **TIMING 2 h**

**93|** Prepare a series of fourfold dilutions of the RT sample in $H_2O$, as shown below. Test PCR amplification (a total of 21 amplification cycles) of these dilutions will be used to determine the appropriate number of PCR cycles to use in full-scale amplification at Step 105. The use of 2 µl of RT sample in dilution 1 leaves 24 µl of RT sample for full-scale amplification at Step 105.

| Dilution | Amount of cDNA | Amount of $H_2O$ (µl) | Equivalent full-scale PCR cycles (Step 105) |
|---|---|---|---|
| 1 | 2 µl RT sample | 6 | 17 |
| 2 | 2 µl Dilution 1 | 6 | 15 |
| 3 | 2 µl Dilution 2 | 6 | 13 |
| 4 | 2 µl Dilution 3 | 6 | 11 |

▲ **CRITICAL STEP** As only 6 µl out of the total 8 µl of dilution 1 is used for PCR, this is equivalent to using 1.5 µl (6/8 × 2) of the original 26-µl RT sample, which is 16-fold less than the remaining 24-µl RT sample. Thus, to account for the 16-fold-higher amount of starting material in the full-scale amplification, the number of PCR cycles needs to be reduced by four compared with the test PCR of dilution 1—i.e., 17 cycles instead of 21. Each remaining dilution in the fourfold dilution series will need to be corrected by a further two cycles compared with the previous dilution—i.e., 15, 13 and 11 cycles for dilutions 2–4, respectively.

**94|** Prepare a test PCR mix. One test PCR amplification will be performed for each dilution prepared in Step 93 (**Supplementary Table 8**).

| Reagents | Volume per reaction (µl) | Final concentration |
|---|---|---|
| DEPC-$H_2O$ | 5 | |
| HF buffer (5×) | 4 | 1× |
| Betaine (5 M) | 4 | 1 M |
| dNTP mix (12.5 mM each) | 0.4 | 250 µM each |
| RP1 primer (25 µM) | 0.2 | 250 nM |
| RPI-1 primer (25 µM) | 0.2 | 250 nM |
| Phusion DNA polymerase | 0.2 | 0.02 units per µl |

**95|** Add 14 µl of the PCR mix to 6 µl of each diluted test sample (dilutions 1–4).

**96|** Use the following thermal cycling to perform test PCR amplification (a total of 21 amplification cycles).

| Cycle number | Denature (95 °C) | Anneal | Extend (72 °C) |
|---|---|---|---|
| 1 | 2 min | | |
| 2–6 | 30 s | 56 °C for 30 s | 30 s |
| 7–22 | 30 s | 65 °C for 30 s | 30 s |
| 23 | | | 10 min |

**Gel analysis of test PCR ● TIMING 2 h**

**97|** Add 2.2 µl of 10× Orange G dye to the 20-µl PCR reactions, and load 20 µl of the samples onto a 2.2% agarose gel in 1× TAE.

**98|** Load 8 µl of 100-bp DNA ladder on a separate lane.

**99|** Run the gel at 100 V for 15 min and then run it at 130 V for up to 45 min.
▲ **CRITICAL STEP** Orange G dye runs at 50 bp. Stop the electrophoresis before the dye runs out of the gel.

**100|** Add 15 µl of SYBR Gold to 150 ml of 1× TAE. Place the gel in this solution and stain it for 30 min on a shaker.

**101|** Image the gel with 485-nm illumination or with UV light. Examine the gel and determine the dilution (and therefore the equivalent full-scale PCR amplification cycle) with desired amplification characteristics (sufficient amount of product, not overamplified and having 50–75% of unused primers). For example, if the lane in the agarose gel with dilution 3 has the desired amplification characteristics, then the optimal number of PCR cycles (optimized cycle, OC) for full-scale amplification is 13 (Step 93). See **Figure 2a**,**b** for an example gel image.
**? TROUBLESHOOTING**

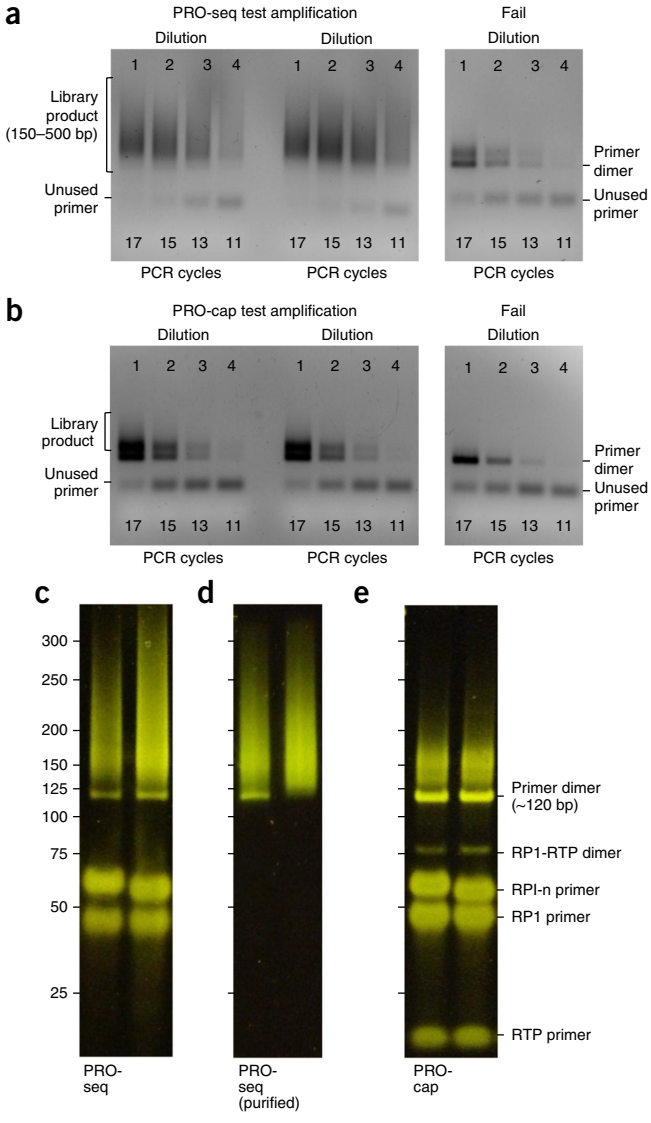


**Figure 2** | Gel images of library products. (**a**) PRO-seq libraries at the test amplification stage (Step 101). Three different samples are loaded on the agarose gel, each at four dilutions. From the left of each series, the four dilutions are equivalent to 17, 15, 13 and 11 cycles of full amplification. The gel image in the left panel shows two successful PRO-seq libraries. Optimal amplification cycles are determined by comparing both the intensities of library products and the unused primers. The optimal amplification cycle is reached when 50–75% of the primers remain unused (13 cycles for this example). A gel image of a failed library preparation is shown in the right-hand panel; only primer dimers are detected. (**b**) PRO-cap libraries at the test amplification stage (Step 101). Library products are smaller in size and amount because most PRO-cap RNA molecules are from paused RNA polymerase. Fifteen cycles were optimal for these samples. (**c**) Two PRO-seq libraries after full amplification, analyzed by 8% PAGE (Step 118). Labels on the left indicate DNA sizes in base pairs. Note the presence of unused primers ~50 bp and primer-dimer bands ~120 bp. The library product is the smear above the primer-dimer band. (**d**) Two PRO-seq libraries after the size selection, analyzed by 8% PAGE (Step 119). The PRO-seq library on the left still has some residual primer dimer. (**e**) Two PRO-cap libraries after the full amplification, analyzed by 8% PAGE (Step 118). Note that there are additional primer and primer-dimer bands compared with PRO-seq.

**Full-scale PCR amplification** ● **TIMING 2.5 h**

**102|** Prepare a full-scale amplification PCR mix according to the table below. For processing multiple samples, scale up accordingly (**Supplementary Table 9**).

| Reagents | Volume per reaction (μl) | Final concentration |
|---|---|---|
| DEPC-$H_2O$ | 3 | |
| HF buffer (5×) | 10 | 1× |
| Betaine (5 M) | 10 | 1 M |
| dNTP mix (12.5 mM each) | 1 | 250 μM each |
| RP1 primer (25 μM) | 0.5 | 250 nM |
| Phusion DNA polymerase | 1 | 0.04 units per μl |

**103|** Add 25.5 μl of the PCR mix to the 24 μl of the remaining RT product (from Step 93).

**104|** Add 0.5 μl of a different RPI-n (25 μM) primer to the different libraries so that each library is differentially barcoded.
▲ **CRITICAL STEP** Different barcodes are needed only if the libraries are to be pooled for sequencing.

**105|** Use the following thermal cycling for full-scale PCR amplification as determined in Step 101.

| Cycle number | Denature (95 °C) | Anneal | Extend (72 °C) |
|---|---|---|---|
| 1 | 2 min | | |
| 2–6 | 30 s | 56 °C for 30 s | 30 s |
| 7 (OC + 1) | 30 s | 65 °C for 30 s | 30 s |
| (OC + 1)–(OC + 2) | | | 10 min |

■ **PAUSE POINT** The PCR product can be stored for up to a month at –20 °C.

**106|** Add 231 μl of $H_2O$, 18 μl of 5 M NaCl and 1 μl of GlycoBlue to the 50 μl of full-amplification PCR product.

**107|** Add 750 μl of 100% ethanol (vol/vol) and vortex thoroughly.

**108|** Centrifuge the mixture at 14,000*g* for 20 min at 4 °C.

**109|** Remove the liquid and the wash the pellet once in 75% (vol/vol) ethanol by repeating Steps 45–48.
■ **PAUSE POINT** The DNA pellet in 75% (vol/vol) ethanol can be stored for up to several months at –80 °C.

**110|** Re-dissolve the DNA pellet in 18 μl of $H_2O$.

**Library size selection by PAGE** ● **TIMING 5 h to 1 d**

▲ **CRITICAL** We describe PAGE purification for size selection of the library. However, a Pippin Prep (Sage Science) can also be used in place of Steps 111–130; follow the manufacturer's instruction for selecting a size range of 140–350 bp.

**111|** Add 2 μl of 10× Orange G loading dye to 18 μl of DNA from Step 110.

**112|** Prepare a medium-size (10-cm running length) native PAGE gel as shown below.

| Reagents | Volume | Final concentration |
|---|---|---|
| DEPC-$H_2O$ | 31.67 ml | |
| Acrylamide (30%) | 13.3 ml | 8% |
| TBE (5×) | 5 ml | 0.5× |
| APS (10%) | 500 μl | 0.1% |
| TEMED | 50 μl | |

**113|** Prerun the gel for 15 min at a constant current of 30 mA.

**114|** Load the samples. Also load 2 µl of 10-bp DNA ladder and 8 µl of 100-bp DNA ladder.

**115|** Run the gel at 15 mA for 30 min until the DNA has entered the gel, and then run it at 30 mA for 1.5 h. Stop electrophoresis 10 min after the Orange G dye has run off the gel.

**116|** During the electrophoresis, puncture the bottom of a sterile, nuclease-free, 0.5-ml centrifuge tube using a 21-gauge needle (heated in a Bunsen flame) to create a hole or several holes in the bottom of the tube. Place the 0.5-ml microtube into a sterile, round-bottom, nuclease-free, 2-ml microtube.

**117|** After the electrophoresis, pry apart the gel cassette and stain the gel with SYBR Gold (10 µl of SYBR Gold per 100 ml of 1× TBE buffer) in a clean container for 5–10 min.

**118|** Visualize the gel on a Dark Reader transilluminator.

**119|** Using a clean scalpel or razor, cut the gel from 140 bp (20 bp just above the 120-bp adaptor dimer) up to 350 bp (**Fig. 2c**–**e**).

**120|** Split the gel fragment vertically, and place the pieces into the 0.5-ml microtube.

**121|** Centrifuge the stacked tubes at 10,000*g* for 2 min at room temperature to shred the gel through the holes into the 2-ml tube (there is no liquid at this point).

**122|** If some gel remains in the top tube, add 100 µl of gel elution buffer and spin it at 10,000*g* again for another 2 min.

**123|** Add 600 µl of gel elution buffer and incubate for 2 h in a rotating incubator at 37 °C.
■ **PAUSE POINT** The elution can continue overnight.

**124|** Spin down the gel pieces for 1 min at max speed in a benchtop centrifuge.

**125|** Transfer all liquid possible to a new tube.

**126|** Add 400 µl of gel elution buffer to the remaining gel pieces.

**127|** Incubate the mixture for 1 h in a rotating incubator at 37 °C.

**128|** After 1 h incubation, spin at the maximum speed in a benchtop centrifuge for 1 min; take the supernatant and pool it with the first elution from Step 125.

**129|** Rinse the gel pieces with 250 µl of $H_2O$; spin and add the rinsed liquid to the pool.

**130|** Transfer the pooled eluate, which may contain small pieces of gel debris, to the top of a Spin-X Filter.

**131|** Centrifuge the filter for 1–2 min at 6,000–7,000*g* at room temperature. Collect the filtrate. If the volume exceeds the filter capacity, use multiple filters or split into batches and repeat filtering a couple of times, pooling the filtrates.

**132|** Lyophilize (on medium setting) the sample using a SpeedVac dryer and reduce the volume to ~400 µl (takes 45 min–2 h). If the volume decreases below 400 µl, bring the volume up to 400 µl by adding DEPC-$H_2O$.

**133|** Add an equal volume of buffered phenol:chloroform, and vortex the mixture thoroughly.

**134|** Centrifuge the mixture at 14,000*g* for 5 min at 4 °C.

**135|** Collect the aqueous layer in a clean tube.

**136|** Add an equal volume of chloroform to the aqueous layer and vortex the mixture thoroughly.

**137|** Centrifuge the mixture at 14,000*g* for 5 min at 4 °C.

**138|** Collect the aqueous layer in a clean tube.

**139|** Add 1 µl of GlycoBlue to the aqueous layer.

**140|** Add 2.5× volume of room-temperature 100% (vol/vol) ethanol.

**141|** Vortex thoroughly and incubate the mixture at room temperature for 10 min.

**142|** Centrifuge the mixture at 14,000*g* for 20 min at 4 °C.

**143|** Remove the liquid, and wash the DNA pellet once in 75% (vol/vol) ethanol by repeating Steps 45–48.

**144|** Re-dissolve the pellet in 12 µl of $H_2O$.

**145|** Use 2 µl of the library DNA for quantification using Qubit or Bioanalyzer. The expected concentration of the library is between 1 and 20 ng/µl.

**146|** If required, dilute the samples to 5 ng/µl. Send ~10 ng to the sequencing facility for sequencing. If the libraries are barcoded, pool the barcoded libraries that are to be sequenced simultaneously.

**High-throughput sequencing ● TIMING 24 h**

**147|** Sequence pooled PRO-seq or PRO-cap libraries using an Illumina TRU-seq-compatible sequencing platform. A sequencing depth of ~20 million and ~50 million reads provides good coverage for insect cells and mammalian cells, respectively.

**Data analysis ● TIMING variable**

▲ **CRITICAL** In PRO-seq, the 3′ end of the nascent RNA corresponds to the genomic position of the RNA polymerase active site. The modified RNA adaptors were designed to enable sequencing of the reverse complement of nascent RNAs. Therefore, the 3′ end of the reverse complement of the sequencing reads reflects the RNA polymerase active site position. In PRO-cap, conventional RNA adaptors are used, and the 5′ ends of each sequence read reflect the TSSs in the same direction. Below, we outline the three major stages of a simple processing pipeline.

**148|** *Pre-process the raw sequence data*. Filter out low-quality reads and trim potential adaptor sequences (TGGAATTCTCGGGTGCCAAGG) from the sequence reads. Tools such as 'cutadapt' are publicly available for this purpose. Depending on the quality of the library, sequences containing only the adaptor sequences (adaptor dimers) may take up to 5% of total reads.

**149|** *Map or align the sequence reads to the genomic sequence*. As most of the nascent RNA reads are captured before RNA processing and splicing, they do not contain large gaps in alignment. Therefore, many alignment programs based on the Burrows–Wheeler transformation algorithm—such as 'bwa' or 'bowtie'—work well. Reads with multiple alignments are usually discarded, unless they are used for studying specific target regions that are repeated more than once. Sometimes reads aligning to the ribosomal DNA sequence can be prefiltered, as they can account for 30–40% of all the transcriptional activity. On average, ~55–70% of the raw sequence reads are aligned uniquely to the genome. The alignment results are commonly stored in 'sam' or 'bam' formats.

**150|** *Generate the coverage of the aligned sequence reads*. A common way to do this using publicly available tools is as follows: first, sort the 'bam' file using 'samtools sort'; then process the sorted 'bam' file using 'bedtools genomecov' with '-ibam' (use bam file input), '-strand' (strand-specific coverage) and '-5' (5′ position coverage)' options. For the PRO-seq data, swap the plus and minus strand data for the correct orientation. These data can be visualized in genome browsers (**Fig. 3**) and used in further downstream analyses.

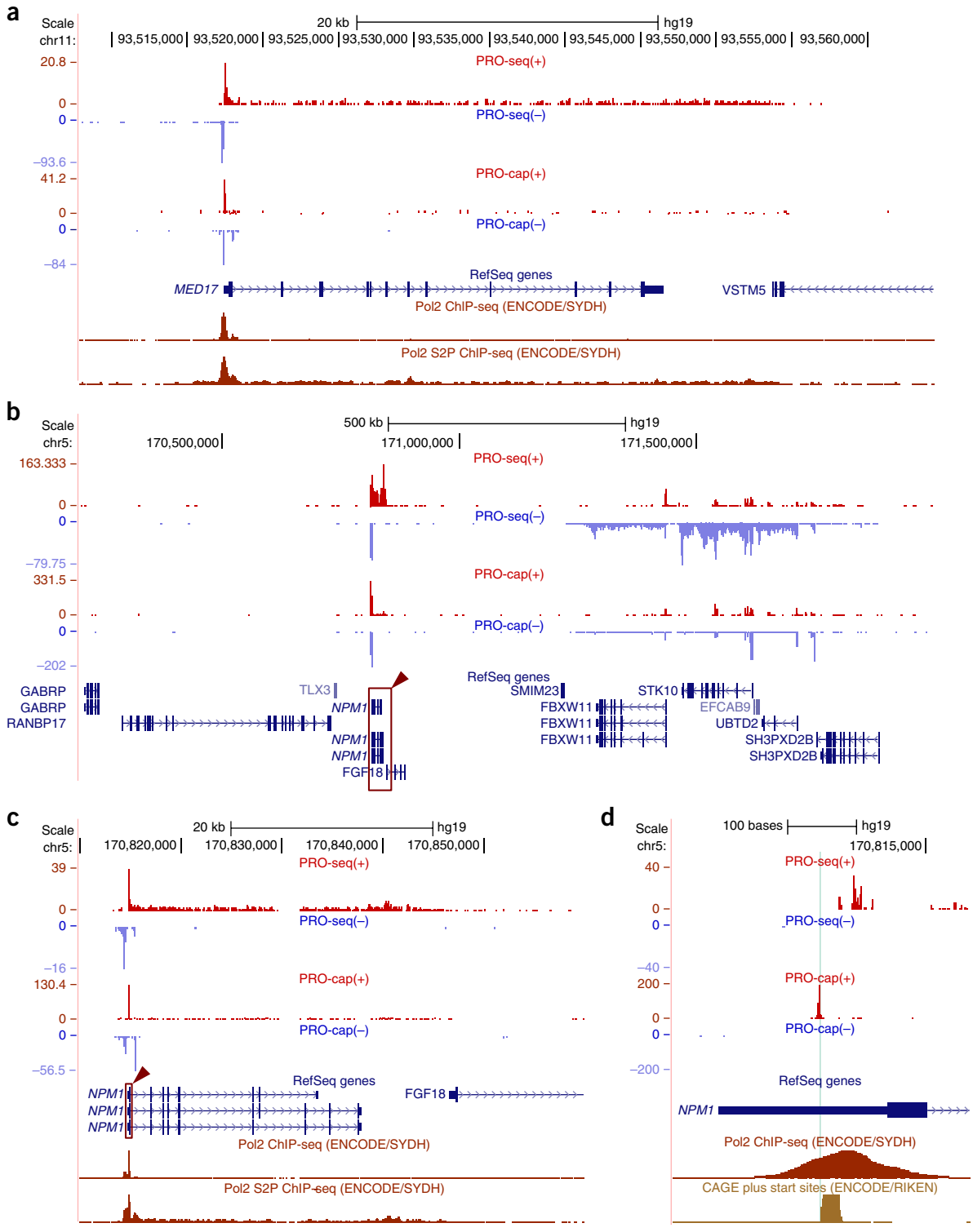


**Figure 3** | Genome browser examples of PRO-seq and PRO-cap results. (**a**) Sample of PRO-seq and PRO-cap data viewed on the University of California at Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu). A region on chromosome 11 encompassing the *MED17* gene showing PRO-seq (top), PRO-cap (middle) and Pol II ChIP-seq and serine-2 phosphorylated Pol II (Pol II S2P) ChIP-seq (bottom) reads aligned to the human genome (hg19). PRO-seq and PRO-cap reads on the plus strand (left to right), red; PRO-seq and PRO-cap reads on the minus strand (right to left), light blue; RefSeq gene annotations, dark blue; and ChIP-seq reads, dark red. The Pol II and Pol II S2P ChIP-seq tracks are from the ENCODE public data on GM12878 cells using 8WG16 and ab5095 antibodies, respectively. *y* axis represents raw read counts displayed on the default setting of the UCSC genome browser. (**b**) Sample of a PRO-seq and PRO-cap data view on the UCSC genome browser. A region on chromosome 5 encompassing the *NPM1* gene showing PRO-seq (top) and PRO-cap (bottom) reads aligned to the human genome (hg19). Red boxes with arrowheads mark the region magnified in subsequent panels. (**c**) PRO-seq (top), PRO-cap (middle) and Pol II ChIP-seq and Pol II S2P ChIP-seq (bottom) data across the gene body of the *NPM1* gene. (**d**) A close-up view around the annotated TSS of *NPM1* gene. ENCODE RIKEN CAGE data (bottom) is shown to illustrate the position of the mRNA cap site relative to the PRO-seq, PRO-cap and Pol II ChIP-seq data around the annotated TSS of the *NPM1* gene.

## ? TROUBLESHOOTING

Troubleshooting advice is provided in **Table 2**.

**TABLE 2** | Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 44 | Small or no pellet | Incomplete precipitation | Re-centrifuge using higher speed<br>If a pellet is still not recovered, add 0.4× volume of isopropanol and re-centrifuge |
| | | Incomplete separation of the organic phase | Perform an additional chloroform extraction after Step 42 |
| | Blue jelly ball-like pellet | Incomplete separation of the organic phase | Re-dissolve the pellet in 100 µl of DEPC-$H_2O$ and extract RNA once more using Trizol LS<br>If the problem persists, add additional chloroform extraction after Step 42 |
| 101 | No library product or the library product amplifies at later cycles | Insufficient amount or quality of the nuclei sample | Adjust the nuclei amount and monitor the quality. A typical batch of active nuclei can incorporate 1–5% of total radioactive CTP [$\alpha$-$^{32}$P] under the nuclear run-on condition |
| | | RNA degradation | Replace all reagents using RNA-grade materials and carry out the steps at 4 °C |
| | | Incomplete RNA extraction during biotin RNA enrichment | Resuspend the bead thoroughly in Step 31<br>Add additional RNA extraction by repeating Step 38 |
| | | Inefficient RNA-modifying enzymes | Monitor enzyme activities according to the manufacturers' instructions |

## ● TIMING

Step 1, cell culture: 24 h
Step 2, sample preparation: 1 h
Steps 3–7, nuclear run-on: 1.5 h
Steps 8–17, RNA extraction: 1 h
Steps 18–22, RNA fragmentation by base hydrolysis (PRO-seq only): 0.5 h
Steps 23–48, biotin RNA enrichment: 3 h
Steps 49–54, 3′ RNA adaptor ligation: 4.5 h (0.5 h hands-on and 4 h incubation; can be stored overnight (suggested end of the first day))
Steps 55 and 56, second biotin RNA enrichment: 3 h
Steps 57–63, enzymatic modification of the RNA 5′ ends: 3.5–4 h
Steps 64–74, hydroxyl repair (PRO-seq only): 2 h
Steps 75–80, 5′ RNA adaptor ligation: 4.5 h (0.5 h hands-on and 4 h incubation; can be stored overnight (suggested end of the second day))
Steps 81 and 82, third biotin RNA enrichment: 3 h
Steps 83–92, reverse transcription: 2 h
Steps 93–96, test PCR amplification: 2 h
Steps 97–101, gel analysis of test PCR: 2 h (suggested end of the third day)
Steps 102–110, full-scale PCR amplification: 2.5 h
Steps 111–146, library size selection by PAGE: 5 h to 1 d
Step 147, high-throughput sequencing: 24 h
Steps 148–150, data analysis: variable
**Box 1**, degradation of RNA containing 5′-monophosphate and removal of 5′-triphosphate and monophosphate from the RNA for PRO-cap: 4 h

## ANTICIPATED RESULTS

The final product of the PRO-seq or PRO-cap method is genome-wide maps of RNA polymerase active sites or TSSs, respectively (**Fig. 3**). In general, the PRO-seq profile of a gene exhibits several features, such as higher read density at the promoter-proximal pause site as compared with the gene body representing paused Pol IIs; uniform read density across exons and introns; reads beyond the polyadenylation site; and divergent PRO-seq reads at the promoter of mammalian genes, indicating divergent transcription. The enhancer regions, which are present in intergenic and intragenic regions, are also characterized by divergent PRO-seq reads. In PRO-cap, read density is very high at TSSs and very low, almost at background levels, in gene bodies.

An indication of whether library preparation has been successful can be obtained at the test amplification stage (Steps 93–101) through estimates of yield and quality (**Fig. 2**). Spiky PRO-seq read coverage along the gene body indicates lower library complexity, which may arise from the use of too few nuclei or permeabilized cells or from the use of too many PCR amplification cycles. Libraries that require fewer amplification cycles (<13 cycles) provide high-quality results, and those requiring between 14 and 18 cycles provide meaningful results, but have some potential for amplification biases. These amplification biases manifest as low library complexity in general and high repetition of certain sequence reads.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

**ACKNOWLEDGMENTS** We thank all the present and former members of the Lis lab who provided advice and support for this work. We especially thank N. Fuda, a former member of our lab, for help at many steps of this protocol. Research reported in this publication was supported by National Institutes of Health (NIH) grant R01GM25232 to J.T.L. and European Research Council Advanced Grant ERCadv-671274 to I.H.J. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

**AUTHOR CONTRIBUTIONS** H.K., L.J.C. and J.T.L. conceived the method and designed the experiments. H.K. and D.B.M. carried out the experiments that generated the data. G.T.B., I.H.J., R.K.P., C.T.W., K.M. and L.J.C. carried out experiments that optimized the protocol. C.G.D. contributed in generating the pipelines for the computational analysis. H.K., D.B.M. and J.T.L. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS** The authors declare no competing financial interests.



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