

# Genome-wide Mapping of 5'-monophosphorylated Ends of Mammalian Nascent RNA Transcripts

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## Abstract

In eukaryotic cells, RNA biogenesis generally requires processing of the nascent transcript as it is being synthesized by RNA polymerase. These processing events include endonucleolytic cleavage, exonucleolytic trimming, and splicing of the growing nascent transcript. Endonucleolytic cleavage events that generate an exposed 5'-monophosphorylated (5'-PO<sub>4</sub>) end on the growing nascent transcript occur in the maturation of rRNAs, tRNAs, and mRNAs. These 5'-PO<sub>4</sub> ends can be a target of further processing or be subjected to 5'-3' exonucleolytic digestion that may result in termination of transcription. Here, we describe how to identify 5'-PO<sub>4</sub> ends of intermediates in nascent RNA metabolism. We capture these species via metabolic labeling with bromouridine followed by immunoprecipitation and specific ligation of 5'-PO<sub>4</sub> RNA ends with the 3'-hydroxyl group of a 5' adaptor (5'-PO<sub>4</sub> Bru-Seq) using RNA ligase I. These ligation events are localized at single nucleotide resolution via high-throughput sequencing, which identifies the position of 5'-PO<sub>4</sub> groups precisely. This protocol successfully detects the 5'-monophosphorylated ends of RNA processing intermediates during production of mature ribosomal, transfer, and micro RNAs. When combined with inhibition of the nuclear 5'-3' exonuclease Xrn2, 5'-PO<sub>4</sub> Bru-Seq maps the 5' splice sites of debranched introns and mRNA and tRNA 3' end processing sites cleaved by CPSF73 and RNaseZ, respectively.

## Key features

- Metabolic labeling for brief periods with bromouridine focuses the analysis of 5'-PO<sub>4</sub> RNA ends on the population of nascent transcripts that are actively transcribed.
- Detects 5'-PO<sub>4</sub> RNA ends on nascent transcripts produced by all RNA polymerases.
- Detects 5'-PO<sub>4</sub> RNA ends at single nucleotide resolution.

**Keywords:** 5'-PO<sub>4</sub> Bru-Seq, 5'-monophosphorylated ends, Nascent transcripts, RNA, Bromouridine labeling, Immunoprecipitation

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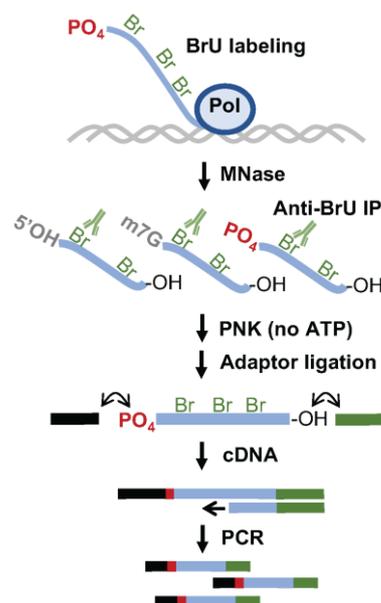
## Background

Tracking genome-wide active transcription and its regulation has been made possible by several complementary approaches (Wissink et al., 2019). These include isolation of chromatin-associated RNAs (Bhatt et al., 2012, Mayer et al., 2015, Weber et al., 2014), RNA polymerase-associated RNAs (Churchman and Weissman, 2011, Nojima et al., 2015, Fong et al., 2017), or nascent transcripts pulse-labeled with nucleoside analogs such as 4-thiouridine (Schwalb et al., 2016, Kenzelmann et al., 2007, Rabani et al., 2011, Herzog et al., 2017, Muhar et al., 2018, Schofield et al., 2018), 5-ethynyluridine (Jao and Salic, 2008), or bromouridine (Paulsen et al., 2014).

In eukaryotic cells, endonucleolytic cleavage of the nascent transcript is used to release fully transcribed RNAs from chromatin or to release small RNAs from longer precursors during transcription [i.e., micro RNAs (miRNAs) and intron-encoded small nucleolar RNAs (snoRNAs)]. Most of these cleavage events are carried out by nucleases that leave 5'-phosphate (5'-PO<sub>4</sub>) and 3' OH ends, including CPSF73, Int11, RNaseP, RNaseZ, and Drosha.

Recently, POINT-5 technology identified 5' ends generated at cleavage sites of RNA pol II-associated transcripts from runoff products of 5' RACE in reverse transcription reactions (Sousa-Luis et al., 2021), but this approach does not inform on the identity of the chemical group at these 5' RNA ends. Previously, methods have been developed to specifically map 5'-PO<sub>4</sub> RNA ends by virtue of their ability to be ligated to adaptors by RNA ligase I (Harigaya and Parker, 2012, German et al., 2008).

Here, we describe 5'-PO<sub>4</sub> Bru-Seq for direct detection of 5'-PO<sub>4</sub> groups in nascent transcripts produced by all RNA polymerases in the cell (Figure 1). The method was previously validated and used to identify targets of the Xrn2 exonuclease (Cortazar et al., 2022). In this method, total RNA is fragmented with Micrococcal Nuclease (MNase) that produces 5' OH and 3' PO<sub>4</sub> ends, and nascent transcripts are enriched by immunoprecipitation of 5-bromouridine pulse-labeled molecules, followed by repair of 3' phosphates with T4 polynucleotide kinase, ligation of 5' and 3' adaptors, and PCR amplification of sequencing libraries. Only RNA fragments with native 5'-PO<sub>4</sub> ends can be incorporated into these libraries (Harigaya and Parker, 2012). Unique molecular identifiers (UMIs) are included to allow removal of PCR duplicates. The molecular events that result in 5'-PO<sub>4</sub> groups on nascent RNA ends detected by this method potentially include cleavage by endonucleases, intron debranching, and decapping.



**Figure 1. The 5'-PO<sub>4</sub> Bru-Seq protocol.** 5'-PO<sub>4</sub> Bru-Seq enriches transcripts being extended by actively transcribing polymerases via metabolic labeling with bromouridine, fragmentation by micrococcal nuclease (MNase), and immunoprecipitation. The 3'-ends generated by MNase cleavage events are repaired by T4 polynucleotide kinase (PNK) in the absence of ATP. 5'-PO<sub>4</sub> ends of nascent transcripts are then ligated to the 3'-hydroxyl group of a 5' adaptor, amplified by PCR, and deep sequenced using Illumina sequencing technology.

## Materials and reagents

1. Mammalian cell type of interest (e.g., HEK293, ATCC, catalog number: CRL-1573)
2. Cell culture media supplemented with 10% serum (e.g., DMEM supplemented with 10% FBS; DMEM, Thermo Fisher Scientific, catalog number: 11995040; FBS, VWR, catalog number: 97068-085)
3. 10 cm tissue culture dishes (e.g., Genesee Scientific, catalog number: 25-200)
4. 5/10 mL sterile serological pipets (e.g., Genesee Scientific, catalog numbers: 12-102, 12-104)
5. 15/50 mL conical bottom centrifuge tubes (e.g., Corning, catalog numbers: 05-538-59A, 05-526B)
6. 1.7 mL microcentrifuge tubes (e.g., Genesee Scientific, catalog number: 22-282)
7. TRIzol reagent (Thermo Fisher Scientific, catalog number: 15596018)
8. Chloroform (Fisher Scientific, catalog number: BP1145-1)
9. Isopropanol (Fisher Scientific, catalog number: BP2618-1)
10. 80% ethanol, molecular biology grade (e.g., Thermo Fisher Scientific, catalog number: T08204K7)
11. GeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific, catalog number: FERSM1333)
12. 5 M NaCl, RNase-free (Thermo Fisher Scientific, catalog number: AM9760G)
13. Micrococcal nuclease (MNase) (2,000,000 gel units/mL) (New England Biolabs, catalog number: M0247S)
14. 10× MNase reaction buffer (New England Biolabs, catalog number: B0247S)
15. 0.5 M EGTA, pH 8.0, DNase and RNase Free (Thermo Fisher Scientific, catalog number: NC1874048)
16. Nuclease-free water (Thermo Fisher Scientific, catalog number: AM9939)
17. 10× phosphate-buffered saline (PBS), pH 7.4, RNase-free (Thermo Fisher Scientific, catalog number: AM9624)
18. 5-bromouridine (Sigma-Aldrich, catalog number: 850187)
19. Triton X-100 (Sigma-Aldrich, catalog number: X100)
20. 1 M DTT (Thermo Fisher Scientific, Catalog number: P2325)
21. Superase-In™ RNase inhibitor (Invitrogen, AM2696)
22. Protein G magnetic beads (Pierce Thermo Scientific, catalog number: 88848)
23. Purified mouse anti-BrdU antibody (clone 3D4, RUO) (BD Pharmingen, catalog number: 555627)
24. Qubit™ RNA High Sensitivity Assay kit (Thermo Fisher Scientific, catalog number: Q32852)
25. Invitrogen™ Qubit™ assay tubes (Thermo Fisher Scientific, catalog number: Q32856)
26. T4 polynucleotide kinase (Thermo Fisher Scientific, catalog number: EK0031)
27. 10× reaction buffer A (Thermo Fisher Scientific, catalog number: EK0031)
28. RNA Clean & Concentrator™-5 kit (ZYMO RESEARCH, catalog number: R1013, R1014)
29. QIAseq miRNA library kit (Qiagen, catalog number: 331502)
30. QIAseq miRNA Index kit IL UDI (e.g., UDI-B-96, Qiagen, catalog number: 331625)
31. 10× TBE Buffer (Thermo Fisher Scientific, catalog number: AM9863)
32. 1:20 dilution of MNase (100,000 gel units/mL) (see Recipes)
33. 50 mM 5-bromouridine (see Recipes)
34. IP buffer (see Recipes)

## Software

1. Removal of UMI duplicates: UMI-tools (Smith et al., 2017)
2. Removal of adaptor sequences: Cutadapt (Martin, 2011)
3. Genomic alignment of sequencing reads: Bowtie2 (Langmead and Salzberg, 2012)
4. BAM file processing: Samtools (Li et al., 2009)
5. Integrative Genomics Viewer (IGV) (Robinson et al., 2011)
6. Conversion of BAM files to BED files: bedtools (Quinlan and Hall, 2010)
7. Conversion of bedGraph files to BigWig: bedGraphToBigWig (Kent et al., 2010)

## Equipment

1. 1.5 mL microcentrifuge magnet stand (e.g., Thermo Fisher Scientific, catalog number: 12321D)
2. Centrifuge 5430 R (e.g., Eppendorf, catalog number: 022620601)
3. Thermal cycler (e.g., Bio-Rad, model: C1000 Touch, catalog number: 1851148)
4. Qubit fluorometer (Thermo Fisher Scientific, catalog number: Q33238)

## Procedure

### A. Labeling of nascent transcripts

The following steps achieve labeling of nascent transcripts with bromouridine to enable isolation of these transcripts via immunoprecipitation in Section D. 5-bromouridine is incorporated by all RNA polymerases actively transcribing during incubation of cells with 5-bromouridine.

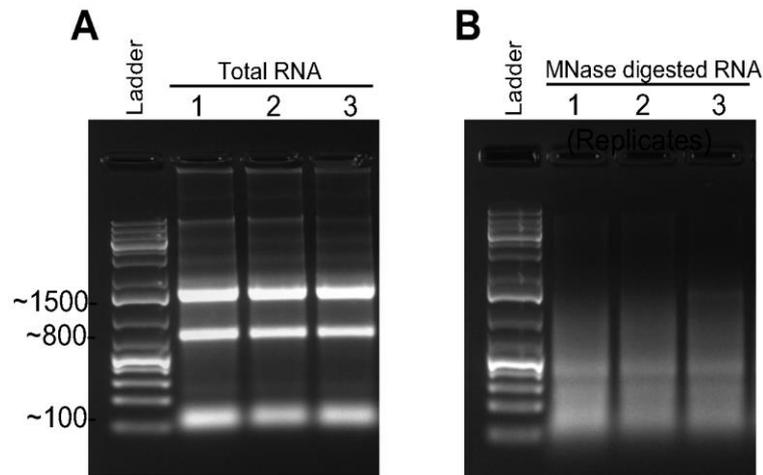
1. Grow cells in 10 cm culture plates in a total of 10 mL of culture medium. For section C, 250 µg of total RNA is required. Scale the number of plates according to the type of cell line and yield from the protocol of total RNA extraction. For HEK293 cells, use a total of 3 × 10 cm plates or a single 15 cm plate per condition (~20 × 10<sup>6</sup> cells).
2. When the cell population has reached approximately 70% confluency, remove the cell medium and replace it with 10 mL of fresh cell medium containing 400 µL of 50 mM 5-bromouridine (2 mM 5-bromouridine final concentration). If using a 15 cm plate, scale up accordingly by using 20 mL of fresh cell medium containing 800 µL of 50 mM 5-bromouridine.
3. Place plate with cells in a humidifying incubator at 37 °C and 5% CO<sub>2</sub> for 30 min (see Note 1).
4. Obtain a *no-bromouridine* negative control sample to rule out unspecific enrichment of RNA after immunoprecipitation. Perform the labeling protocol with the number of plates used per condition, excluding 5-bromouridine from the fresh cell medium in step A2.
5. Remove cell medium, add 1 mL of TRIzol reagent to dissociate cells from the culture plate, and transfer to a 1.5 mL microcentrifuge tube. If using a 15 cm plate, harvest in at least 3 mL of TRIzol reagent.
6. Pipette the lysate up and down to homogenize.

The following sections should be performed with special care to not contaminate RNA samples, which could result in RNA degradation by RNases. Use RNase-free certified plasticware and filter tips.

### B. Total RNA extraction

1. Add 0.2 mL of chloroform per 1 mL of TRIzol lysate, securely cap the tube, and thoroughly mix by shaking for ~30 s.
2. Centrifuge the sample at 12,000× *g* for 15 min at 4 °C.
3. Transfer the aqueous phase containing the RNA to a new 1.5 mL microcentrifuge tube by angling the tube at 45° and pipetting the solution out.
4. Add an equal volume of isopropanol to the aqueous phase, mix by pipetting up and down, and incubate for 10 min at 4 °C.
5. Centrifuge at 12,000× *g* for 10 min at 4 °C.
6. Total RNA precipitate forms a white, gel-like pellet at the bottom of the tube.
7. Remove the solution by pipetting. Avoid removing the RNA pellet, which should be located below the hinge of the microcentrifuge tube as a white pellet.
8. Gently add 1 mL of ice-cold 80% ethanol and centrifuge at 12,000× *g* for 5 min at 4 °C.
9. Remove the 80% ethanol solution by pipetting out and pulse-spin the 1.7 mL microcentrifuge tube in a mini-centrifuge, to bring any residual ethanol from the sides of the tube down to the bottom.

10. Using a P20 pipette and tip, remove the remaining solution.
11. Resuspend the RNA pellet in 100  $\mu$ L of RNase-free water, or in 300  $\mu$ L if starting from a 15 cm plate.
12. Quantify the concentration of RNA using a Nanodrop spectrophotometer.
13. We suggest ruling out RNA fragmentation during the extraction of total RNA by analyzing RNA quality by your method of choice (i.e., Bioanalyzer, TapeStation, or RNA gel electrophoresis analysis). For RNA gel electrophoresis, we load 2  $\mu$ L of the total RNA sample and 3  $\mu$ L of GeneRuler 1 kb Plus DNA ladder into a 1% agarose gel in 1 $\times$  TBE buffer and apply 140 V for 1 h (Figure 2A) (see Note 2). Store the RNA samples in a freezer at -80  $^{\circ}$ C or continue with Section C.



**Figure 2. Gel electrophoresis of MNase digested total RNA.** Nascent transcripts of HEK293 cells were pulse-labeled with 5-bromouridine for 30 min and total RNA was extracted. Shown is the purified total RNA sample before (A) and after fragmentation with MNase (B), for three biological replicates.

### C. RNA fragmentation with MNase

Fragmentation of total RNA with MNase before immunoprecipitation results in enrichment of only the region of pre-mRNA molecules that is labeled with 5-bromouridine. In addition, MNase digestion creates RNA molecules of a suitable size for library preparation in Section G.

The procedures described in Sections C and D have been performed uninterrupted. Before starting, consider that this protocol does not include a stopping point between these sections. If a stopping point is necessary, we propose to complete step C4 in this section and place the fragmented RNA on ice until this sample is used in step D6 within 24 h.

1. Mix 250  $\mu$ g of total RNA, 25  $\mu$ L of 5 M NaCl, and 50  $\mu$ L of 10 $\times$  MNase reaction buffer in a 1.7 mL tube and bring to a total volume of 500  $\mu$ L with nuclease-free water. Mix by pipetting up and down.
2. Incubate samples at 37  $^{\circ}$ C for 5 min.
3. Add 5  $\mu$ L of a 1:20 dilution of MNase (100,000 gel units/mL), mix by pipetting up and down, and incubate for 1 min.
4. Stop the reaction by adding 10  $\mu$ L of 0.5 M EGTA. Mix well and place sample on ice until completing step D5.
5. Evaluate RNA fragmentation by performing a gel electrophoresis using 2–4  $\mu$ L of fragmented RNA (1–2  $\mu$ g). RNA fragments should range from ~1.5 kb to ~100 bp in length. (Figure 2B).

### D. Immunoprecipitation of nascent transcripts

The following steps enrich bromouridine-labeled nascent RNA transcripts via specific binding to a mouse BrdU antibody conjugated to protein G magnetic beads while washing away unlabeled RNA.

1. Wash protein G magnetic beads with IP buffer. Transfer 50  $\mu$ L of well-mixed protein G magnetic beads-containing solution into a 1.7 mL tube and follow the steps below to wash the beads:
  - a. Place the 1.7 mL tube on the magnet stand to immobilize the beads.
  - b. When the solution has cleared completely and all beads are immobilized on the wall of the tube, remove the supernatant by pipetting out and add 500  $\mu$ L of IP buffer.
  - c. Remove the tube from the magnet stand and resuspend beads by pipetting up and down gently until all beads are dissociated and no clumps of beads are observed. Place back on the magnet stand.
  - d. When the solution has cleared completely and all beads are immobilized on the wall of the tube, remove the supernatant by pipetting out.
2. Add 1 mL of IP buffer to the washed beads and remove from the magnet stand.
3. Conjugate mouse anti-BrdU antibody to protein G magnetic beads. Add 8  $\mu$ L of anti-BrdU antibody (4  $\mu$ g) to the 1 mL of IP buffer containing the protein G magnetic beads, mix by pipetting up and down, and incubate the sample at 4  $^{\circ}$ C rotating end-to-end for 1 h. If the stopping point proposed in section C is included, this incubation can be performed overnight.
4. Wash conjugated anti-BrdU antibody and beads with IP buffer by performing the wash procedure described in step D1 (a–d) three times.
5. Add 1 mL of IP buffer to the beads and remove tube from the magnet stand.
6. Add the fragmented RNA from step C4 to the tube containing the beads, mix by pipetting up and down, and incubate the sample at 4  $^{\circ}$ C rotating end-to-end for 1 h (see Note 3).
7. Wash beads with IP buffer by performing the wash procedure described in step D1 (a–d) three times. Leave the tube on the magnet stand after the last wash.
8. Without disturbing the beads, add 500  $\mu$ L of RNase-free 1 $\times$  PBS, incubate for 30 s, and remove the supernatant by pipetting out.
9. Remove tube from the magnet stand, add 20  $\mu$ L of nuclease-free water, resuspend beads by pipetting up and down, and transfer to a PCR tube.
10. Place the tube on a thermal cycler and run the following program to elute the antibody and nascent transcripts from the beads: 90  $^{\circ}$ C for 5 min, 12  $^{\circ}$ C for 30 s, with heated lid at 105  $^{\circ}$ C.
11. Vortex and transfer the sample to a 1.5 mL tube.
12. Place the 1.5 mL tube on the magnet stand and, when the solution has cleared, transfer the supernatant into a clean 1.5 mL tube.
13. Measure the RNA concentration of the sample using the Qubit<sup>TM</sup> RNA High Sensitivity Assay kit. The expected concentration of specific enrichment of nascent transcript from HEK293 cells following this protocol is ~9–15 ng/ $\mu$ L with a total RNA yield of ~180–300 ng. The *no-bromouridine* negative control sample should not contain detectable signal by the Qubit Assay (< 0.2 ng/ $\mu$ L). Detection of RNA in this negative control suggests low efficiency of the washes after the immunoprecipitation.

## E. Repair of MNase-fragmented RNA 3'-end

RNA fragmentation by MNase creates 3' phosphate groups (Alexander et al., 1961). These can be converted to 3'-OH RNA ends by T4 polynucleotide kinase, required for ligation to a 3' adaptor during library preparation in section G. This reaction is performed in the absence of ATP, precluding installation of *non-native* 5'-PO<sub>4</sub> groups on RNA molecules by this enzyme.

1. Transfer 150 ng of nascent transcripts into a 1.5 mL tube, add 5  $\mu$ L of 10 $\times$  reaction buffer A, add RNase-free water to bring the solution up to 49  $\mu$ L, and mix by pipetting up and down.
2. Add 1  $\mu$ L of T4 polynucleotide kinase (10 U) and incubate at 30  $^{\circ}$ C for 30 min.

## F. In-column RNA purification

Purify the RNA from the in vitro reaction using any affinity micro-column purification protocol that elutes the RNA in a small quantity (< 20  $\mu$ L). We used the RNA Clean & Concentrator<sup>TM</sup>-5 cleanup kit described below.

1. Add two volumes of RNA binding buffer to each sample and mix by pipetting up and down.
2. Add an equal volume of ethanol (95%–100%) and mix by pipetting up and down.

3. Transfer the sample to the Zymo-Spin<sup>TM</sup> IC column in a collection tube and centrifuge at 12,000× *g* for 30 s. Discard the flowthrough.
4. Skip the DNase I treatment step, add 400 μL of RNA prep buffer to the column, and centrifuge at 12,000× *g* for 30 s. Discard the flowthrough.
5. Add 700 μL of RNA wash buffer to the column and centrifuge at 12,000× *g* for 30 s. Discard the flowthrough.
6. Add 400 μL of RNA wash buffer to the column and centrifuge at 12,000× *g* for 1 min. Ensure complete removal of the wash buffer. Carefully, transfer the column into a RNase-free tube.
7. Add 15 μL of DNase/RNase-free water directly to the column matrix and centrifuge at 12,000× *g* for 1 min to elute the RNA.
8. Measure the RNA concentration of the sample using the Qubit<sup>TM</sup> RNA High Sensitivity Assay kit (expected to be approximately 6 ng/μL). Continue to section G or freeze the RNA sample at -80 °C.

## G. Preparation of 5'-PO<sub>4</sub> Bru-Seq libraries

Preparation of 5'-PO<sub>4</sub> Bru-Seq libraries can be achieved using a library preparation protocol to detect miRNAs. In this protocol, we employed the QIAseq miRNA library kit, including 10-base UMIs. Transcripts with 5' ends other than a monophosphate, including cap structures, are excluded because they cannot be ligated to the 5' adaptor. Follow the steps in the manufacturer's protocol with the specific conditions described below:

1. Prepare reagents required for the 3' ligation reactions. Thaw QIAseq miRNA NGS 3' adapter, QIAseq miRNA NGS 3' buffer, 2× miRNA ligation activator, and nuclease-free water at room temperature (15–25 °C). Mix each solution by flicking the tubes. Centrifuge the tubes briefly to collect any residual liquid from the sides of the tubes and keep at room temperature.
2. Use 10–50 ng of nascent RNA transcripts using a 1:5 dilution of the 3' adapter according to the QIAseq miRNA library kit Table 5 (Dilution of the QIAseq miRNA NGS 3' Adapter).
3. On ice, prepare the 3' ligation reaction according to Table 1. Briefly centrifuge, mix by pipetting up and down 15–20 times, and centrifuge briefly again.

**Table 1. Setup of 3' ligation reactions**

Components	Volume/reaction
Nuclease-free water	Variable
QIAseq miRNA NGS 3' adapter	1 μL
QIAseq miRNA NGS RI	1 μL
QIAseq miRNA NGS 3' ligase	1 μL
QIAseq miRNA NGS 3' buffer	2 μL
2x miRNA ligation activator	10 μL
Template RNA (added in step G4)	Variable
<b>Total volume</b>	<b>20 μL</b>

4. Add template RNA to each tube containing the 3' ligation master mix. Briefly centrifuge, mix by pipetting up and down 15–20 times, and centrifuge briefly again.
5. Incubate for 1 h at 28 °C.
6. Incubate for 20 min at 65 °C.
7. Hold at 4 °C for at least 5 min.
8. Prepare reagents required for the 5' ligation reactions. Thaw QIAseq miRNA NGS 5' adapter and QIAseq miRNA NGS 5' buffer at room temperature. Mix by flicking the tube. Centrifuge the tube briefly to collect residual liquid from the sides of the tube and keep at room temperature.
9. Use a 1:2.5 dilution of the 5' adapter according to the QIAseq miRNA library kit Table 7 (Dilution of the QIAseq miRNA NGS 5' Adapter).
10. On ice, prepare the 5' ligation reaction according to Table 2, adding the components in the order listed. Briefly centrifuge, mix by pipetting up and down 10–15 times, and centrifuge briefly again.

**Table 2. Setup of 5' ligation reactions**

Component	Volume/reaction
3' ligation reaction (already in the tube)	20 $\mu$ L
Nuclease-free water	15 $\mu$ L
QIAseq miRNA NGS 5' buffer	2 $\mu$ L
QIAseq miRNA NGS RI	1 $\mu$ L
QIAseq miRNA NGS 5' ligase	1 $\mu$ L
QIAseq miRNA NGS 5' adapter	1 $\mu$ L
<b>Total volume</b>	<b>40 <math>\mu</math>L</b>

11. Incubate for 30 min at 28 °C.
12. Incubate for 20 min at 65 °C.
13. Hold at 4 °C and proceed immediately to step G14.
14. Prepare reagents required for the reverse transcription reactions. Thaw QIAseq miRNA NGS RT initiator, QIAseq miRNA NGS RT buffer, and QIAseq miRNA NGS RT primer at room temperature. Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes and keep at room temperature.
15. Add 2  $\mu$ L of QIAseq miRNA NGS RT initiator to each tube. Briefly centrifuge, mix by pipetting up and down 15–20 times, and centrifuge briefly again.
16. Incubate the tubes as described in Table 3.

**Table 3. Incubation of tubes with QIAseq miRNA NGS RT initiator**

Time	Temperature
2	75 °C
2	70 °C
2	65 °C
2	60 °C
2	55 °C
5	37 °C
5	25 °C
$\infty$ *	4 °C

\*Hold until setup of the RT reaction

17. Use a 1:5 dilution of the QIAseq miRNA NGS RT primer according to the QIAseq miRNA library kit Table 10 (Dilution of the QIAseq miRNA NGS RT Primer).
18. On ice, prepare the reverse transcription reaction according to Table 4. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

**Table 4. Setup of reverse transcription reactions**

Component	Volume/reaction
5' ligation reaction + QIAseq miRNA NGS RT initiator (already in the tube)	42 $\mu$ L
QIAseq miRNA NGS RT primer	2 $\mu$ L
Nuclease-free water	2 $\mu$ L
QIAseq miRNA NGS RT buffer	12 $\mu$ L
QIAseq miRNA NGS RI	1 $\mu$ L
QIAseq miRNA NGS RT enzyme	1 $\mu$ L
<b>Total volume</b>	<b>60 <math>\mu</math>L</b>

19. Incubate for 1 h at 50 °C.
20. Incubate for 15 min at 70 °C.

21. Hold at 4 °C for at least 5 min.
22. Prepare QIAseq miRNA NGS beads (QMN beads). Thoroughly vortex QIAseq beads and QIAseq miRNA NGS bead binding buffer to ensure that the beads are in suspension and homogenously distributed. Do not centrifuge the reagents. Important: QIAseq beads need to be homogenous. This necessitates working quickly and thoroughly resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads again.
23. Carefully add 400 µL of QIAseq beads (bead storage buffer is viscous) to a 2 mL microfuge tube. This quantity of beads is sufficient to perform “Protocol: cDNA Cleanup” and the cleanup associated with library amplification for one sample. Briefly centrifuge and immediately separate beads on a magnet stand.
24. When beads have fully migrated, carefully remove and discard the supernatant.
25. Remove the tube from the magnet stand and carefully pipette (buffer is viscous) 150 µL of QIAseq miRNA NGS bead binding buffer onto the beads. Thoroughly vortex to completely resuspend the bead pellet. Briefly centrifuge and immediately separate the beads on a magnet stand.
26. When beads have fully migrated, carefully remove and discard the supernatant.
27. Remove the tube from the magnet stand and carefully pipette 400 µL of QIAseq miRNA NGS bead binding buffer onto the beads (buffer is viscous). Thoroughly vortex to completely resuspend the bead pellet. Preparation of the QMN beads is now complete. If the beads will not be used immediately, store them on ice or at 2–8 °C.
28. Perform a cDNA cleanup. Centrifuge the tubes containing the cDNA reactions and add 143 µL of QMN beads to tubes containing the cDNA reactions. Vortex for 3 s and centrifuge briefly.
29. Incubate for 5 min at room temperature.
30. Place the tubes on a magnet stand for ~4 min or until the beads have fully migrated.
31. Discard the supernatant and keep the beads.
32. With the beads still on the magnet stand, add 200 µL of 80% ethanol. Immediately remove and discard the ethanol wash.
33. Repeat the wash by adding 200 µL of 80% ethanol. Immediately remove and discard the second ethanol wash. Important: completely remove all traces of ethanol after the second wash. Briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol with a 200 µL pipette first, and then use a 10 µL pipette to remove any residual ethanol.
34. With the beads still on the magnetic stand, air-dry at room temperature for 10 min. Residual ethanol can hinder amplification efficiency in the subsequent library amplification reactions. Depending on humidity, extended drying time may be required.
35. With the beads still on the magnetic stand, elute the DNA by adding 17 µL of nuclease-free water to the tubes. Subsequently close/cover and remove the tubes/plates from the magnetic stand.
36. Carefully pipette up and down until all the beads are thoroughly resuspended, briefly centrifuge, and incubate at room temperature for 2 min.
37. Return the tubes to the magnetic stand for ~2 min or until the beads have fully migrated. The completed cDNA cleanup product can be stored at -20 °C; alternatively, continue with the steps below.
38. Prepare reagents required for the library amplification reactions. Thaw QIAseq miRNA NGS library buffer, QIAseq miRNA NGS ILM library forward primer, and required index primer(s). Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes.
39. On ice, prepare the library amplification reaction according to Table 5. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

**Table 5. Setup of library amplification reactions when using tube indices**

Component	Volume/reaction
Product from “Protocol: cDNA Cleanup”	15 µL
QIAseq miRNA NGS library buffer	16 µL
HotStarTaq DNA polymerase	3 µL
QIAseq miRNA NGS ILM library forward primer	2 µL
QIAseq miRNA NGS ILM IPD1 through IPD48 (Index Primer)	2 µL
Nuclease-free water	42 µL

Total volume	80 $\mu$ L
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40. Program the thermal cycler according to Table 6.

**Table 6. Library amplification protocol**

Step	Time	Temperature
Hold	15 min	95 °C
3-step cycling (18 cycles)		
Denaturation	15 s	95 °C
Annealing	30 s	60 °C
Extension	15 s	72 °C
Hold	2 min	72 °C
Hold	$\infty$	4 °C

41. Place the library amplification reaction in the thermal cycler and start the run. Upon completion of the protocol, hold at 4 °C for at least 5 min.
42. Add 75  $\mu$ L of QMN beads to tubes. Ensure the QMN beads are thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.
43. Briefly centrifuge the 80  $\mu$ L library amplification reactions and transfer 75  $\mu$ L to the tubes containing the QMN beads. Vortex for 3 s and briefly centrifuge.
44. Incubate for 5 min at room temperature.
45. Place tubes on a magnet stand for approximately 4 min or until the beads have fully migrated.
46. Keep the supernatant and transfer 145  $\mu$ L of the supernatant to new tubes. Discard the tubes containing the beads. Important: do not discard the supernatant at this step.
47. To the 145  $\mu$ L supernatant, add 130  $\mu$ L of QMN beads. Vortex for 3 s and briefly centrifuge.
48. Incubate at room temperature for 5 min.
49. Place the tubes on a magnet stand until beads have fully migrated.
50. Discard the supernatant and keep the beads.
51. With the beads still on the magnet stand, add 200  $\mu$ L of 80% ethanol. Immediately remove and discard the ethanol wash.
52. Repeat the wash by adding 200  $\mu$ L of 80% ethanol. Immediately remove and discard the second ethanol wash. It is important to completely remove all traces of the ethanol wash after the second wash. Briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol with a 200  $\mu$ L pipette first, and then use a 10  $\mu$ L pipette to remove any residual ethanol.
53. With the beads still on the magnetic stand, air-dry at room temperature for 10 min.
54. With the beads still on the magnetic stand, elute the DNA by adding 17  $\mu$ L of nuclease-free water to the tubes. Subsequently close and remove the tubes from the magnetic stand.
55. Carefully pipette up and down until all beads are thoroughly resuspended; briefly centrifuge and incubate at room temperature for 2 min.
56. Place the tubes on the magnetic stand for ~2 min (or until beads have cleared).
57. Transfer 15  $\mu$ L of eluted DNA to new tubes. This is the 5'-PO<sub>4</sub> Bru-Seq sequencing library. Store sequencing libraries at -20 °C.
58. Submit libraries for Illumina sequencing. It is recommended to use paired-end sequencing. The 5' end of the sequenced Read 1 informs on the position of the ligation event (5'-PO<sub>4</sub> RNA end) and the first 12 nucleotides at the 5' end of Read 2 inform on the identity of the UMI.

## Data analysis

1. Extract UMIs from the Illumina sequencing run files (e.g., Read1.fastq, Read2.fastq) and obtain a new Read1.UMI.fastq file using UMI-tools (see Note 4).

```
umi_tools extract \
-I Read2.fastq \
--extract-method=string \
--bc-pattern=NNNNNNNNNNNN \
--read2-in=Read1.fastq \
--read2-out=Read1.UMI.fastq \
> Read2.UMI.fastq
```

2. Remove adaptor sequences using Cutadapt.

```
cutadapt \
-a 'AACTGTAGGCACCATCAAT' \
-A 'GATCGTCGGACTGTAGAACTCTGAAC' \
-o Read1.trimmed.fastq \
-p Read2.trimmed.fastq \
Read1.UMI.fastq \
Read2.UMI.fastq \
```

3. Use Bowtie (e.g., Bowtie 2) to align reads in the Read1.trimmed.fastq file to the human genome index (e.g., GRCh37/hg19).

```
bowtie2 \
-x bowtie_index \
-U Read1.trimmed.fastq \
-S Read1.sam
```

4. Convert the “Read1.sam” file to BAM format.

```
samtools view -S -b Read1.sam > Read1.bam
```

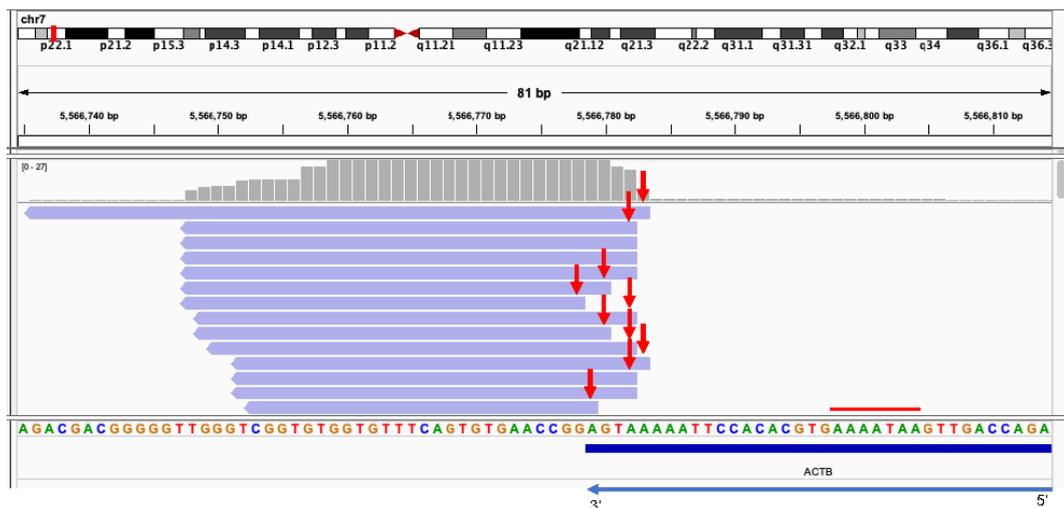
5. Sort the Read1.bam file.

```
samtools sort Read1.bam -o Read1.sorted.bam
```

6. Remove UMI duplicates using UMI-tools.

```
umi_tools dedup \
--method unique \
--read-length \
-I Read1.sorted.bam \
-S Read1.filtered.bam
```

7. Use the Read1.filtered.bam and the Read1.filtered.bam.bai files to visualize sequence reads on IGV. Figure 3 below shows sequenced reads mapped to the poly(A) site of the ACTB gene, where the first 5' nucleotide in the sequenced read contains the single nucleotide coordinates of the 5'-PO<sub>4</sub> RNA end in the nascent transcript immediately downstream of the poly(A) cleavage site (red arrows).



**Figure 3. Integrative Genomics Viewer (IGV) screenshot showing sequenced 5'-PO<sub>4</sub> Bru-Seq reads mapped to the 3' end of the ACTB gene, where the localized 5'-monophosphorylated nucleotides in the nascent transcript are indicated by read arrows. The poly(A) signal is indicated by a red line above the sequence track.**

8. Convert the “Read1.filtered.bam” file to BED format using bedtools.

```
bedtools bamtobed \
-i Read1.filtered.bam \
> Read1.filtered.bed
```

9. Collapse read coordinates in the “Read1.filtered.bed” file to the 5' single nucleotide coordinate, which corresponds to the 5'-PO<sub>4</sub> RNA single nucleotide coordinate, and create coverage BEDGRAPH files with strand specificity using the “chrom.sizes” file associated with the reference genome that was used for mapping.

```
bedtools genomecov \
-strand + \
-5 \
-i Read1.filtered.bed \
-bg chrom.sizes \
> Read1.5p.positive.bg
bedtools genomecov \
-strand - \
-5 \
-i Read1.filtered.bed \
-bg chrom.sizes \
> Read1.5p.negative.bg
```

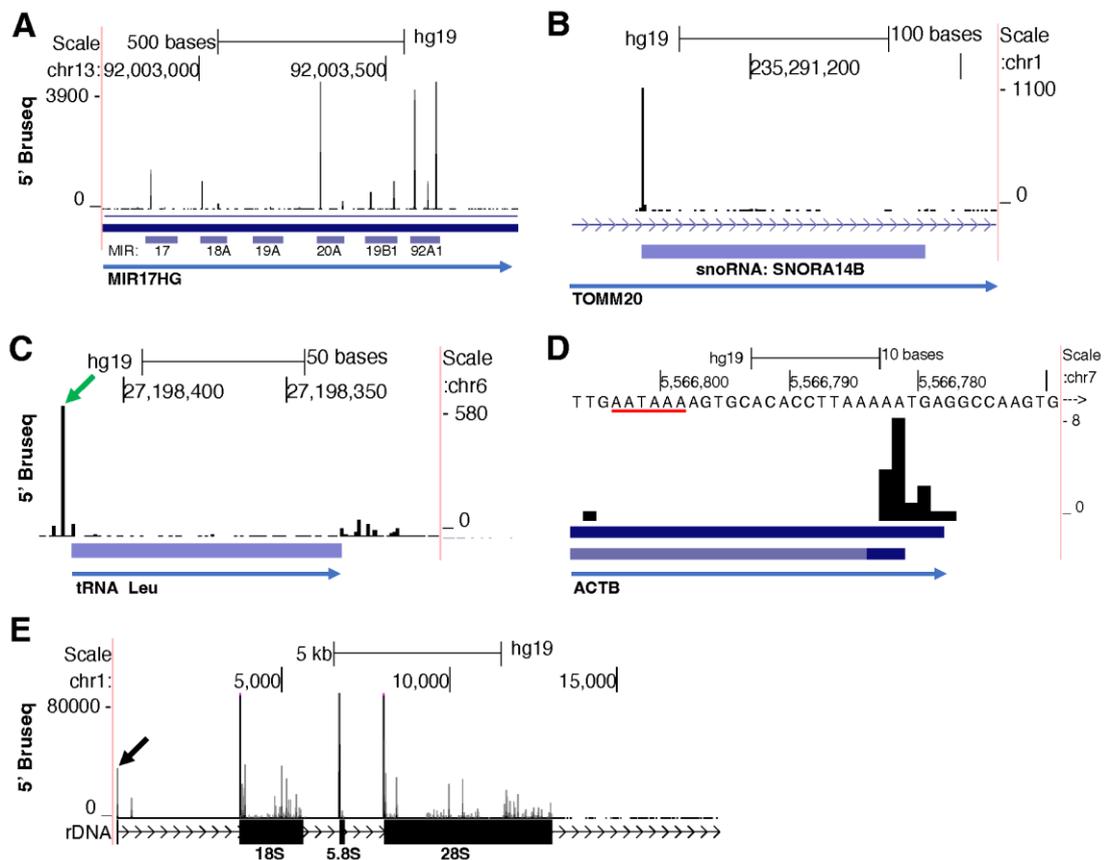
10. Sort the output BEDGRAPH files.

```
sort -k1,1 -k2,2n Read1.5p.positive.bg \
> Read1.5p.sorted.positive.bg
sort -k1,1 -k2,2n Read1.5p.negative.bg \
> Read1.5p.sorted.negative.bg
```

- Use bedGraphToBigWig to create BigWig files for visualization of genomic data on the University of California Santa Cruz (UCSC) Genome Browser.

```
bedGraphToBigWig \
Read1.5p.sorted.positive.bg \
chrom.sizes \
Read1.5p.positive.bw
bedGraphToBigWig \
Read1.5p.sorted.negative.bg \
chrom.sizes \
Read1.5p.negative.bw
```

- Confirm enrichment of 5'-PO<sub>4</sub> Bru-Seq signal by detection of 5'-PO<sub>4</sub> RNA ends at microprocessor cleavage sites of the MIR17HG miRNA cluster (Figure 4A), at the 5' end of the SNORA14B snoRNA (Figure 4B), the 5' end of the primary tRNA transcript prior to RNaseP cleavage (Figure 4C, red arrow), and 5'-PO<sub>4</sub> ends generated after cleavage at the poly(A) site on the ACTB gene (Figure 4D). 5'-PO<sub>4</sub> signal can be further validated by detection of the 5' end of the 47S rRNA precursor (Wang and Pestov, 2011) (Figure 4E, red arrow). Ribosomal signal can be visualized by mapping to the human ribosomal repeating unit reference sequence (GenBank accession no. U13369). Finally, relative quantification of peak intensities across libraries can be made by normalizing to the total number of mapped reads or by normalization to an appropriate internal control region [e.g., the mitochondrial genome (Cortazar et al., 2022)].



**Figure 4. 5'-PO<sub>4</sub> Bru-Seq maps known 5'-PO<sub>4</sub> RNA ends of nascent transcripts.** Shown are UCSC genome browser screenshots of 5'-PO<sub>4</sub> Bru-Seq signal at: (A) the MIR17HG miRNA cluster, (B) the SNORA14B snoRNA, (C) a tRNA gene where the 5' end of the primary tRNA transcript is indicated by a green arrow, and

(D) the 3' end of the ACTB gene where the poly(A) signal is underlined upstream of the poly(A) cleavage site. The peaks represent the nucleotide with the 5'-PO<sub>4</sub> group in the RNA fragment downstream of the poly(A) cleavage site, and (E) the ribosomal RNA transcription unit zoomed in y-axis values (0–8,000), to visualize the 5' end of the 47S rRNA precursor indicated by a black arrow. The y-axis indicates the number of mapped reads per one million of mitochondrial 5'-PO<sub>4</sub> Bru-Seq sequenced reads.

## Notes

1. Incubation of cells with 2 mM 5-bromouridine for a period of 30 min has been previously validated to result in mainly nascent transcript signal (Paulsen et al., 2013). Shorter incubation times can be performed (~20 min), although the yield of nascent transcripts is substantially decreased. Longer incubation times are not recommended, given that labeled nascent transcripts are processed, accumulate in the population of mature RNA, and contaminate nascent transcript signal.
2. The population of intact RNA molecules in high-quality samples show distinct bands that correspond to the 28S and 18S rRNA species, respectively. A fuzzy and faint band at the bottom of the gel corresponding to the population of tRNAs can also be visualized depending on the amount of RNA loaded (Figure 2A). The qualitative 28S/18S band intensity ratio value of 2 has been historically considered to indicate intact RNA.
3. Shorter incubations during immunoprecipitation of bromo-labeled RNA reduce the potential for RNA fragmentation.
4. By default, UMI\_tools extracts UMIs from the “Read1.fastq” input file using argument “-I.” Given that the UMI is contained in Read 2, you can simply switch the input file provided for argument “--read2-in.”

## Recipes

### 1. 1:20 dilution of MNase (100,000 gel units/mL)

Mix 1 µL of MNase (2,000,000 gel units/mL) with 19 µL of 1× MNase reaction buffer.

### 2. 50 mM 5-bromouridine

Dissolve 808 mg of 5-bromouridine in 50 mL of RNase-free 1× PBS and store at -20 °C.

### 3. IP Buffer

0.05% Triton X-100, 1 mM DTT, supplemented with Superase-In<sup>TM</sup> RNase inhibitor in RNase-free 1× PBS. Mix 25 µL of Triton X-100, 50 µL of 1 M DTT, 50 µL of Superase-In<sup>TM</sup> RNase inhibitor, and 50 mL of RNase-free 1× PBS.

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## Competing interests

The authors declare no competing interests.

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