Selective amplification and sequencing of cyclic phosphate–containing RNAs by the cP-RNA-seq method

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RNA digestions catalyzed by many ribonucleases generate RNA fragments that contain a 2′,3′-cyclic phosphate (cP) at their 3′ termini. However, standard RNA-seq methods are unable to accurately capture cP-containing RNAs because the cP inhibits the adapter ligation reaction. We recently developed a method named cP-RNA-seq that is able to selectively amplify and sequence cP-containing RNAs. Here we describe the cP-RNA-seq protocol in which the 3′ termini of all RNAs, except those containing a cP, are cleaved through a periodate treatment after phosphatase treatment; hence, subsequent adapter ligation and cDNA amplification steps are exclusively applied to cP-containing RNAs. cP-RNA-seq takes ~6 d, excluding the time required for sequencing and bioinformatics analyses, which are not covered in detail in this protocol. Biochemical validation of the existence of cP in the identified RNAs takes ~3 d. Even though the cP-RNA-seq method was developed to identify angiogenin-generating 5′-tRNA halves as a proof of principle, the method should be applicable to global identification of cP-containing RNA repertoires in various transcriptomes.

INTRODUCTION

The advent of next-generation sequencing technologies has largely unveiled the cellular transcriptome, leading to great advances in our understanding of gene expression, biogenesis mechanisms and the molecular functions of coding and noncoding RNAs. The current standard RNA-seq methods, particularly those that target small noncoding RNAs, include an adapter ligation step in which two different oligonucleotide adapters of known sequences are ligated to the 5′ and 3′ ends of a targeted population of RNA molecules. Any RNAs that fail to ligate with both adapters are not amplified through subsequent reverse transcription (RT)-PCRs; hence, they do not appear in the resulting sequencing data. Because adapter ligation reactions require that the targeted RNA substrates contain a 5′-terminal phosphate (P) and a 3′-terminal hydroxyl group (OH), standard RNA-seq methods limit their utility for sequencing RNAs with terminal modifications that inhibit adapter ligation.

A 2′,3′-cP at the 3′ end of RNA is one such modification with which RNAs are not ligated to a 3′ adapter. RNA fragments containing a 3′-terminal cP are produced from RNA digestions catalyzed by many endoribonucleases such as the following from various organisms: tRNA splicing endonuclease[1], inositol-requiring enzyme-1 (Ire1)[2], RNase T_2 (ref. 3), RNase L[4], angiogenin (ANG)[5], MazF[6], general control protein 4 (GCN4)[7], placental protein 11 (PP11)[8], nidoviral uridylate-specific endoribonuclease (NendoU)[9] and colicin D[10]. Ribozymes[11,12] and Mpn1 exonuclease[13] also form a cP-containing 3′ end in their cleaved RNA fragments. Such cP formations are not just the result of specific ribonuclease digestions, but cP formations themselves can have a functional significance: cP formations regulate RNA-protein interactions, RNA stability and turnover, and cell proliferation[7,14–16]. In tRNA splicing, 3′-terminal cP of 5′ exons is an important intermediate, which is processed by a cyclic phosphodiesterase for ligation with 3′ exons[17].

cP-containing RNAs have been reported to have a physiological significance in various biological processes. For example, U6 small nuclear RNA (snRNA) is an evolutionarily conserved component that is essential for mRNA splicing; U6 snRNA contains a 3′-cP that is formed by Mpn1, and it enhances the affinity of the RNA for its interacting proteins[13,14]. Also belonging to cP-containing RNAs are the 5′ halves of tRNAs produced by ANG-mediated tRNA cleavage upon various stress stimuli, which are known as tRNA-derived stress-induced RNAs (tiRNAs)[18]. These 5′-tiRNAs promote stress granule formation[19], inhibit translation[18,20,21] and trigger cellular stress responses and apoptosis in neurodevelopmental disorders[22]. Our recent study demonstrated the occurrence of a distinct class of ANG-generating tRNA halves, sex hormone–dependent tRNA-derived RNAs (SHOT-RNAs), whose expression is promoted by sex hormones and their receptors in breast and prostate cancers[5]. 5′-SHOT-RNAs, corresponding to 5′-tRNA halves, are cP-containing RNAs and have a functional significance in cell proliferation[5].

Given that cP-containing RNAs are expressed as functional molecules, capturing the whole repertoire of cP-containing RNAs would reveal significant biological events that have not been identified using the current standard RNA-seq methods.

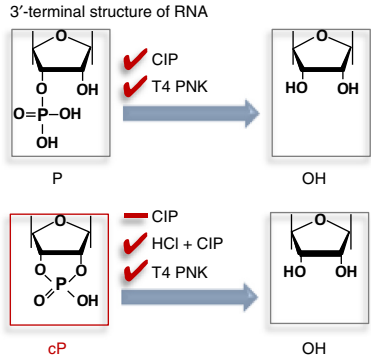

Figure 1 | A schematic representation of the reactivity of a 3′-P and a 3′-cP with enzymatic treatments. T4 PNK removes both a 3′-P and 3′-cP to form a 3′-OH end. A phosphatase such as CIP removes a 3′-P (and 5′-P) but not a 3′-cP. HCl treatment followed by CIP treatment (HCl + CIP) can remove a 3′-cP because the HCl treatment converts 3′-cP to a 3′-P.

Although an increasing number of RNA sequencing data are accelerating large-scale comparative analyses of transcriptomes and greatly contributing to the identification of significant RNA species in biological phenomena and diseases[23,24], cP-containing RNAs remain a hidden component in such analyses. In the course of investigating SHOT-RNAs, we recently developed a method named cP-RNA-seq, which is able to selectively amplify and sequence cP-containing RNAs[5]. Here we describe a detailed cP-RNA-seq protocol that can be adapted for global exploration and identification of cP-containing RNAs in any RNA samples derived from various organisms, tissues and cells.

Different reactivity of 3′-terminal phosphate and cyclic phosphate of RNAs to enzymatic treatments

The different reactivity of 3′-P and 3′-cP of RNAs to enzymatic treatments (**Fig. 1**) is used as the basis of cP-RNA-seq. The T4 polynucleotide kinase (T4 PNK) has 3′-terminal phosphatase activity that removes both a P and cP from the 3′ end of RNAs to form a 3′-OH end[25]. In contrast, a widely used phosphatase such as calf intestinal phosphatase (CIP) removes a 5′-P and 3′-P but not a 3′-cP. Although CIP treatment alone cannot remove a 3′-cP, a combination treatment of acid, such as HCl, followed by CIP treatment can remove a 3′-cP because the acid treatment hydrolyzes a 3′-cP and converts it to a 3′-P (ref. 26).

Overview of the protocol

The entire procedure of cP-RNA-seq is illustrated in **Figure 2**. Gel-purified RNAs with approximate targeted lengths are treated with a phosphatase (CIP), followed by treatment with a periodate ($NaIO_4$). These two treatments disrupt the 3′ ends of the RNAs that originally contain 3′-P and 3′-OH ends. The RNAs containing a 3′-cP survive the treatments, and therefore they are exclusively ligated to adapters after their cP is removed by T4 PNK treatment. Subsequent RT-PCR amplification and next-generation sequencing of the amplified cDNAs thus selectively identify the sequences of 3′-cP–containing RNAs. After identification, the actual presence of 3′-cP in the representative identified RNAs should be biochemically validated. For the validation, total RNA is treated with T4 PNK, CIP or acid (HCl) followed by CIP treatment, and then northern blots are performed to examine the mobility of the targeted RNA bands. The cP-RNA-seq procedure takes ~6 d, excluding the time required for sequencing and bioinformatics analyses. In addition, the biochemical validation takes ~3 d to complete.

Application of the protocol

To identify 5′-SHOT-RNAs, we applied the cP-RNA-seq method to total RNA isolated from BT-474 breast cancer cells[5]. We successfully obtained ~33 million reads aligned to the human genome, of which ~85% were actually derived from 5′-tRNA halves, indicating the high specificity, sensitivity and credibility of the method[5]. We expect that the concept of the cP-RNA-seq method will be widely applicable to the identification of cP-containing RNA repertoire in any cells or tissues from any organism, and even in valuable clinical samples, provided that RNA samples are available. Identification of the cP-containing RNA repertoire would

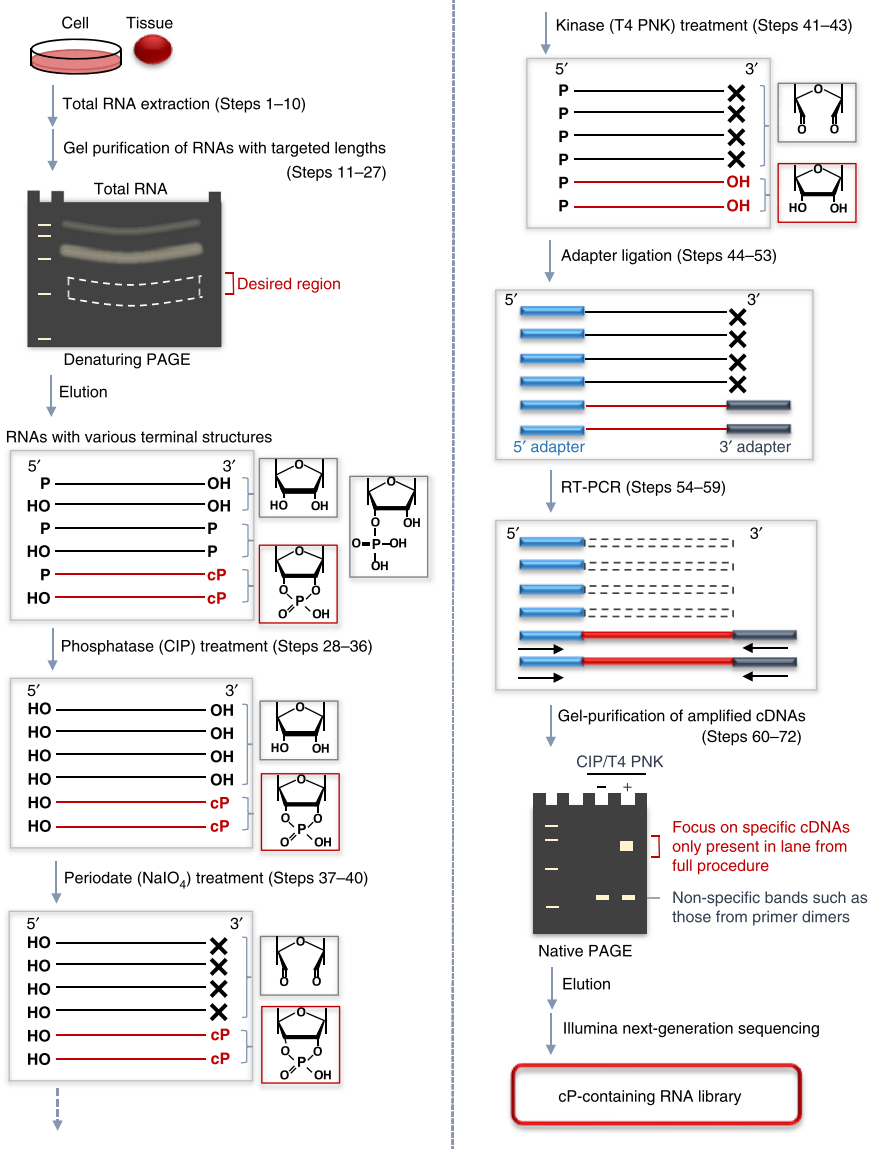

Figure 2 | A schematic representation of the cP-RNA-seq procedure for selective amplification and sequencing of cP-containing RNAs. Total RNA is extracted from cell lines or tissues (Steps 1–10), and then the RNAs with targeted lengths are gel-purified using denaturing PAGE (Steps 11–27). The purified RNAs are subjected to CIP treatment (Steps 28–36), periodate treatment (Steps 37–40) and T4 PNK treatment (Steps 41–43). By using an Illumina kit, the treated RNAs are ligated to adapters (Steps 44–53) and their cDNAs are amplified by RT-PCR (Steps 54–59). The amplified cDNAs are gel-purified using native PAGE (Steps 60–72), and then they are subjected to Illumina next-generation sequencing.

complement the information obtained using standard RNA-seq, and it may identify novel RNA processing events.

Comparison with other methods

To date, there is only one alternative sequencing-based method reported for the identification of cP-containing RNAs. By using *Arabidopsis thaliana* tRNA ligase, whose ligation activity is specific to cP-containing RNAs, Schutz *et al.*[27] specifically ligated an adapter to cP-containing RNAs and identified U6 snRNAs and specific tRNA fragments as cP-containing RNA species in human brain total RNA. The method requires the recombinant tRNA ligase and the 2′-phosphotransferase, which they expressed and purified by themselves; it also involves many steps including three gel purifications. The cP-RNA-seq method is efficient, specific and only requires commercially available reagents, as described below.

Limitations

The selective amplification of 3′-cP-containing RNAs is dependent on the periodate-mediated cleavage of any other RNAs with a 3′-OH end after CIP treatment. Because the requirement for periodate cleavage is the presence of a 2′,3′-diol structure of ribose, post-transcriptional ribose modifications that displace the diol structure will prevent the cleavage. To date, plant miRNAs[28], plant and animal siRNAs[29,30] and animal PIWI-interacting RNAs (piRNAs)[31–34] are known to possess a 2′-*O*-methyl ribose modification that displaces the diol structure. Despite the absence of 3′-cP, these modified RNAs would not be cleaved via a periodate treatment, and therefore they would be sequenced by cP-RNA-seq. This point should always be remembered, especially when 20- to 30-nt small RNAs are subjected to the cP-RNA-seq method.

Experimental design

RNA sample extraction and preparation (Steps 1–27). The necessary quantity and quality of starting RNA materials can vary widely depending on the expression levels of the targeted cP-containing RNAs in the total RNA. To identify 5′-SHOT-RNAs in BT-474 cells, we used 50–100 μg of total RNA as a starting material and gel-purified 30- to 50-nt RNAs from the total RNA using denaturing PAGE[5]. Because we failed to amplify 5′-SHOT-RNAs directly from the total RNA without the gel-purification step (data not shown), it is recommended to concentrate the targeted cP-containing RNA species (if known) using gel purification or column chromatography before enzymatic and chemical treatments of the RNAs. If the targeted RNA species are unknown and exploration of the cP-containing RNAs is the objective, depletion of at least rRNA using a kit such as the Ribo-Zero rRNA removal kit (Illumina) or the RiboMinus eukaryote kit for RNA-seq (Life Technologies) would help improve the amplification efficiency of cP-RNA-seq. Extraction of smaller RNAs using a kit such as mirVana (Life Technologies) would also be an option.

Enzymatic and chemical treatments of RNAs (Steps 28–43). Gel-purified RNA populations are supposed to be a mixture of RNAs with various terminal phosphate states: i.e., either 5′-P/3′-OH, 5′-OH/3′-OH, 5′-P/3′-P, 5′-OH/3′-P, 5′-P/3′-cP or 5′-OH/3′-cP. The CIP treatment is used to remove a P from both the 5′ and 3′ ends of the RNAs, although the state of cP is not changed. Eventually, the RNA pools contain two subgroups: one with 5′-OH/3′-OH and a second with 5′-OH/3′-cP. Subsequent periodate treatment cleaves the *cis*-diol group of the 3′-OH end of the former group to generate 2′,3′-dialdehydes that no longer serve as a substrate for adapter ligation[35]. The latter group survives periodate treatment because of the presence of a 3′-cP. Therefore, after removal of the 3′-cP and addition of a 5′-P by T4 PNK treatment, the latter group becomes the only RNA that bears a 5′-P and 3′-OH ends for 5′- and 3′-adapter ligations. These enzymatic and chemical treatments enable selective adapter ligation to the RNAs that originally contained a 3′-cP.

cDNA library preparation and next-generation sequencing (Steps 44–72). The treated RNAs are subjected to the adapter ligation reaction, followed by the RT-PCR amplification of cDNAs for next-generation sequencing. When identifying 5′-SHOT-RNAs, as described in this protocol, we used an Illumina TruSeq small RNA preparation kit[5]. The Illumina kit can be substituted with any cDNA library preparation kit for next-generation sequencing as long as it is based on adapter ligations to RNAs. Sequencing and bioinformatics analyses of cP-RNA-seq data will depend on the goals of the investigator and on the target RNA species. Sequence analyses of 5′-SHOT-RNAs are described in Honda *et al.*[5].

Many RNA post-transcriptional modifications are known to affect RT[36], which will cause biased sequencing results of heavily modified RNAs, such as tRNAs and their fragments. Recent studies have succeeded in removing key RT-impairing modifications, such as m^1A, using AlkB demethylase and thus in improving the efficiency and quality of the amplification and sequencing of heavily modified RNAs[37,38]. Therefore, if the targeted RNAs are expected to contain modifications, pretreatment of the RNA samples with the demethylase would be an option for better efficiency and quality of cP-RNA-seq.

Controls. To ensure that the sequences obtained from cP-RNA-seq are derived from cP-containing RNAs and not from ribose-modified RNAs, our protocol calls for a negative control experiment that lacks two steps: CIP treatment and T4 PNK treatment. Because the 'CIP/T4 PNK–' procedure can specifically amplify the modified small RNAs with the 5′-P and 3′-ribose (5′-/3′-ribose) modification, the appearance of cDNA bands from the full procedure, but not from the negative control procedure as shown in **Figure 3**, would strongly suggest that the amplified bands are derived from cP-containing RNAs. The cDNA band pattern also indicates

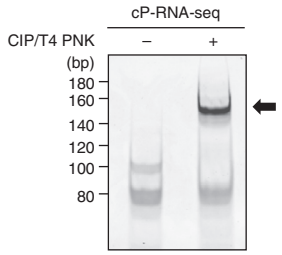

Figure 3 | Selective amplification of mouse 5′-tRNA halves using cP-RNA-seq. Total RNA extracted from mouse spleen was subjected to the cP-RNA-seq method. As shown by an arrow, the method amplified ~150- to 155-bp cDNA products corresponding to the targeted 5′-tRNA halves (5′ adapter, 55 bp; 3′ adapter, 63 bp; and therefore inserted sequences ~32–37 bp). The cDNA bands were absent from the negative control procedure without CIP and T4 PNK treatment.

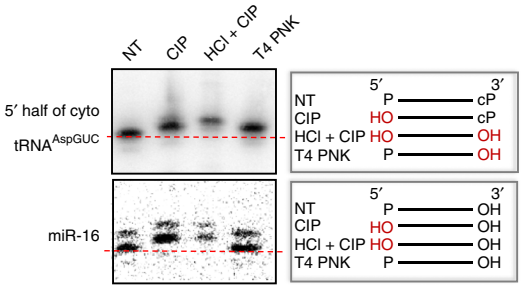

Figure 4 | Biochemical validation of the presence of a cP in a mouse 5′-tRNA half. Mouse spleen total RNA was treated with T4 PNK, CIP or acid followed by CIP treatment (HCl + CIP). NT designates nontreated sample used as a negative control. The treated or nontreated RNAs were subjected to northern blotting targeting the cytoplasmic (cyto) 5′-tRNA^{AspGUC} half and miR-16. The expected terminal structures of the targeted RNA in the treated total RNA samples are shown on the right. In the analyses of cyto 5′-tRNA^{AspGUC} half, the band was similarly shifted up by the CIP treatment and by the T4 PNK treatment, and a further upshift was observed by the HCl + CIP treatment, indicating the presence of a 5′-P and a 3′-cP. In the control analyses of miR-16, as expected, the CIP and HCl + CIP treatments identically shifted the band up, whereas the T4 PNK treatment caused no shift, indicating the presence of a 5′-P and a 3′-OH. A dotted red line indicated the position of the main band in the NT sample, which helps to show band mobility differences.

complete periodate-mediated cleavage for the RNAs containing 3′-OH ends, which is the key to the cP-RNA-seq method.

Biochemical validation of the presence of cP in the identified RNAs (Steps 73–96). Because of the limitation discussed above, the actual presence of 3′-cP in the representative identified RNAs should be biochemically validated. For validation, total RNA is first treated with T4 PNK, CIP or acid (HCl), followed by CIP treatment. By subsequent northern blot for targeted RNA, the difference in the band mobility due to the changed 3′-cP state is then analyzed. Because the HCl + CIP treatment removes 3′-cP but the CIP treatment alone cannot, as shown in **Figure 4**, the upshift of the HCl + CIP band compared with the CIP band indicates the original presence of 3′-cP in the targeted RNAs. In addition, because T4 PNK removes 3′-cP, the T4 PNK band should be shifted up compared with the nontreatment band. This validation method is effective when the targeted RNAs are short enough for discriminating band mobility between the RNAs with and without cP or P. The presence of cP in long RNAs should be validated by a different method such as that using poly-A-polymerase[39,40].

MATERIALS

REAGENTS

- TRIsure (Bioline, cat. no. BIO-38032) **! CAUTION** TRIsure is toxic on inhalation. Use a hood, protective clothing, eye protection and gloves.
- Chloroform (Sigma-Aldrich, cat. no. C2432) **! CAUTION** Chloroform is toxic on inhalation. Use a hood, protective clothing, eye protection and gloves.
- Isopropanol (Sigma-Aldrich, cat. no. I9516)
- Ethyl alcohol (Sigma-Aldrich, cat. no. E7023)
- Certified nuclease-free water (BioExpress, cat. no. G-3250)
- Urea (Sigma-Aldrich, cat. no. U5378)
- TBE, 10× liquid concentrate (Amresco, cat. no. 0658)
- Acrylamide/Bis solution, 40%, wt/vol, 19:1 (Bio-Rad, cat. no. 1610144EDU) **! CAUTION** Monomeric acrylamide is a neurotoxin. Use a hood, protective clothing, eye protection and gloves.
- Ammonium persulfate (APS; Sigma-Aldrich, cat. no. A3678)
- UltraPure TEMED (Life Technologies, cat. no. 15524-010) **! CAUTION** TEMED is toxic on inhalation. Use a hood, protective clothing, eye protection and gloves.
- Bromophenol blue (Sigma-Aldrich, cat. no. B0126)
- Xylene cyanol FF (Sigma-Aldrich, cat. no. X4126)
- Low-molecular-weight marker, 10–100 nt (Affymetrix, cat. no. 76410)
- SYBR Gold nucleic acid gel stain (Life Technologies, cat. no. S-11494) **! CAUTION** SYBR Gold is a DNA-binding agent, and thus it is potentially mutagenic. Use protective clothing, eye protection and gloves.
- Sodium acetate (3 M), pH 5.5 (Life Technologies, cat. no. AM9740)
- EDTA (0.5 M), pH 8.0 (Life Technologies, cat. no. AM9260G)
- SDS, 20% (wt/vol) solution (Life Technologies, cat. no. AM9820)
- Alkaline phosphatase, calf intestinal (CIP; New England Biolabs, cat. no. M0290)
- CutSmart buffer, 10× (supplied with CIP)
- RNasin ribonuclease inhibitor (Promega, cat. no. N2111)
- Acid-phenol:chloroform, pH 4.5 (with IAA, 125:24:1; Life Technologies, cat. no. AM9720) **! CAUTION** Phenol and chloroform are toxic on inhalation. Use a hood, protective clothing, eye protection and gloves.
- Linear acrylamide (Life Technologies, cat. no. AM9520)
- Sodium periodate (Sigma-Aldrich, cat. no. 311448)
- T4 polynucleotide kinase (T4 PNK; New England BioLabs, cat. no. M0201)
- T4 polynucleotide kinase reaction buffer, 10× (supplied with T4 PNK)
- ATP (New England BioLabs, cat. no. P0756)
- TruSeq small RNA library preparation kits (Illumina, cat. no. RS-200-0012), containing RNA 3′ adapter, ligation buffer, stop solution, RNA 5′ adapter, 10 mM ATP, T4 RNA ligase, 25 mM dNTP mix, RNA RT primer, PCR mix, RNA PCR primer, RNA PCR primer index and high resolution ladder
- T4 RNA ligase 2, truncated (New England BioLabs, cat. no. M0242L)
- SuperScript III reverse transcriptase (Life Technologies, cat. no. 18080-044) containing 5× first-strand buffer and 100 mM DTT
- RNaseOUT (Life Technologies, cat. no. 10777-019)
- TAE, 50× (AMRESCO, cat. no. K915)
- phiX174 DNA-HaeIII digest (New England BioLabs, cat, no. N3026S)
- Loading dye, 6× (Affymetrix, cat. no. 76715)
- Hydrochloric acid (Sigma-Aldrich, cat. no. H1758) **! CAUTION** Hydrochloric acid is toxic on inhalation, and it can cause eye and skin burns. Use a hood, protective clothing, eye protection and gloves. Do not store it in a metal container.
- ATP, [γ-^{32}P] 6,000 Ci/mmol, 10 mCi/ml, 250 µCi (PerkinElmer, cat. no. BLU002Z250UC) **! CAUTION** All radioisotopes should be used in strict accordance with the regulations and guidelines of one's institution. ^{32}P is a high-energy beta emitter. All steps should be conducted behind Plexiglas shielding. Use protective clothing, eye protection and gloves.
- illustra MicroSpin G-25 columns (GE Healthcare, cat. no. 27-5325-01)
- PerfectHyb plus hybridization buffer (Sigma-Aldrich, cat. no. H7033)
- Extra thick blot filter paper, precut, 19 × 18.5 cm (Bio-Rad, cat. no. 1703969)
- Saline sodium citrate (SSC) buffer, 20× liquid concentrate (Amresco, cat. no. 0804)
- DMEM, high glucose (Life Technologies, cat. no. 11965-092)
- FBS, Optima, heat inactivated (Atlanta Biologicals, cat. no. S12450H)
- Penicillin-streptomycin (Life Technologies, cat. no. 15140-122)
- BT-474 cell line (American Type Culture Collection, cat. no. HTB-20) **! CAUTION** The BT-474 cells were cultured in DMEM containing 10% (vol/vol) FBS and penicillin-streptomycin. The absence of *Mycoplasma* was regularly checked every month.
- C57BL/6J mice (The Jackson Laboratory, stock no. 000664) **! CAUTION** Mice were treated under appropriate protocols according to relevant institutional and governmental regulations and approved by the institutional animal care and use committee at Thomas Jefferson University. Spleens were obtained from male adult mice at ~3 months of age.

EQUIPMENT

- RNase-free microcentrifuge tubes, 1.7 and 0.6 ml
- RNase-free round-bottom, microcentrifuge tube, 2.0 ml
- RNase-free microcentrifuge tube, 0.2 ml for PCR
- Vortex
- Filter tips
- Disposable pipettes
- Pipet-Aid (Corning)
- Thermoblock with shaking (Eppendorf Thermomixer R)
- Corning Costar Spin-X centrifuge tube filters, 0.45-µm pore (Sigma-Aldrich, cat. no. CLS8162)
- Spectrometers (TECAN, infinite 200 Pro series)
- Vacuum filter unit, 0.22-µm pore (BioExpress, cat. no. F-2980)
- Refrigerated benchtop centrifuge (Eppendorf, cat. no. 5424R)
- SE410 tall air-cooled vertical electrophoresis unit (Hoefer, cat. no. SE410)
- SE400 air-cooled vertical electrophoresis unit (Hoefer, cat. no. SE400)
- Mini-PROTEAN tetra cell casting stand with clamp kit for single core system (Bio-Rad, cat. no. 1658052)
- Mini-PROTEAN tetra electrode assembly (Bio-Rad, cat. no. 1658037)
- Buffer tank and lid (Bio-Rad, cat. no. 1658040)
- Short plates (Bio-Rad, cat. no. 1653308)
- Spacer plates with 1.0 mm integrated spacers (Bio-Rad, cat. no. 1653311)
- Mini-PROTEAN comb, 10-well, 1.0 mm, 44 µl (Bio-Rad, cat. no. 1653359)
- Power supply unit
- Gel imaging system (Bio-Rad, ChemiDoc, cat. no. 1708280)
- Blue light transilluminator (Syngene UltraSlim-LED)
- Vacuum concentrator (Eppendorf Vacufuge plus, cat. no. 2231000079)
- Thermal cycler (Labnet MultiGene OptiMax thermal cycler, cat. no. TC9610)
- Blade
- Needle, 21G
- Cross-linker, shortwave UV (UVP, cat. no. 95-0174-01)
- Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad, cat. no. 1703940)
- Liquid scintillation and gamma counters (Hidex, Triathler)
- Amersham Hybond-N+ (GE Healthcare, cat. no. RPN303B)
- Deluxe ProBlot hybridization systems (Labnet)
- Hybridization bottles
- Wrap
- Imaging screen-K (Kodak; Bio-Rad, cat. no. 1707843)
- Exposure cassette-K (Bio-Rad, cat. no. 1707861)
- Phosphorimager (GE Healthcare, Typhoon FLA 7000)

REAGENT SETUP

Acrylamide gel solution, 15% (wt/vol) containing 7 M urea Prepare as tabulated below. After filtration using a vacuum filter unit, the solution can be stored at 4 °C for up to 3 months with shielding from light.

Reagent	Volume/amount
Acrylamide:bisacrylamide (19:1), 40% (wt/vol)	187.5 ml
TBE, 10×	50 ml
Urea	210.21 g
Milli-Q water	Up to 500 ml
Total volume	500 ml

▲ **CRITICAL** Change the acrylamide concentration depending on the targeted RNA length.

Loading buffer, 2× Prepare as tabulated below:

Reagent	Final concentration
Urea	9 M
Xylene cyanol	0.05%
Bromophenol blue	0.05%

RNA elution buffer Prepare as tabulated below:

Reagent	Volume	Final concentration
SDS solution, 20% (wt/vol)	50 µl	0.10% (wt/vol)
EDTA, 0.5 M, pH 8.0	2 µl	0.1 mM
Nuclease-free water	Up to 10 ml	
Total volume	10 ml	

Acrylamide gel solution, 8% (wt/vol) for native PAGE Prepare as tabulated below:

Reagent	Volume
Acrylamide:bisacrylamide (19:1), 40% (wt/vol)	4 ml
TAE, 50×	400 µl
Milli-Q water	Up to 20 ml
Total volume	20 ml

▲ **CRITICAL** Change the acrylamide concentration depending on the targeted cDNA length.

PROCEDURE

RNA extraction ● TIMING 1 h

1| Suspend cells or tissues in 1 ml of TRIsure (the volume depends on the sample amount), add 200 µl of chloroform and vortex the mixture for 15 s.

2| After incubation at room temperature (20–25 °C) for 3 min, centrifuge the tube at >15,000*g* for 15 min at 4 °C.

3| Transfer the aqueous phase to a new tube.

4| Add 500 µl of isopropanol, vortex the tube well and incubate it for 10 min at −20 °C.

5| Centrifuge at >15,000*g* for 30 min at 4 °C.

6| Remove the supernatant and discard it, without dislodging the pellet.

7| Add 1 ml of 70% (vol/vol) ethanol and centrifuge the tube at >7,500*g* for 10 min at 4 °C.

8| Remove the supernatant completely and discard it, without dislodging the pellet.

9| Evaporate the residual supernatant from the pellet for ~45 s at room temperature using a centrifugal vacuum concentrator.

10| Dissolve the pellet in an appropriate amount (expected resultant concentration: 1–10 µg/µl) of nuclease-free water.
▲ **CRITICAL STEP** To avoid pellet insolubility, do not dry the pellet for a long time, and immediately add nuclease-free water. Perform pipetting over 100 times to completely dissolve the pellet. Do not dissolve by vortexing and tapping.
▲ **CRITICAL STEP** We recommend using fresh RNA samples for cP-RNA-seq. Do not use old RNA samples that are stored in a freezer for a long time, because cP is not very stable in aqueous solution and it is susceptible to background hydrolysis.

Gel purification of RNAs with targeted lengths ● **TIMING 2 d**
11| Assemble a gel apparatus (e.g., Mini-PROTEAN, 7 cm × 10 cm; Bio-Rad).

12| Add 75 µl of 10% (wt/vol) ammonium persulfate solution and 5 µl of UltraPure TEMED per 10 ml of the 15% (wt/vol) acrylamide gel solution containing 7 M urea (see Reagent Setup), gently mix, and then immediately pour the mixture into the gel apparatus. To polymerize, incubate for 1–2 h at room temperature.

13| After prerunning for 30 min at 200 V, wash the wells using a 21G needle with a syringe, and then apply the extracted total RNAs from Step 10 mixed with the same volume of the 2× loading buffer.
▲ **CRITICAL STEP** The total RNA amount depends on the amount of targeted RNA in the sample. The amount of targeted RNAs could be estimated by northern blots. In our 5′-SHOT-RNA analyses, 5′-SHOT-RNA^{AspGUC} and 5′-SHOT-RNA^{HisGUG} were clearly detected by northern blotting using 5 µg of BT-474 total RNA[5]. We loaded 50–100 µg of BT-474 total RNA for this procedure.

14| Run the gel at 200 V for 1 h until bromophenol blue reaches the bottom of the gel.
▲ **CRITICAL STEP** Change the electrophoresis time depending on the targeted RNA length. The above conditions are appropriate for 20- to 50-nt targeted RNAs. If the targets have longer lengths such as 50–100 nt, increase the running time and run the gel until xylene cyanol reaches the bottom of the gel.

15| Disassemble the apparatus and stain the gel with 0.01% (vol/vol) SYBR Gold in 1× TBE for 15 min.

16| Visualize the gel on a LED blue light transilluminator and then cut the gel region containing the RNAs with the targeted lengths.

17| Make a gel-breaker tube by puncturing the bottom (three or four places) of a 0.6-ml microcentrifuge tube with a 21G needle, and then place the tube into a round-bottom 2.0-ml microcentrifuge tube.

18| Place the cut gel fractions into the gel breaker tube, and break the gel by centrifuging at >20,000*g* for 5 min at 4 °C.

19| Add 300–800 µl of the RNA elution buffer (see Reagent Setup) into the produced gel debris in the 2.0-ml microcentrifuge tube.
▲ **CRITICAL STEP** Change the volume of the elution buffer depending on the volume of gel debris. The volume of elution buffer should be approximately three times more than that of gel debris.

20| Rotate or shake the tube overnight at 4 °C.
▲ **CRITICAL STEP** Confirm that the debris is floating in the buffer and not sticking at the bottom of the tube during rotation.

21| Centrifuge the tube at >15,000*g* for 5 min at 4 °C, and then transfer the eluate into a centrifuge tube filter with a 0.45-µm pore.

22| Centrifuge at 600*g* for 2 min at 4 °C, and then collect the eluate for complete removal of the gel debris.

23| Add a 10% volume of 3 M sodium acetate and 1–2 µl of linear acrylamide and vortex. Next, add one volume of isopropanol, vortex the mixture well and incubate it for 30 min at –20 °C.

24| Centrifuge the tube at >15,000*g* for 30 min at 4 °C and remove the supernatant and discard it, without dislodging the pellet.
▲ **CRITICAL STEP** After terminating the centrifugation, remove the supernatant as quickly as possible without losing the pellet, which might only be faint white.
? TROUBLESHOOTING

25| Add 1 ml of 70% (vol/vol) ethanol, followed by centrifugation at 7,500*g* for 5 min at 4 °C.

26| Remove the ethanol completely and dry the sample for 45 s at room temperature using a vacuum centrifuge concentrator.
▲ **CRITICAL STEP** Remove the supernatant as quickly as possible and do not dry the pellet for a long time.

27| Dissolve the pellet in nuclease-free water; the amount is dependent on the expected amount of targeted RNA. In our 5′-SHOT-RNA identification, we dissolved the pellet in 50 µl of water.

Phosphatase (CIP) treatment ● TIMING 2 h
28| Add the following 30 µl of reaction mixture into 20 µl of the gel-purified RNA from Step 27, and incubate it for 40 min at 37 °C.

Reagent	Volume (µl) per reaction	Final concentration
CutSmart buffer, 10×	5	1×
RNasin	0.5	0.2–0.4 U/µl
CIP	1	0.2 U/µl
Nuclease-free water	23.5	
Total volume	30	

29| Add 50 µl of nuclease-free water and 100 µl of acid phenol-chloroform.

30| Vortex the mixture for 10 s and centrifuge it at >15,000*g* for 5 min at room temperature.

31| Transfer the upper aqueous phase into a new tube.
▲ **CRITICAL STEP** Completely avoid contamination from the lower organic fraction.

32| Add 200 µl of nuclease-free water, 1 µl of linear acrylamide and 30 µl of 3 M sodium acetate, and then vortex the mixture.

33| Add 750 µl of ethanol, vortex the mixture and place the tube for 30 min at –80 °C.

34| Centrifuge the tube at 15,000*g* for 30 min at 4 °C.

35| Remove the supernatant completely and discard it, without dislodging the pellet, and dry the sample for 45 s at room temperature using a vacuum centrifuge concentrator.
▲ **CRITICAL STEP** Remove the supernatant as quickly as possible and do not dry the pellet for a long time.

36| Dissolve the pellet in 45 µl of nuclease-free water.

Periodate ($NaIO_4$) treatment ● TIMING 2 h
37| Prepare 100 mM $NaIO_4$ solution by dissolving it in nuclease-free water.
▲ **CRITICAL STEP** Prepare a fresh solution for every experiment. $NaIO_4$ is light-sensitive; therefore, the solution and the following reaction mixture containing $NaIO_4$ should not be exposed to light (e.g., cover the tube with aluminum foil).

38| Add 5 µl of 100 mM $NaIO_4$ solution into 45 µl of the CIP-treated RNA from Step 36 (final concentration of $NaIO_4$ is 10 mM), and incubate it for 40 min on ice in the dark.

39| Add 250 µl of nuclease-free water and perform ethanol precipitation, as described in Steps 32–35.

40| Dissolve the pellet in 10 µl of nuclease-free water.
■ **PAUSE POINT** The dissolved RNA can be stored at –80 °C for up to 3 months.

Kinase (T4 PNK) treatment ● TIMING 2 h

41| Add 20 µl of the following T4 PNK reaction mixture into 10 µl of the $NaIO_4$-treated RNA from Step 40, and incubate it for 40 min at 37 °C.

Reagent	Volume (µl) per reaction	Final concentration
T4 PNK reaction buffer, 10×	3	1×
ATP, 10 mM	0.5	166 µM
RNasin	0.5	0.3–0.6 U/µl
T4 PNK	1	0.33 U/µl
Nuclease-free water	15	
Total volume	20	

42| Add 70 µl of nuclease-free water and perform phenol chloroform extraction and ethanol precipitation, as described in Steps 29–35.

43| Dissolve the pellet in 10 µl of nuclease-free water.
■ **PAUSE POINT** The dissolved RNA can be stored at –80 °C for up to 3 months.

Adapter ligation using Illumina TruSeq small RNA preparation kit ● TIMING 3 d

44| Add 1 µl of 3′ adapter (RA3) into 5 µl of the T4 PNK-treated RNA from Step 43.

45| Incubate the tube for 2 min at 70 °C, and then immediately place it on ice and incubate it for 1 min.

46| Add 4 µl of the following mixture.

Reagent	Volume (µl) per reaction
Ligation buffer (HML)	2
RNase inhibitor	1
T4 RNA ligase 2, truncated	1
Total volume	4

47| Incubate the tube for 1 h at 28 °C and then overnight at 4 °C.

48| Add 1 µl of the stop buffer (STP), incubate the mixture for 15 min at 28 °C, and then place the tube on ice.

49| Put 1.1 µl/sample of 5′ adapter (RA5) into a new tube, incubate it for 2 min at 70 °C, and then immediately place the tube on ice and incubate for 1 min.

50| Add 1.1 µl/sample of 10 mM ATP into the tube and mix by pipetting.

51| Add 1.1 µl/sample of T4 RNA ligase and mix by pipetting.

52| Add 3 µl of the mixture of 5′ adapter, ATP and T4 RNA ligase from Step 51 into the 3′ adapter–ligated sample from Step 48.

53| Incubate the tube for 1 h at 28 °C and then overnight at 4 °C.
■ **PAUSE POINT** The dissolved RNA can be stored at –80 °C for up to 3 months.

RT-PCR ● TIMING 2 h

54| In a 0.2-ml PCR tube, add 1 µl of RT primer (RTP) to 6 µl of the adapter ligated-RNA from Step 53.

55| Incubate the tube for 2 min at 70 °C in a thermal cycler and then immediately place the tube on ice.

56| Add 5.5 µl of the following RT reaction mixture and gently mix by pipetting.

Reagent	Volume (µl) per reaction
First-strand buffer, 5×	2
dNTP mix, 12.5 mM	0.5
DTT, 100 mM	1
RNase inhibitor	1
SuperScript III reverse transcriptase	1
Total volume	5.5

57| Incubate for 60 min at 50 °C in a thermal cycler.
■ **PAUSE POINT** The synthesized cDNA can be stored at 4 °C for up to 1 week.

58| Add 37.5 µl of the following PCR mixture.

Reagent	Volume (µl) per reaction
UltraPure water	8.5
PCR mix (PML)	25
RNA PCR primer (RP1)	2
RNA PCR primer index (RPIX)	2

▲ **CRITICAL STEP** Use the different RPIX for multiplex sequencing, allowing multiple samples to be sequenced simultaneously in a single sequencing lane. The barcode sequences in the primer index will be distinguished and sorted during data analysis.

59| Amplify the cDNAs in a thermal cycler using the following PCR cycle conditions:

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	98	30 s	1
Denaturation	98	10 s	11–15
Annealing	60	30 s	
Extension	72	15 s	
Final extension	72	10 min	1
Store	4	∞	1

Gel purification of the amplified cDNAs ● **TIMING 2 d**

60| Assemble a gel cast apparatus (e.g., SE400 air-cooled vertical electrophoresis unit for 18 cm × 16 cm × 0.75 mm gel; Hoefer).

61| Add 150 µl of 10% (wt/vol) ammonium persulfate solution and 10 µl of UltraPure TEMED per 20 ml of the 8% (wt/vol) acrylamide gel solution, gently mix, and then immediately pour the mixture into the gel apparatus. To polymerize, incubate for 1–2 h at room temperature.

62| Add 6× loading dye to the amplified cDNA samples from Step 59 and apply to the gel. As size markers, also apply a high-resolution ladder and phiX174 DNA-HaeIII digest marker.

63| Run the gel at 200 V for ~3 h until the xylene cyanol has moved 75% from the top of the gel.
▲ **CRITICAL STEP** Change the electrophoresis time depending on the targeted cDNA length. The above condition is appropriate for 20- to 50-nt-targeted cDNAs. If the target lengths are longer (50–100 nt), run the gel until the xylene cyanol has moved to the bottom of the gel.

64| Disassemble the apparatus and stain the gel with 0.01% (vol/vol) SYBR Gold in 1× TAE for 15 min.

65| Capture the gel image of strained cDNAs using a gel-imaging system (e.g., ChemiDoc; Bio-Rad).
▲ **CRITICAL STEP** Focus on specific cDNA bands that are only present in a lane from a full reaction with T4 PNK treatment and absent in a lane from a negative control reaction without CIP/T4 PNK treatment.
? TROUBLESHOOTING

66| Visualize the gel on a LED blue light transilluminator and cut the targeted cDNA bands.

67| Break the gel as described in Steps 17 and 18.
■ **PAUSE POINT** The cut or broken gel can be stored at –20 °C for up to 3 months.

68| Add 300–800 µl of nuclease-free water into the gel debris.
▲ **CRITICAL STEP** Change the volume of water depending on the volume of gel debris.

69| Rotate or shake the tube overnight at room temperature.
▲ **CRITICAL STEP** Confirm that the debris is floating in the buffer and not sticking at the bottom of the tube during rotation.

70| Take the eluate and perform ethanol precipitation, as described in Steps 21–26.

71| Dissolve the pellet in 10 µl of nuclease-free water.

72| Perform a quality check, Illumina next-generation sequencing and bioinformatics analyses. See, for example, the analyses of 5′-SHOT-RNAs described in Honda *et al.*[5].

Biochemical validation of the presence of cP in the identified RNAs: acid (HCl) treatment ● TIMING 4 h
73| Incubate the total RNA (1–20 µg) from Step 10 in the following HCl solution for 3 h on ice.

Reagent	Volume (µl) per reaction	Final concentration
Total RNA	Variable; depends on RNA amount	
HCl, 100 mM	5	10 mM
Nuclease-free water	Up to 50 µl	
Total volume	50	

▲ **CRITICAL STEP** To prevent RNA damage from the concentrated acid, mix the total RNA and water first, add 100 mM HCl solution onto the inner wall of the tube and then mix it by vortexing.

74| Add 250 µl of nuclease-free water and 30 µl of 3 M sodium acetate (pH 5.5) and vortex well. Next, add 750 µl of 100% ethanol, vortex the mixture well and incubate it for 30 min at –80 °C.

75| Centrifuge it at >15,000*g* for 30 min at 4 °C.

76| Remove the supernatant completely, and then discard it and dry the pellet for 45 s at room temperature using a centrifugal vacuum concentrator.

77| Dissolve the pellet in an appropriate amount (expected resultant concentration: 1–10 µg/µl) of nuclease-free water, and measure the concentration using a spectrometer (e.g., TECAN infinite 200 Pro series).
▲ **CRITICAL STEP** To avoid pellet insolubility, do not dry the pellet for a long time, and immediately add nuclease-free water.
■ **PAUSE POINT** The dissolved RNA can be stored at –80 °C for up to 3 months.

Phosphatase (CIP) treatment ● TIMING 2 h

78| Incubate a sample of the acid-treated RNA (from Step 77) and a sample of nontreated total RNA (1–20 µg) from Step 10 in the following CIP reaction mixture for 40 min at 37 °C.

Reagent	Volume (µl) per reaction	Final concentration
Total RNA	Variable; depends on RNA amount	
CutSmart buffer, 10×	5	1×
RNasin	0.5	0.2–0.4 U/µl
CIP	1	0.2 U/µl
Nuclease-free water	Up to 50 µl	
Total volume	50	

79| Add 50 µl of nuclease-free water and perform phenol chloroform extraction and ethanol precipitation as described in Steps 30–36 (addition of linear acrylamide is not required).

80| Dissolve the pellet in an appropriate amount (expected resultant concentration: 1–10 µg/µl) of nuclease-free water, and measure the concentration by using a spectrophotometer. These samples using acid treated or nontreated total RNA are referred to as HCl + CIP samples or CIP samples, respectively.

■ **PAUSE POINT** The dissolved RNA can be stored at –80 °C for up to 3 months.

Kinase (T4 PNK) treatment ● TIMING 2 h

81| Incubate total RNA (1–20 µg) from Step 10 in the following T4 PNK reaction mixture for 40 min at 37 °C.

Reagent	Volume (µl) per reaction	Final concentration
Total RNA	Variable; depends on RNA amount	
T4 PNK reaction buffer, 10×	3	1×
RNasin	0.5	0.3–0.6 U/µl
T4 PNK	1	0.3 U/µl
Nuclease-free water	Up to 30 µl	
Total volume	30	

82| Add 70 µl of nuclease-free water and perform phenol-chloroform extraction and ethanol precipitation, as described in Steps 29–35 (addition of linear acrylamide is not required).

83| Dissolve the pellet in an appropriate amount (expected resultant concentration: 1–10 µg/µl) of nuclease-free water, and measure the concentration by using a spectrophotometer. This is referred to as a T4 PNK sample.

■ **PAUSE POINT** The dissolved RNA can be stored at –80 °C for up to 3 months.

Northern blotting ● TIMING 3 d

84| For miRNA (~22 nt) or 5′-SHOT-RNAs (~34 nt), make 18 cm × 24 cm × 0.75 mm 15% (wt/vol) denaturing acrylamide gel (e.g., using a SE410 tall air-cooled vertical electrophoresis unit), as described in Steps 11 and 12.

85| Radiolabel low-molecular-weight, single-nucleotide marker (e.g., low-molecular-weight marker, 10–100 nt; Affymetrix) by incubating the following reaction mixture for 1 h at 37 °C.

Reagent	Volume (µl) per reaction
Low-molecular-weight marker	1
^{32}P-γ-ATP, 6,000 Ci/mmol	1
T4 PNK reaction buffer, 10×	1
T4 PNK	0.5
Nuclease-free water	Up to 10 µl
Total volume	10

86| Purify the labeled marker using illustra MicroSpin G-25 columns. Count the counts per minute (c.p.m.) of the marker solution using a liquid scintillation counter (5,000–10,000 c.p.m. of the labeled marker should be applied in the following step).
! CAUTION Use all radioisotopes in strict accordance with the regulations and guidelines of one's institution.

87| After pre-running the gel from Step 84 for 30 min at 300 V, wash the wells using a 21G needle with a syringe, and apply the RNA samples (labeled marker (from Step 86), nontreatment total RNA (from Step 10) and three treated total RNAs: CIP, HCl + CIP and T4 PNK (from Steps 80 and 83)) mixed with the same volume of the 2× loading buffer.

88| Run the gel at 300 V for 4–5 h in 1×TBE buffer until the xylene cyanol has moved 75% from the top of the gel.
▲ CRITICAL STEP To avoid curving of the bands, do not increase the voltage too much. Change the electrophoresis time depending on the targeted RNA length. The above conditions are appropriate for 20- to 40-nt-targeted RNAs. Xylene cyanol matches ~30-nt RNAs in the 15% (wt/vol) denaturing acrylamide gel. If targeted RNAs are longer, prepare a larger gel (e.g., using Fisher Scientific; sequencing system aluminum 2, cat. no. OW S4S) and increase the running time.

89| Disassemble the apparatus and transfer the RNAs in the gel to Hybond-N+ membrane using a semi-dry blotting system for 30 min at 400 mA in 1× TBE.

90| After drying the membrane, perform UV cross-linking at 120 mJ/cm^2 twice.

91| For prehybridization, rotate and incubate the membrane with PerfectHyb plus hybridization buffer in a hybridization bottle for 1 h at 37 °C.

92| Prepare ^{32}P-labeled probe by incubating the following mixture for 1 h at 37 °C, and then purify the labeled probe using illustra MicroSpin G-25 columns. Count the c.p.m. of the probe using a liquid scintillation counter.

Reagent	Volume (μl) per reaction
DNA probe, 2 μM	2
^{32}P-γ-ATP, 6,000 Ci/mmol	1
T4 PNK reaction buffer, 10×	1
T4 PNK	0.5
Nuclease-free water	Up to 10 μl
Total volume	10

93| Add 1,000,000–2,000,000 c.p.m. of the ^{32}P-labeled probe to the hybridization bottle and incubate it overnight at 37 °C.

94| Briefly shake the membrane in 2× SSC buffer, discard the buffer, and then wash the membrane twice by rotating and incubating it for 20 min at 37 °C in 2× SSC buffer.

95| After drying the membrane, cover it with plastic wrap and expose it to a phosphor-imaging screen for 1 h overnight at room temperature in a cassette.

96| Scan the image using a Phosphorimager (e.g., Typhoon; GE Healthcare), and observe the band differences between samples.
▲ CRITICAL STEP For a 20- to 35-nt target, removal of a cP or P causes an upshifted band, because the effect of the phosphate charge is more crucial than that of the phosphate mass. The balance of the effect between charge and mass will vary depending on the size of target RNAs.
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
24	The RNA pellet is invisible	Low amount of targeted RNA fraction	Add an additional 1 µl of linear acrylamide, vortex and centrifuge again
65	Appearance of many strong bands in the CIP/T4 PNK negative control lane	Insufficient periodate treatment	Use fresh $NaIO_4$ solution prepared immediately before the treatment
	Weak band from the full procedure of cP-RNA-seq	Insufficient amount of targeted RNA	Increase the amount of starting material
		Insufficient enzymatic activity	Change the enzymes. Avoid phenol contamination in the samples by not taking any of the lower organic phase during phenol-chloroform extraction
		RNA degradation during the procedure	Perform experiments with careful attention to RNase contamination in an RNase-free environment. Clean the pipettes, bench top, tube rack, tubes and so on using RNaseZap reagent. Keep the sample tubes on ice during the experiment
96	Insufficient shift of the bands	Insufficient electrophoresis length	Use a larger gel and perform electrophoresis for a longer time so that band mobility differences from the presence or absence of cP or P can be clearly observed
		Targeted RNA is too long	It is difficult to observe the band mobility differences from long targeted RNA. Therefore, try a different method, such as the method using poly-A polymerase[39,40]
		Insufficient enzymatic activity	Change enzymes. Avoid phenol contamination in the samples by not taking any of the lower organic phase during phenol-chloroform extraction

● TIMING

cP-RNA-seq

Steps 1–10, RNA extraction: 1 h
Steps 11–27, gel purification of RNAs with targeted lengths: 2 d
Steps 28–36, phosphatase (CIP) treatment: 2 h
Steps 37–40, periodate ($NaIO_4$) treatment: 2 h
Steps 41–43, kinase (T4 PNK) treatment: 2 h
Steps 44–53, adapter ligation using Illumina TruSeq small RNA preparation kit: 3 d
Steps 54–59, RT-PCR: 2 h
Steps 60–72, gel purification of the amplified cDNAs: 2 d

Biochemical validation of the presence of cP in the identified RNAs

Steps 73–77, acid (HCl) treatment: 4 h
Steps 78–80, phosphatase (CIP) treatment: 2 h
Steps 81–83, kinase (T4 PNK) treatment: 2 h
Steps 84–96, northern blotting: 3 d

ANTICIPATED RESULTS

Selective amplification of 5′-tRNA halves using the cP-RNA-seq procedure

As an example, we show the native PAGE result from a cP-RNA-seq procedure applied to mouse spleen total RNA (**Fig. 3**). As indicated by an arrow, the method successfully amplified ~150- to 155-bp cDNA products corresponding to the targeted 5′-tRNA halves (~32–37 bp). The absence of a band from the negative control procedure without CIP and T4 PNK treatment suggested that the band is from cP-containing RNAs.

Biochemical validation of the presence of a cP in a mouse 5′-tRNA half

As an example, we show the northern blot results using enzyme-treated mouse spleen total RNAs to validate the presence of a cP in a 5′-tRNA half derived from cytoplasmic $tRNA^{AspGUC}$ (**Fig. 4**). NT designates the nontreated sample used as a negative control. The band was shifted up by the CIP treatment, suggesting the presence of a P in the 5′-tRNA half. The T4 PNK treatment similarly shifted the band up, and further upshift was observed by the HCl + CIP treatment, indicating the additional presence of a cP. These results indicate that the 5′-$tRNA^{AspGUC}$ half contains a 5′-P and a 3′-cP. Detection of mouse miRNA-16 (miR-16) was used as a control. Because miR-16 contains 5′-P and 3′-OH, the CIP and HCl + CIP treatments identically shifted the band up, whereas the T4 PNK treatment caused no shift.

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