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tRIBO-seq: Selective profiling of translationally active tRNAs V.1

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GitHub: <https://github.com/novoalab/tRIBO-seq/tree/main>

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We use this protocol and it's working

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Abstract

Here we describe **tRIBO-seq**, a simple and robust nanopore-based method for the selective capture of ribosome-associated native tRNA populations (ribo-tRNAs) from actively translating ribosomes. This approach enables the simultaneous assessment of tRNA abundance, modification status, and fragmentation within a single experiment. The protocol details the recovery of ribosome-embedded tRNAs, which can subsequently be sequenced using nanopore or Illumina platforms.

Guidelines

tRIBO-seq uses the **RiboLace Starter Kit** (Immagina Biotech, #RL00S-04), which relies on a proprietary puromycin derivative, **3P**. This molecule retains the ability of puromycin to intercalate into the catalytic site of actively translating ribosomes while being covalently linked to biotin. Samples are first treated with cycloheximide (CHX) to stall ribosomes on translating mRNAs (recommended step), followed by cell lysis and nuclease digestion to generate ribosome-protected fragments (RPFs) embedded within translating ribosomes. In parallel, magnetic beads are functionalized with 3P and, after nuclease digestion, are added to the lysate to selectively capture active ribosomal complexes via rapid magnetic separation. Owing to the use of the 3P molecule, this protocol is compatible only with mammalian systems.

Optimal Workflow Recommendations

- Please run up to 6 samples in parallel. Longer manipulation time may introduce an unwanted variability between the first and the last sample.
- Allocate at least 1 day for the completion of the entire workflow.
- If possible, please perform a preliminary lysis experiment to set the lysis volume following the suggested AU operational range.
- The Beads Functionalization and the Nuclease Digestion can be performed in parallel, to shorten the protocol length.
- This protocol has been optimized to perform the RPF pulldown starting with 0.9 AU (Abs260nm) of lysate. However, due to the adjustability of the protocol, it is possible to tailor the reagents to any input amount between 0.1 and 1.2 AU.
- Here, we detail the protocol for adherent cells. For other starting materials, please consult Immagina Biotech documentation at [this link](#).

Below some information before getting started with the protocol!



Materials

- Cycloheximide stock solution (10 mg/mL)
- RNase-free water
- PBS (ice-cold)
- IMMAGINA RiboLace Starter Kit (#RL00S-04)
- Sodium deoxycholate (SDC), 10% (w/v) stock
- DNase I (1 U/ μ L)
- RNaseOUT™ (40 U/ μ L)
- Suprase•In™ RNase Inhibitor
- RNase Inhibitor, Murine (40,000 units/mL; NEB #M0314L)
- 1.5 mL RNase-free microcentrifuge tubes (pre-chilled)
- 0.22 μ m filter membranes
- Magnetic rack for bead separation
- Zymo RNA Clean & Concentrator-5 Kit (Zymo #R1015 or #R1016)
- RNeasy MinElute Cleanup Kit (Qiagen #74204)
- RWT Buffer (Qiagen #1067933)
- DNase TURBOTM (2 U/ μ L; Thermo Fisher #AM2238)
- Absolute ethanol (Merck #100983250)
- Qubit™ Fluorometer with Qubit RNA HS Assay Kit (Thermo Fisher)
- Agilent TapeStation system with High Sensitivity RNA ScreenTape

Before start

Sample amount recommendations

The amount of RNA that can be isolated from a sample is strongly affected by its translational state and must be considered when programming experiments. For instance, two lysates similarly concentrated (i.e., similar Abs260nm) but from different cell types or specimens (e.g. human vs mouse, brain vs liver, or immortalized vs primary), or with different treatments (e.g. drugs and transfection reagents) could have completely different amounts of translating ribosomes, leading to opposite outcomes.

It is not possible to provide a minimal sample size as a defined number of cells or weight of tissue. As a general indicator 5 million non-treated cells, coming from an immortalized line (such as HeLa, HEK, CHO, and K562) at 70 to 80% confluence represent a comfortable starting point. Given specimen-to-specimen variability, as a preliminary experiment, we suggest testing the lysis step on different sample amounts, recording the corresponding total A.U., and using it to fine-tune volumes and sample size during the real experiment.

AU calculation - Input lysate quantification

Cells and tissues should be lysed as described in protocol below. The AU of your sample is measured using a spectrophotometer, most commonly a Nanodrop. Set the instrument so to measure the Abs at 260 nm (usually Nucleic Acid function) and measure the absorbance of your lysate using the Supplemented Lysis Buffer (SLB) as blank. The use of different lysis buffers is strongly discouraged because it may interfere with the efficiency of ribosome pull-down and with the AU calculation (some components may absorb at 260 nm).

If the instrument does not allow to use of the SLB as blank, please use water instead, then record the absorbance of both the SLB and the lysate and subtract the absorbance of the SLB to the lysate.

Example:

* Supplemented Lysis buffer SLB Abs260nm = 7 AU

* Specimen Abs260nm = 17 AU

* Absorbance value of lysate = $17 - 7 = 10$ AU

Lysis volume selection

It is important to lyse the specimen in an appropriate volume to obtain a lysate with an optimal range of Abs at 260 between 7 to 15 AU. Lower or higher values may affect the efficiency and reproducibility of the kit since using smaller amounts or using more diluted lysate could cause quantification and/or pipetting errors.

The resuspension values suggested in Table 1, should set you within the optimal AU range. For instance, starting with 5 million immortalized cells lysed in 300 µL of lysis buffer an absorbance between 7 to 15 AU is expected after blank subtraction.

	Specimen	Quantity	Lysis buffer	Volume of supplemented LB (uL)
	Cell	0.3 - 1 million cells	# RL00S-04	50
	Cell	1 - 5 million cells	# RL00S-04	150
	Cell	> 5 million cells	# RL00S-04	300
	Tissue	< 10 mg	# IBT0032	500
	Tissue	> 10 mg	# IBT0032	800

Table 1: The quantity of lysis buffer depends on specimen amount.



Calculate the volume of lysate needed for the pulldown.

The absorbance of your sample depends on your sample characteristics (type of cell/tissue and amount) and the volume in which it has been resuspended. Given this volume dependence, it is possible to consider the AU read out as a concentration, and we can decide arbitrarily to set it as AU/mL.

To calculate the volume of lysate to utilize to pipet 0.9 AU, follow the example below:

Example: Nanodrop absorbance value of lysate at 260 nm = 10 AU.

This means that, arbitrarily, we set the absorbance of the lysate at 10 AU/ml, which is divided by 1000 $\mu\text{L}/\text{mL}$ to get the concentration per μL = 0.01AU/ μL .

□ To start with 0.9 AU use: $0.9\text{AU}/0.01\text{ AU}/\mu\text{L} = 90\ \mu\text{L}$ of lysate

Cellular culture and lysis

- 1 Prepare fresh supplemented lysis buffer (SLB) by combining reagents Table 2. If SDC appears white/cloudy make a fresh stock. Please see comments in 'Before starting' for deciding on the appropriate SLB volume.

	n	Lysis buffer (LB)	Sodium deoxycholate (SDC) 10% (w/v)	DNase I 1 U/uL	RiboLock RNase Inhibitor 40 U/uL	Final volume
	N = 1	267 uL	30 uL	1.5 uL	1.5 uL	300 uL
	N = -					

Table 2: Supplemented lysis buffer (SLB).

- 2 Treat cells with **10 ug/mL** cycloheximide (CHX) for 5 min at 37 C before lysis. Cells need to be at 70-80% confluency.



Warning: Cycloheximide (CHX) is highly toxic. Always handle in a fume hood using appropriate PPE.

- 3 After incubation place the cells on ice and wash them 2X quickly with cold PBS containing CHX (**20 ug/mL**).

- 4 Remove all residual PBS to cells to not dilute the lysis buffer.

- 5 Perform the lysis directly adding 300 uL of complete **supplemented lysis buffer** to each cell dish and scrape vigorously. Mechanical scraping helps the downstream processing by disrupting the cell membrane and releasing the cellular contents, including ribosomes.

- 6 Collect the lysate in a pre-chilled 1.5 mL Eppendorf microcentrifuge tube.

- 7 Pellet the cell debris and nuclei by centrifugation at 20,000 g for 5 min at 4°C.

- 8 Transfer the supernatant to a fresh tube and keep on ice for 20 min vortexing every 2-3 minutes to aid the lysis.

- 9 Check the absorbance of the cell lysate at 260 nm, we suggest using a Nanodrop setting the "nucleic acid" function and using 1.5 µL of the supplemented lysis buffer as blank. If the sample is not processed the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing.



See notes in 'Before starting' section.

Bead functionalisation

- 10 *During bead functionalisation, it is essential to handle the beads carefully to preserve their integrity. The beads should never be allowed to dry out nor spun down, as centrifugation can cause them to collapse. As with most magnetic beads, it is best to avoid pipetting whenever possible, as they readily adhere to the plastic of pipette tips.*
- Place samples and **RsP** to thaw on ice
- 11 Prepare the probe by adding 324 μL ice-cold B-buffer to 76 μL concentrated RsP
- This step is only completed once per kit. If not using all diluted RSP (dRSP) make aliquots and store in -80 .*
- 12 Remove the RiboLace magnetic beads (RmB) from 4°C and place the tube at RT for at least 30 min
- 13 Vortex the RmB beads tube thoroughly for > 30 sec
- 14 *From here onwards volumes indicated are for 1 sample, please scale up as needed except where indicated not to.*
- Place **144** μL of RmB in a new 2 mL tube.
- 15 Place the tube on a magnet to separate the RmB. Visually inspect that all the beads are attached to the magnet and remove the supernatant.
- 16 Remove the tube from the magnet and wash the RmB with **270** μL of **OH-buffer (OH)** for 5 min shaking at 1,400 rpm at RT.
- 17 Place back the tube and the magnet and remove the supernatant.
- 18 Wash with **1000** μL of **nuclease-free water** by shaking for 2 min at 1,400 rpm at RT, place the tube on the magnet, and remove the supernatant.
- Do not scale up this volume by number of samples.*
- 19 Wash the RmB with **270** μL of **B-buffer (BB)**, shaking for 3 min at 1,400 rpm at RT. Place the tube on the magnet for at least 1 minute and remove the supernatant.
- 20 Repeat the wash once again with the same **270** μL of volume of BB.
- 21 Keep at least 2 μL of diluted RiboLace smart probe for quality control.
- 22 Resuspend the RmB beads with **81** μL of diluted RiboLace smart probe (**dRsP**).



- 23 Incubate for 1h at RT in a shaker at 1,400 rpm. Do not allow beads to sediment.
- 24 Place mPEG to thaw on ice during incubation.
- 25 **During this incubation we start the nuclease digestion of the lysates.**
- 26 After the incubation, place the tube on a magnet and remove 3 μL of the supernatant (unbound probe) for the security checkpoint. Leave beads shaking.
- By comparing the difference in absorbance measure at 270nm on Nanodrop for the unbound probe and the starting solution of the dRSP we should see a reduction in the signal. Between 10% and 50% absorbance reduction in the unbound probe compared to the starting solution is expected. If no decrease is observed, incubate beads for up to 2 h and check absorbance again. The measurement is taken in the custom mode in the Nanodrop UV-Vis at 270 nm with baseline correction at 750 nm. First, blank with water and then measure just water to ensure there is no signal. The dRSP value should be between 7-9 and not below 5.*
- 27 Measure the absorbance of dRSP and unbound probe at 270 nm.
- 28 Add **7.5 μL** of **mPEG** and mix in a shaker at 1,400 rpm at RT for 15 min. Do not allow the beads to precipitate. mPEG covers the bead surface not bound to the probe to remove non-specific binding.
- 29 Place the tube on a magnet for 2–3 min, discard the supernatant and wash **1000 μL** of **nuclease-free water**, for 2 min with shaking at 1,400 rpm at RT. Put back on the magnet and remove the supernatant.
- Do not scale up this volume by number of samples.*
- 30 Wash the functionalized RmB beads two times with **1000 μL** of **W-buffer (WB)** for 2 min with shaking at 1,400 rpm at RT. After the first wash, put the tube on the magnet to remove the supernatant before adding the solution. After the second wash, place the tube on the magnet and remove completely the supernatant.
- Do not scale up this volume by number of samples.*
- 31 Resuspend the functionalized RmB beads with **100** of W-buffer (**WB**).
- 32 If the beads were functionalized for more than one reaction, equally divide the functionalized beads in individual tubes according to the (N) number of samples you are processing.
- 33 The beads are now functionalized and ready to be placed in contact with the digested lysate. To avoid drying the beads, please, **remove the WB buffer just before adding the digested lysate.**

Nuclease digestion

- 34 Start with a total volume of lysate corresponding to 0.9 A.U. (260 nm) (see Introduction for calculation) diluted in W-buffer (**WB**) to the final volume of 450 μ L.

Optional: If total RNA sequencing is also desired, retain **10 μ g of RNA** from the lysate. This amount is sufficient to perform short-long RNA enrichment, which improves sequencing output. If this amount is not available and **Nanopore sequencing** is used, as little as **1 μ g of RNA** can be retained.

- 35 *The digestion conditions described here represent the default settings. When working with a new cell line, digestion efficiency should be evaluated by running pre- and post-digestion samples on a 15% TBE-urea gel. Incomplete digestion significantly reduces the efficiency of ribosome pull-down. For example, while a 1 $\times$ nuclease concentration is sufficient for HEK293T cells, A549 cells require a 2 $\times$ nuclease concentration to achieve optimal digestion. If digestion efficiency is uncertain, retain aliquots of the lysate before and after digestion for downstream gel-based troubleshooting. For more details, please consult Immagina Biotech documentation at [this link](#).*

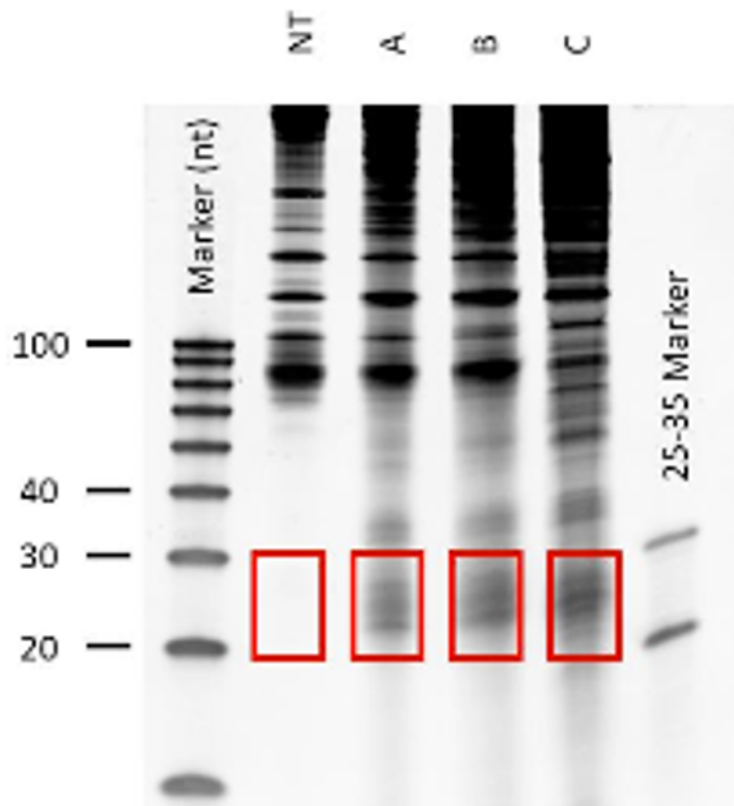


Figure 1: Example of RNA extracted after ribosome pull-down and resolved on a 15% TBE-urea gel. NT, non-treated control. A, B, and C correspond to increasing nuclease concentrations. Optimal digestion is indicated by enrichment of RNA fragments migrating within the 25–35 bp size range, corresponding to ribosome-protected footprints.

Load an equal amount of protein diluted in W-buffer to the final volume of 450 μ L.



- 36 Add 0.9 μL of **Nux Enhancer (NE)**.
- 37 Dilute 1.5 μL of **Nuclease (Nux)** by adding 98.5 μL W-buffer (**WB**). Pipette up and down 5 times to mix well the diluted Nux solution (dNux).
- 38 Digest the sample in a 1.5 mL tube for 45 min at 25 °C shaking with 4.5 μL of the diluted Nuclease (**dNux**) prepared before. Trash the remaining diluted Nux solution, for experiments performed on other days, prepare fresh diluted Nux.
- 39 Stop digestion with 1.5 μL of **SUPERaseIn** for 10 min on ice.

Ribosome pull-down

- 40 Remove the W-buffer (WB) from washed beads only immediately before adding the cell lysate to avoid drying. 
- 41 Discard the supernatant of the functionalised beads and add digested cell lysate. Mix well.
- 42 Incubate for 70 min, on a wheel in slow motion (3-10 rpm) at 4°C.
- 43 Place magnetic rack on ice to pre-chill.
- 44 Remove the tubes from the wheel. **DO NOT CENTRIFUGE** but allow the entire solution with the beads to settle at the bottom of the tube. Place the tubes on ice. Place the magnet in an ice bucket before putting the tubes on it. If residual solution is present on the lid, pull down the beads by flipping the magnet so that the supernatant captures beads that are on the lid.
- 45 Keep working on ice and separate the beads with a magnet.
- 46 **DO NOT REMOVE THE BEADS FROM THE MAGNET, NEVER TOUCH THE BEADS IN THE NEXT WASHING STEPS. NO PIPETTING UP AND DOWN- THE BOND IS NOT COVALENT.** 
- 47 Transfer the supernatant to a fresh Eppendorf and keep as the FT for downstream troubleshooting
- 48 Carefully wash the beads twice with 1000 μL W-buffer (WB). Do not remove the samples from the magnet. Carefully add the WB on the opposite side of the Eppendorf to where the beads are present. Carefully remove the supernatant without disturbing the beads.
- 49 Remove completely the W-buffer (WB) before removing the beads from the magnet. Proceed immediately with extraction steps without drying the beads for too long to avoid cracking them.

The ribosomes are attached to the beads now. Proceed with the RNA extraction and the protein extraction.

RNA extraction

- 50 This is completed with Zymo Clean and Concentrator (Zymo catalog. no. R1015 or R1016)
- 51 Extract the RNA by directly adding 200 μ L of the Zymo RNA Binding Buffer (**ZBB***) to the beads pipetting up and down.
- 52 Transfer the bead suspension to a new nuclease-free 1.5 mL tube.
- 53 Incubate the beads suspension at RT for 5 min with shaking at 600 rpm.
- 54 After the incubation, place the tube on a magnet and **collect the supernatant**, transferring it to a new nuclease-free 1.5 mL tube. Discard the beads.
- 55 Add 200 μ L of EtOH 95-100% mixing the solution by pipetting.
- 56 Transfer the mixture to the **Zymo-Spin TM Column** and centrifuge for 30 seconds at 12000xg at RT. **Discard the flow-through.**
- 57 Add 400 μ L **RNA Prep Buffer** to the column and centrifuge for 30 seconds at 12000xg at RT. **Discard the flow-through.**
- 58 Add 700 μ L **RNA Wash Buffer** to the column and centrifuge for 30 seconds at 12000xg at RT. Discard the flow-through.
- 59 Add 400 μ L **RNA Wash Buffer** to the column and centrifuge for 1 minute at 12000xg at RT to ensure complete removal of the wash buffer. **Discard the flow-through.**
- 60 Carefully, transfer the column into a new RNase-free tube.
- 61 Add 12 μ L of **nuclease free water (NFW)** directly to the column matrix, allow to sit in the column for 1 min and centrifuge for 30 seconds at 12000xg at RT.
- 62 The extracted RNA is present in the flow-through. **Keep the Eppendorf with the flow through.**
- 63 With Nanodrop, measure the absorbance of each sample at 260 nm (set up the "nucleic acid" function of the Nanodrop), using 1 μ L of NFW as blank.

Enrichment of short RNAs

64 The efficiency of the short clean-up is typically around **5%**. In practice, we use **5 µg** of the ribosome-embedded eluate for the clean-up of ribo-tRNAs and **10 µg** of cell lysate for the clean-up of total tRNAs. We proceed directly with short enrichment from the lysate without performing a prior clean-up of the lysate.

Note: This step is recommended but not essential. If the pull-down yields less than **5 µg**, it is preferable to proceed directly to sequencing. Additionally, if you intend to sequence the mRNA from the pull-down, ensure that at least **1.5 µg** is available to obtain high-quality libraries.

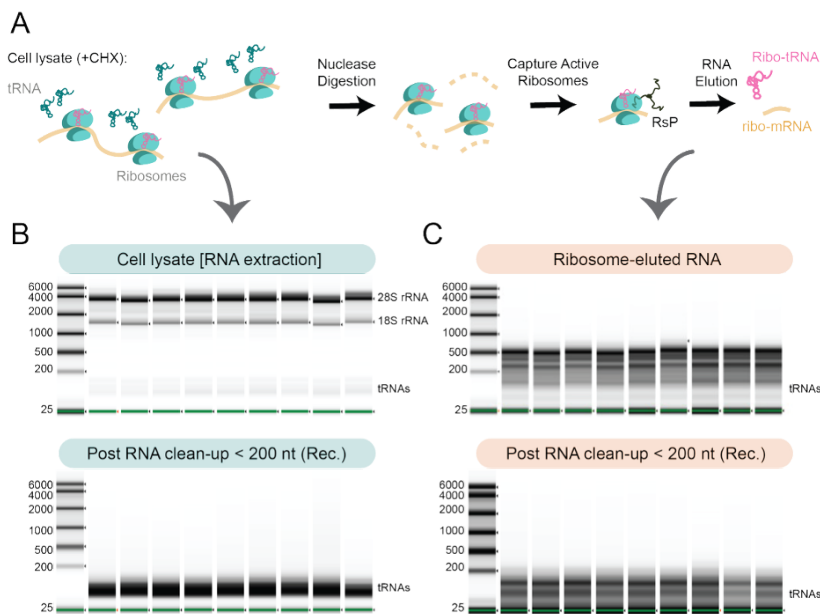


Figure 2: Short-long RNA enrichment strategy used in tRIBO-seq. (A) Schematic overview of the tRIBO-seq workflow, illustrating the key steps in capturing and sequencing ribosome-associated tRNAs. A total of 9 independent biological samples from HEK293T cells are shown. (B) TapeStation RNA profiles showing total RNA from cell lysate samples and corresponding <200 nt fractions obtained after clean-up (see Methods). These samples were used as input for total-tRNA (Nano-tRNAseq) library preparation. (C) TapeStation RNA profiles of ribosome-captured RNAs, used as input for ribo-tRNAs (tRIBO-seq) library preparation. Ribosome-bound RNA samples exhibit a marked enrichment of ribosomal RNA (rRNA) and tRNAs compared to total input RNA, highlighting selective recovery of actively engaged RNAs. Rec.: Recommended. As the adaptors selectively capture tRNAs, the clean-up step is optional when material is limited.

- 65 Prepare fresh ethanol solutions:
- For each sample, prepare **225 uL of 70% ethanol** and keep at RT
 - For each sample, prepare **500 uL of 80% ethanol** and keep at RT

66 Place MinElute columns at RT for at least 30 min

67 Prepare 5 ug per ribo-tRNA sample and 10 ug per total tRNA sample in a fresh eppendorf tube. Complete volume to 50 uL with nuclease-free water.



- 68 Add 175 μ L Buffer RLT to the 50 μ L sample of RNA. Pipette slowly to mix well. Final volume = 225 μ L.
- 69 Add 225 μ L 70% Ethanol (1X) to the mix. Final volume = 450 μ L.
- 70 Transfer up to 700 μ L of sample into an RNeasy Minispin column in a 2 mL collection tube.
- 71 Close the lid gently and centrifuge at $\geq 8000 \times g$ ($\geq 10,000$ rpm) for 30 s at RT
- 72 Store the columns in the -80. This holds the **long RNA** and can be used downstream for troubleshooting.
- 73 Take the flowthrough (short RNA) for clean-up
- 74 Add 292,5 μ L 100% Ethanol to the flowthrough (0.65X). Final volume = V=742,5 μ L.
- 75 Transfer the liquid into an RNeasy MinElute column in a 2 mL collection tube and close the lid gently.
- 76 Centrifuge for 30 s at $8000 \times g$ ($\geq 10,000$ rpm). **Discard the flow-through.**
- 77 Add 700 μ L RWT buffer into the RNeasy MinElute column and close the lid gently
- 78 Centrifuge for 30 s at $8000 \times g$ ($\geq 10,000$ rpm). **Discard the flow-through.**
- 79 Pipet 500 μ L Buffer RPE into the RNeasy MinElute column and close the lid gently.
- 80 Centrifuge for 30 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). **Discard the flow-through.**
- 81 Add 500 μ L of 80% ethanol to the RNeasy MinElute spin column and close the lid gently.
- 82 Centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to dry the spin column membrane. **Discard the flow-through** and collection tube.
- 83 Place the RNeasy MinElute spin column into a new 2 mL collection tube.
- 84 Open the lid and centrifuge for 5 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm)



- 85 Place the RNeasy MinElute spin column into a lobind 1.5 mL collection tube
- 86 Pipet 15 μ L RNase free water onto the spin column membrane, lose the lid gently and incubate for 1 min at RT
- 87 Centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm)
- 88 Measure concentration by Qubit RNA HS and assess integrity using TapeStation.

*Ideally, 100-200 ng per sample needed for high-quality nano-tRNA-seq libraries.
Please proceed with library preparation.*

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