Ribo-tRNAseq PRO kit

Product	Catalog no	Samples	Barcode
Ribo-tRNAseq PRO Kit with demultiplexing	RTPRO-12	12	12

Shipping: Dry ice

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: Ribo-tRNAseq PRO enables the pulldown of actively translating ribosomes, the extraction of the tRNAs associated to the ribosome and their native, single-molecule, full-length tRNA sequencing on the Oxford Nanopore platform, with flexible multiplexing of up to 12 samples per flow cell. By sequencing multiple samples in the same run, the kit eliminates run-to-run technical variability and significantly reduces per-sample sequencing costs.

After sequencing, the integrated analysis pipeline provides: (i) quantification of tRNA abundances, (ii) identification of tRNA modification sites, and (iii) detection of modification circuits across tRNA molecules.

The protocol is optimized for the RNA004 chemistry and RNA flow cells and is designed for sequencing on the MinION platform. A minimum of 300,000 reads per sample is recommended for robust downstream analysis. For experiments requiring higher output or involving more than 8 multiplexed samples, sequencing on the PromethION platform is also supported.

Suitable for: Eukaryotic cells and tissues with annotated genome.

What's Included:

1. **RiboLace pulldown kit** — tool for isolation of actively translating ribosomes and the associated tRNAs. Its unique puromycin derivative (3P) binds active ribosomes, captured by magnetic beads for easy separation.
2. **Library Prep Kit** — Reagents and protocol for preparing your tRNA library for sequencing on the Oxford Nanopore platform**.**
3. **IT-Based Demultiplexing Tool** — A powerful software tool that enables demultiplexing of your sequencing run, accessible for 40 days post-activation.

For Internal Research and Service Use Only. Not Intended for Diagnostic or Therapeutic Use.

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Kit storage information

	Quantity	Storage
4°C components	1 box	4°C
-20°C components	2 boxes	-20°C
-80°C components	1 box	-80°C
USB pen drive with instructions on how to perform the demultiplexing step after sequencing.	1	RT

Additionally Required Material:

- PBS
- Sodium deoxycholate 10% solution in DNase/RNase-free water
- Cycloheximide (Sigma-Aldrich, catalog no. C4859-1ML)
- DNase I (Thermo Scientific catalog no. 89836)
- RiboLock RNase Inhibitor (Thermo Scientific catalog no. EO0381 or EO0382)
- SUPERaseIn (Invitrogen, catalog no. AM2696 or AM2694)
- Nanodrop ND-1000 UV-VIS Spectrophotometer
- Microcentrifuge and non-stick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- Automatic wheel (rotator)
- Magnetic stand for 1.5mL tube
- RNA Clean & Concentrator™-5 (Zymo catalog. no. R1015 or R1016)
- Nuclease free water (NFW)
- Ethanol 95-100%
- RNase Inhibitor, Murine 40000 units/ml (NEB, cat. no. M0314S or M0314L)
- Qubit™ HS RNA Assay Kit (Thermo Fisher Scientific, Q32852 or Q32855)
- Qubit™ HS dsDNA Assay Kit (Thermo Fisher Scientific, Q32851 or Q32854)
- Direct RNA Sequencing SQK-RNA004 (Oxford Nanopore)
- FLO-MIN004RA flow cell (Oxford Nanopore)
- T4 DNA Ligase (New England Biolabs, cat. no. M0202T or M0202M)
- NEBNext® Quick Ligation Reaction Buffer (New England Biolabs, cat. no. B6058S)
- Agencourt RNA Clean XP for RNA Purification (Beckman Coulter, cat. no. A63987)

Optional Material:

- Agilent 2100 Bioanalyzer
- Agilent RNA 6000 Nano Kit (or equivalent) (Agilent Technologies, cat. no. 5067-1511)

INTRODUCTION

The Ribo-tRNAseq PRO Kit is a complete solution combining Immagina's **RiboLace** technology for the isolation of active ribosome with the **nano-tRNAseq** technology for the preparation of efficient and precise Oxford Nanopore libraries of tRNAs associated with the ribosomes. The synergy between those two technologies results in a fast, simple, and robust workflow. It is fast because ribosome isolation takes 1 day and the entire workflow from 3 to 4 days depending on the organization of the working time.

At the heart of the **RiboLace** method lies a proprietary puromycin derivative, called 3P, that retains the ability to interact with active ribosomes while being covalently linked to a biotin molecule. The samples are first exposed to cycloheximide to clamp ribosomes on the mRNA fragments (recommended step), then lysate and nuclease digested to produce individual ribosomes. In parallel, magnetic beads are functionalized with the 3P, and once the digestion is over, they are added to the reaction mix to pull down the active ribosomal complex. Ribosomes are thus purified by affinity purification and magnetic separation, and the ribosomes are extracted from the ribosomal complex. The original proof-of-concept of the technology was published on Cell Report in 2018.

nano-tRNAseq is the only product that enables the comprehensive sequencing of tRNA molecules in their full-length, native state, allowing for the simultaneous assessment of tRNA abundances and modification status. Studying tRNAs is particularly challenging due to their extensive post-transcriptional modifications and dynamic roles in translation. For these reasons, traditional sequencing methods such as sequencing by synthesis often fail to capture such complexities in tRNAs. Other NGS-based methods are complementary to nano-tRNAseq since those are cDNA and PCR based. Building upon the pioneering work of the Dr. Eva Novoa Lab at Centre for Genomic Regulation (CRG), Barcelona, our method bypasses the need for cDNA/PCR sequencing (Nature Biotech in 2024).

The first step in nano-tRNAseq comprises small RNA enrichment from total RNA. The tRNAs are bound with adaptors and subsequently to barcodes, allowing for multiplexing of up to 12 samples. The native tRNA is then sequenced on the Oxford Nanopore Technologies (ONT) platform. As tRNA is transversing the pore, each base generates an electrical current, which is converted into the tRNA sequence. After data analysis, information on tRNA abundance, coverage and post-transcriptional chemical modifications are obtained.

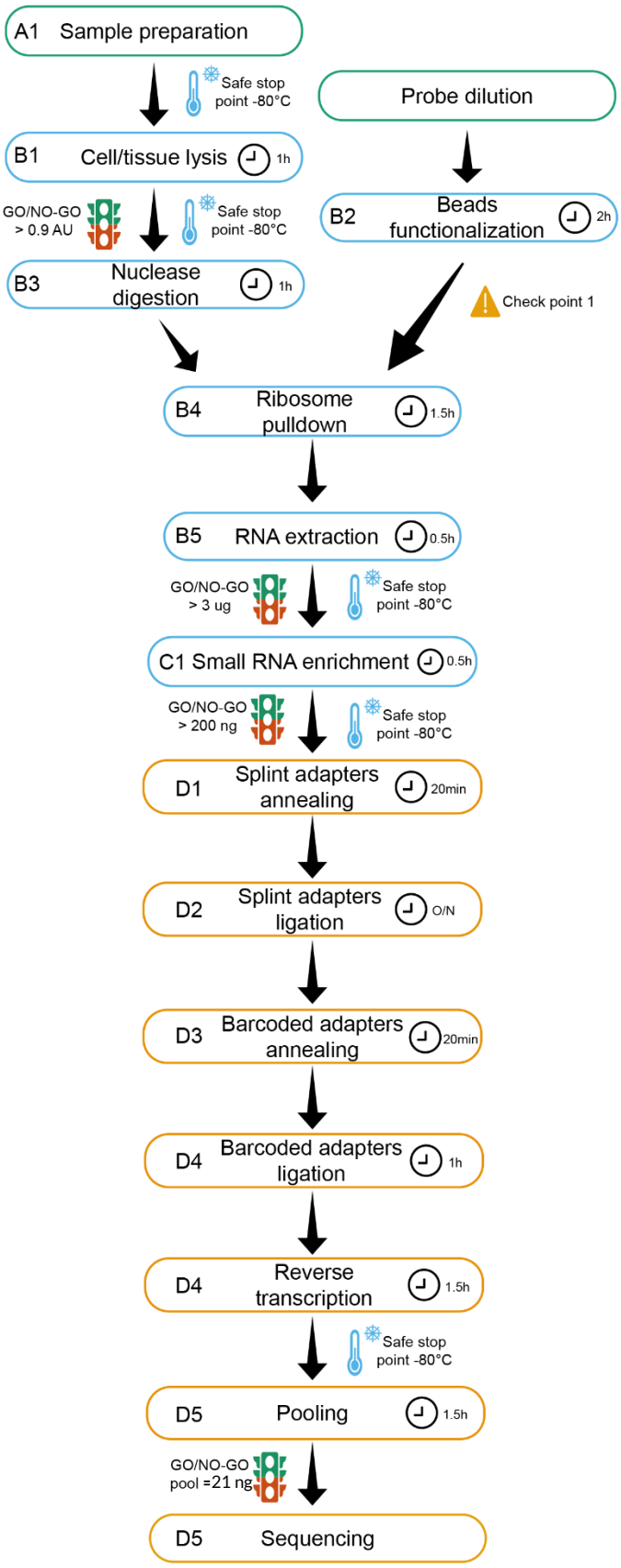


Figure 1. Overview of the Ribo-tRNAseq PRO Kit workflow. *In each box, steps are present in the left corner, while the time to complete the step is listed in the right corner (clock picture). Safe stopping points (thermometer pictures) and GO/NO-GO conditions (traffic lights) are placed right at the bottom of the corresponding steps. Optional and required checkpoints (caution signal) are also indicated on the right.*

Optimal Workflow Recommendations

- Please avoid performing more than 6 pulldowns in parallel. Longer manipulation time may introduce an unwanted variability between the first and the last samples. Pulldown RNA can be stored at -80°C for up to two weeks before performing library preparation.
- The Ribo-tRNAseq PRO Kit has been optimized for the multiplexing of up to 12 samples per library. Multiplexing requires a combination of barcoded adaptors as reported on page 25.
- Please note that the reagent volumes included in this kit for the section D (tRNA library prep) are intended for processing 12 samples total. These can be processed all at once, or in a maximum of 4 runs of 3 samples each. Processing samples in smaller groups is not recommended, as each mastermix preparation requires an additional dead volume, which may cause some reagents to run out before all 12 samples are processed.
- Allocate at least 3 days for the completion of the entire workflow.
- Please make sure to purchase all the additionally required materials needed for the protocol before starting the experiment.
- If possible, please perform a preliminary lysis experiment to set the lysis volume following the suggested AU operational range (see section A3).
- The Beads Functionalization and the Nuclease Digestion (Steps B2 and B3) can be performed in parallel, to shorten the protocol length.
- This protocol has been optimized to perform the RPF pulldown and the library preparation starting with 0.9 AU (Abs260nm) of lysate, and 3 µg of RNA after RPF's pulldown. Crucially, if the sample amount does not allow it to reach 0.9 AU, it is still possible but not advisable, to lower the lysate input down to 0.6 AU without the need of modifying the kit stoichiometry.
- Starting with 0.9 AU of specimen lysate after RPF pulldown we expect to obtain at least 1.5 µg of RNA.
- This protocol is optimized for tRNA extraction starting from at least 3 µg* of pulldown RNA.
- At least 200 ng* of small RNA are needed after small RNA enrichment step to move forward with the "Splinter Adaptor Ligation" reaction (step D2).
- Please be aware that the "Splint Adaptor Ligation" reaction (step B2) can be performed starting from 200 to 500 ng of input.
- The final library pool must comprise exactly 21 ng of material, equally divided among the number of samples you decided to multiplex.
- The pooled tRNA library MUST be sequenced right away and cannot be stored for later processing.
- Before starting the sequencing, please select "Flow cell type": MIN004-RA, "Kit selection": Direct RNA Sequencing Kit, please deactivate "Basecalling" and be sure to select the saving of the .POD5 file formats.
- Note that at least 300.000 raw reads per sample are suggested to perform robust data analysis.
- A set of tables is available in the Appendixes to allow for a step-by-step approach while running the experiment. To improve user experience, we suggest printing them and having them available during the actual manipulations.

** If you are not able to reach at least 3 µg of pulldown RNA and/or 200 ng of small RNA please contact us at techsupport@immaginabiotech.com*

A. SAMPLE PREPARATION

The amount of Ribosomes that can be isolated from a sample is strongly affected by its translational state and must be considered when programming experiments with the IMMAGINA Ribo-tRNAseq PROkit. 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.

While it is not possible to provide a minimal sample size as a defined number of cells or weight of tissue, two indicators can be used as a checkpoint and go/no-go at two convenient stages during the protocol:

- The first is the total AU after cell lysis (Step B1), and before the Ribosome pulldown.
- The second, is the amount of RNA retrieved after Ribosome pulldown (Step B5).

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. For tissues (such as liver and brain) we suggest starting with 30 mg of material.

Given specimen-to-specimen variability, as a preliminary experiment, we suggest testing the lysis step on different sample amounts, recording the corresponding total AU, and using it to fine-tune volumes and sample size during the real experiment (See Table 1 for lysis buffer volumes).

A.2 AU Calculation - Input lysate Quantification

A.2.1 Measure Lysate AU

Cells and tissues should be lysed following Step B1 a, b, or c instructions depending on your specimen type. 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 (see Before starting the experiment – Lysis Buffer Supplementing & Table 2). 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*

A.2.2 Lysis Volume Selection

It is important to lysate the specimen in an appropriate volume to obtain a lysate with an optimal range of Abs at 260 between 7 to 15 AU. It is still possible to use the kit with an AU as low as 2 and as high as 30. 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. Feel free to modify those resuspension values according to your sample behavior.

Specimen	Quantity	Lysis buffer	Volume of supplemented LB (µL)
Cell	0.3 – 1 million	# IBT0033	50 µL
Cell	1 – 5 million cells	# IBT0033	150 µL
Cell	> 5 million cells	# IBT0033	300 µL
Tissue	< 10 mg	# IBT0032	500 µL
Tissue	> 10 mg	# IBT0032	800 µL

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

A.3 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 examples below.

Example 1: 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 µL/mL to get the concentration per µL = 0.01AU/µL.

- ☐ To start with 0.9 AU use: 0.9AU/0.01 AU/µL = 90 µL of lysate

Example 2: Nanodrop absorbance value of lysate at 260 nm = 4 AU.

This means that, arbitrarily, we set the absorbance of the lysate at 4 AU/ml (=0.004 AU/µl).

- ☐ To start with 0.9 AU use: 0.9AU/0.004 AU/µL = 225 µL of lysate

B. ACTIVE RIBOSOME PULLDOWN

Ribo-tRNAseq PRO kit components and additional required materials needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
B1	Lysis buffer (LB)	# IBT0033	18 mL	-20°C	Bottle	--
B1	SDC 10%	Additionally Required Material				
B1	DNAse I	Additionally Required Material				
B1	RiboLock RNase Inhibitor	Additionally Required Material				
B1	Cycloheximide (CHX)	Additionally Required Material				
B1	PBS	Additionally Required Material				
B2	Binding Buffer (BB)	# IBT0021	10 mL	4°C	Bottle	--
B2	RiboLace magnetic Beads v2.1 (RmB v2.1)	# IBT0042	1.8 mL	4°C	Vial	clear
B2	OH-buffer (OH)	# IBT0051	10 mL	4°C	Bottle	--
B2	mPEG	# IBT0061	120 µL	-20°C	Vial	clear
B2/B5	Nuclease free water	Additionally Required Material				
B2*	RiboLace smart probe (RsP)	# IBT0012	200 µL	-20°C	Vial	clear
B2	diluted RiboLace smart probe (dRsP)	Dilute Aliquot from RsP		-80°C		
B2/B3/B4	Wash Buffer (WB)	# IBT0071	2 x 25 mL	4°C	Bottle	--
B3	Nuclease (Nux)	# IBT0091	21 µL	-20°C	Vial	clear
B3	Diluted Nuclease (dNux)	Dilute Aliquot from Nux				
B3	Nux Enhancer (NE)	# IBT0081	15 µL	-20°C	Vial	clear
B3	SuperRNAse In	Additionally Required Material				

Step B1. CELL LYSIS

⚠ **Before starting the experiment – Supplemented Lysis Buffer (SLB)**

To ensure optimal reproducibility, for both cell and tissue lysis buffer, we recommend producing a fresh Supplemented Lysis Buffer (SLB) aliquot for each new experiment, right before proceeding with the Lysis Step. Working on ice, combine the SLB by following Table 2 instructions and multiply the volumes according to the number of samples being processed (N). please combine the different reagents following the left-to-right order.

	Lysis buffer (LB)	Sodium deoxycholate (SDC) 10% (W/V)	DNase I 1 U/µL	RiboLock RNase Inhibitor 40 U/µL	Final Volume
N=1	267 µL	30 µL	1.5 µL	1.5 µL	300 µL
N=___	____	____	____	____	____

Table 2. *Recipe for the supplementation of the provided lysis buffer or tissues lysis buffer.*

The SLB final concentration is Sodium deoxycholate (1%), DNase I (5U/mL), and RiboLock RNase Inhibitor (200 U/mL).

If the SLB appears as a whiteish and cloudy solution, do not proceed and check Appendix 3.

Please note that the Lysis buffer # IBT0033, the Tissue Lysis buffer #IBT0032, and W-buffer # IBT0071 contain CHX (10 µg/mL, 100 µg/mL and 10 µg/mL respectively).

Adherent Cells lysis

☐ **B1.1a** Treat the cells with 10 µg/mL of **cycloheximide (CHX)** for 5 min at 37°C before lysis. We recommend using cells at 70-80% confluence. CHX treatment is suggested – but it is not mandatory - to increase the efficiency of the ribosomes' affinity purification. CHX treatment could induce the accumulation of ribosomes within the first 10 codons. Should you not wish to add CHX check Appendix 2 for the alternative protocol.

☐ **B1.2a** After incubation, place the cells on ice and wash them quickly with **cold PBS** containing CHX (20 µg/mL).

☐ **B1.3a** Remove all residual PBS with P200 pipette. **All the PBS must be removed before proceeding with the lysis to avoid diluting the lysis buffer.**

☐ **B1.4a** Perform the lysis directly adding the complete **Supplemented Lysis Buffer** (for resuspension volumes check the guidelines in section A.3 - Input lysate preparation and quantification - & Table 1) 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.

To ensure good lysis, follow these guidelines for mechanical scraping:

- Before scraping, make sure you are working in a sterile environment using appropriate aseptic techniques.
- Prepare your sample by adding the necessary lysis buffer or solution as per the protocol.
- Using a suitable tool such as a cell scraper, spatula, or pipette tip, gently scrape the surface of the cell culture dish or tissue to dislodge the cells.
- Apply consistent but gentle pressure to ensure thorough scraping while avoiding excessive force that may introduce debris.
- Scrape in a systematic manner, covering the entire surface area to ensure an even distribution of lysed cells.
- Continue scraping until you observe the desired level of cell detachment and release of cellular material.
- Transfer the lysate to a suitable collection vessel, such as a microcentrifuge tube, for further processing or analysis.

☐ **B1.5a** Collect the cell lysate in a 1.5 mL microcentrifuge tube and pellet the cell debris and nuclei by centrifugation at 20,000 g for 5 min at 4°C.

☐ **B1.6a** Transfer the supernatant to a new tube and keep it on ice for 20 min.

☐ **B1.7a** 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 (for troubleshooting check A.3 AU calculation - Input lysate quantification). 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.

Suspension Cells lysis

☐ **B1.1b** Treat the cells with 10 µg/mL of **cycloheximide (CHX)** for 5 min at 37°C before lysis, should you not wish to add CHX check Appendix 2 for the alternative protocol. CHX treatment is suggested – but it is not mandatory - to increase the efficiency of the ribosomes’ affinity purification. CHX treatment could induce the accumulation of ribosomes within the first 10 codons. Should you not wish to add CHX check Appendix 2 for the alternative protocol.

☐ **B1.2b** Collect the cells and centrifuge at 950 g for 5min at 4°C, remove the media, and wash with **cold PBS** containing CHX (20 µg/mL).

☐ **B1.3b** Collect and centrifuge at 950 g for 5 min at 4°C. Remove the supernatant completely.

☐ **B1.4b** Resuspend cell pellet in **Supplemented Lysis Buffer** (for resuspension volumes check the guidelines in section A.3 - Input lysate preparation and quantification - & Table 1).

☐ **B1.5b** Lysate cells by passing them through a G26 needle ~20 times (please note that if the volume is below 50 µL, using the syringe will lead to the loss of specimen, as a possibility you could pipette up and down ~20 times avoiding creating bubbles).

☐ **B1.6b** Pellet the nuclei and cell debris by centrifugation at 20,000 g for 5 min at 4°C.

☐ **B1.7b** Transfer the supernatant to a new tube. Leave on ice for 20 min.

☐ **B1.8b** 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 (for troubleshooting check A.3 AU calculation - Input lysate quantification). 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.

Tissues lysis

☐ **B1.1c** Pulverize the tissue under liquid nitrogen with mortar and pestle. Recover the powder in a 1.5 mL tube.

☐ **B1.2c** Resuspend with 800 µL of **Tissues Lysis Buffer** (not included - Immagina catalog no. #IBT0032) supplemented as per instruction in the section “Before starting the experiment – Lysis Buffer Supplementing” & Table 1.

☐ **B1.3c** Centrifuge at max speed (20,000 g) for 2 min at 4°C to remove tissue and membrane debris and collect the supernatant.

☐ **B1.4c** Centrifuge again the supernatant for 5 min at max speed (20,000 g) at 4°C and collect the supernatant. Keep on ice for 20 min.

☐ **B1.5c** 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 (for troubleshooting check A.3 AU calculation - Input lysate quantification). 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.

Step B2. BEADS FUNCTIONALIZATION

⚠ **DO NOT LET THE BEADS DRY OUT AT ANY POINT!**

⚠ **First-time opening of the kit – RiboLace Smart Probe (RsP) dilution and aliquoting.**

The RiboLace smart probe (RsP) present in the kit is concentrated and is NOT intended to be used without proper dilution. Before starting the experiment, as first time opening the kit, please thaw on ice the 200 µL of concentrated RiboLace smart probe (RsP) and add 800 µL of B-buffer (4°C, you can keep it on ice during the procedure) to create the diluted RiboLace smart probe (dRsP). To avoid more than two freeze-thaw cycles, we suggest aliquoting the diluted probe and storing the solution at -80°C in ready-to-use aliquots. For simplicity, we suggest making 166 µL aliquots as this approach allows you to conduct two experiments per aliquot with a lysate input of 0.9 AU per experiment.

Beads functionalization steps:

The amount of beads that need to be functionalized per experiment depends on the number of samples it is composed of and on the concentration of the lysate of the samples (expressed in AU). For clarity, the following steps refer to one reaction at the canonical concentration of 0.9 AU per sample. For multiple samples, it is possible to functionalize beads for more than one reaction in one single tube (within its capacity). To ensure an effortless and thorough process we suggest you print the checklist in Appendix 5, fill it with your specific volumes and mark each completed step during the manipulation.

- ☐ **B2.1** Remove the **RiboLace magnetic beads (RmB)** from 4°C and place the tube at RT for at least 30 min.
- ☐ **B2.2** Vortex the RiboLace magnetic beads (RmB) tube thoroughly for > 30 sec.
- ☐ **B2.3** Put 144 µL of RiboLace magnetic beads (RmB) in a new 1.5 mL tube. 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.
- ☐ **B2.4** 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. Place back the tube and the magnet and remove the supernatant.
- ☐ **B2.5** 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. If RmB are binding to the plastic tube, you can add Triton X-100 to a final concentration of 0.1%.
- ☐ **B2.6** 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. If RmB are binding to the plastic tube, you can add Triton X-100 to a final concentration of 0.1%. Repeat the wash once again with the same 270 µL of volume of BB.
- ☐ **B2.7** Keep at least 2 µL of diluted RiboLace smart probe (**dRsP, see "First Time Opening – RiboLace Smart Probe (RsP) dilution and aliquoting"** above) for security checkpoint (see grey box below).

☐ **B2.8** Resuspend the RmB beads with 81 µL of diluted RiboLace smart probe (**dRsP**).

☐ **B2.9** Incubate for 1h at RT in a shaker at 1,400 rpm. Do not allow beads to sediment.

During the incubation, we suggest starting the Nuclease Digestion (STEP.B3).

☐ **B2.10** After the incubation, place the tube on a magnet and remove 3 µL of the supernatant (unbound probe) for the security checkpoint (see below). Keep the remaining volume in the vial.

☐ **B2.11** Add 7.5 µL of **mPEG** to the tube and mix in a shaker at 1,400 rpm at RT for 15 min. Do not allow the beads to precipitate.

☐ **B2.12** 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.

☐ **B2.13** 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.

☐ **B2.14** Resuspend the functionalized RmB beads with 100 µL of W-buffer (**WB**).

☐ **B2.15** 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.

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** (End of Step B3, Beginning of Step B4).

⚠Security Check Point

You can check for proper bead functionalization by following the instructions in Appendix 6. This step is optional, and it is useful to validate the proper execution of the above-mentioned functionalization steps.

Step B3. NUCLEASE DIGESTION

Individual Ribosomes are generated during the Nuclease Digestion step (in the process called ribosome footprinting for generating the ribosome protected fragments used in general for RiboSeq experiment). The suggested Nuclease amount and digestion timing are well-suited for most organisms and tissues. Nonetheless, please note that the concentration of the nuclease is critical for the outcome as, using the incorrect quantity, might lead to varying effects on the read length distribution. ⚠ **Should you need to perform a titration curve to assess the proper quantity of Nux to add to your (non-conventional) sample, you can check the guidelines in Appendix 8 - Optional Nuclease Optimization.**

- ☐ **B3.1** Start with a total volume of lysate corresponding to 0.9 A.U. (260 nm) (see Section A2.3 for calculation) diluted in W-buffer (**WB**) to the final volume of 450 µL.
- ☐ **B3.2** Add 0.9 µL of **Nux Enhancer (NE)**.
- ☐ **B3.3** Dilute 1.5 µL of **Nuclease (Nux)** by adding 98.5 µL W-buffer (**WB**). Pipet up and down 5 times to mix well the diluted Nux solution (dNux).
- ☐ **B3.4** Digest the sample in a 1.5 mL tube for 45 min at 25 °C 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.
- ☐ **B3.5** Stop digestion with 1.5 µL of **SUPERaseIn** for 10 min on ice.

Step B4. RIBOSOMES PULLDOWN

Remove the W-buffer (WB) from Step B2.14 only immediately before adding the cell lysate!

☐ **B4.1** Add the **digested cell lysate** to the functionalized beads (to avoid dilution, discard the supernatant of the beads before adding the cell lysate) and mix well.

☐ **B4.2** Incubate for 70 min, on a wheel in slow motion (3-10 rpm) at 4°C.

☐ **B4.3** Remove the tubes from the wheel. **DO NOT CENTRIFUGATE** but allow the entire solution with the beads to settle at the bottom of the tube. If residual solution is present on the lid, pull down the beads by gently flicking down the tube by hand 2 or 3 times. Place the tubes on ice. Place the magnet in an ice bucket before putting the tubes on it.

☐ **B4.4** Keep working on ice and separate the beads with a magnet.

☐ **DO NOT REMOVE THE BEADS FROM THE MAGNET and NEVER TOUCH THE BEADS IN THE NEXT WASHING STEPS.**

☐ **B4.5** Remove the supernatant. 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.

☐ **B4.6** Remove completely the W-buffer (WB) before removing the beads from the magnet and resuspend them in 400 µL of W-buffer (WB).

☐ **B4.7** Transfer the bead suspension to a new nuclease-free 1.5 mL tube.

⚠ ***Your ribosomes are attached to the beads now, do NOT discard them!***

Step B5. RNA EXTRACTION

The reagents are part of the RNA Clean & Concentrator™-5 kit (Zymo catalog. no. R1015 or R1016)

- ☐ **B5.1** Extract the RNA by directly adding 200 µL of the Zymo RNA Binding Buffer (ZBB) to the beads pipetting up and down.
- ☐ **B5.2** Transfer the bead suspension to a new nuclease-free 1.5 mL tube.
- ☐ **B5.3** Incubate the beads suspension at RT for 5 min with shaking at 600 rpm.
- ☐ **B5.4** 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.
- ☐ **B5.5** Add 200 µL of EtOH 95-100% mixing the solution by pipetting.
- ☐ **B5.6** Transfer the mixture to the Zymo-Spin™ Column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **B5.7** Add 400 µl RNA Prep Buffer to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **B5.8** Add 700 µl RNA Wash Buffer to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **B5.9** Add 400 µl RNA Wash Buffer to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **B5.10** To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 g at RT. Discard the flow-through. Carefully, transfer the column into a new RNase-free tube.
- ☐ **B5.11** Add **12 µL** of **nuclease free water** (**NFW**) directly to the column matrix and wait 1 min.
- ☐ **B5.12** Centrifuge for 30 seconds at 12,000 g at RT. The extracted RNA is present in the flow-through. Keep the Eppendorf with the flow through.
- ☐ **B5.13** With Nanodrop, measure the concentration of each sample at 260 nm (set up the “nucleic acid” function of the Nanodrop), using 1 µL of NFW as blank. To start with library preparation, the suggested quantity ranges from 3 to 5 µg of extracted RNA. It is still possible to perform the reaction if you retrieved at least 200 ng of small RNA after small RNA enrichment in step.

⚠ **SAFE STOPPING POINT (store at -80°C)**

C. tRNA EXTRACTION

Components and additional materials needed in this section:

Step N	Additional Material	Type
C1	RNA Clean & Concentrator™-5	Required
C1	Nuclease free water (NFW)	Required
C1	Ethanol	Required
C1	Qubit™ HS RNA Assay Kit	Required

NOTE: After total pulldown RNA extraction, it is important to enrich only the small RNA fraction to retrieve high quality tRNAs. Please proceed with at least 3 µg of pulldown RNA to get enough material for library preparation.

If you are not able to reach at least 3 µg of pulldown RNA please contact us at techsupport@immaginabiotech.com

Step C1. SMALL RNA (<200nt) ENRICHMENT

Small RNA Enrichment is achieved using the **RNA Clean & Concentrator™-5 kit (Zymo catalog. no. R1015 or R1016).** Perform all steps at room temperature and centrifugation at 12,000 g for 30 seconds, unless otherwise specified.

☐ C1.1 Add nuclease free water to the extracted RNA (at least 3 µg) from step B5.13 to a final total volume of 50 µL.

☐ C1.2 Prepare **adjusted RNA Binding Buffer** by mixing 50 µL of **Zymo RNA Binding Buffer** (**ZBB**) and 50 µL of ethanol (95-100%) for each sample you want to process.

☐ C1.3 Add 100 µL of **adjusted RNA Binding Buffer** (from step C1.2) to each sample and mix.

☐ C1.4 Transfer the mixture to the **Zymo-Spin™ Column** and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**

☐ C1.5 Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

☐ C1.6 Add 400 µL **Zymo RNA Prep Buffer** to the column and centrifuge. Discard the flow-through.

☐ C1.7 Add 700 µL **Zymo RNA Wash Buffer** to the column and centrifuge. Discard the flow-through.

☐ C1.8 Add 400 µL **Zymo RNA Wash Buffer** to the column and centrifuge for 1 minute to ensure complete removal of the wash buffer. Carefully, transfer the column into a new RNase-free tube.

☐ C1.9 Add 11 µL of **nuclease-free water** directly to the column matrix, wait for 1 minute at RT and centrifuge.

☐ **C1.10** The small RNAs are present in the flow-through. Keep the reaction tube containing the flow-through.

☐ **C1.11** Quantify 1 µL of extracted small RNA using a Qubit™ HS RNA Assay Kit. The recovered material should be at least 200 ng. *If you are not able to reach at least 200 ng of small RNA, please contact us at techsupport@immaginabiotech.com*

D. tRNA LIBRARY PREPARATION

Ribo-tRNAseq PRO Kit components needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
D1/D3	Annealing buffer 1 (AB1)	IBT0541	50 µL	-20°C	vial	Yellow
D1/D3	Annealing Buffer 2 (AB2)	IBT0542	50 µL	-20°C	vial	Yellow
D1	Splint adapter 1 (SA1)	IBT0602	50 µL	-80°C	strip	
D1	Splint adapter 2 (SA2)	IBT0602	50 µL	-80°C	strip	
D2	PEG 8000 (PEG)	IBT0251	300 µL	-20°C	vial	Yellow
D2	Buffer T1 (BT1)	IBT0521	50 µL	-20°C	vial	Yellow
D2	T1 enzyme (T1)	IBT0531	27 µL	-20°C	vial	Yellow
D3	Barcoded Adapter BCA FWD (AF)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCA REV (AR)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCB FWD (BF)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCB REV (BR)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCC FWD (CF)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCC REV (CR)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCD FWD (DF)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCD REV (DR)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCE FWD (EF)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCE REV (ER)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCF FWD (FF)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCF REV (FR)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCG FWD (GF)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCG REV (GR)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCH FWD (HF)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCH REV (HR)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCI FWD (IF)	IBT0602	10 µL	-80°C	strip	
D3	Barcoded Adapter BCI REV (IR)	IBT0602	10 µL	-80°C	strip	

D3	Barcoded Adapter BCJ FWD (JF)	IBT0602	10 µL	-80°C	strip		
D3	Barcoded Adapter BCJ REV (JR)	IBT0602	10 µL	-80°C	strip		
D3	Barcoded Adapter BCK FWD (KF)	IBT0602	10 µL	-80°C	strip		
D3	Barcoded Adapter BCK REV (KR)	IBT0602	10 µL	-80°C	strip		
D3	Barcoded Adapter BCL FWD (LF)	IBT0602	10 µL	-80°C	strip		
D3	Barcoded Adapter BCL REV (LR)	IBT0602	10 µL	-80°C	strip		
D4	Buffer T2 (BT2)	IBT0561	65 µL	-20°C	vial		Red
D4	T2 enzyme (T2)	IBT0571	20 µL	-20°C	vial		Red
D4	dNTPs	IBT0301	30 µL	-20°C	vial		Green
D4	Buffer T3 (BT3)	IBT0581	110 µL	-20°C	vial		Green
D4	T3 enzyme (T3)	IBT0591	10 µL	-20°C	vial		Green

Additional materials needed in this section:

Step N	Additional Material	Type
D1/D2/D3/D4	RNase Inhibitor, Murine	Required
D2/D4/D5	Agencourt RNA Clean XP for RNA Purification	Required
D2/D4	Ethanol	Required
D2/D3/D4/D5	Nuclease Free Water (NFW)	Required
D4	Qubit™ HS dsDNA Assay Kit	Required
D5	Direct RNA sequencing SQK-RNA004 (Oxford Nanopore)	Required
D5	FLO-MIN004RA flow cell (Oxford Nanopore)	Required
D5	T4 DNA Ligase	Required
D5	NEBNext® Quick Ligation Reaction Buffer	Required

Step D1. SPLINT ADAPTERS ANNEALING

Please note that a single 10 µL reaction for the annealing of splint adapters (D1.1) will be sufficient to handle 5 or 2 samples according to the input RNA that you wish to use in reaction D2.1 (200 or 500 ng, respectively), so you may need to perform more than one reaction from section D1.1 depending on the number of samples you decide to process in a single experiment.

☐ **D1.1** Mix the following reagents in a 0.2 mL reaction tube. Please note that the volumes in Table 3 are intended for one single reaction of splint adapters annealing. Plan the number of reactions according to the number of samples that you wish to process, and the RNA input you plan to use in reaction D2.1.

Reagent	Volume (µL)
Annealing Buffer 1 (AB1)	1
Annealing Buffer 2 (AB2)	1
Splint Adapter 1 (SA1)	3.75
Splint Adapter 2 (SA2)	3.75
RNase Inhibitor, Murine	0.5
Total volume	**10**

Table 3. *Components' volumes to use in step D1 in one single reaction of splint adapters annealing.*

☐ **D1.2** Mix the reactions well by pipetting, then heat to 75°C for 15 sec, and ramp down to 25°C at 0.1°C/s. Store on ice until further use. Once used, please toss the leftovers.

Step D2. SPLINT ADAPTERS LIGATION

NOTE: Start the library preparation with a minimum of **200 ng to a maximum of 500 ng** of small RNA. Adjust the amount of annealed Splint Adapter to use according to the input material (see Table 4 below).

D2.1 Mix the following reagents in a 1.5 mL reaction tube. For clarity, volumes indicated in Table 4 are to be considered for one reaction only and must be repeated for each sample.

	200 ng of RNA	**500 ng of RNA**	**Formula**
Reagent	**Amount (µL)**	**Amount (µL)**	**Amount (µL)**
Small RNA from step C1.11	200 ng (X µL)	500 ng (X µL)	Y ng (X µL)
Annealed Splint Adapter from step D1.2	**1.5**	**3.8**	**0.0076*Y (Z µL)**
PEG 8000	10	10	10
Buffer T1 (BT)	2.5	2.5	2.5
T1 Enzyme (T1)	2	2	2
RNase Inhibitor, Murine	0.5	0.5	0.5
H_2O	8.5 - X	6.2 - X	10 - X - Z
Total volume	**25**	**25**	**25**

Table 4. *Components' volumes to use in step B2 for one reaction. The columns show the volumes of reagents according to the input of small RNA chosen (200 ng, 500 ng or any quantity between 200 to 500 ng).*

☐ D2.2 Incubate the reaction overnight at 4°C.

☐ D2.3 Purify the reaction by adding 50 µL of vortexed **Agencourt RNA Clean XP** beads **(warm the beads at RT for 30 minutes before use)** to each sample and mix well by pipetting the entire volume up and down at least 10 times.

☐ D2.4 Incubate at RT for 10 minutes.

☐ D2.5 Place the tubes on a magnetic rack and discard the supernatant when clear and colorless.

tRNAs are now attached to the beads!

☐ D2.6 Keep the tubes on the magnetic rack. Add 200 µL of EtOH 70% freshly prepared to the beads. Incubate for 30 seconds and then remove the supernatant.

☐ D2.7 Repeat the washing step.

☐ D2.8 Remove supernatant and let the beads pellet dry on the magnetic rack at room temperature for ~2-4 minutes.

Avoid over-drying the beads (pellet cracked) as this will significantly decrease elution efficiency.

☐ D2.9 Remove the tubes from the magnetic rack and resuspend the beads in 9 µL of nuclease-free water. Mix thoroughly by pipetting up and down to ensure complete bead dispersion. Incubate at RT for 10 minutes.

☐ D2.10 Place the tubes on a magnetic rack until the solution is completely clear. **tRNAs are now in the supernatant!**

☐ D2.11 Remove the 9 µL of supernatant and place into a clean 0.2 mL nuclease-free tube.

Step D3. BARCODED ADAPTERS ANNEALING

NOTE1: Each pair of barcoded adapters (FWD and REV) needs to be annealed following the passages below. For clarity, Table 5 shows an example for the barcode pair **AF** and **AR**. Please, repeat the reaction for every pair of barcodes needed (up to 12 per library preparation).

NOTE2: This resource provides recommendations for optimizing output when pooling indexed libraries. Pooling combines at least two libraries to sequence in one run.

Barcodes:

- Group 1: A, B, C
- Group 2: D, E, F
- Group 3: G, H, I
- Group 4: J, K, L

Use the following barcode combinations when pooling samples:

- 2–3 samples: use Group 1 barcodes (A, B, C)
- 4–6 samples: use Group 1 (A, B, C) and Group 2 (D, E, F)
- 7–12 samples: use Group 1 (A, B, C), Group 2 (D, E, F), Group 3 (G, H, I) and Group 4 (J, K, L)

At this step, work only with the barcoded adapters (Forward and Reverse) corresponding to the number of samples you intend to multiplex.

☐ **D3.1** Mix the following reagents in a 0.2 mL reaction tube:

Reagent	Volume (µL)
Annealing Buffer 1 (AB1)	1
Annealing Buffer 2 (AB2)	1
Barcoded Adapter A FWD (AF)	1.5
Barcoded Adapter A REV (AR)	1.5
H_2O	4.5
RNase Inhibitor, Murine	0.5
Total volume	**10**

Table 5. *Components' volumes to use in step D3.1.*

☐ **D3.2** Mix the reactions well by pipetting, then heat to 75°C for 15 sec, and ramp down to 25°C at 0.1°C/s. Store on ice until further use. Once used, please toss the leftovers.

Step D4. BARCODED ADAPTERS LIGATION AND REVERSE TRANSCRIPTION

☐ D4.1 Mix the following reagents in a 0.2 mL nuclease-free reaction tube. **Please perform separate reactions for each sample/barcoded adapter you are processing.**

Reagent	Volume (µL)
Buffer T2 (BT2)	3
tRNA from step D2.11	8.5
Annealed Barcoded Adapter* from step D3.2	1.5
RNase Inhibitor, Murine	0.5
T2 Enzyme (T2)	1.5
Total volume	**15**

***Please use different Barcoded Adapters for different samples**

Table 6. *Components' volumes to use in step D4.1. Please perform separate reactions for each sample/barcoded adapters you are processing.*

☐ D4.2 Incubate the reaction for 10 minutes at RT.

☐ D4.3 Meanwhile, prepare the reverse transcription master mix as follows**.** Please consider that the volumes in Table 7 are to be considered for one single sample and corresponding barcoded adapters.

Reagent	Volume (µL)
H_2O	14.5
Buffer T3 (BT3)	8
dNTPs	2
Total volume	**24.5**

Table 7. *Components' volumes to use in step B4.3. Volumes in this table are to be considered for one single sample and corresponding barcoded adapters.*

☐ D4.4 Add the master mix to the reaction tube containing the barcoded adapters-ligated tRNA from step D4.2. Mix by pipetting.

☐ D4.5 Add **0.5 µL of T3 enzyme (T3)** to the reaction and mix by pipetting.

☐ D4.6 Incubate at 60°C for 30 minutes, then at 85°C for 5 minutes, and bring to 4°C before proceeding with the next step.

☐ D4.7 Purify the reaction by adding 80 µL of vortexed **Agencourt RNA Clean XP** beads **(warm the beads at RT for 30 min before use)** to each sample and mix well by pipetting the entire volume up and down at least 10 times.

☐ D4.8 Incubate at RT for 10 minutes.

- ☐ **D4.9** Place the tubes on a magnetic rack and discard the supernatant when clear and colorless. **tRNAs are now attached to the beads!**
- ☐ **D4.10** Keep the tubes on the magnetic rack. Add 200 µL of EtOH 70% freshly prepared to the beads. Incubate for 30 seconds and then remove the supernatant.
- ☐ **D4.11** Repeat the washing step.
- ☐ **D4.12** Remove supernatant and let the beads pellet dry on the magnetic rack at room temperature for ~2-4 minutes. **Avoid over-drying the beads (pellet cracked) as this will significantly decrease elution efficiency.**
- ☐ **D4.13** Remove the tubes from the magnetic rack and resuspend the beads in 8.5 µL of nuclease-free water. Mix thoroughly by pipetting up and down to ensure complete bead dispersion. Incubate at room temperature for 10 minutes.
- ☐ **D4.14** Place the tubes on a magnetic rack until the solution is completely clear. **tRNAs are now in the supernatant!**
- ☐ **D4.15** Remove the 8.5 µL of supernatant and place into a clean 1.5 mL reaction tube.
- ☐ **D4.16** Quantify 2 µL of the eluate using a Qubit™ HS dsDNA Assay Kit.

⚠ **SAFE STOPPING POINT (store at -80°C for up to one week)**

Step D5. RNA POOLING, RMX LIGATION AND SEQUENCING

☐ **D5.1** Pool in a 1.5 mL tube the barcoded samples from step **D4.16** so that the total RNA amount is 21 ng in 23 µL (if necessary, use nuclease-free water to reach the requested volume). Use the following table 8 as a guide:

Samples barcoded with	Pool 3	Pool 6	Pool 9	Pool 12	Volume (µL)
	Quantity (ng)				
A	7	3.5	2.3	1.75	
B	7	3.5	2.3	1.75	
C	7	3.5	2.3	1.75	
D		3.5	2.3	1.75	
E		3.5	2.3	1.75	
F		3.5	2.3	1.75	
G			2.3	1.75	
H			2.3	1.75	
I			2.3	1.75	
J				1.75	
K				1.75	
L				1.75	
NFW	if needed				
Total	**21**	**21**	**21**	**21**	**23**

Table 8. *Calculated volumes required per barcoded sample based on the total number of pooled samples. Each specified volume should be combined into the final pool, with nuclease-free water added to bring the total volume to 23 µL.*

Please note that the reagents indicated by an asterisk (*) are part of the Direct RNA Sequencing SQK-RNA004 (Oxford Nanopore)

☐ **D5.2** In the same 1.5 mL tube, combine reagents as follows, and mix by pipetting:

Reagent	Volume (µL)
Pooled RNA	23
NEB Next Quick Ligation Reaction Buffer	8
RNA Ligation Adapter (RLA) *	6
T4 DNA Ligase	3
Total volume	**40**

Table 9. *Components' volumes to use in step D5.3. Volumes in this table are to be considered for one single sample. For pooled RNA volumes greater than 23 µL, see Appendix 1.*

☐ **D5.3** Mix by pipetting and incubate the reaction at RT for 10 minutes.

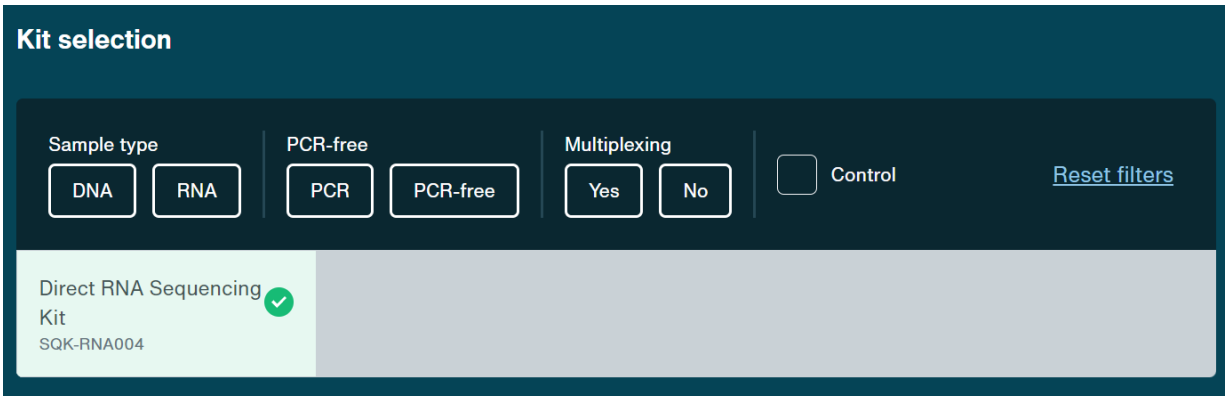
- ☐ **D5.4** Let the **Agencourt RNA Clean XP beads** equilibrate at RT for 30 minutes, then resuspend by vortexing.
- ☐ **D5.5** Add 80 µL of Agencourt RNA Clean XP beads to the reaction and mix by pipetting.
- ☐ **D5.6** Incubate on a rotator mixer at RT for 5 minutes.
- ☐ **D5.7** Spin the sample down and pellet on a magnet. Discard the supernatant when the solution is completely transparent.
- ☐ **D5.8** Add 150 µL of Wash Buffer (WSB)* to the beads. Close the cap and resuspend by flicking the tube. Return the tube to the magnetic rack, allow the beads to pellet and remove the supernatant when the solution is completely transparent. Repeat this step twice.
- ☐ **D5.9** Spin the tube down and return it to the magnetic rack until the beads have pelleted. Remove completely any remaining Wash Buffer (WSB)*.
- ☐ **D5.10** Remove the tube from the magnetic rack and resuspend in 13 µL of RNA Elution Buffer (REB)* by gently flicking the tube. Incubate at RT for 10 minutes.
- ☐ **D5.11** Pellet the beads on the magnet until the supernatant is completely transparent.
- ☐ **D5.12** Retain the 13 µL of eluate and place into a clean 1.5 mL tube.
- ☐ **D5.13** Proceed following from section 4 (Priming and loading the MinION/GridION Flow Cell) of Library preparation from Oxford Nanopore SQK-RNA004 protocol. **Please, make sure to carefully follow the guidelines given below BEFORE starting the sequencing.**

IMPORTANT: The tRNA library must be sequenced immediately and cannot be stored for later use.

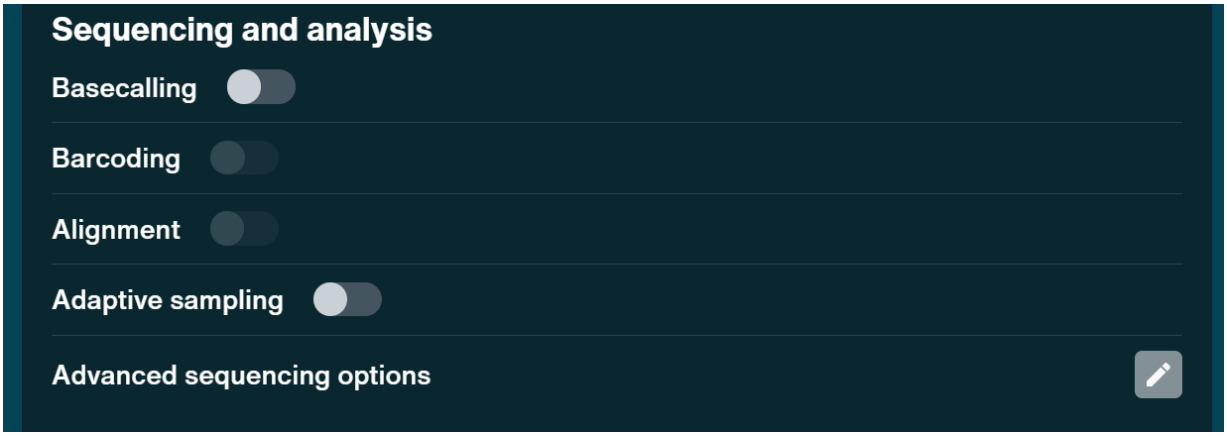
IMPORTANT: BEFORE STARTING THE SEQUENCING

⚠ Select “Flow cell type”: FLO-MIN004-RA

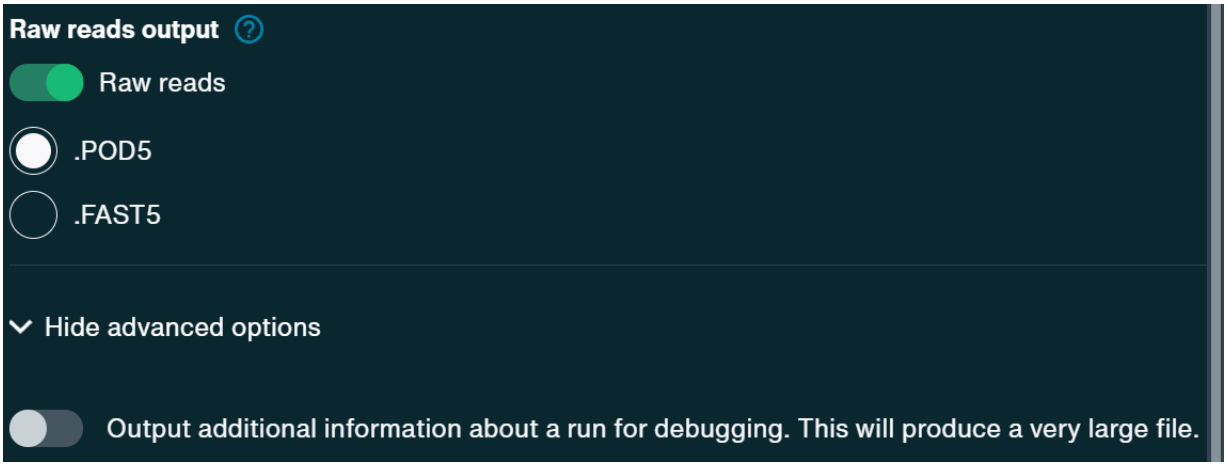
⚠ Select “Kit selection”: Direct RNA Sequencing Kit (SQK-RNA004).



⚠ Deactivate “Basecalling”.



⚠ Select the saving of the .POD5 file format in the software before starting the sequencing.



All the information related to the demultiplexing step are present in the USB pen drive that has been provided with the kit. Please, make sure to read all the instructions before loading the library on the flow cell.

During demultiplexing, all possible barcodes are evaluated; therefore, a small number of background reads may map to barcodes that were not used in the experiment.

Table 10 below provides the cross-reference matrix to link your physical library barcodes to the final demultiplexed file names. Note that the demultiplexing software outputs files in alphanumeric order (BC01, BC02, etc.), whereas your experimental layout used alphabetical naming (A, B, etc.). As a reference, Barcode A maps to BC13.

Group	Barcodes (F / R)	Conversion matrix		
1	A · B · C	A = BC13	B = BC03	C = BC07
2	D · E · F	D = BC09	E = BC01	F = BC10
3	G · H · I	G = BC08	H = BC06	I = BC02
4	J · K · L	J = BC11	K = BC12	L = BC04
Spare	—	**BC05** — *internal use · lot quality assurance only*		

Table 10. *Demultiplexing matrix for sample recognition as demultiplexing software outputs files in alphanumeric order, whereas your experimental layout used alphabetical naming. BC05 is reserved for internal QC and lot tracking as it is not present in the kit provided.*

APPENDIX

App.1 Guidelines for sample input amount optimization

For guidelines supporting any custom protocol optimization, please refer to the RiboLace Starter kit protocol available on our website.

App.2 Alternative CHX removal protocol

It is worth mentioning that CHX treatment could lead to the accumulation of ribosomes within the first 10 codons. Therefore, if you decide to use CHX treatment, be aware of this potential effect on ribosome distribution along the CDS (coding sequence). CHX treatment is recommended, but not mandatory, to enhance the efficiency of ribosome affinity purification. If you choose to avoid CHX treatment, it is crucial to ensure the prompt and proper flash freezing of the sample. Flash freezing helps to preserve the sample's integrity and minimize potential degradation. To achieve this, follow these steps:

- After collecting the sample (e.g., detaching or pelleting the cells), transfer it to a suitable container or tube.
- Pellet the cells and remove the media.
- Wash with cold PBS and remove completely the liquid.
- Place the container in a liquid nitrogen bath or use a dry ice and ethanol mixture for rapid freezing.
- Ensure that the sample is fully submerged in the liquid nitrogen or surrounded by the dry ice mixture to facilitate rapid cooling.
- Allow the sample to freeze rapidly for a few minutes until it reaches a fully frozen state.
- Once the sample is completely frozen, store it at -80°C or in a cryogenic storage system to maintain its stability until further processing.
- Once ready to perform the experiment, defrost the cell pellet in ice and proceed with treating the sample from step 1.4b (lysing the pellet cells in supplemented lysis buffer).

App.3 Lysis buffer supplementation issues

Please check if, after adding Sodium deoxycholate a whiteish and cloudy solution appears. If so, please do not proceed with the lysis of the sample and toss the supplemented LB. Subsequently, warm up the SDC at RT and add it to a new aliquot of the not-supplemented LB. If the whiteish and cloudy solution persists, please contact our tech support (techsupport@immaginabiotech.com).

App.4 Sample Lysis output summary table

The following table allows for recording the amount of sample and the relative amount obtained after the Cell Lysis Step - B1.

Sample number	Sample name	Amount utilized (n° cells or mg of tissue)	SLB V utilized (µL)	AU/mL (after blank subtraction)	AU/µL	V for 0.9 AU (µL)
1						
2						
3						
4						
5						
6						

Table 9. *Sample Lysis Output Summary*

App.5 Beads functionalization checklist

Reagent	N=1 0.9 A.U.	N=___ 0.9 A.U.	Needed in Step	Step-by-Step Checklist
			B2.1	□ Place RmB v2-1 at RT for 30 min
			B2.2	□Vortex 30'
RiboLace magnetic beads (RmB)	144 µL	____	B2.3	□Add "___" Beads in 1.5 / 2 mL Tube □Place on magnet □REMOVE Supernatant
OH-buffer (OH)	270 µL	____	B2.4	□Remove from magnet □Wash OH Buffer: add "____" µL □Shake 5min 1400 rpm RT □Place on magnet □REMOVE Supernatant
Nuclease-free water	1000 µL	1000 µL	B2.5	□Remove from magnet □Wash N.F. Water: add 1000 µL □Shake 2min 1400 rpm RT □Place on magnet □REMOVE Supernatant
B-Buffer (BB)	270 µL	____	B2.6	□Remove from magnet □Wash B Buffer: add "____" µL □Shake 3min 1400 rpm RT □Place on magnet □REMOVE Supernatant □Repeat wash 2nd time
			B2.7	□Store 2 µL of diluted RsP for control
Diluted RiboLace Smart Probe (RsP)	81 µL	____	B2.8	□Remove from magnet □Resuspend in diluted RsP: "___" µL
			B2.9	□Incubate 1h shacking 1400 rpm RT
At this point, you can start the Nuclease digestion (step B3) in parallel				
			B2.10	□Place on magnet □Store 3 µl of Supernatant for control □Remove from magnet
mPEG	7.5 µL	____	B2.11	□Add mPEG "____" µL □Incubate 15min shacking 1400 rpm RT
Nuclease-free water	1000 µL	1000 µL	B2.12	□Place on magnet □REMOVE Supernatant □Remove from magnet □Wash with N.F. water: add 1000 µL □Shake 2 min 1400 rpm RT
W-buffer (WB)	1000 µL	1000 µL	B2.13	□Place on magnet □REMOVE Supernatant □Remove from magnet □Wash with W buffer 1000 µL □Shake 2 min 1400 rpm RT □Repeat wash 2nd time
W-buffer (WB)	100 µL	____	B2.14	□Place on magnet □REMOVE Supernatant □Resuspend in "____"µL w-Buffer
			B2.15	□Aliquot in 105 µL of equal volumes in N tubes

Table 10. *Components' volumes to use for the Bead Functionalization Step B2. N = number of reactions. The table is intended as a guideline to follow when dealing with non-standard bead amounts and multiple samples.*

App.6 Check proper beads functionalization (for Step B2 – Beads functionalization)

Comparing the difference in the absorbance measured at A 270 nm (Nanodrop ND-1000) for the unbound probe (collected in Step B2.10) and the staring solution of the diluted RiboLace smart probe (RsP) (collected in Step B2.7) allows an estimation of the binding efficiency.

reduction in % = $(1 - \frac{\text{Step B2.10 } A_{270}}{\text{Step B2.7 } A_{270}}) * 100$

Between 10% and 50% absorbance reduction in the unbound probe compared to the starting solution is expected. If the decrease in absorbance is not observed, please incubate beads for up to 2 hours and check again the absorbance.

Sample number	Sample name	AU 270 Before Step B2.7	AU 270 After Step B2.10	Reduction %
1				
2				
3				
4				

Table 11. *Sample beads functionalization summary*

App.7 Nuclease digestion checklist

Table 12 provides the output from Step Lysis B1 with the possibility, if needed, to list the amount of W-buffer to reach the reaction amount.

Sample number	Sample name	AU/µL	V for 0.9 AU (µL)	V of W-Buffer up to 450 (µL)
1				
2				
3				
4				
5				
6				

Table 12. *Summary of the volumes to utilize for the dilution of lysate Step B3.*

Table 13 is intended as a guideline to follow for digesting the lysate.

Reagent	0.9 A.U	Needed in Step	Step-by-Step Checklist
W-Buffer	____	B3.1	□ If needed, dilute the lysate calculated following Step A2.1 in W-Buffer up to "___"
Nux Enhancer (NE)	0.9 µL	B3.2	□ Add 0.9 µL of NE to the lysate
Nux (Nux) + W-Buffer		B3.3	□ Dilute 1.5 µL of Nux in 98.5 µL W-buffer to create diluted Nux (dNux)
Diluted Nux (dNux)	4.5 µL	B3.4	□ Add 4.5 µL of dNux to the lysate □ Incubate 45 min at 25°C
SUPERase•In	1.5 µL	B3.5	□ Stop the reaction by adding 1.5 µL of SUPERase•In into the lysate □ Incubate for 10 min on ice

Table 13. *Components' volumes to use for the Digestion of lysate Step B3. The table is intended as a guideline to follow when dealing with non-standard nuclease amounts.*

App.8 Optional Nuclease optimization (for Step B3 – Nuclease Digestion)

The quantity of Nuclease (Nux) to utilize for lysing the sample could be optimized before proceeding with the pulldown. The kit contains a concentrated vial of Nux (#IBT0091) that is intended to be diluted before use, and that needs to be added to the lysate sample in a fixed quantity, depending on the amount of AU as starting material. This quantity is suitable for most cell lines; however, it can be modulated depending on the needs and type of specimen. To optimize this quantity, after lysing the sample in Step B1, start with 0.3 AU as the starting material and W-buffer up to a final volume of 150 µL. Perform a titration assay, by adding to each reaction different quantities of Nux, below is an example:

Starting lysate	Quantity of diluted Nux (Step. 3.3)	Sample
0.3 AU	0	NT
0.3 AU	0.3 µL	A
0.3 AU	3 µL	B
0.3 AU	30 µL	C

Table 14. *Components' volumes to use for the Nuclease Optimization of lysate. The table is intended as a guideline to follow when dealing with non-standard samples that require ad hoc digestion.*

- ☐ Digest the samples for 45 min at 25°C.
- ☐ Stop digestion with 1.5 µL of SUPERaseIn for 10 min on ice.
- ☐ Add 300 µL of the Zymo RNA Binding Buffer (ZBB).
- ☐ Incubate the solution at RT for 5 min with shaking at 600 rpm.
- ☐ Add 450 µL of EtOH 95-100% mixing the solution by pipetting.
- ☐ Transfer 700 µL of the mixture to the Zymo-Spin™ Column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ Transfer the remaining volume of the mixture to the Zymo-Spin™ Column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ Add 400 µl RNA Prep Buffer to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ Add 700 µl RNA Wash Buffer to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ Add 400 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
- ☐ To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 g at RT. Discard the flow-through. Carefully, transfer the column into a new RNase-free tube.
- ☐ Add **11 µL** of **Nuclease Free Water** directly to the column matrix and wait 1 minute.
- ☐ Centrifuge for 30 seconds at 12,000 g at RT. The extracted RNA is present in the flow-through. Collect the flow through.

- ☐ With Nanodrop, measure the absorbance of each sample at 260 nm (set up the “nucleic acid” function of the Nanodrop), using 1 µL of Nuclease Free Water as blank.
- ☐ Extracted RNA needs to be run on a 15% TBE-urea gel.
- ☐ Pre-run the gel at 200 V for 30 min in TBE prepared with nuclease-free water. Clean well the gel wells with a syringe to remove UREA residuals before loading the samples.
- ☐ Prepare samples: add Gel Loading Buffer II to 1.5 µg of RNA (1:1 volume).
- ☐ Use an ultra-low range molecular weight marker as reference.
- ☐ Load the samples and the Marker on 15% TBE-urea polyacrylamide gel and run the gel for 1 h at 200V until the bromophenol blue band reaches the bottom of the gel.
- ☐ Stain the gel for 5 minutes with SYBR Gold in TBE and visualize the RNA using a UV-Transilluminator.

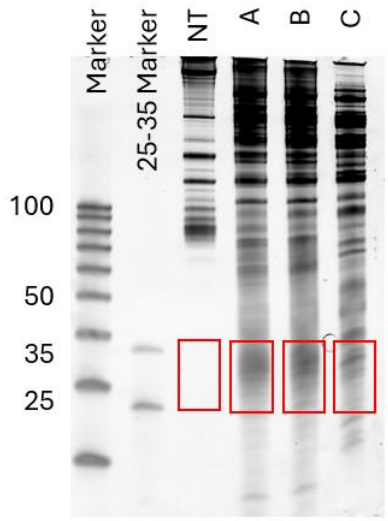


Fig.2 Example of RNA extracted after nuclease titration run on 15% TBE-Urea gel. In the red square the sizes are between 25-35 nt.

As depicted in the figure above, not digested (NT) sample does not present the typical enrichment of fragments at 25-35 nt (red square in Fig.2). Under-digested sample (A) does display an enrichment of RPFs but they are not well resolved, while the over-digested sample (C) should display a ladder-like pattern of bands below 50 nt and the disappearance of the signal on the top part of the gel. In this example, the quantity of Nux used in sample B needs to be utilized for all the reactions as it displays a sharper definition of peaks between 25-35 nt.

By conducting the titration assay, you can determine the ideal amount of Nux required for efficient lysis of your sample according to your specific needs and specimen characteristics. Furthermore, if nucleic acid from your non-digested (NT) sample is degraded, you may observe a ladder-like pattern of bands below 50 nt and the disappearance of the signal on the top part of the gel. In such cases, it is advisable to restart the experiment since the poor quality of the sample can significantly impact the results. It is crucial to ensure that the sample's integrity is maintained for reliable and accurate data during the experiment.

App.9 Low output from Step B4 BARCODED ADAPTERS LIGATION AND REVERSE TRANSCRIPTION

If the total RNA amount of the barcoded samples from step D4.16 intended for pooling does not reach 21 ng in 23 µL, it is advisable to restart the procedure. If restarting is not feasible, all available material may be pooled (e.g. 6 µL per sample). In this scenario, the pool will not be balanced, as differences in sample amount may occur. Furthermore, the total volume of the pooled libraries will exceed 23 µL (e.g. up to 36 µL if 6 samples are pooled at 6 µL each). Note that deviating from the recommended volume may affect sequencing throughput and the outcome cannot be guaranteed.

The usage is to add more volume in step D5.2 as in the example below:

☐ **D5.2b** In the same 1.5 mL tube, combine reagents as follows, and mix by pipetting:

Reagent	Volume (µL)	Example Volume (µL)
Pooled RNA	23 + X	36
H_2O	13 - X	0
NEB Next Quick Ligation Reaction Buffer	12	12
RNA Ligation Adapter (RLA) *	9	9
T4 DNA Ligase	4.5	4.5
Total volume	61.5	**61.5**

☐ **D5.3b** Mix by pipetting and incubate the reaction at RT for 10 minutes.

☐ **D5.4b** Let the **Agencourt RNA Clean XP beads** equilibrate at RT for 30 minutes, then resuspend by vortexing.

☐ **D5.5b** Add 123 µL of Agencourt RNA Clean XP beads to the reaction and mix by pipetting.

☐ **D5.6b** Incubate on a rotator mixer at RT for 5 minutes.

☐ **D5.7b** Spin the sample down and pellet on a magnet. Discard the supernatant when the solution is completely transparent.

☐ **D5.8b** Add 150 µL of Wash Buffer (WSB)* to the beads. Close the cap and resuspend by flicking the tube. Return the tube to the magnetic rack, allow the beads to pellet and remove the supernatant when the solution is completely transparent. Repeat this step twice.

☐ **D5.9b** Spin the tube down and return it to the magnetic rack until the beads have pelleted. Remove completely any remaining Wash Buffer (WSB)*.

☐ **D5.10b** Remove the tube from the magnetic rack and resuspend in 13 µL of RNA Elution Buffer (REB)* by gently flicking the tube. Incubate at RT for 10 minutes.

☐ **D5.11b** Pellet the beads on the magnet until the supernatant is completely transparent.

☐ **D5.12b** Retain the 13 µL of eluate and place into a clean 1.5 mL tube.

☐ **D5.13b** Proceed following from section 4 (Priming and loading the MinION/GridION Flow Cell) of Library preparation from Oxford Nanopore SQK-RNA004 protocol. **Please, make sure to carefully follow the guidelines given below BEFORE starting the sequencing.**

IMPORTANT: The tRNA library must be sequenced immediately and cannot be stored for later use.

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Notes: