nano-tRNAseq PRO kit

High-Multiplex nano-tRNAseq kit protocol (12 Barcodes)

nano-tRNAseq PRO Kit: a complete, end-to-end solution for native full-length direct tRNA sequencing, combining wet-lab library preparation with an integrated computational demultiplexing app for high-throughput, multiplexed sequencing and analysis of tRNAs on Oxford Nanopore Technologies (ONT) platforms.

Product	Catalog no	Samples	Barcode
nano-tRNAseq PRO Kit with Demultiplexing Tool	NTPRO-12	12	12

Shipping: Dry ice

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: nano-tRNAseq PRO kit enables 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/prokaryotic cells and tissues with annotated genome.

What's Included:

1. **Library Prep Kit** — Reagents and protocol for preparing your tRNA library for sequencing on the Oxford Nanopore platform**.**
2. **Computational Demultiplexing Tool** — A powerful software tool for demultiplexing of multiplexed sequencing runs, 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
-20°C components	1 box	-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:

- RNA Clean & Concentrator™-5 (Zymo, cat. 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

Nano-tRNAseq is an Immagina's technology that marks a significant milestone in tRNA and translatomics research. It enables the comprehensive sequencing of tRNA molecules in their full-length, native state, allowing for the simultaneous assessment of tRNA abundances and modification status. The **nano-tRNAseq PRO kit** is the only product that allows for the sequencing of native full-length tRNA molecules with an easy, fast and robust workflow.

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 laboratory at Centre for Genomic Regulation (CRG), Barcelona, our method bypasses the need for cDNA/PCR sequencing (https://doi.org/10.1038/s41587-023-01743-6)

The first step in nano-tRNAseq comprises deacylation (1) and small RNA enrichment from total RNA (2). Next, the tRNAs are bound with adaptors and subsequently to barcodes (3), allowing for multiplexing of up to 12 samples. The native tRNA is then sequenced on the Oxford Nanopore Technologies (ONT) platform (4). As tRNA is transversing the pore, each base generates an electrical current, which is converted into the tRNA sequence. After demultiplexing and data analysis (5), information on tRNA abundance, coverage and post-transcriptional chemical modifications are obtained (Figure 1).

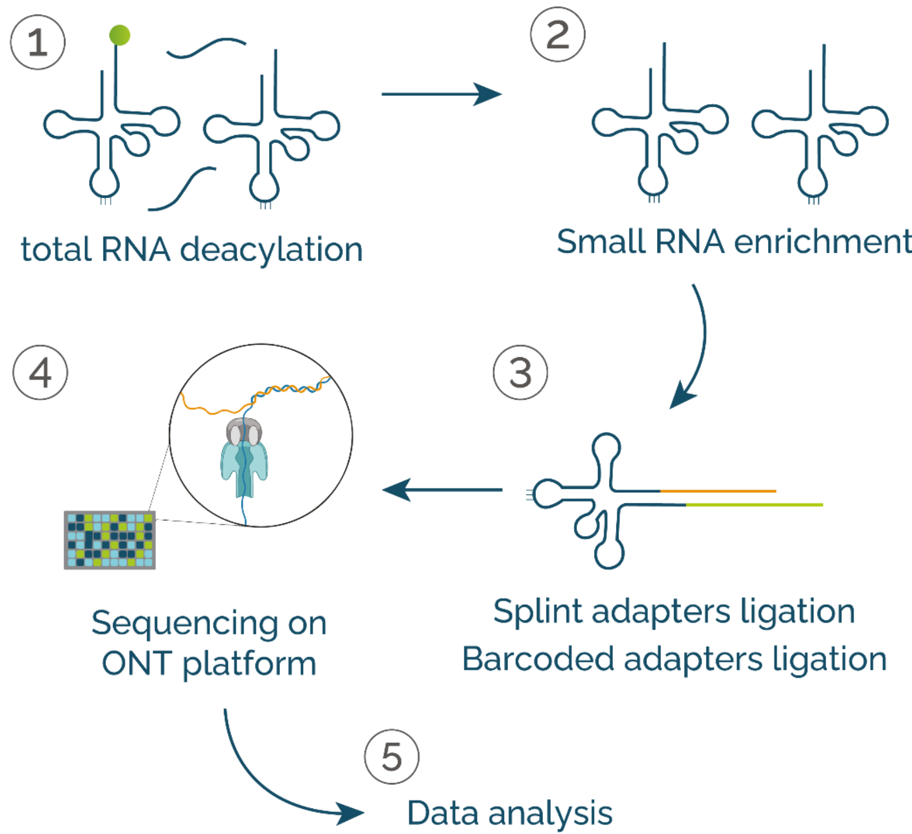


Figure 1. *Simple overview of the nano-tRNAseq protocol.*

PROTOCOL WORKFLOW

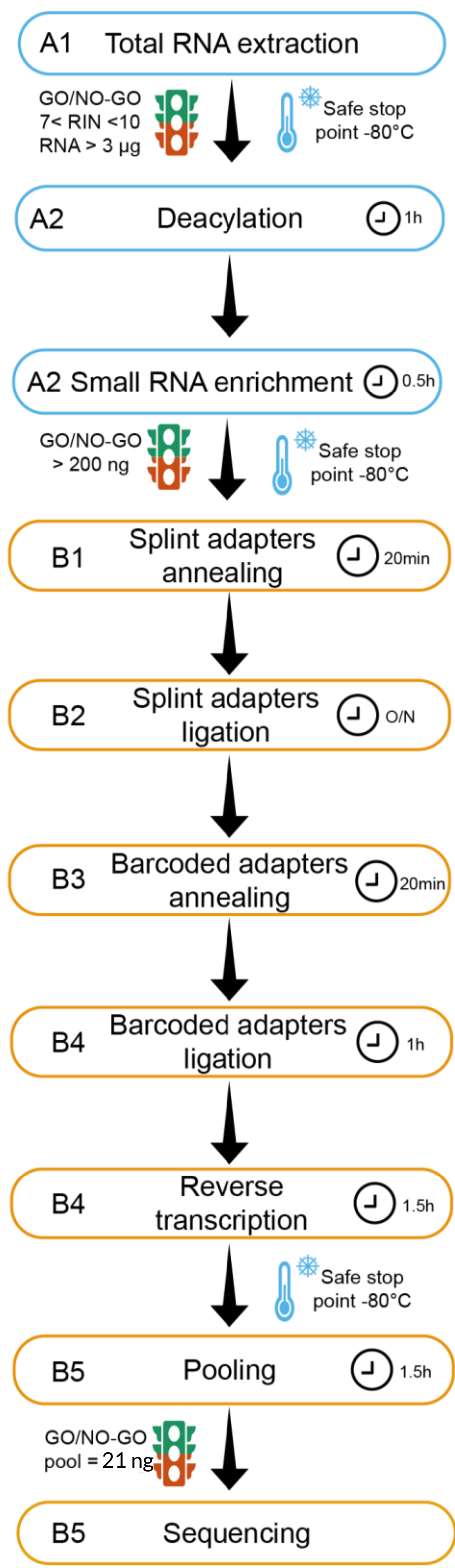


Figure 2. Overview of the nano-tRNAseq Pro Kit workflow. *In each box, steps are present in the left corner, while the time to complete the step is listed in the right corner (clock picture). Safe stopping points (thermometer pictures) and GO/NO-GO conditions (traffic lights) are placed right at the bottom of the corresponding steps. Optional and required checkpoints (caution signal) are also indicated on the right.*

Optimal Workflow Recommendations

- The nano-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 14.
- Please note that the reagent volumes included in this kit 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 2 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.
- This protocol is optimized for tRNA extraction starting from at least 3 µg* of total RNA.
- At least 200 ng* of small RNA are needed after the deacylation step (step A2) to move forward with library preparation.
- 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.

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

A. tRNA EXTRACTION

Components and additional materials needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
A3	Deacylation Buffer (DB)	IBT0551	1300 µL	-20°C	vial	clear

Step N	Additional Material	Type
A1	Agilent RNA 6000 Nano Kit	Optional
A1	Agilent 2100 Bioanalyzer	Optional
A2/A3	RNA Clean & Concentrator™-5	Required
A1/2/3	Nuclease free water (NFW)	Required
A1/2/3	Ethanol	Required
A3	Qubit™ HS RNA Assay Kit	Required

Step A1. TOTAL RNA EXTRACTION

A1.1 Start extracting total RNA from your samples with any kit available at your facility. It is important to elute the extracted RNA in 12 µL of nuclease-free water for downstream application.

After quantification, it is important to check for proper RNA integrity before proceeding with deacylation and small RNA enrichment. The best approach is evaluating the extracted RNA by **Agilent 2100 Bioanalyzer** using the Agilent RNA 6000 Nano Kit (for non-eukaryotic species please use equivalent). An expected RNA Integrity Number (RIN) between 7 and 10 is needed for producing informative libraries.

SAFE STOPPING POINT (store at -80°C)

Step A2. DEACYLATION and SMALL RNA (<200nt) ENRICHMENT

NOTE: After total RNA extraction deacylation needs to be performed on at least 3 µg of total RNA to get enough material for library preparation. If you are not able to reach at least 3 µg of total RNA please contact us at techsupport@immaginabiotech.com

- ☐ **A2.1** Add 90 µL of deacylation buffer (DB) to 10 µL of extracted RNA, to a final volume of 100 µL.
- ☐ **A2.2** Incubate the reaction 30 minutes at 37°C.

Purify deacylated small RNA 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.

- ☐ **A2.3** Prepare **adjusted RNA Binding Buffer** by mixing 100 µL of **Zymo RNA Binding Buffer** (**ZBB**) and 100 µL of ethanol (95-100%) for each sample you want to process.
- ☐ **A2.4** Add 200 µL of **adjusted RNA Binding Buffer** (from step **A2.3**) to each sample and mix.
- ☐ **A2.5** Transfer the mixture to the **Zymo-Spin™ Column** and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**
- ☐ **A2.6** Add 300 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.
- ☐ **A2.7** Add 400 µL **Zymo RNA Prep Buffer** to the column and centrifuge. Discard the flow-through.
- ☐ **A2.8** Add 700 µL **Zymo RNA Wash Buffer** to the column and centrifuge. Discard the flow-through.
- ☐ **A2.9** 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.
- ☐ **A2.10** Add 8 µL of **nuclease-free water** directly to the column matrix, wait for 1 minute at RT and centrifuge.
- ☐ **A2.11** The small RNAs are present in the flow-through. Keep the reaction tube containing the flow-through.
- ☐ Quantify 1 µL from **A2.11** using a Qubit™ HS RNA Assay Kit. The recovered material should be at least 200 ng of deacylated small RNA. *If you are not able to reach at least 200 ng of small RNA, please contact us at techsupport@immaginabiotech.com*

⚠ **Please use deacylated small RNA for library preparation (step B) within the same day.**

B. tRNA LIBRARY PREPARATION

nano-tRNAseq Pro Kit components needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
B1/B3	Annealing buffer 1 (AB1)	IBT0541	50 µL	-20°C	vial	Yellow
B1/B3	Annealing Buffer 2 (AB2)	IBT0542	50 µL	-20°C	vial	Yellow
B1	Splint adapter 1 (SA1)	IBT0602	50 µL	-80°C	strip	
B1	Splint adapter 2 (SA2)	IBT0602	50 µL	-80°C	strip	
B2	PEG 8000 (PEG)	IBT0251	300 µL	-20°C	vial	Yellow
B2	Buffer T1 (BT1)	IBT0521	50 µL	-20°C	vial	Yellow
B2	T1 enzyme (T1)	IBT0531	27 µL	-20°C	vial	Yellow
B3	Barcoded Adapter BCA FWD (AF)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCA REV (AR)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCB FWD (BF)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCB REV (BR)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCC FWD (CF)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCC REV (CR)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCD FWD (DF)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCD REV (DR)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCE FWD (EF)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCE REV (ER)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCF FWD (FF)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCF REV (FR)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCG FWD (GF)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCG REV (GR)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCH FWD (HF)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCH REV (HR)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCI FWD (IF)	IBT0602	10 µL	-80°C	strip	
B3	Barcoded Adapter BCI REV (IR)	IBT0602	10 µL	-80°C	strip	

B3	Barcoded Adapter BCJ FWD (JF)	IBT0602	10 µL	-80°C	strip		
B3	Barcoded Adapter BCJ REV (JR)	IBT0602	10 µL	-80°C	strip		
B3	Barcoded Adapter BCK FWD (KF)	IBT0602	10 µL	-80°C	strip		
B3	Barcoded Adapter BCK REV (KR)	IBT0602	10 µL	-80°C	strip		
B3	Barcoded Adapter BCL FWD (LF)	IBT0602	10 µL	-80°C	strip		
B3	Barcoded Adapter BCL REV (LR)	IBT0602	10 µL	-80°C	strip		
B4	Buffer T2 (BT2)	IBT0561	65 µL	-20°C	vial		Red
B4	T2 enzyme (T2)	IBT0571	20 µL	-20°C	vial		Red
B4	dNTPs	IBT0301	30 µL	-20°C	vial		Green
B4	Buffer T3 (BT3)	IBT0581	110 µL	-20°C	vial		Green
B4	T3 enzyme (T3)	IBT0591	10 µL	-20°C	vial		Green

Additional materials needed in this section:

Step N	Additional Material	Type
B1/B2/B3/B4	RNase Inhibitor, Murine	Required
B2/B4/B5	Agencourt RNA Clean XP for RNA Purification	Required
B2/B4	Ethanol	Required
B2/B3/B4/B5	Nuclease Free Water (NFW)	Required
B4	Qubit™ HS dsDNA Assay Kit	Required
B5	Direct RNA sequencing SQK-RNA004 (Oxford Nanopore)	Required
B5	FLO-MIN004RA flow cell (Oxford Nanopore)	Required
B5	T4 DNA Ligase	Required
B5	NEBNext® Quick Ligation Reaction Buffer	Required

Step B1. SPLINT ADAPTERS ANNEALING

Please note that a single 10 µL reaction for the annealing of splint adapters (B1.1) will be sufficient to handle 6 or 2 samples according to the input RNA that you wish to use in reaction B2.1 (from 200 to 500 ng), so you may need to perform more than one reaction from section B1.1 depending on the number of samples you decide to process in a single experiment.

☐ B1.1 Mix the following reagents in a 0.2 mL reaction tube. Please note that the volumes in Table 1 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 B2.1.

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 1. *Components' volumes to use in step B1 in one single reaction of splint adapters annealing.*

☐ B1.2 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 B2. SPLINT ADAPTERS LIGATION

NOTE: Start the library preparation with at least **200** to a maximum of **500 ng** of deacylated small RNA. Adjust the amount of annealed Splint Adapter to use according to the input material (see Table 2 below).

B2.1 Mix the following reagents in a 1.5 mL reaction tube. For clarity, volumes indicated in Table 2 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 A2.11	200 ng (X µL)	500 ng (X µL)	Y ng (X µL)
Annealed Splint Adapter from step B1.2	**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 2. *Components' volumes to use in step B2 for one reaction. The columns show the volumes of reagents according to the input of small RNA deacylated chosen (200 ng, 500 ng or any quantity between 200 to 500 ng).*

- ☐ B2.2 Incubate the reaction overnight at 4°C.
- ☐ B2.3 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.
- ☐ B2.4 Incubate at RT for 10 minutes.
- ☐ B2.5 Place the tubes on a magnetic rack and discard the supernatant when clear and colorless.

  tRNAs are now attached to the beads!
- ☐ B2.6 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.
- ☐ B2.7 Repeat the washing step B2.5.
- ☐ B2.8 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.
- ☐ B2.9 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.
- ☐ B2.10 Place the tubes on a magnetic rack until the solution is completely clear. **tRNAs are now in the supernatant!**
- ☐ B2.11 Collect the 9 µL of supernatant and place into a clean 0.2 mL nuclease-free tube.

Step B3. BARCODED ADAPTERS ANNEALING

NOTE1: Each pair of barcoded adapters (FWD and REV) needs to be annealed following the passages below. For clarity, Table 3 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.

☐ **B3.1** 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 3. *Components' volumes to use in step B3.*

☐ **B3.2** 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 B4. BARCODED ADAPTERS LIGATION AND REVERSE TRANSCRIPTION

☐ B4.1 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 B2.11	8.5
Annealed Barcoded Adapter* from step B3.2	1.5
RNase Inhibitor, Murine	0.5
T2 Enzyme (T2)	1.5
Total volume	**15**

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

Table 4. *Components' volumes to use in step B4.1. Please perform separate reactions for each sample/barcoded adapters you are processing.*

☐ B4.2 Incubate the reaction for 10 minutes at RT.

☐ B4.3 Meanwhile, prepare the reverse transcription master mix as follows. Please consider that the volumes in Table 5 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 5. *Components' volumes to use in step B4.3. Volumes in this table are to be considered for one single sample and corresponding barcoded adapters.*

☐ B4.4 Add the master mix to the reaction tube containing the barcoded adapters-ligated tRNA from step B4.2. Mix by pipetting.

☐ B4.5 Add **0.5 µL of T3 enzyme (T3)** to the reaction and mix by pipetting.

☐ B4.6 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.

☐ B4.7 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.

☐ B4.8 Incubate at RT for 10 minutes.

☐ B4.9 Place the tubes on a magnetic rack and discard the supernatant when clear and colorless.

tRNAs are now attached to the beads!

☐ B4.10 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.

☐ B4.11 Repeat the washing step.

☐ B4.12 Remove supernatant and let the beads 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.**

☐ B4.13 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.

☐ B4.14 Place the tubes on a magnetic rack until the solution is completely clear. **tRNAs are now in the supernatant!**

☐ B4.15 Collect the 9 µL of supernatant and place it into a clean 1.5 mL reaction tube.

☐ B4.16 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 B5. RNA POOLING, RMX LIGATION AND SEQUENCING

☐ **B5.1** Pool in a 1.5 mL tube the barcoded samples from step **B4.16** 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 6 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 6. *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)

☐ **B5.2** 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 7. *Components' volumes to use in step B5.3. Volumes in this table are to be considered for one single sample. For pooled RNA volumes greater than 23 µL, see Appendix 1.*

☐ **B5.3** Mix by pipetting and incubate the reaction at RT for 10 minutes.

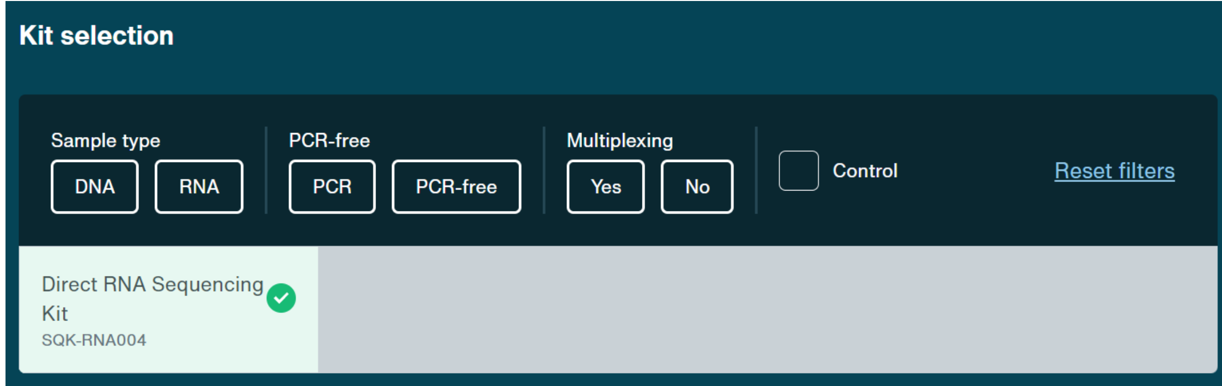
- ☐ B5.4 Let the **Agencourt RNA Clean XP beads** equilibrate at RT for 30 minutes, then resuspend by vortexing.
- ☐ B5.5 Add 80 μL of Agencourt RNA Clean XP beads to the reaction and mix by pipetting.
- ☐ B5.6 Incubate on a rotator mixer at RT for 5 minutes.
- ☐ B5.7 Spin the sample down and pellet on a magnet. Discard the supernatant when the solution is completely transparent.
- ☐ B5.8 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.
- ☐ B5.9 Spin the tube down and return it to the magnetic rack until the beads have pelleted. Remove completely any remaining Wash Buffer (WSB)*.
- ☐ B5.10 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.
- ☐ B5.11 Pellet the beads on the magnet until the supernatant is completely transparent.
- ☐ B5.12 Retain the 13 μL of eluate and place into a clean 1.5 mL tube.
- ☐ B5.13 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.

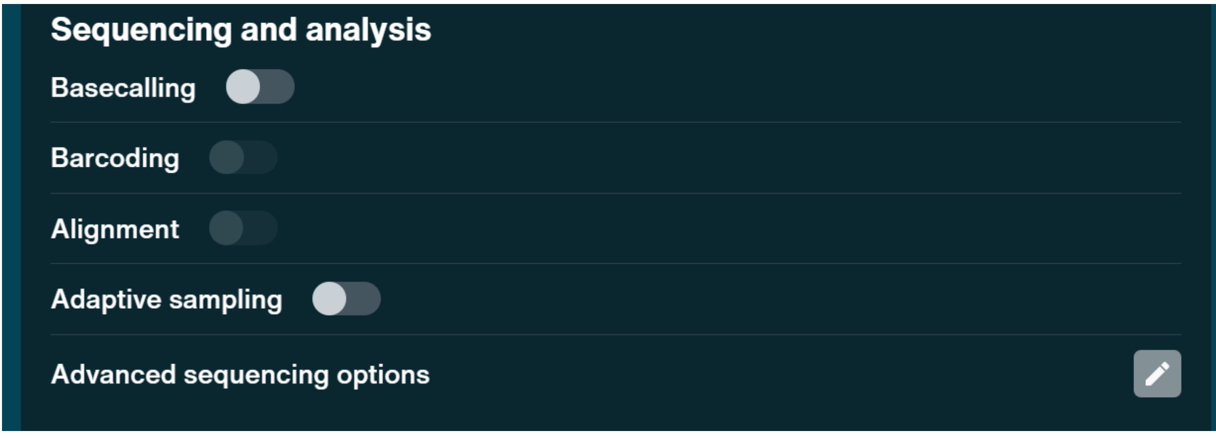
IMPORTANT: BEFORE STARTING THE SEQUENCING

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

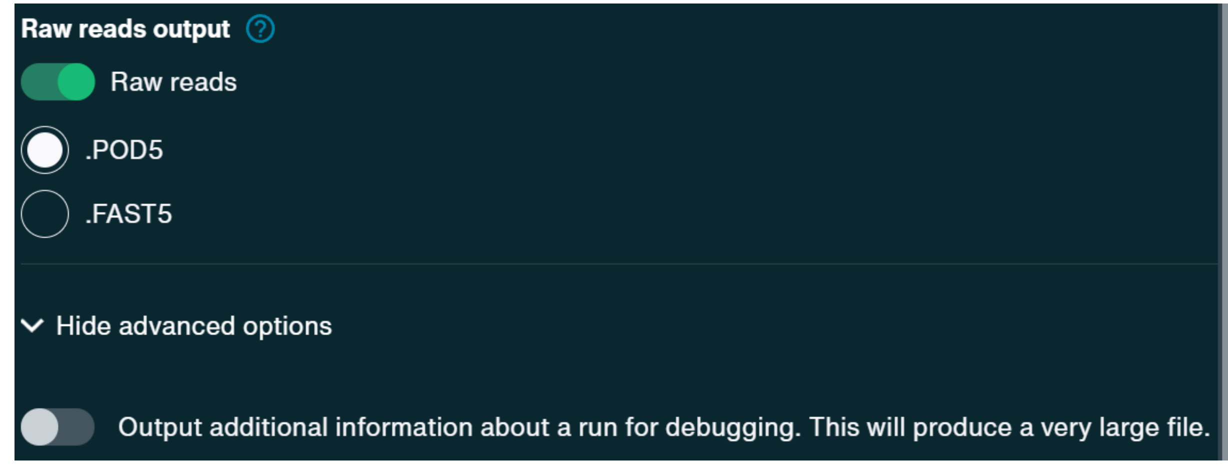
⚠ Select “Kit selection”: Direct RNA Sequencing Kit (SQK-RNA004).



⚠ 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 8 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 8. *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 Low output from Step B4 BARCODED ADAPTERS LIGATION AND REVERSE TRANSCRIPTION

If the total RNA amount of the barcoded samples from step B4.16 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.

In this case, adjust the volume accordingly in step B5.2, as shown in the example below:

☐ **B5.2b** 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**

☐ **B5.3b** Mix by pipetting and incubate the reaction at RT for 10 minutes.

☐ **B5.4b** Let the **Agencourt RNA Clean XP beads** equilibrate at RT for 30 minutes, then resuspend by vortexing.

☐ **B5.5b** Add 123 µL of Agencourt RNA Clean XP beads to the reaction and mix by pipetting.

☐ **B5.6b** Incubate on a rotator mixer at RT for 5 minutes.

☐ **B5.7b** Spin the sample down and pellet on a magnet. Discard the supernatant when the solution is completely transparent.

☐ **B5.8b** 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.

☐ **B5.9b** Spin the tube down and return it to the magnetic rack until the beads have pelleted. Remove completely any remaining Wash Buffer (WSB)*.

☐ **B5.10b** 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.

☐ **B5.11b** Pellet the beads on the magnet until the supernatant is completely transparent.

☐ **B5.12b** Retain the 13 µL of eluate and place into a clean 1.5 mL tube.

☐ **B5.13b** 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: