# nano-tRNAseq Kit

**Our tRNA Sequencing Solution:** the perfect blend of physical and digital methodologies with our nano-tRNAseq kit, a complete solution designed for streamlined tRNA multiplexed sequencing.

| Product | Catalog no | Rxns. |
|---|---|---|
| nano-tRNAseq Kit with demultiplexing | NTRSQ-12 | 12 |

Shipping: Dry ice

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: nano-tRNAseq enables single molecule sequencing of native full-length tRNA using the Oxford Nanopore platform.

(i) quantification of tRNA abundances,

(ii) infer on tRNA modifications sites and

(iii) detect modification “circuits”.

Updated for RNA004 kit/RNA flow cell chemistry (2024).

Suitable for: Eukaryotic/prokaryotic cells and tissues with annotated genome.

**What’s Included:**

1. **Library Prep Kit** — Comprehensive tools for preparing your tRNA library ready for sequencing with Oxford Nanopore reagents.
2. **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 |
|---|---|---|
| -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)
- Agencourt AMPure XP Beads for DNA Cleanup (Beckman Coulter, cat. no. A63881)
- 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 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 Lab 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 6 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 data analysis (5), information on tRNA abundance, coverage and post-transcriptional chemical modifications are obtained (Figure 1).

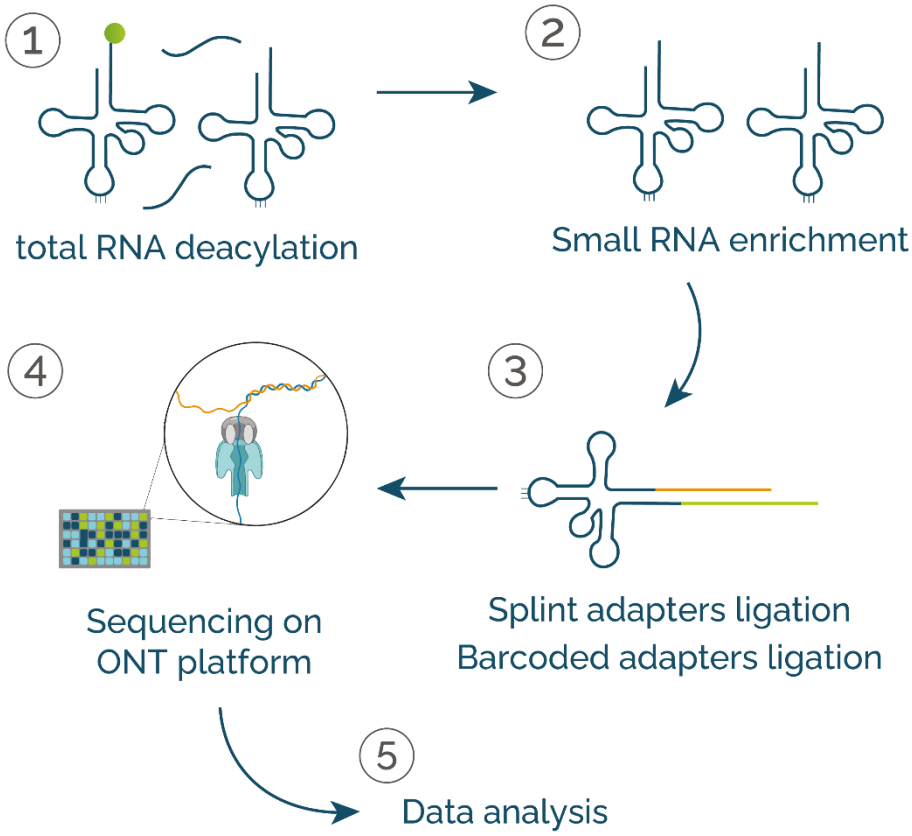



**Figure 1.** *Simple overview of the nano-tRNAseq protocol.*

# PROTOCOL WORKFLOW

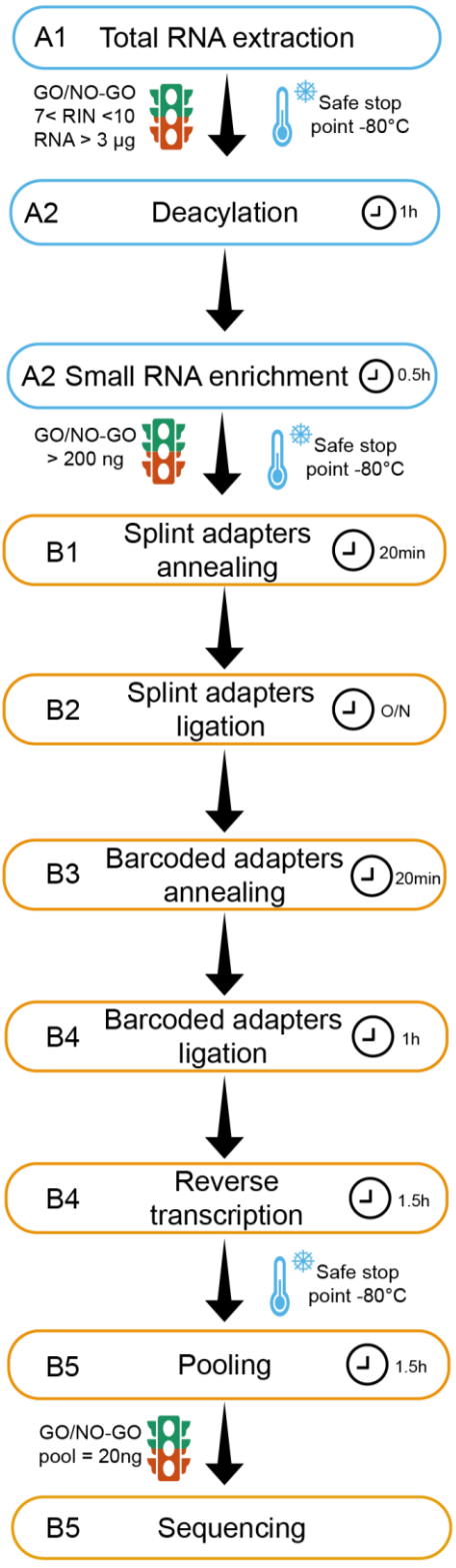



**Figure 2. Overview of the nano-tRNAseq Kit workflow.** *In each box, steps are present in the left corner, while the time to complete the step is listed in the right corner (clock picture). Safe stopping points (thermometer pictures) and GO/NO-GO conditions (traffic lights) are placed right at the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

## Optimal Workflow Recommendations

- ⚠ The nano-tRNAseq Kit has been optimized for the multiplexing of up to 6 samples per library.
- The multiplexing protocol allows you to process a total of 12 samples by generating **pooled libraries composed of up to 6 samples** each. If you wish, you can multiplex less samples per reaction. In case you want to multiplex less than 6 samples, we suggest you to use combinations of the barcoded adapters in the following order BC1 > BC2 > BC3 > BC4 > BC5 > BC6.
- 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 to move forward with library preparation.
- Please be aware that the “Splinter Adaptor Ligation” reaction (step B2) can be performed starting from 200 to 500 ng of input.
- The final library pool must comprise exactly 20 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 1.5M raw reads are needed to perform data analysis when multiplexing 6 samples.

** 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 | Volume | Storage | Type | Vial cap color |
|---|---|---|---|---|---|
| A3 | Deacylation Buffer (DB) | 1300 µL | -20°C | Vial | clear |

| Step N | Additional Material | Type |
|---|---|---|
| A1 | Agilent RNA 6000 Nano Kit | Optional |
| A2/A3 | RNA Clean & Concentrator™-5 | Required |
| A1/2/3 | Nuclease free water (NFW) | Required |
| A1/2/3 | Ethanol | Required |
| A2 | Agilent Small RNA Kit | Optional |
| 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** To 10 µL of extracted RNA, add 90 µL of **deacylation buffer (DB)** so that the total volume is 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 2 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 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 A (SA) | IBT0601 | 50 µL | -80°C | strip | |
| D1 | Splint adapter B (SB) | IBT0601 | 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 BC1 FWD (1 F) | IBT0601 | 25 µL | -80°C | strip | |
| D3 | Barcoded Adapter BC1 REV (1 R) | IBT0601 | 25 µL | -80°C | strip | |
| D3 | Barcoded Adapter BC2 FWD (2 F) | IBT0601 | 25 µL | -80°C | strip | |
| D3 | Barcoded Adapter BC2 REV (2 R) | IBT0601 | 25 µL | -80°C | strip | |
| D3 | Barcoded Adapter BC3 FWD (3 F) | IBT0601 | 25 µL | -80°C | strip | |
| D3 | Barcoded Adapter BC3 REV (3 R) | IBT0601 | 25 µL | -80°C | strip | |
| D3 | Barcoded Adapter BC4 FWD (4 F) | IBT0601 | 25 µL | -80°C | strip | |
| D3 | Barcoded Adapter BC4 REV (4 R) | IBT0601 | 25 µL | -80°C | strip | |
| D3 | Barcoded Adapter BC5 FWD (5 F) | IBT0601 | 25 µL | -80°C | strip | |
| D3 | Barcoded Adapter BC5 REV (5 R) | IBT0601 | 25 µL | -80°C | strip | |
| D3 | Barcoded Adapter BC6 FWD (6 F) | IBT0601 | 25 µL | -80°C | strip | |
| D3 | Barcoded Adapter BC6 REV (6 R) | IBT0601 | 25 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 |
|---|---|---|
| B1/B2/B3/B4 | RNase Inhibitor, Murine | Required |
| B2/B4 | Agencourt AMPure XP | 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 |
| B5 | Agencourt RNA Clean XP for RNA Purification | 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 5 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 1.5 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 A (SA) | 3.75 |
| Splint Adapter B (SB) | 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 maximum **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** | **1.52** | **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 |
| $H_2O$ | 8.48 - 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 AMPure 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.

☐ **B2.8** 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

**NOTE:** 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 **1** F and **1** R. Please, repeat the reaction for every pair of barcodes needed (up to 6 per library preparation).

☐ **B3.1** Mix the following reagents in a 1.5 mL reaction tube:

| Reagent | Volume (µL) |
|---|---|
| Annealing Buffer 1 (AB1) | 1 |
| Annealing Buffer 2 (AB2) | 1 |
| Barcoded Adapter BC1 FWD (1 F) | 1.5 |
| Barcoded Adapter BC1 REV (1 R) | 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 AMPure 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 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.**
- ☐ **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 8.5 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

**NOTE: At this step it is possible to decide how many samples to pool together as long as 20 ng of total material is reached (for troubleshooting see Appendix 1).**

☐ **B5.1** Pool in a 1.5 mL tube the barcoded samples from step **B4.16** so that the total RNA amount is 20 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 | Quantity (ng) | Volume (µL) |
|---|---|---|
| BC1 | 3.3 | |
| BC2 | 3.3 | |
| BC3 | 3.3 | |
| BC4 | 3.3 | |
| BC5 | 3.3 | |
| BC6 | 3.3 | |
| NFW | if needed | |
| **Total** | **20** | **23** |

**Table 6.** *Calculate the volume corresponding to 3.3 ng of each barcoded sample. This volume needs to be added in the final pool of 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.*

☐ **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.

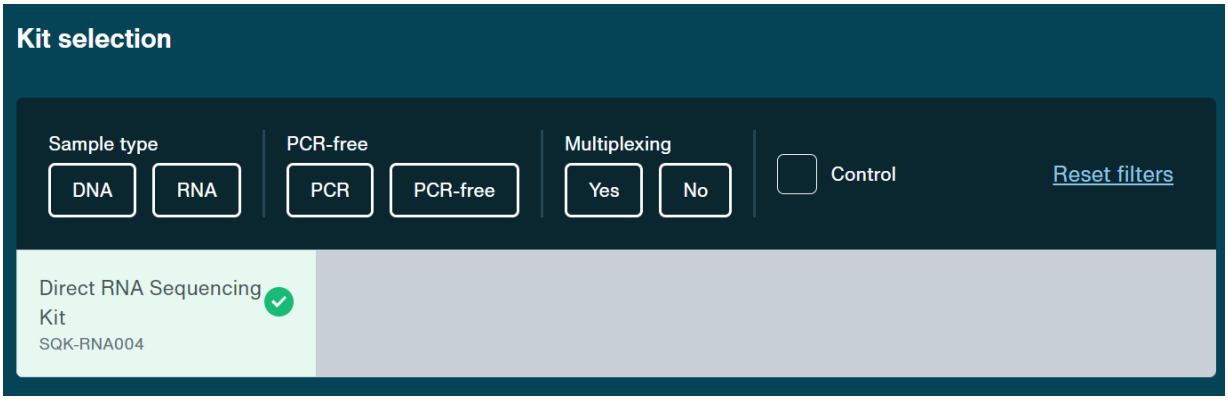
- ☐ **B5.6** Incubate on a rotator mixer at RT for 5 minutes.
- ☐ **B5.7** Spin the sample down and pellet on a magnet. Discard the supernatant when the solution is completely transparent.
- ☐ **B5.8** Add 150 µL of Wash Buffer (WSB)* to the beads. Close the cap and resuspend by flicking the tube. Return 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 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.**

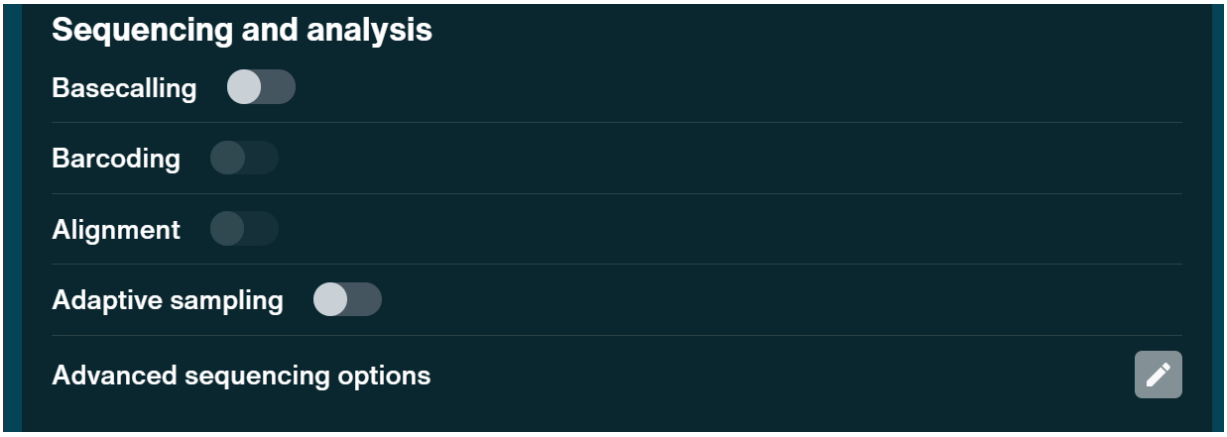
## ⚠ IMPORTANT: BEFORE STARTING THE SEQUENCING

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

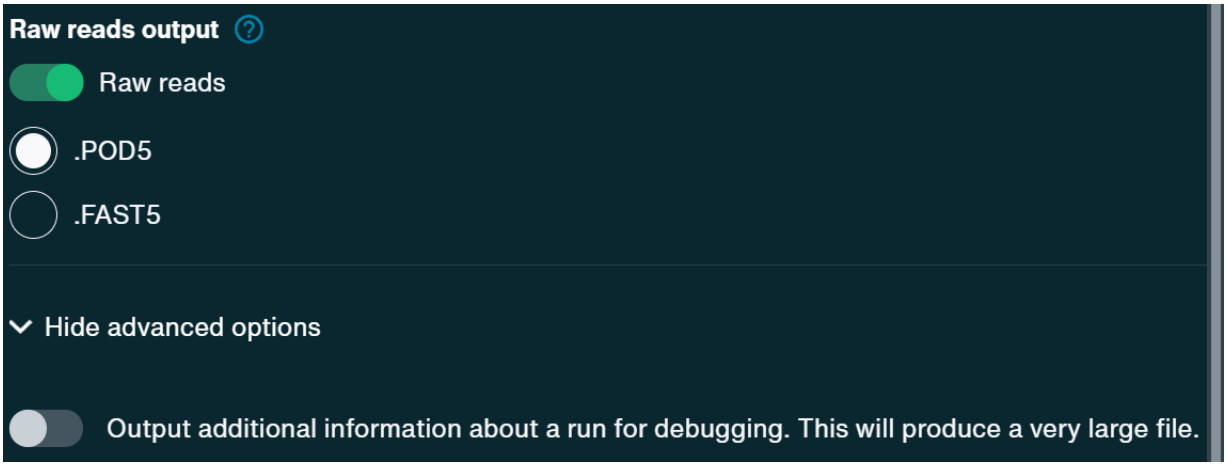

⚠ Select “Kit selection”: Direct RNA Sequencing Kit (SQK-RNA004).




⚠ 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.**

# APPENDIX

## App.1 Low output from Step B4 BARCODED ADAPTERS LIGATION AND REVERSE TRANSCRIPTION

If the total RNA amount of the sum of the barcoded samples from step **B4.16** that are intended to be pooled together is not 20 ng in 23 µL it is advisable to start again with the procedure. However, if you do not wish to start the procedure again, you could pool together all the material produced (e.g. 6 µL for each sample). In this scenario, the pool will not be balanced, as differences in the sample amount can be encountered. Moreover, the volume of the pooled libraries will result in more than 23 µL (e.g. 36 µL maximum if 6 samples are pooled together with 6 µL each). Note that changing the volume might affect the sequencing throughput and the outcome cannot be guaranteed.

The usage is to add more volume in step B5.2 as 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 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: