Adhere to the following instructions for successful completion of your project. This document will provide guidelines on RNA sample handling and quality requirements for Oxford Nanopore Technologies (ONT) sequencing at CNAG.

Contents

STEPS:

1. Project/Subproject Creation – by CNAG Project Management (experimental design, sequencing protocol, number of samples, analysis, invoicing matters, prioritization, deadlines).

2. Barcoded Tubes Shipment – by CNAG Biorepository (shipment logistics, support in selecting RNA extraction methods).

3. Samples Shipment to CNAG - by the Collaborator.

4. Samples Reception – by CNAG Biorepository.

5. Sample Quality Control and Report by CNAG Biorepository (QC report, replacement issues).

6. Samples Selection Approval – by the Collaborator.

7. Library Preparation and Sequencing – by CNAG Long-Read Sequencing Team.

8. Data QC and Transfer – by CNAG Production Bioinformatics Team.

1. General considerations

- After your project has been reviewed and approved by CNAG Project Management and Long-Read Sequencing Team, CNAG Biorepository will contact you to provide the materials and a URL link to the submission site for sample data collection.
- Refer to INS-110 for specific information about sample submission and shipment to CNAG.
- Use only the sample shipment materials provided by CNAG (e.g., tubes and racks). Protective outer packaging (e.g., Styrofoam boxes or other wrapping materials) is not provided by CNAG and should be supplied by the sender if needed.
- Questions related to RNA sample requirements and shipment details should be directed to the CNAG Biorepository (Lídia Agueda, Biorepository Laboratory Manager, lidia.agueda@cnag.eu or Ana González, ana.gonzalez@cnag.eu) or to the Long-Read Sequencing Manager Laura Aguilera (laura.aguilera@cnag.eu). Other questions regarding experimental design, quotations, sample number change, etc. should be addressed to CNAG Project Management team (projectmanager@cnag.eu).

2. Guidelines for RNA extraction and handling

For most of the sample types we recommend using TRIzol based extraction protocols or RNeasy kits (Qiagen).

In general, the following precautions need to be taken when handling RNA:

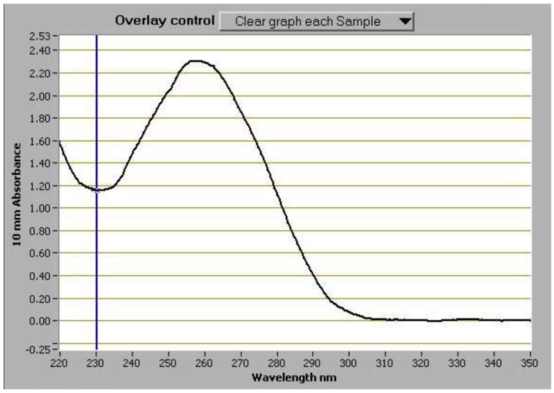
- Immediately snap-freeze your specimen in liquid nitrogen and then store at -80°C to avoid the action of endogenous, intracellular RNases. It is also possible to proceed immediately to RNA extraction.
- Thoroughly homogenize samples. If you use frozen samples, never let them thaw before homogenization. The freeze-thaw process can disrupt cellular compartments where RNases are sequestered, giving them access to the RNA. Ideally, the sample should be ground while still frozen and then placed into lysis buffer.
- Reduce exposure to environmental RNases. Make sure you work in an RNase free environment, if possible, create an RNA-only handling space.
- Include a DNase treatment.
- Avoid over-drying of RNA. Allow the RNA to air dry. Do not heat when drying in a speed-vac.
- Always store your RNA at -80°C and minimize freeze-thaw cycles. Each cycle causes RNA fragmentation.
- High concentrations of EDTA must be removed prior to library preparation. A low-TE buffer is suitable for ONT sample preparation (0.1mM EDTA).

3. RNA sample quality assessment

The total RNA or polyA-enriched RNA quality check is required prior to submitting RNA. The following recommendations can be used to assess RNA integrity, purity, and concentration:

1) RNA integrity
   - For accurate fragment size distribution and integrity assessment, automated capillary electrophoresis systems are recommended, such as TapeStation systems (Agilent) using RNA Analysis assays.
   - We recommend that the total RNA has a RIN value between 9 and 10.
   - After the polyA RNA enrichment the electrophoretic profile should demonstrate that the majority of RNA fragments are >500 nt.

2) RNA purity
   - The purity of RNA may be assessed using a Nanodrop spectrophotometer (for samples with concentration >20 ng/µl).
   - Chemical impurities such as detergents, denaturants, chelating agents and high concentrations of salts should be avoided as these may affect the efficiency of enzymatic steps. Other contaminants such as DNA, proteins and dyes may also reduce the efficiency of steps in the library preparation.
   - The RNA sample should have 260/280 ~ 2.0 and 260/230 ~2.0-2.2.
     - A 260/280 which is lower than ~2.0 indicates the presence of DNA.
     - A 260/280 which is lower than ~1.8 can indicate the presence of protein or phenol.
     - If the 260/230 is significantly lower than 2.0-2.2, this indicates the presence of contaminants, and the RNA may need additional purification.



The absorbance spectrum of the RNA sample above indicates a high purity with close to ideal A260/280 and A260/230 ratios. Also note that the concentration is within the reliable range of the Nanodrop ND-1000.

3) RNA concentration
   - Always use Qubit® fluorometer together with Qubit RNA assays for quantification purposes.

4. Sample requirements

Required RNA input amount varies depending on the application. See below the required material in each case, multiply the amounts needed by the number of flow cells required.

- **direct RNA – 850 ng polyA RNA (50-100ng/ul) or 2.5ug of total RNA RIN 10 (150-200ng/ul) per flow cell.**
- **cDNA PCR - 50 ng polyA RNA (10-100ng/ul) or 1.5ug of total RNA RIN 10 (50-120ng/ul) per flow cell.**

Note: The RNA concentration is crucial since the input volume for library preparation is limited.

The best sequencing results and yields are obtained when starting with polyA RNA, but it is also possible to use total RNA as input material for the library preparation. Starting with total RNA, the yield will be approximately 50% lower compared to starting with polyA-enriched RNA.

Note: For the direct RNA protocol, when multiplexing several samples on one flow cell, it is only possible to start from polyA enriched RNA. The protocol has not been validated for total RNA as input material.

CNAG will not perform rRNA depletion or poly(A) selection unless this has been agreed upon in advance with the CNAG project manager, including prior confirmation that total RNA will be sent to CNAG for on site ribodepletion.The minimal recommended total RNA amounts for polyA selection is in general 15-40ug with the minimal concentration 120ng/ul for direct cDNA protocol or 30-100ug for one experiment with the direct RNA protocol. However, it is recommended to consider the transcriptional activity for the particular tissue in order to estimate correctly the representation of the transcribed mRNA.

Important: Both the cDNA and direct RNA sequencing protocols selectively sequence only polyadenylated RNA molecules. Consequently, prokaryotic or viral RNA samples that do not naturally contain poly(A) tails must be polyadenylated by the Collaborator before submission.

5. Shipment

- The RNA should be sent on dry ice, avoid shipments during local public holiday and weekend .
- It is important to ship an aliquot of the RNA elution buffer, if other than water, that will be used for nanodrop blank.
- Refer to INS-110 for specific information about sample submission and shipment to CNAG.