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

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STEPS:

1. . Project/subproject Creation – by CNAG Project Management (experimental design, sequencing protocol, number of samples, analysis (if required), invoicing matters, prioritization, deadlines…)
2. Barcoded Tubes shipment – by CNAG Biorepository (shipment logistics, Support in selecting DNA extraction methods, …)
3. Samples shipment to CNAG - by the Collaborator.
4. Samples reception by CNAG Biorepository.
5. Samples Quality Control and Report - by CNAG Biorepository (QC results, replacement issues…)
6. Samples selection approval – by the Collaborator.
7. Libraries 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 material provided by CNAG for sample shipment.
- Questions related to DNA 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@cnag.eu). Other questions regarding experimental design, quotations, change in number of samples, etc… should be addressed to CNAG Project Management team (projectmanager@cnag.eu).

2. Guidelines for handling High Molecular Weight DNA (HMW DNA)

The precautions need to be taken when handling DNA:

- Use wide bore pippette tips and avoid vortexing.
- If precipitating the DNA is needed through the extraction process, avoid overdrying. Allow the DNA to air dry.
- Avoid eluting in RNase-free H_2O or unbuffered solutions. DNA should be eluted and stored in a neutral, buffered solution such as low TE (10 mM Tris, pH 8, 0.1mM EDTA) or TE (10 mM Tris, pH 8, 1mM EDTA). High concentrations of EDTA must be removed (accepted up to 1mM EDTA).
- To help to resuspend the DNA, carefully invert the tube several times after adding buffer and/or tap the tube gently. Alternatively, allow the DNA to stand in buffer overnight at 25°C to resuspend.
- After DNA extraction, inactivate DNAse as recommended by the vendor kit if necessary. Avoid heat incativation when possible since overheating can introduce DNA damage. An alternative is AMPure beads purification.
- In case you are working with gel extracted DNA (i.e. when working with amplicons), it can result in lower sequencing performance due to the damage inherently caused by intercalating dyes such as ethidium bromide and exposure to UV radiation. Sequencing amplicons stained with SYBR dyes is untested, and therefore cannot be recommended. If working with a gel-extracted product that has been stained with a dye, it is recommended to bring it through additional rounds of amplification to remove damage and/or dyes prior to library prep and sequencing.
- DNA storage conditions: 4°C (short-term); –20°C / –80°C (long-term).
- The DNA should be sent on 4ºC.

To maximize read length and quality, it is essential that the DNA sample:

- is double-stranded; single-stranded DNA or single-strand nicked DNA cannot be used to generate the sequencing template.
- has not undergone multiple freeze-thaw cycles as they can lead to DNA damage.
- has not been exposed to high temperatures (e.g. > 65ºC for 1 hour) or pH extremes (< 6 or > 9).
- has an OD260/OD280 ratio of 1.8 to 1.9.
- has an OD260/OD230 ratio of 2.0-2.2.
- has an OD260/OD270 ratio between 1.2-1.3. (Important for the extractions from plants)
- does not contain insoluble material.
- does not contain RNA contamination.
- does not contain denaturants (e.g., guanidinium salts or phenol) or detergents (e.g., SDS or Triton-X100).
- does not contain carryover contamination from the original organism/tissue (e.g., heme, humic acid, polyphenols, etc.)

3. HMW DNA sample quality assessment

A thorough DNA quality check is required prior to submitting DNA. The following recommendations to ascertain DNA integrity, purity, and concentration are recommended:

1) HMW gDNA sample integrity:

gDNA integrity can be initially assessed by agarose gel electrophoresis; however, this method provides limited resolution for HMW gDNA. For accurate fragment size distribution and integrity assessment, automated capillary electrophoresis systems are recommended. The preferred method is the Femto Pulse System (Agilent), which allows high-resolution analysis of HMW DNA. Alternatively, the Fragment Analyzer or TapeStation systems (Agilent) together with genomic DNA assays can be used.

For high-quality gDNA, the expected fragment size is >48 kb.

To achieve the best results, DNA samples should not show any signs of degradation, which can be observed through smeared DNA or even bands. When using lower resolution assays, the presence of one predominant band, showing high-molecular-weight DNA with no degradation, is optimal. When running agarose gels, it is good practice to include relevant marker sizes and indicate the amount of sample and ladder loaded.

2) Purity of HMW gDNA sample:

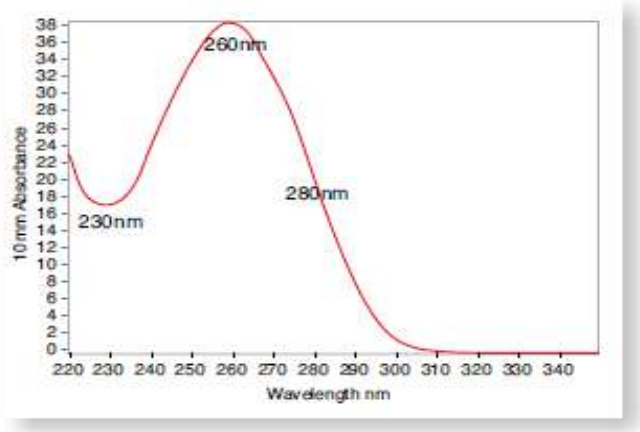
DNA purity should be determined by NanoDrop® instrument or equivalent. Readings of both A260/A280, A260/A230 need to be obtained:

260/280: The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~**1.8** is generally accepted as “pure” for DNA, but is dependent on the nucleotide composition of the submitted sample.

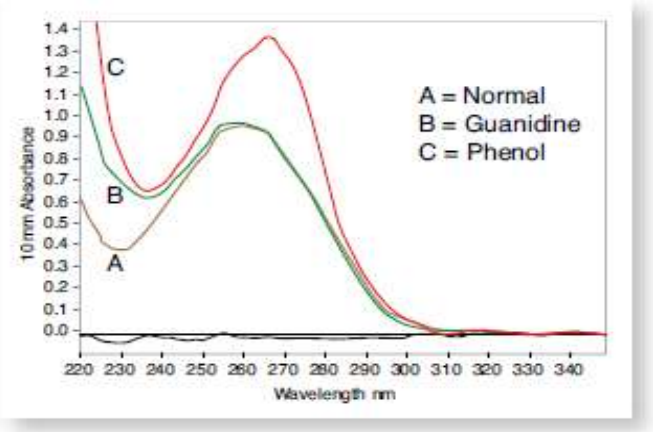
260/230: The 260/230 ratio provides a secondary measurement of DNA purity to make inferences about the quality of sample extraction. Readings to determine purity are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of **2.0-2.2**.

In addition to evaluating the Nanodrop absorbance ratios, it is critical that the gDNA concentration measured by Nanodrop does not exceed the concentration measured by fluorometric based methods by more than 150% (ratio Nanodrop concentration/fluorescence concentration ≤1.5). This ratio indicates that the majority of nucleic acids present are double-stranded and that significant amounts of other 260 nm–absorbing contaminants (e.g., RNA) are not present. If the NanoDrop/fluorescence ratio is greater than 1.5, additional purification steps should be performed before proceeding.

It is useful to evaluate the Nanodrop absorption spectra from 220 to 350 nm that reveals the presence of contaminants.



Typical nucleic acid sample spectrum



Comparison of nucleic acid sample spectra with and without 2 common contaminants

Thermo Scientific NanoDrop Spectrophotometers, Rev 11/2010

Note: It is necessary to ship an aliquot of the DNA elution buffer, that will be used for nanodrop blank.

3) Concentration of HMW gDNA sample:

It is highly recommended to use a fluorescence-based assay such as the PicoGreen® assay or a Qubit® fluorimeter for quantitation purposes. Traditional spectrophotometric assays cannot determine DNA concentrations <5 ng/µl. More importantly, almost all spectrophotometric assays do not distinguish between different types of nucleotides (e.g., double stranded DNA, RNA, dNTPs, and single-stranded DNA).

Please closely follow the recommended guidelines provided by the respective vendors when carrying out quantitation assays. Perform replicate readings of concentration and use the average of replicates as the final concentration whenever possible.

4. Sample requirements

Required DNA amount varies depending on the application. See below the required material in each case:

- Human gDNA for WGS sequencing (if shearing of the DNA to 15-20kb is acceptable for the analysis): **6-10ug** total DNA, concentration range 150-300ng/ul per flow cell.
- WGS for de novo assemblies, especially for difficult-to-sequence species: **16ug** total DNA, concentration range 150-300ng/ul per flow cell.
- If multiplexing several samples in one flow cell, the total DNA amount must be at least 6-10ug for human gDNA or 16ug for difficult-to-sequence species. However, requirements will be discussed case by case.
- DNA can be dissolved in Tris buffer (e.g., 10 mM Tris, pH 7.0 – pH 8.0). Do not use nuclease-free water as this is insufficient for long-term DNA stabilization. For long-term storage of HMW gDNA we recommend the use of TE buffer or low TE buffer. High concentrations of EDTA must be removed prior to library preparation as this is not compatible with some enzymatic reactions (Mg++ dependent). EDTA concentration accepted up to 1mM.

Note: Input requirements also depend on DNA quality, particularly fragment size distribution. High molecular weight DNA is strongly recommended, as degraded or shorter fragments may require adjusted input amounts. DNA purity is also critical, as contaminants (e.g., proteins, salts, or residual solvents) may interfere with library preparation and sequencing performance.

The following table summarizes the minimal sample requirements for HMW DNA Oxford Nanopore sequencing:

	Quality requirements
HMW DNA input	6-16 ug per flow cell (quantified by fluoresecence-based method)
Fragment size distribution	Most of the DNA is ≥48 kb
Nanodrop ratios	OD260/OD280 = 1.8-1.9. OD260/OD230 = 2.0-2.2. Nanodrop concentration/fluorescence concentration ≤1.5
Buffers accepted	Tris buffer (e.g., 10 mM Tris, pH 7.0 – pH 8.0) Low TE buffer (10mM Tris, 0.1mM EDTA)