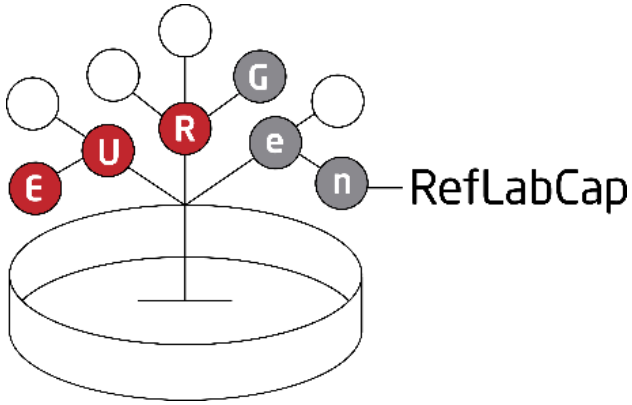
EURGen-RefLabCap

Best practice workshop – Nanopore sequencing

Second day

Wednesday, 6 December 2023

09:00 – 17:00 CET



Funded by the European Union

Previously…

First day (virtual) – Thursday 30 November 2023, 9:00 - 12:30 CET

- Biochemical principles of Oxford Nanopore Technologies
- Comparison between ONT and Illumina sequencing
- State-of-the-art – literature on ONT sequencing applied to microbiology research and public health surveillance
- DNA extraction and library preparation for ONT sequencing
- Troubleshooting ONT runs
- Introduction of bioinformatics analyses of ONT data
- Explanation regarding purchase of Nanopore sequencer and reagents

Funded by the European Union

Agenda for today

Second day (physical) – Wednesday 6 December 2023, 9:00 - 17:00 CET

9:00 – 9:10: Introduction and agenda for the day (Ana Rita Rebelo, DTU)

9:10 – 10:00: Overview of the DNA extraction protocol and preparation for lab work (Ana Rita Rebelo, DTU)

10:00 – 14:00: Laboratory work – DNA extraction (including *ad hoc* lunch)

14:00 – 15:30: Laboratory work – DNA quality control with Qubit

15:30 – 16:00: Coffee break

16:00 – 16:30: Other options for DNA quality control (Niamh Lacy-Roberts and Jette Sejer Kjeldgaard, DTU)

16:30 – 17:00: Q&A, wrap-up (Ana Rita Rebelo, DTU)

17:00: Bus to the restaurant

Funded by the European Union

Ana Rita Rebelo
anrire@food.dtu.dk

Overview of the DNA extraction protocol

Funded by the European Union

DNA extraction

We will use protocol:


INSTRUCTION MANUAL

NEW ENGLAND BioLabs Inc.

be INSPIRED drive DISCOVERY stay GENUINE

Monarch® HMW DNA Extraction Kit for Tissue

NEB #T3060S/L

5/50 preps

Version 2.1_4/21

Funded by the European Union

DNA extraction

The full protocol has 33 pages. What's important for us:

Table of Contents

Funded by the European Union

DNA extraction

And then pages 25-33:

- Homogenization of HMW DNA
- Measuring & Analyzing HMW DNA
- Troubleshooting

Funded by the European Union

DNA extraction

We will not use the big protocol.

We prepared a 6-page shortened protocol with the most important information.

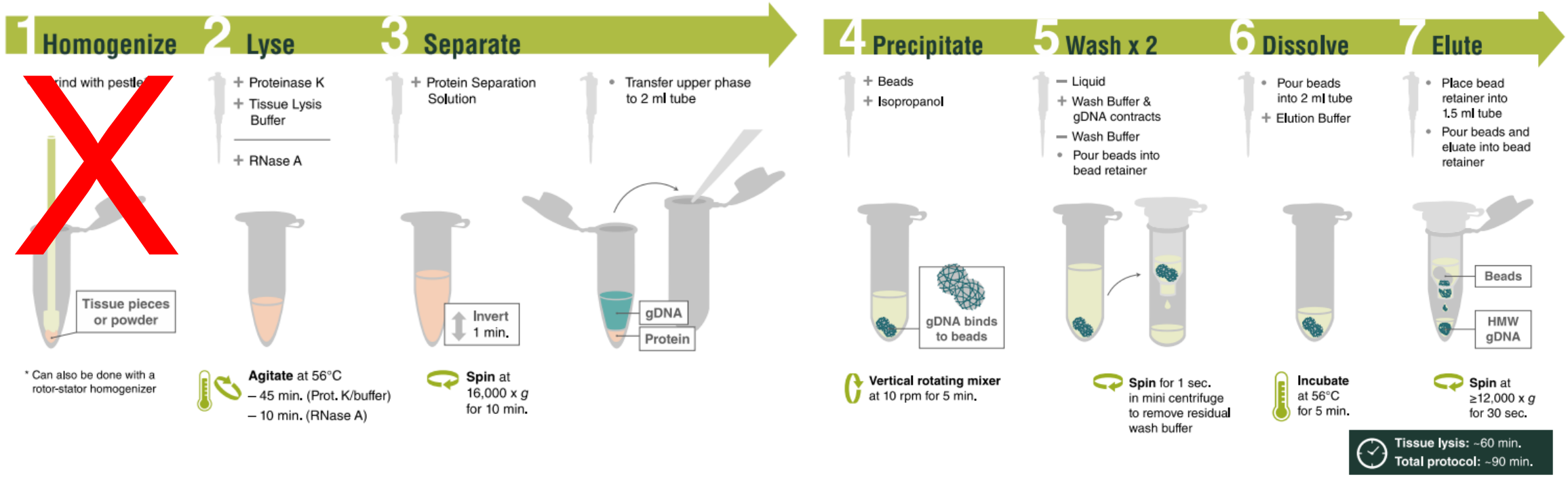
This short protocol has been optimized at DTU:

- input material and amount (e.g. bacterial colonies from agar plates instead of ON liquid culture)
- volumes of some reagents (e.g. lysozyme)
- agitation times and speeds
- centrifugation times and temperatures

Funded by the European Union

DNA extraction

Figure 2: Workflow for Tissue Samples



Funded by the European Union

DNA extraction

https://www.youtube.com/watch?v=B4x4oQBrscU&t=214s

Funded by the European Union

DNA extraction

Very important:

- manual inversions need to be gentle

- it's probable that you will need to centrifuge longer for phase separation

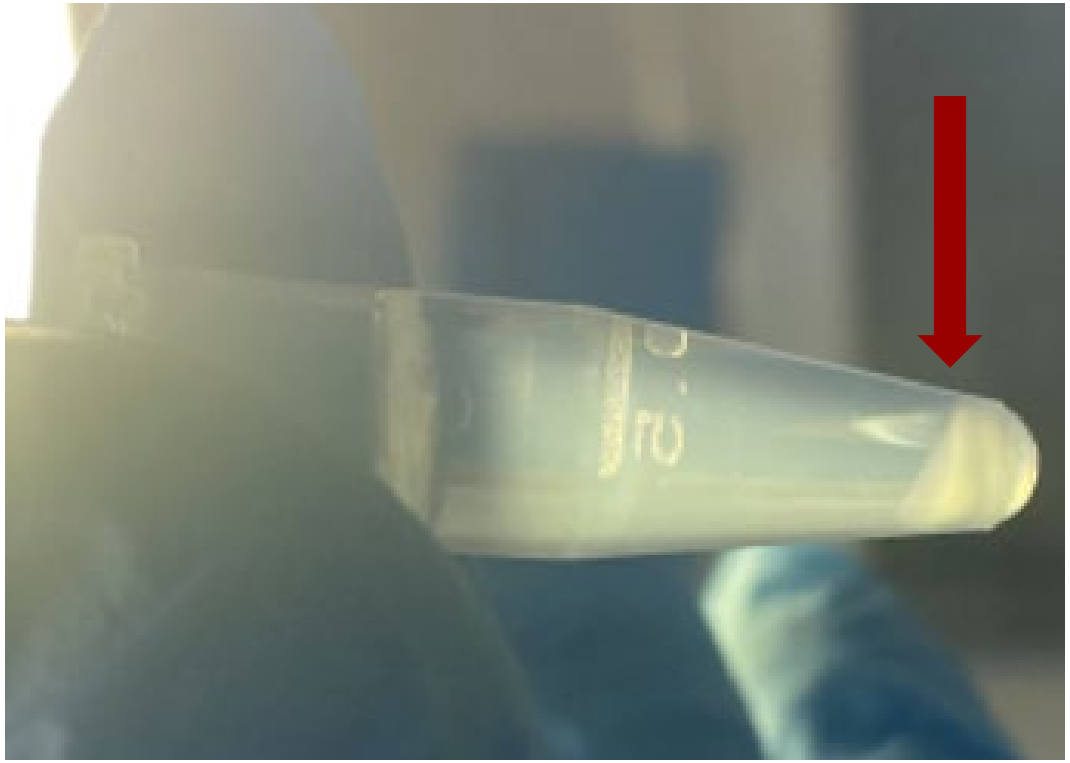
- inversions after adding the isopropanol needs to be **extra gentle – 5-6 seconds** per inversion

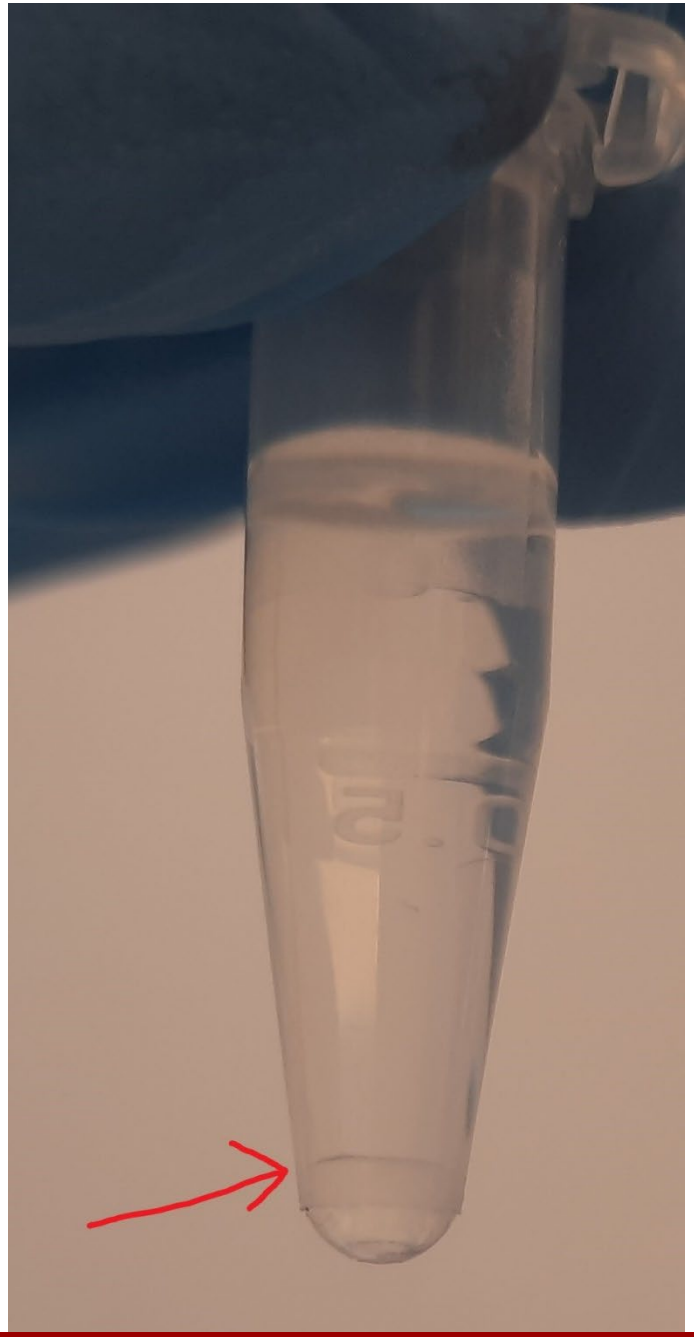
- If you're unsure about what do discard (beads? eluate?) - ask

Funded by the European Union

DNA extraction

How the phase separation will look:

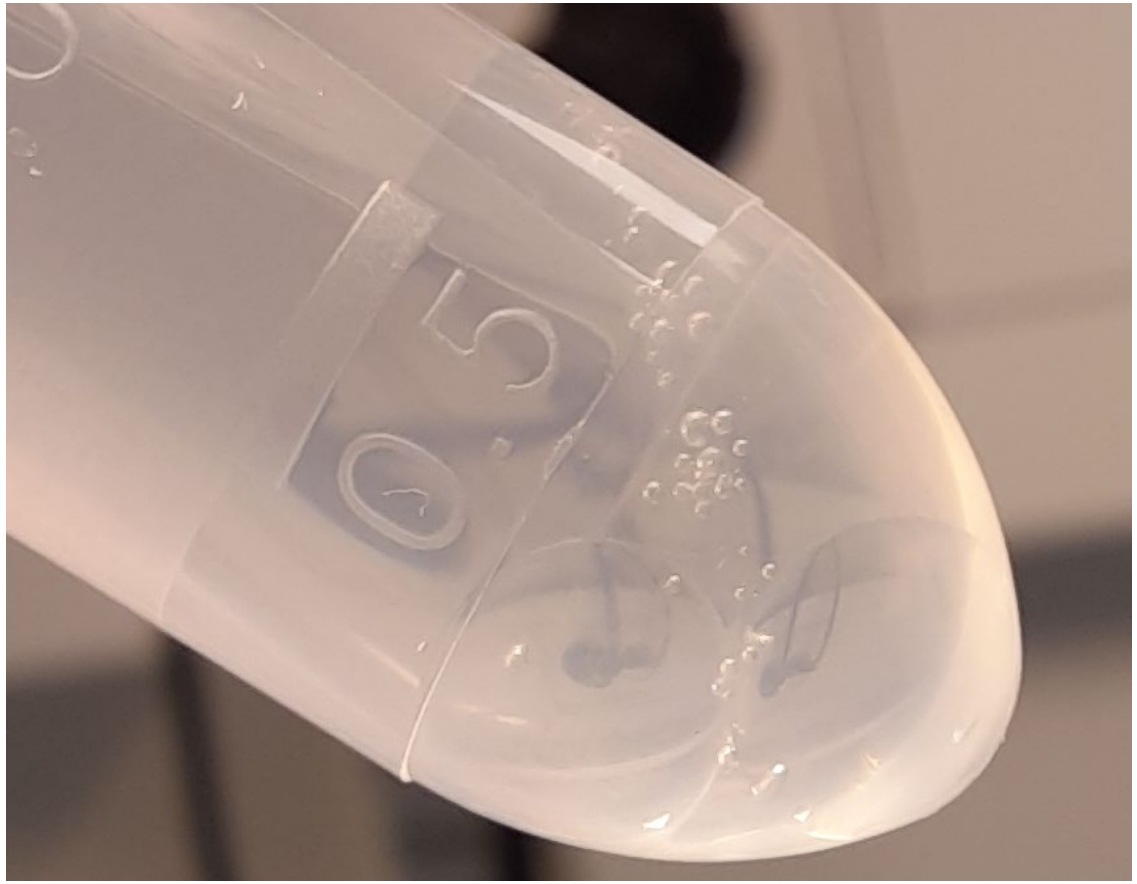




Funded by the European Union

DNA extraction

How the DNA will look wrapped around the beads:



Funded by the European Union

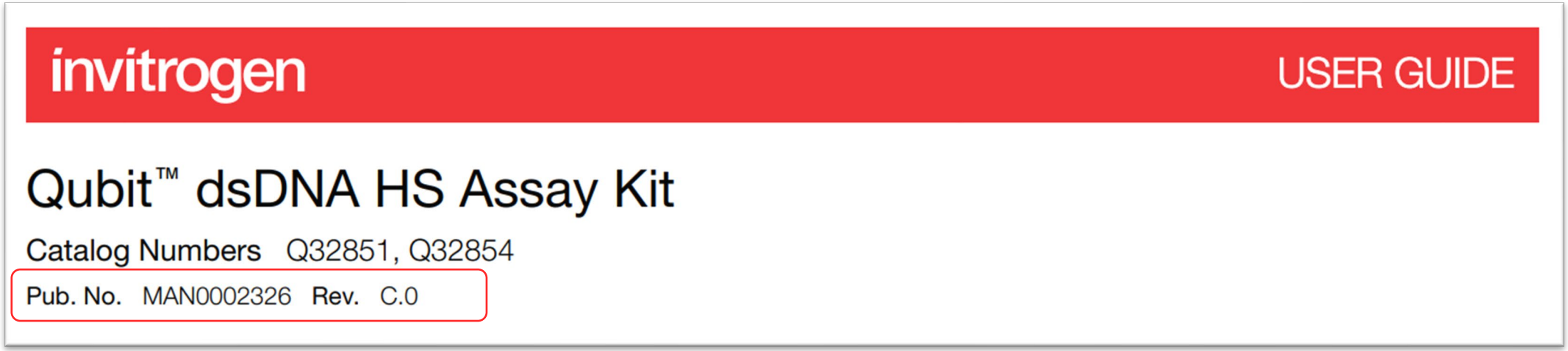
Ana Rita Rebelo
anrire@food.dtu.dk

Overview of DNA quality control with Qubit

Funded by the European Union

DNA QC

We will use protocol:


invitrogen

USER GUIDE

Qubit™ dsDNA HS Assay Kit

Catalog Numbers Q32851, Q32854

Pub. No. MAN0002326 Rev. C.0

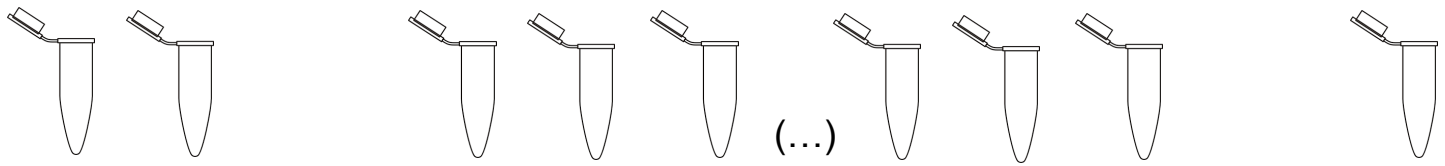
We will not use the big protocol (8 pages).

We prepared a 2-page shortened protocol with the most important information.

Funded by the European Union

DNA QC

PREPARING THE WORKING SOLUTION:



2 standards (always)

All your DNA samples

One extra (for pipetting losses)

N assays

Volume of reagent to use = ***N*** µL

Volume of buffer to use = ***N*** x 199 µL

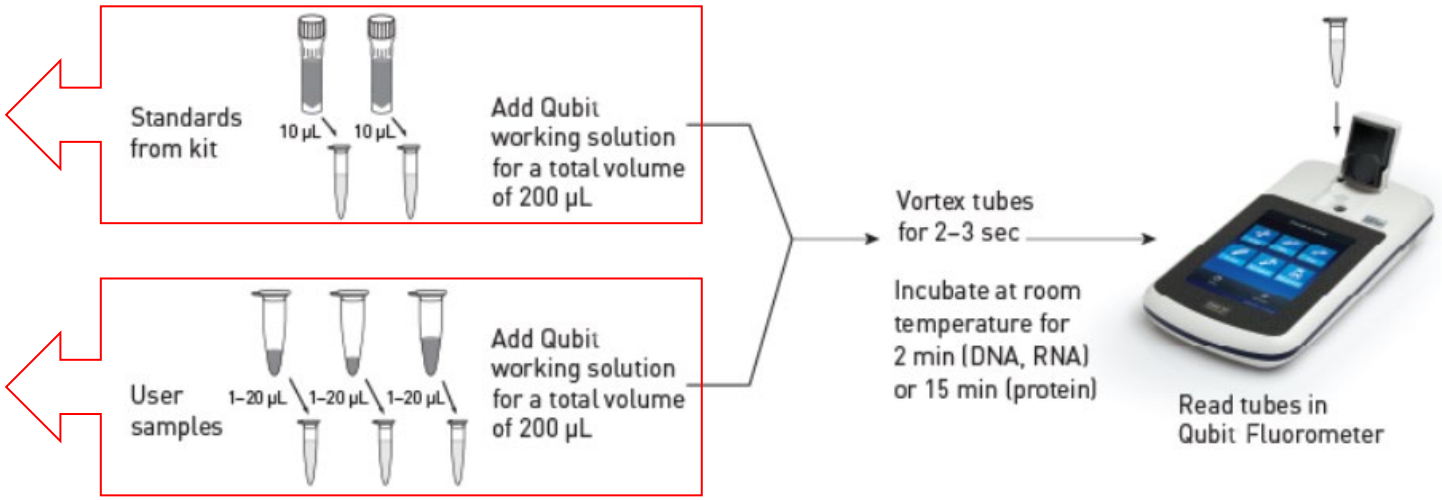
Funded by the European Union

DNA QC

DISTRIBUTING WORKING SOLUTION AND DNA:

190 µL of solution
+ 10 µL of DNA

198 µL of solution
+ 2 µL of DNA



Or other combinations for a total of 200 µL
(DNA sample must be between 1-20 µL)

GOAL:
Min 10 ng/µL
Max 100 ng/µL

Funded by the European Union

Niamh Lacy-Roberts and Jette Sejer Kjeldgaard

nlac@food.dtu.dk
jetk@food.dtu.dk

DNA Quality Control

Funded by the European Union

Which quality control (QC) is performed for sequencing(!?)

DNA quantity

DNA integrity

DNA purity

For WGS/NGS we have two check points:

1. DNA Quality Control (DNA QC)
2. Library Quality Control (Library QC)

Funded by the European Union

Why is it important to quality control DNA?

- It is important that you check your input DNA for quality before and during library preparation.
- Low molecular weight, incorrectly quantified and/or contaminated DNA can have a significant impact on downstream processes and your sequencing runs.
- 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 single stranded DNA, RNA, proteins, dyes and beads may also reduce the efficiency of steps in the library preparation.

Why is it important to quality control DNA?

- It’s important that the nanopores are kept filled with DNA to minimize the time they are idle between strands – for maximum sequencing yield.

- The less DNA goes into the flow cell, the less DNA is captured by the pores. This means the pores will be searching for DNA for longer, and if the pores are not always sequencing throughput may be compromised. Shorter fragments can also have a similar effect.

- Therefore, you can measure:
  - Mass (concentration in ng/ul)
  - Quality (purity)
  - Fragment Length

- ONT recommends the Qubit fluorometer to determine the mass of the sample, for quality use nanodrop and for fragment size you could use: gels (e.g. Bio-Rad CHEF system), Agilent devices (e.g. Bioanalyzer or Tapestation), or FEMTO Pulse from AATI.

Measuring concentration with Qubit

- Quantifies 1 µl DNA per sample
- Broad range (0.01 – 5 µg/mL) and High sensitivity dsDNA kits (1 – 500 ng/mL)
- Should be accurate in the presence of RNA, salts, solvents, proteins, and free nucleotides

Image at http://vitechltd.vn/en/qubit-4-fluorometer-with-wifi.html

Measuring quality with Nanodrop

- Quantifies 1 µl DNA per sample
- Also measures concentration (but always refer to qubit for concentration over nanodrop)
- ONT recommends that sample DNA has a 260/280 ~ 1.80 and a 260/230 ~ 2.0-2.2.
- A 260/280 which is higher than ~1.8 indicates the presence of RNA.
- A 260/280 which is lower than ~1.8 can indicate the presence of protein or phenol.
- A 260/230 significantly lower than 2.0-2.2 indicates the presence of contaminants, and the DNA may need additional purification.

Image at https://www.fishersci.com/shop/products/nanodrop-one-spectrophotometer-wifi-qubit-4-fluorometer/13400525

Measuring fragment length

- Nanopore sequencing devices generate reads that reflect the lengths of the fragments loaded into the flow cell.

- To have control over the size of the fragments generated in the library prep, it is important to begin with high molecular weight (HMW) DNA.

- The shearing of HMW DNA can be minimised by:
  - Using wide-bore pipette tips to handle the gDNA
  - Mixing gently but thoroughly by flicking the tube, as opposed to vortexing or pipetting
  - Avoiding unnecessary freeze-thaw cycles
  - Avoiding pH <6 and >9
  - Avoiding high temperatures, which can lead to degradation

- Conventional agarose gels cannot resolve DNA fragments greater than 15–20 kb, but the molecular weight of starting material can be measured by pulsed-field gel analysis.

Assessing fragmentation

- The quality of the fragmented material may be assessed by different methods e.g. Agilent Bioanalzyer.
- Quantifies 1 µl DNA per sample – up to 12,000bp
- Takes 30 minutes, 12 samples at a time

Image at https://community.nanoporetech.com/docs/prepare/library_prep_protocols/input-dna-rna-qc/v/idi_s1006_v1_revb_18apr2016/assessing-input-dna

Thanks for listening!

Any Questions? ☺

EURGen-RefLabCap@food.dtu.dk

Thank you on behalf of the EURGen-RefLabCap team

Funded by the European Union