# Protocol Packet

## for Sentinel Sites in SeqAfrica

from single isolate to sequencing reads

V. 2.0

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Title: Protocol Packet for sentinel sites in SeqAfrica – from single isolate to sequencing reads V.2.0
Authors: Natasia Rebekka Thornval, Niamh Lacy-Roberts and Pernille Nilsson.
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# Content of protocol packet

This protocol packet was developed for the SeqAfrica sentinel sites in Ghana and Senegal focusing on antimicrobial resistance (AMR) surveillance and contains protocols for all the steps required to move from the DNA extraction of cultured bacteria to the generation of raw Oxford Nanopore sequence reads and analysis of sequence data using a freely available bacterial genomes analysis workflow.

- Protocol: DNA extraction from Gram negative single isolates
- Protocol: DNA extraction from Gram positive single isolates
- Protocol: Measuring DNA concentration using Qubit
- Protocol: ONT sequencing with multiplexing
- Protocol: Data analysis using EPI2ME's Bacterial Genomes wf

# Figures in the protocol packet

Most figures in this protocol packet have been created in Biorender. The details on the figures and the associated publication license for these figures is listed below.

Nilsson, P. (2025). Figures in Protocol Packet for Sentinel Sites in SeqAfrica from single isolate to sequencing reads. Created in BioRender. https://BioRender.com/nw737nm

**Protocol: DNA extraction from Gram negative single isolates**

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Page 7: Figure 2 Culture plates.
Page 7: Figure 3 The tubes found in kit.
Page 12: Figure 4 Bacterial lysis and cleaning of lysis.
Page 13: Figure 5 Elution.

**Protocol: DNA extraction from Gram positive single isolates**

Page 6: Figure 1 Material needed for extracting DNA.
Figures 2, 3, 4 and 5 in this protocol as above.

**Protocol: Measuring DNA concentration using Qubit**

Figure 1. Overview of material needed for measuring DNA.
Figure 2 Overview of steps measuring DNA concentration with Qubit.

**Protocol: ONT sequencing with multiplexing**

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# Protocol

## DNA extraction from Gram negative single isolates

for Sentinel Sites in SeqAfrica

V. 1.4

| Version | Date | Author | Edits | Contact |
|---|---|---|---|---|
| 1.4 | 05.05.25 | Pernille Nilsson & Natasia R. Thornval | Minor edits to text and clarifications in the methods. | pnil@food.dtu.dk; nareth@food.dtu.dk |
| 1.3 | 20.09.24 | Pernille Nilsson | Updated format and minor edits of the methods. | pnil@food.dtu.dk |
| 1.2 | 06.07.2024 | Natasia Thornval | Revised several steps in methods. | nareth@food.dtu.dk |
| 1.1 | 14.06.2024 | Pernille Nilsson | Format change, title change from “Extraction of DNA from Gram negative single isolates using”, figures created in BioRender | pnil@food.dtu.dk |
| 1.0 | 23.05.2024 | Natasia R. Thornval & Niamh Lacy-Roberts | | nareth@food.dtu.dk; nlac@food.dtu.dk |

# Table of contents

# Summary

This protocol describes DNA extraction using the Qiagen DNeasy Blood & Tissue kit and has been modified to yield DNA suitable for long-read sequencing.

A brief note on sample preparation (i.e. of your bacteria) is covered in the section “Preparing for DNA extraction”.

Sequencing is not included in this protocol. Please refer to the “ONT sequencing with multiplexing” protocol for sequencing after DNA extraction.

Please note that throughout the protocol commas are used for digit grouping of thousands (e.g., 10,000 rpm) while decimal points are placed as separators before the fractional part of the decimal number (e.g., 4.8 ml).

SeqAfrica

Step 1

# Preparing for DNA Extraction

# Material needed for extracting DNA

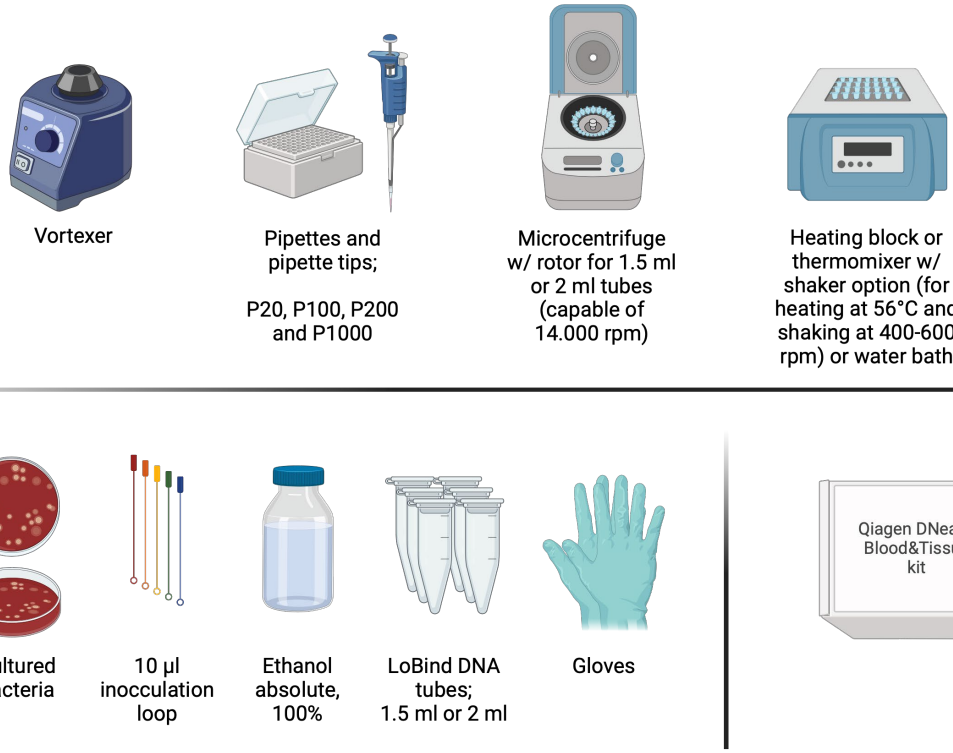


*Figure 1. Overview of materials needed for the protocol.* Created in Biorender. Nilsson, P. (2025) https://BioRender.com/nw737nm

## Why not denatured ethanol?

Denatured ethanol contains additives, including emetic agents to discourage its consumption. Some of the additives can interfere with downstream applications of the extracted DNA. If you do not have access to ethanol absolute, substitute with molecular grade ethanol approved for PCR or similar.

## Why LoBind DNA tubes?

When stored in standard tubes, a large portion of the extracted DNA may bind to the inside surface of the tubes. LoBind tubes have a hydrophilic surface (non-silicone) which minimizes surface binding of the DNA and prevents DNA loss. You should always use LoBind tubes for steps involving your DNA sample.

## What is rpm?

rpm = revolutions per minute. You can convert rpm to g force/rcf (relative centrifugal force). The conversion depends on the size of the rotor in your centrifuge.

The link below or the QR code takes you to a website where you can do the conversion:
http://www.endmemo.com/bio/grpm.php

# Culturing

Please note that sample material is grown on solid media overnight (16-24h) at 37±1°C in ambient air unless species require other growth environment (e.g., *Campylobacter spp.* should be grown at 41±1°C under microaerobic conditions in a tri-gas incubator (10% CO2, 5% O2, 85% N2) or similar). We recommend growth on TSA Agar plates with 5% calves' or sheep's blood. We also recommend the use of cultures that are not older than 24 hrs, as secondary metabolites in the bacteria builds up (due to lack of nutrients) which can interfere with the downstream analysis.

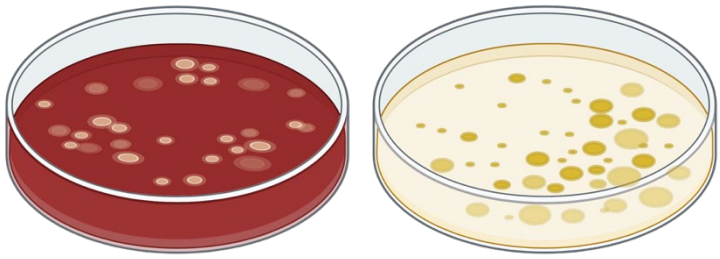

*Figure 2.* Created in Biorender. Nilsson, P. (2025) https://BioRender.com/nw737nm

# Labelling

Label the culture plates clearly. This will help you stay organized and help you match metadata with the correct sample. If label stickers are not available in your lab you can mark culture plates, LoBind tubes and spin-columns with a permanent marker.

Samples originating from the same source (e.g., a patient sample), can be indicated e.g., in a [] field on the labels by writing the number of the other sample(s) originating from the same source

**Remember to label/mark the following:**

- All LoBind tubes containing your bacterial suspension during the DNA extraction as you will be centrifugating your tubes several times.
- All spin-column **lids**, as you will be discarding collection tubes during the process.
- The DNA-containing LoBind tubes, once you have finished extracting your DNA.

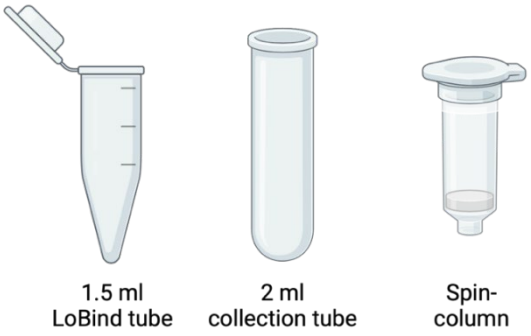


*Figure 3.* Created in Biorender. Nilsson, P. (2025) https://BioRender.com/nw737nm

# Preparing your workspace

### Avoiding environmental contaminants

- Make sure you are working on a clean surface.
  - Wipe surface down with 70% ethanol.
  - If a Laminar Air Flow (LAF)-bench or other sterile environment is accessible, perform extraction here.

  NOTE
  ! *Depending on which pathogens you are extracting DNA from, you may have to work in a LAF bench. Consult your laboratory safety representative if you are not sure of how the pathogen in question is classified in your laboratory.*
- Wear gloves throughout the entire DNA extraction.
- Keep petri dishes closed and upside down when not interacting with them.
- If using a water bath for temperature control, make sure you do not submerge the tubes completely in the water bath. Make sure the tube lids are above the water and avoid water from the water bath getting around the lid opening of your tubes.

# Before you begin to extract DNA

- [ ] Prepare fresh EtOH

  Dilute the 100% absolute ethanol to 96-98% ethanol using nuclease free water or milliQ water.

  For example, if preparing fresh 96% EtOH for 24 DNA extractions you would use:

  - [ ] 4.8 **ml** 100% EtOH
  - [ ] 200 **µl** nuclease free water or milliQ water

  You can do this in a bigger batch and store aquilots in the freezer at -18 to -20 ºC to avoid preparing EtOH each time.

- [ ] Buffer ATL and Buffer AL may form a precipitate upon storage. If necessary, warm to 56ºC until the precipitate has fully dissolved.

- [ ] Buffer AW1 and Buffer AW2 of the DNeasy Blood & Tissue kit are provided as concentrates. Before using for the first time, add the appropriate amount of ethanol (96-100%) as indicated on the bottle to obtain a working solution.

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Step 2

# Performing DNA Extraction

# DNA extraction for Gram negative bacteria

## Step 1. Bacterial lysis

- ☐ Set your heating block to 56°C.
- ☐ Add 180 µl Buffer ATL to 1.5ml Lobind tube.
- ☐ Add 20 µl proteinase K to the Buffer ATL.
- ☐ Suspend one full 1 µl loop bacteria in the 180 µl Buffer mix.

- ☐ Vortex suspension immediately until biomass is fully dispensed in the buffer mix.
- ☐ Incubate the suspension at 56°C for 60-90 minutes or until the suspension turns clear, while shaking (300-600 rpm). If the suspension is not clear extend the incubation step by 30-60 minutes. If more convenient, you can leave the solution overnight.

  If you do not have a shaking heatblock, mix the solution during incubation by turning the tube every 15 minutes.

  NOTE

  ! After incubation you should NEVER vortex the sample, as it breaks the DNA strands. Instead pipette up-and-down or flick the tube to mix.

- ☐ Remove the tube from the heating block.
- ☐ Spin down briefly (4-6 sec) in a bench top centrifuge at 200-400 rpm to collect any condensation from the lid.
- ☐ Add 200 µl Buffer AL.
- ☐ Pipette up-and-down to mix.
- ☐ Incubate for 10 minutes at 70°C. The suspension may be viscous but should not be gelatinous. If gelatinous precipitate appears, prolong the incubation by 10 additional minutes.
- ☐ Spin down briefly (4-6 sec) in a bench top centrifuge at 200-400 rpm to collect any condensation from the lid.
- ☐ Add 200 µl ethanol 96-98%.
- ☐ Pipette up-and-down to mix.

  NOTE

  ! After the addition of ethanol, a white precipitate may form. It could be an indication of having too much starting material (bacteria) and incomplete bacterial lysis. This precipitate may clog your spin-column. Try using less starting material or incubate at 70°C for 10 minutes before moving to step 2.

## Step 2. Cleaning the bacterial lysate

- ☐ Prepare a DNeasy spin-column and its 2 ml collection tube for each sample.
- ☐ Pipette the bacterial suspension from your 1.5 ml LoBind tube from Step 1 into the prepared DNeasy spin-column.

  NOTE

  ! It is important that the membrane in the spin-column does NOT touch the flow through after any of the centrifuge steps below. If the membrane does touch the flow-through, please repeat the centrifugation step (e.g., 1 min at 10,000 rpm or 3 min at 14,000 rpm depending on the step you where you stopped).
  ! Remember to balance the centrifuge.
- ☐ Centrifuge for 1 min at **10,000** rpm (or ≥12,000 x g).
- ☐ Discard the flow-through and collection tube.

  Flow-through refers to the liquid which has passed through the spin-column into the collection tube during centrifugation. **Do not discard the spin-column! This contains your DNA.**
- ☐ Place the spin-column in a new 2 ml collection tube.
- ☐ Add 500 µl buffer AW1 to the spin-column.
- ☐ Centrifuge for 1 minute at **10,000** rpm (or ≥12,000 x g).
- ☐ Discard the flow-through and collection tube.
- ☐ Place the spin-column in a new 2 ml collection tube.
- ☐ Add 500 µl Buffer AW2 to the spin-column.
- ☐ Centrifuge for 3 minutes at **14,000** rpm (or ≥20,000 x g).
- ☐ Discard the flow-through and collection tube.

  NOTE

  ! The spin column membrane should be dry after centrifugation. If any ethanol from the AW1 and AW2 buffers remains, empty the collection tube and centrifuge for 1 min at 14,000 rpm (or ≥20,000 x g). Residual ethanol may interfere with downstream workflows and analysis.

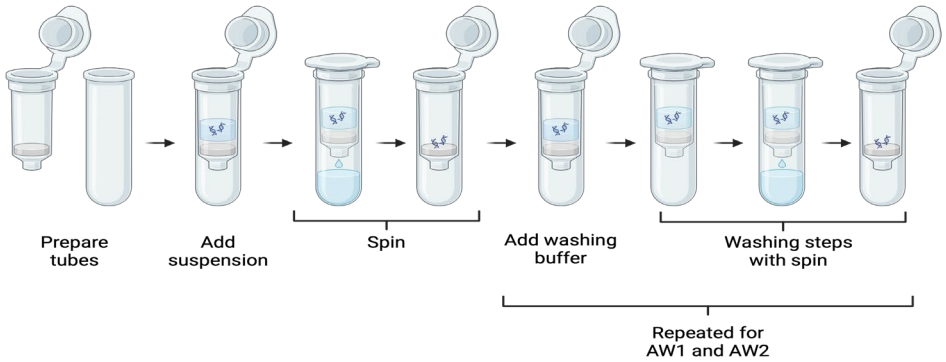


*Figure 4.* Created in Biorender. Nilsson, P. (2025) https://BioRender.com/nw737nm

## Step 3. Elution of the DNA

- ☐ Place the spin-column in a 1.5 ml LoBind tube.
- ☐ Add 100 µl Buffer AE to the spin-column.
- ☐ Centrifuge for 2 min. at **10,000** rpm (or ≥12,000 x g).
- ☐ Discard the spin-column while being careful not to let the membrane touch the flow-through in the 1.5 ml LoBind tube.
- ☐ Close the LoBind tube.

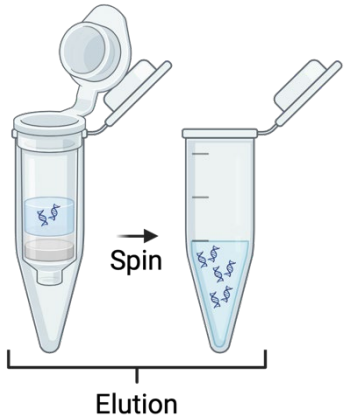


*Figure 5*. Created in Biorender. Nilsson, P. (2025) https://BioRender.com/nw737nm

## Step 4 Checking your DNA

- ☐ Check the eluted DNA for a pellet in the bottom of the tube. If there is a visible pellet, transfer the supernatant to a new LoBind tube and discard the old LoBind tube containing the pellet.

The supernatant in the 1.5 ml LoBind tube contains your extracted DNA.

The DNA extraction is now ready to be measured with a tool such as a Qubit. Store the DNA in the refrigerator at 4ºC or in the freezer at -20ºC (long time storage).

Please refer to the “Measuring DNA concentration using Qubit” protocol for quantifying DNA concentration.

# Protocol

## DNA extraction from Gram positive single isolates

for Sentinel Sites in SeqAfrica

V. 1.2

| Version | Date | Author | Edits | Contact |
|---|---|---|---|---|
| 1.2 | 05.05.25 | Pernille Nilsson & Natasia R. Thornval | Minor edits to text and clarifications in the methods. | pnil@food.dtu.dk; nareth@food.dtu.dk |
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| 1.0 | 23.05.2024 | Natasia R. Thornval & Niamh Lacy-Roberts | | nareth@food.dtu.dk; nlac@food.dtu.dk |

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This protocol describes DNA extraction using the Qiagen Dneasy Blood & Tissue kit and has been modified to yield DNA suitable for long-read sequencing.

A brief note on the sample preparation (i.e. your bacteria) is covered in the section “Preparing for DNA extraction”.

Sequencing is not included in this protocol. Please refer to the “ONT sequencing with multiplexing” protocol for sequencing after DNA extraction.

Please note that throughout the protocol commas are used for digit grouping of thousands (e.g., 10,000 rpm) while decimal points are placed as separators before the fractional part of the decimal number (e.g., 4.8 ml).

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Step 1

# Preparing for DNA Extraction

# Material needed for extracting DNA

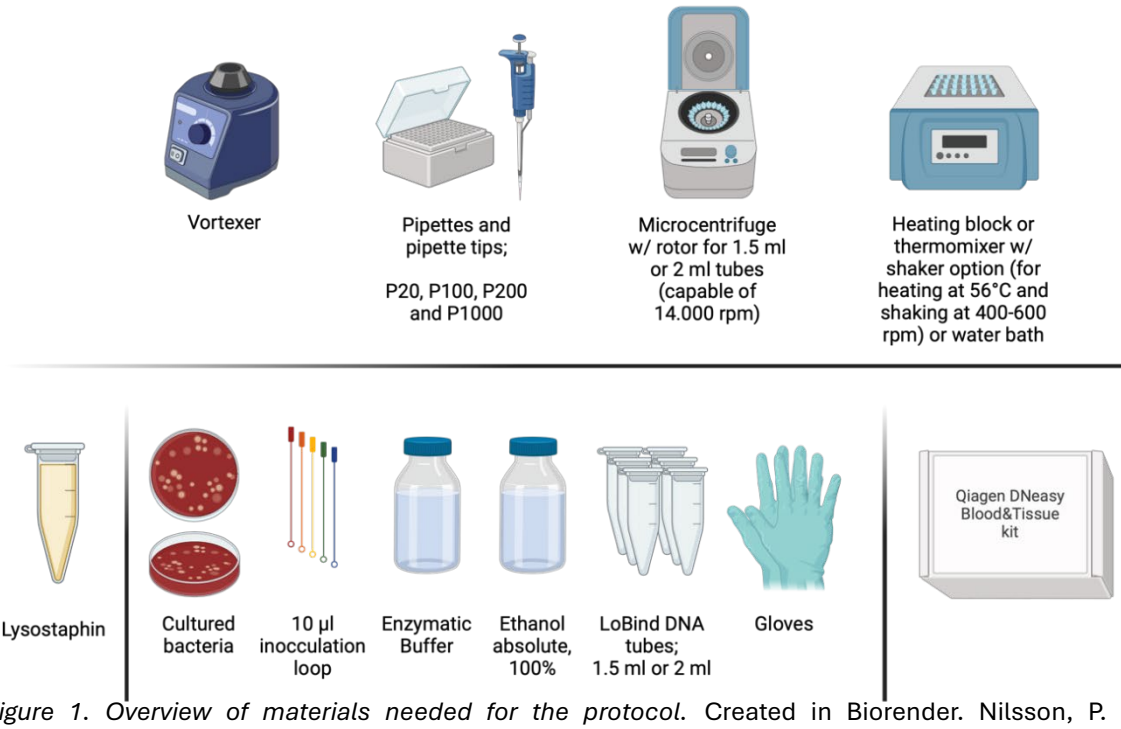


*Figure 1. Overview of materials needed for the protocol.* Created in Biorender. Nilsson, P. (2025) https://BioRender.com/nw737nm

## Why not denatured ethanol?

Denatured ethanol contains additives, including emetic agents to discourage its consumption. Some of the additives can interfere with the downstream applications of the extracted DNA. If you do not have access to ethanol absolute, substitute with molecular grade ethanol approved for PCR or similar.

## Why LoBind DNA tubes?

When stored in standard tubes, a large portion of the extracted DNA may bind to the inside surface of the tubes. LoBind tubes have a hydrophilic surface (non-silicone) which minimizes surface binding of the DNA and prevents DNA loss. You should always use LoBind tubes for steps involving your DNA sample.

## What is rpm?

rpm = revolutions per minute. You can convert rpm to g force/rcf (relative centrifugal force). The conversion depends on the size of the rotor in your centrifuge.

The link below or the QR code takes you to a website where you can do the conversion:
http://www.endmemo.com/bio/grpm.php

**Please note that lysostaphin is only required when working with** Staphylococcus.spp¡

# Culturing

Please note that sample material is grown on solid media overnight (16-24h) at 37±1°C in ambient air unless species require another growth environment. We recommend growth on TSA Agar plates with 5% calves' or sheep's blood. We also recommend the use of cultures that are not older than 24 hrs, as secondary metabolites in the bacteria builds up (due to lack of nutrients) which can interfere with the downstream analysis.

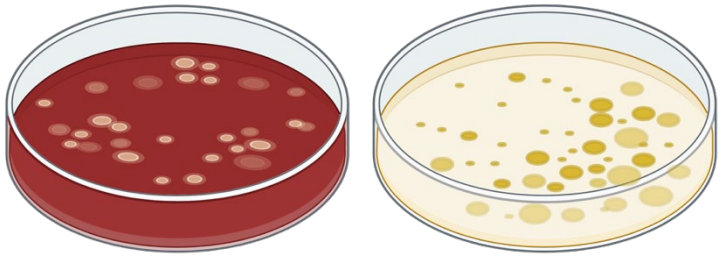

*Figure 2*. Created in Biorender. Nilsson, P. (2025) https://BioRender.com/nw737nm

# Labelling

Label the culture plates clearly. This will help you stay organized and help you match metadata with the correct sample. If label stickers are not available in your lab you can mark culture plates, LoBind tubes and spin-columns with a permanent marker.

Samples originating from the same source (e.g., a patient sample), can be indicated e.g., in a [] field on the labels by writing the number of the other sample(s) originating from the same source.

**Remember to label/mark the following:**

- All LoBind tubes containing your bacterial suspension during the DNA extraction as you will be centrifugating your tubes several times.
- All spin-column **lids**, as you will be discarding collection tubes during the process.
- The DNA-containing LoBind tubes, once you have finished extracting your DNA.

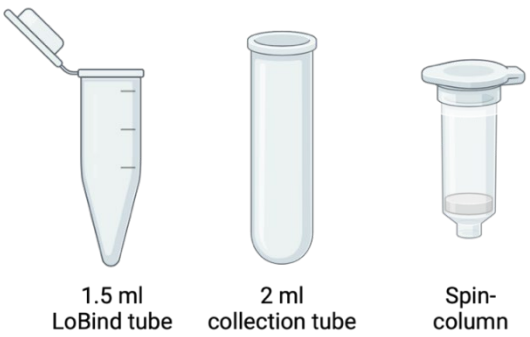


*Figure 3*. Created in Biorender. Nilsson, P. (2025) https://BioRender.com/nw737nm

# Preparing your workspace

### Avoiding environmental contaminants

- Make sure you are working on a clean surface.
  - Wipe surface down with 70% ethanol.
  - If a Laminar Air Flow (LAF)-bench or other sterile environment is accessible, perform extraction here.

  NOTE
  ! *Depending on which pathogens you are extracting DNA from, you may have to work in a LAF bench. Consult your laboratory safety representative if you are not sure of how the pathogen in question is classified in your laboratory.*
- Wear gloves throughout the entire DNA extraction.
- Keep petri dishes closed and upside down when not interacting with them.
- If using a water bath for temperature control, make sure you do not submerge the tubes completely in the water bath. Make sure the tube lids are above the water and avoid water from the water bath getting around the lid opening of your tubes.

# Before you begin to extract DNA

- [ ] Prepare fresh EtOH

  Dilute the 100% absolute ethanol to 96-98% ethanol using nuclease free water or milliQ water.

  For example, if preparing fresh 96% EtOH for 24 DNA extractions you would use:

  - [ ] 4.8 **ml** 100% EtOH
  - [ ] 200 **µl** nuclease free water or milliQ water

  You can do this in a bigger batch and store aquilots in the freezer at -18 to -20 ºC to avoid preparing EtOH each time.

- [ ] Buffer AL may form a precipitate upon storage. If necessary, warm to 56ºC until the precipitate has fully dissolved.

- [ ] Buffer AW1 and Buffer AW2 of the DNeasy Blood & Tissue kit are provided as concentrates. Before using for the first time, add the appropriate amount of ethanol (96-100%) as indicated on the bottle to obtain a working solution.

- [ ] Prepare Enzymatic Buffer for gram-positives by mixing the following reagents (store at 4°C):

  - [ ] 192.8 ml nuclease free water or milliQ water
  - [ ] 4.0 ml Tris HCl 1M pH 8.0
  - [ ] 2.0 ml EDTA 0.5M
  - [ ] 2.4 Triton X 100

  You can do this in a bigger batch and store at 4°C.

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Step 2

# Performing DNA Extraction

# DNA extraction for Gram positive bacteria

## Step 1. Bacterial lysis

- ☐ Set your heating block to 37ºC.
- ☐ Add 180 µl Enzymatic Buffer and 20 µl lysozyme (10 mg/ml) in a 1.5 ml LoBind tube.
  - ☐ If extracting DNA from *Staphylococcus spp.*, add 25 µl lysostaphin (2 mg/ml) to the bacterial suspension.

NOTE

! Lysozyme and lysostaphin cannot be premixed but must be added just before adding the bacteria.

- ☐ Suspend one full 1 µl loop bacteria in the LoBind tube containing the buffer mix.
- ☐ Vortex suspension immediately until biomass is fully dispensed in the buffer mix.
- ☐ Incubate the suspension at 37ºC for 60 minutes. The solution should appear clear.
- ☐ Remove the tube from the heating block.
- ☐ Set your heating block to 56ºC.
- ☐ Add 25 µl proteinase K to suspension in tube.
- ☐ Add 200 µl Buffer AL to suspension tube. Please note that the Buffer AL should be pure without ethanol added.
- ☐ Vortex suspension.
- ☐ Incubate the suspension at 56 ºC for 30 minutes, while shaking (400-600 rpm).

If you do not have a shaking heatblock, mix the solution during incubation by turning the tube every 15 minutes.

NOTE

! After incubation you should NEVER vortex sample, as it breaks the DNA strands. Instead pipette up-and-down or flick tube to mix.

- ☐ Remove tube from heating block.
- ☐ Spin down briefly (4-6 sec) in a bench top centrifuge at 200-400 rpm to collect any condensation from the lid.
- ☐ Add 200 µl ethanol 96-98%.
- ☐ Pipette up-and-down to mix thoroughly.

NOTE

! After the addition of ethanol, a white precipitate may form. It could be an indication of having too much starting material (bacteria) and incomplete bacterial lysis. This precipitate may clog your spin-column. Try using less starting material or incubate at 70ºC for 10 minutes before moving to step 2.

## Step 2. Cleaning the bacterial lysate

- ☐ Prepare a DNeasy spin-column and its 2 ml collection tube for each sample.
- ☐ Pipette the bacterial suspension from your 1.5 ml LoBind tube from Step 1 into the prepared DNeasy spin-column.

  NOTE

  ! It is important that the membrane in the spin-column does NOT touch the flow through after any of the centrifuge steps below. If the membrane does touch the flow-through, please repeat the centrifugation step (e.g., 1 minute at 10,000 rpm or 3 minutes at 14,000 rpm depending on the step you where you stopped).
  ! Remember to balance the centrifuge.
- ☐ Centrifuge for 1 min at **10,000** rpm (or ≥12,000 x g).
- ☐ Discard the flow-through and collection tube.

  Flow-through refers to the liquid which has passed through the spin-column into the collection tube during centrifugation. **Do not discard the spin column! This contains your DNA.**
- ☐ Place the spin-column in a new 2 ml collection tube.
- ☐ Add 500 µl buffer AW1 to the spin-column.
- ☐ Centrifuge for 1 minute at **10,000** rpm (or ≥12,000 x g).
- ☐ Discard the flow-through and collection tube.
- ☐ Place the spin-column in a new 2 ml collection tube.
- ☐ Add 500 µl Buffer AW2 to the spin-column.
- ☐ Centrifuge for 3 minutes at **14,000** rpm (or ≥20,000 x g).
- ☐ Discard the flow-through and collection tube.

  NOTE

  ! The spin column membrane should be dry after centrifugation. If ethanol from AW1 and AW2 buffers remain, empty the collection tube and centrifuge 1 minute at 14,000 rpm (or ≥20,000 x g). Residual ethanol may interfere with downstream workflows and analysis.

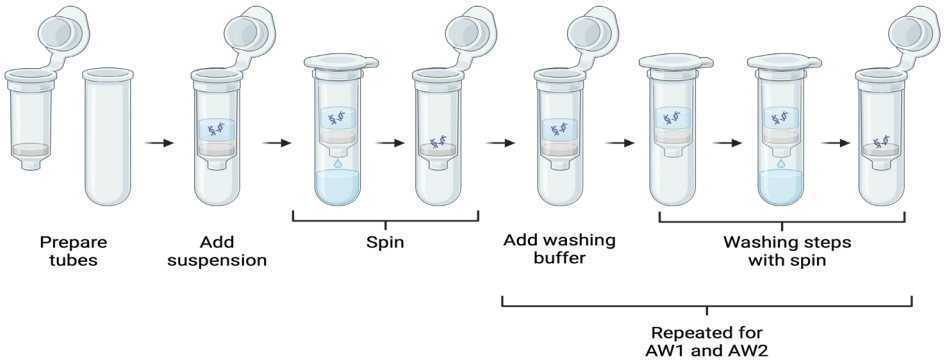


*Figure 4.* Created in Biorender. Nilsson, P. (2025) https://BioRender.com/nw737nm

## Step 3. Elution of the DNA

- ☐ Place the spin-column in a 1,5 ml LoBind tube.
- ☐ Add 100 µl Buffer AE to the spin-column.
- ☐ Centrifuge for 2 min. at **10,000** rpm (or ≥12,000 x g).
- ☐ Discard the spin-column while being careful not to let the membrane touch the flow-through in the 1.5 ml LoBind tube.
- ☐ Close the LoBind tube.

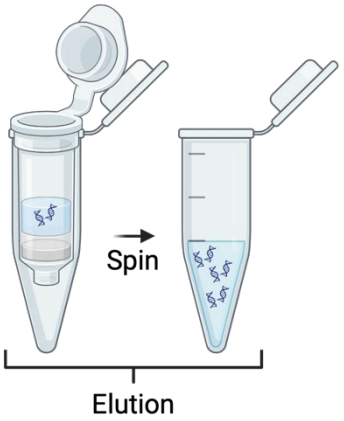


*Figure 5*. Created in Biorender. Nilsson, P. (2025) https://BioRender.com/nw737nm

## Step 4 Checking your DNA

- ☐ Check the eluted DNA for a pellet in the bottom of the tube. If there is a visible pellet, transfer the supernatant to a new LoBind tube and discard the old LoBind tube containing the pellet.

The supernatant in the 1.5 ml LoBind tube contains your extracted DNA.

The DNA extraction is now ready to be measured with a tool such as a Qubit. Store the DNA in the refrigerator at 4ºC or in the freezer at -20ºC (long time storage).

Please refer to the “Measuring DNA concentration using Qubit” protocol for quantifying DNA concentration.

# Protocol

## Measuring DNA concentration using Qubit

for Sentinel Sites in SeqAfrica

V. 1.2

| Version | Date | Author | Edits | Contact |
|---|---|---|---|---|
| 1.2 | 20.09.24 | Pernille Nilsson | Updates to format. | pnil@food.dtu.dk |
| 1.1 | 06.07.2024 | Natasia Thornval | Update of Figure 2 and minor text revisions. | nareth@food.dtu.dk |
| 1.0 | 14.06.2024 | Pernille Nilsson | Figures created in BioRender. | pnil@food.dtu.dk |

# Table of contents

# Summary

This protocol describes how to measure DNA concentration after DNA extraction using the Qubit dsDNA HS Assay Kit and Qubit 4 Fluorometer and has been modified to simplify the standard protocol.

The assay measures DNA concentration using a fluorescent dye that binds to double stranded DNA. The DNA concentration is determined by measuring the strength of the fluorescent signal emitted from the sample.

How to extract DNA from bacterial isolates is not covered in this protocol, please refer instead to the “DNA extraction from Gram negative single isolates” or “DNA extraction from Gram positive single isolates” protocols.

Sequencing is also not covered in this protocol, please refer to the “ONT sequencing with multiplexing” protocol.

Please note that throughout the protocol commas are used for digit grouping of thousands (e.g., 5,174 µl) while decimal points are placed as separators before the fractional part of the decimal number (e.g., 4.8 ml).

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Step 1

# Preparing for measuring DNA concentration

# Material needed for measuring DNA

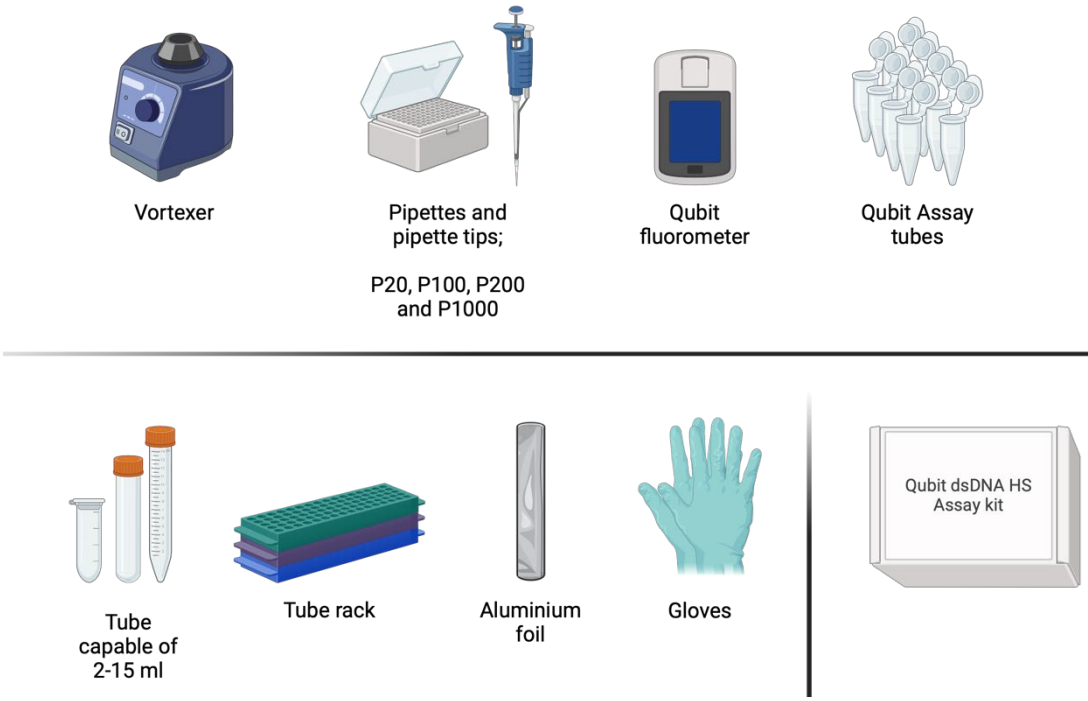



*Figure 1. Overview of materials needed for the protocol. Created in BioRender. Nilsson, P. (2025)* https://BioRender.com/nw737nm

## Why Qubit Assay tubes?

The Qubit Assay tubes are thin-walled, clear 0.5 ml tubes. These types of tubes are required so the fluorescent reading of the DNA concentration is accurate. Other similar PCR tubes can be used if they are thin-walled, clear and fit snuggly in the Qubit fluorometer.

## Why aluminium foil?

The kit uses a fluorescent dye (Qubit Reagent) that binds to the double-stranded DNA. The dye is light sensitive and should be kept in the dark as much as possible. The aluminium foil should be wrapped around the tube of dye and used to cover the other tubes to block out as much light as possible when preparing the working solution and processing samples.

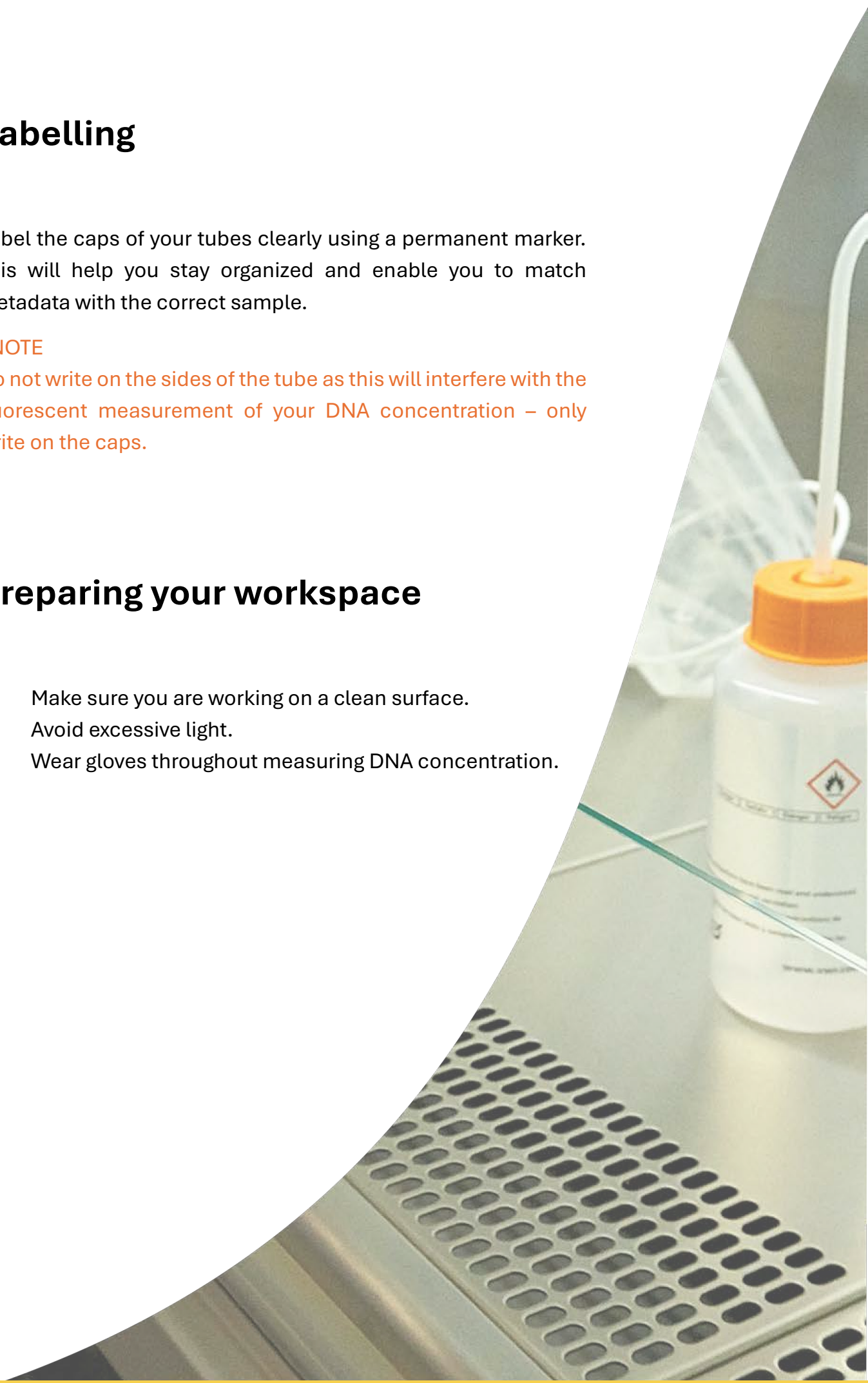
# Labelling

Label the caps of your tubes clearly using a permanent marker. This will help you stay organized and enable you to match metadata with the correct sample.

**!** NOTE
Do not write on the sides of the tube as this will interfere with the fluorescent measurement of your DNA concentration – only write on the caps.

# Preparing your workspace

- Make sure you are working on a clean surface.
- Avoid excessive light.
- Wear gloves throughout measuring DNA concentration.



# Qubit kit –content and storage instructions

| Material | Amount | Concentration | Storage |
|---|---|---|---|
| Qubit Reagent | 250 µl | 200x concentrate in DMSO | • Room temperature<br>• Desiccate<br>• Protect from light |
| Qubit Buffer | 50 ml | Not applicable | • Room temperature |
| Qubit Standard 1 | 1 ml | 0 ng/ µl in TE buffer | • ≤ 4ºC<br>• Avoid freezing |
| Qubit Standard 2 | 1 ml | 100 ng/ µl in TE buffer | |

When stored as directed, kits are stable for 6 months.

### DMSO

Dimethyl sulfoxide (DMSO) is a highly polar organic reagent with solvent properties for organic and inorganic chemicals.

### TE buffer

TE buffer is a buffer solution consisting of Tris (a pH buffer) and EDTA (molecule that chelates cations like $Mg^{2+}$) often used in molecular biology. The purpose is to solubilise DNA while protecting it from degradation.

# Before you begin DNA measurement

- ☐ Bring reagents to room temperature (22-28 ºC).
- ☐ Make sure you have all reagents, equipment and consumables lined up.
- ☐ Perform calculations of how much working solution to prepare. The Qubit working solution is made by mixing the Qubit Buffer with the Qubit Reagent (fluorescent dye) in the following ratios:
  - ☐ 199 x n µl Qubit Buffer
  - ☐ 1 x n µl Qubit Reagent

  Where n = number of standards (2) plus number of samples.

  Example for 24 samples:

  - ☐ 199 x 26 = 5,174 µl Qubit Buffer
  - ☐ 1 x 26 = 26 µl Qubit Reagent

Prepare slightly more Qubit working solution than the number of samples you will be measuring to account for pipetting error.

The Qubit 4.0 has a Reagent Calculator that you can use to quickly determine the correct amount of Qubit Reagent (fluorescent dye) and Buffer required to make the appropriate amount of working solution. The complete Qubit 4 User Guide is available in the SeqAfrica pilot sites' Google Drive folder using this link or the QR code below. This guide also includes information on all features of the instrument, safety instructions and a troubleshooting guide.

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Step 2

# Measuring concentration of DNA extract

# Measuring concentration of DNA extract

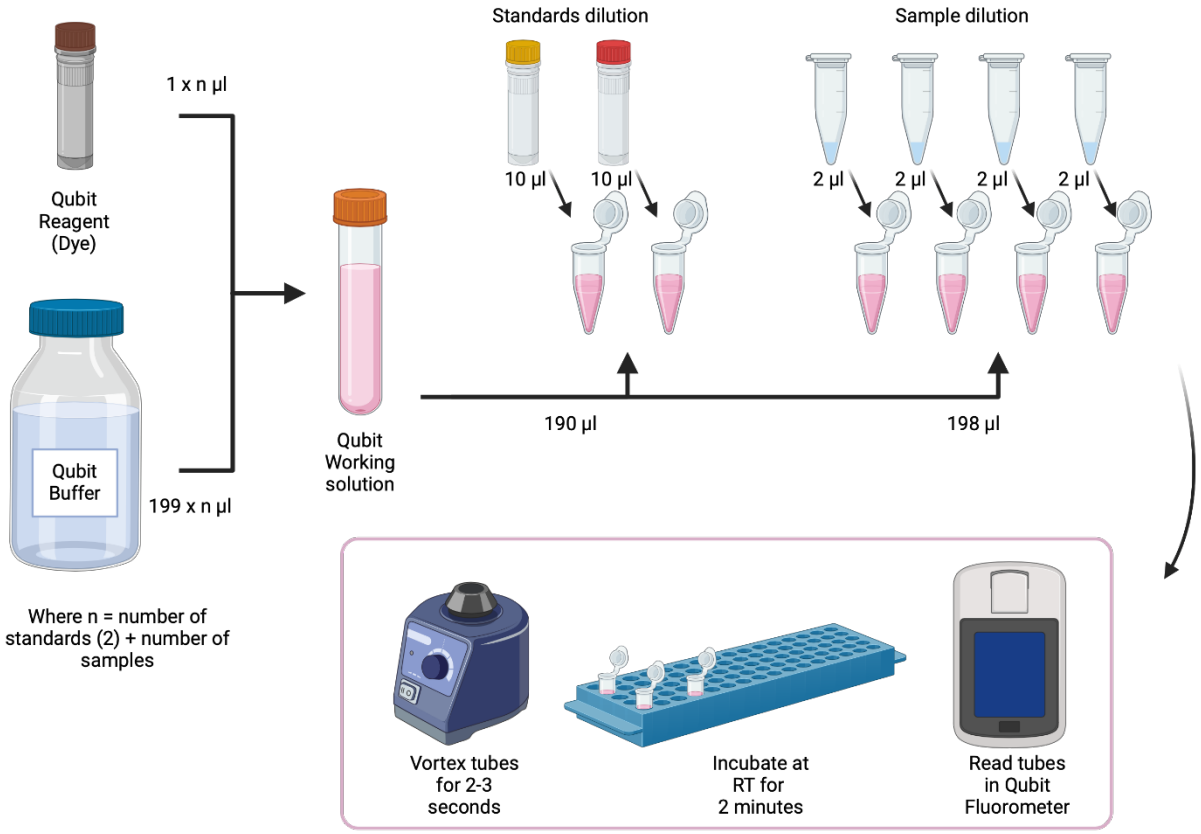


*Figure 2. Overview of steps measuring DNA concentration with Qubit. Created in BioRender. Nilsson, P. (2025)* https://BioRender.com/nw737nm

## Step 1. Prepare samples and standards

- ☐ Set up the required number of 0.5 ml tubes for standards and samples.
- ☐ Label the tube lids/tube caps.
- ☐ Prepare the working solution by mixing the Qubit Buffer and Qubit Reagent in a clean plastic tube.

  TIP
  Always pipette the larger volume first. In this way you can more easily pipette the smaller volume into the fluid in your tube. Careful pipetting is especially critical when preparing the standards to ensure that exactly 10 µl of each standard is added to the working solution in the next steps.

  NOTE
  ! Protect all tubes containing the working solution from light using aluminium foil throughout the assay until measurements are complete. Keep the plastic tube with working solution wrapped in foil and put a sheet of foil over your rack containing standard and sample tubes.
- ☐ Add 190 µl working solution to each of the tubes used for **standards**.
- ☐ Add 10 µl of each Qubit standard (Standard 1 and Standard 2) to the appropriate tube.
- ☐ Vortex each tube for 2-3 seconds.

  NOTE
  ! Be careful not to create bubbles when vortexing as this will interfere with obtaining accurate measurements.
- ☐ Add 198 µl of working solution to each **sample** tube.
- ☐ Add 2 µl of each sample to the respective sample tube containing working solution.
- ☐ Vortex each tube for 2-3 seconds.

  NOTE
  ! Be careful not to create bubbles when vortexing as these will interfere with obtaining accurate measurements.
- ☐ Allow all tubes to incubate at room temperature for 2 minutes.

  The assay is performed at room temperature, and the fluorescent signal is stable for 3 hours.

## Step 2. Reading standards and samples

- ☐ On the home screen of the Qubit Fluorometer press **DNA**, then select **dsDNA High Sensitivity** as the assay type.
- ☐ Press **Read Standards** to proceed.

  NOTE
  ! If you have already performed a calibration for the selected assay, the instrument prompts you to choose between reading new standards and running samples using the previous calibration.

- ☐ Insert tube containing Standard 1 into the sample chamber, close the lid, then press **Read Standard**. Reading takes ~3 seconds.
- ☐ Remove Standard 1.
- ☐ Insert tube containing Standard 2 into the sample chamber, close the lid, then press **Read Standard**. Reading takes ~3 seconds.
- ☐ Remove Standard 2.

  The instrument displays the results on the Read standards screen. For information on interpreting the calibration results, refer to the Qubit 4 *User Guide*.

- ☐ Press **Run samples**.
- ☐ On the assay screen, select the sample volume added to the assay tube [2 µl].
- ☐ On the assay screen, select the units for output sample concentration from the drop-down menu [µg/µl].
- ☐ Insert sample tube into the sample chamber, close the lid, then press **Read tube**. Reading takes ~3 seconds.

  The instrument displays the result on the assay screen. The Qubit 4 performs the calculation automatically: The top value (in large font) is the concentration of the original sample. The bottom value is the dilution concentration (the concentration of the sample in the tube inserted into the Qubit Fluorometer). For information on interpreting the sample results, refer to the Qubit 4 User Guide.

- ☐ Remove the sample tube.
- ☐ Write down the results of the reading.
- ☐ Repeat reading for all sample tubes.

# Protocol

## ONT sequencing with multiplexing

for Sentinel Sites in SeqAfrica

V. 1.4

| Version | Date | Author | Edits | Contact |
|---|---|---|---|---|
| 1.4 | 05.05.25 | Pernille Nilsson & Natasia R. Thornval | Minor updates to methods, addition of QR codes to SeqAfrica videos. | pnil@food.dtu.dk; nareth@food.dtu.dk |
| 1.3 | 20.09.2024 | Pernille Nilsson | Updates to method, Layout and design. | pnil@food.dtu.dk |
| 1.2 | 16.07.2024 | Natasia R. Thornval | Updates to method | nareth@food.dtu.dk |
| 1.1 | 14.06.2024 | Pernille Nilsson | Format change, title generated, figures created in BioRender. | pnil@food.dtu.dk |
| 1.0 | 23.05.2024 | Natasia R. Thornval & Niamh Lacy-Roberts | | nareth@food.dtu.dk; nlac@food.dtu.dk |

# Table of contents

# Summary

This protocol describes library prep and long-read sequencing using the portable MinION device from Oxford Nanopore Technology (ONT). The protocol is specific for performing the library preparation using the Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) or the Rapid Barcoding Kit 96 V14 (SQK-RBK114.96) for multiplexing up to 24 or 96 bacterial samples. Please note these kits are only compatible with R10.4.1 flow cells (FLO-MIN114).

The ONT sequencing protocol describes sequencing of 24 samples per flow cell. This means sites can multiplex 24 samples during library preparation. If sites do not have or want to multiplex 24 different isolates for a run, samples can be run in duplicate or triplicate etc. to achieve 24 samples per flow cell or choose to multiplex fewer samples during the barcoding step with minor adjustments to the step-by-step instructions.

This protocol does not describe DNA extraction or how to determine DNA concentration after DNA extraction. Please refer to the "DNA extraction from Gram negative single isolates", "DNA extraction from Gram positive single isolates" and "Measuring DNA concentration using Qubit" protocols for these steps.

Please note that throughout the protocol commas are used for digit grouping of thousands (e.g., 2,000 µl) while decimal points are placed as separators before the fractional part of the decimal number (e.g., 4.8 ml).

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Step 1

# Preparing for ONT sequencing

# Material needed for ONT sequencing

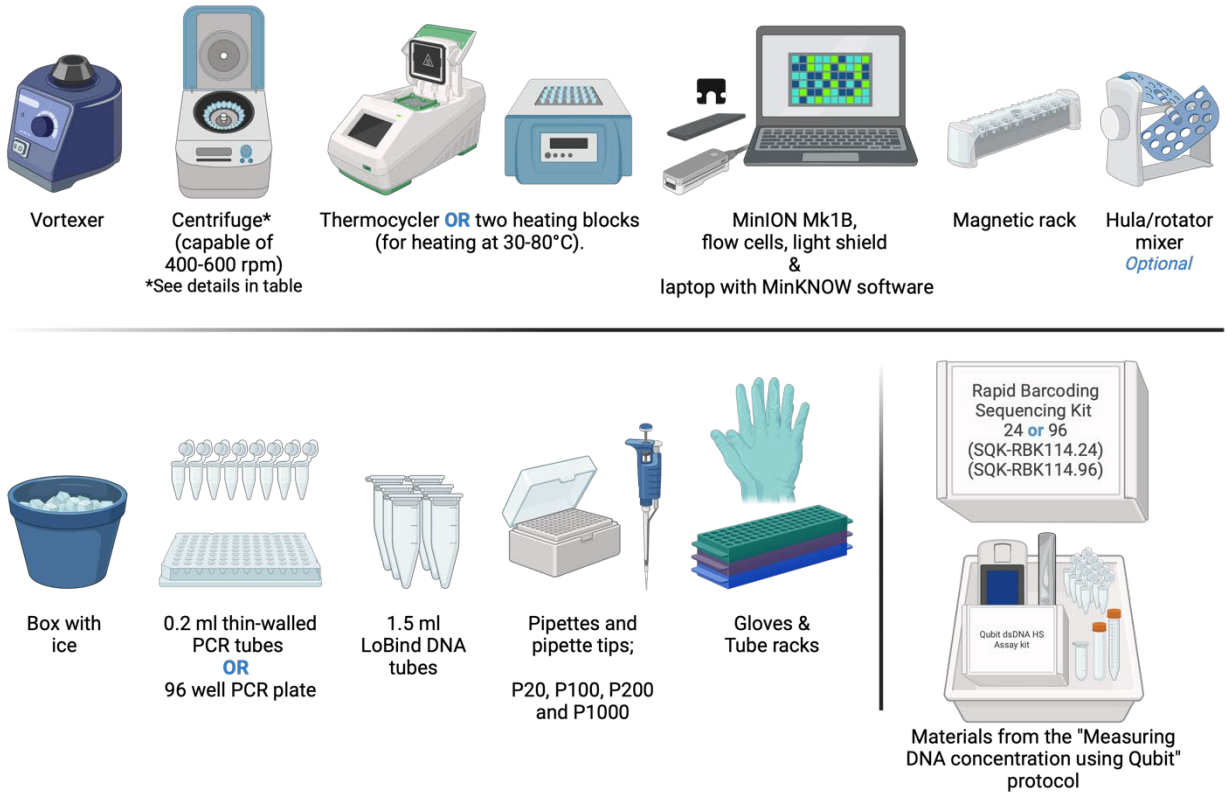



*Figure 1. Overview of materials needed for the protocol.* Created in Biorender. Nilsson, P. (2025) https://BioRender.com/nw737nm

Please note that you can either use PCR tubes, PCR plates or 1.5 ml LoBind tubes for the multiplexing part of the protocol and as a result some of the required equipment varies (see table below). Please choose the option fitting your laboratory equipment and follow the appropriate description under Step 2 (Step 2.A or 2.B).

| Multiplexing in: | Consumables | Storage |
|---|---|---|
| 1.5 ml LoBind tubes (**Step 2.A, p. 12**) | 1.5 ml LoBind tubes | • Two heat blocks for 1.5 ml tubes (capable of 30-80ºC) |
| PCR tubes (**Step 2.B, p. 13**) | 0.2 ml thin-walled PCR tubes | • Microcentrifuge for PCR tubes<br>• Thermal cycler for PCR tubes |
| PCR plate (**Step 2.B, p. 13**) | PCR plate 96 LoBind, semi-skirted (e.g., Eppendorf, cat #0030129504) with heat seals | • Microplate centrifuge<br>• Thermal cycler for PCR plates |

# Labelling

Label the caps of your tubes clearly using a permanent marker. This will help you stay organized as you work your way through the various steps of the library preparation.

If you are performing the multiplex using a 96-well PCR plate, creating a printable template to put underneath the PCR plate while pipetting can help you keep track of which sample goes in which well.

# Preparing your workspace

**Avoiding environmental contaminants**

- Make sure you are working on a clean surface.
- Wear gloves.

# Supporting videos

SeqAfrica has created a couple of supporting videos showing some of the useful techniques described during the library preparation.

- Step 3. Completing your library preparation
  Link: Video tutorial of washing the magnetic beads

  QR code ⟶

- Step 4. Priming and loading the MinION flow cell
  Link: Video tutorial of priming and loading

  QR code ⟶

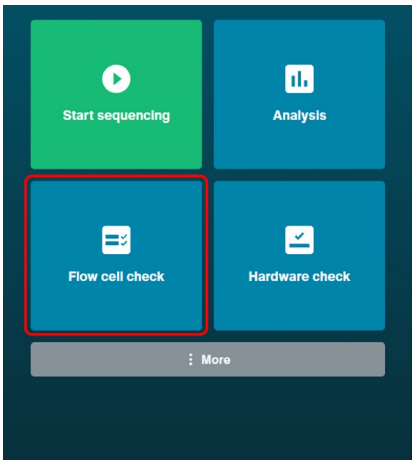
# Flow cell check

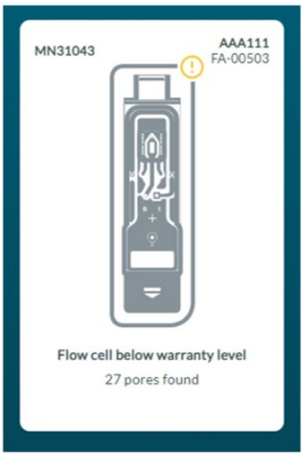
☐ Check **how many active pores** your flow cell has in the MinKNOW software by running a Flow cell check. *The flow cell should have at least 800 active pores.*

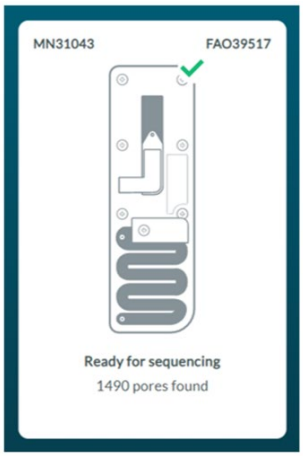
NOTE

! *If the flow cell has less than 800 active pores, take a screenshot of the flow cell check and contact ONT for a replacement flow cell. This is usually done through the online return portal* (link below), but you might have to contact your local distributor from whom you received your flow cells.

https://community.nanoporetech.com/support/contact_us/return-device?from=support




☐ The flow cell is then ready to be used for sequencing or can be and stored for later use according to the manufacturer's recommendations.

! *Flow cell check must be performed within the warranty cover of 12 weeks from purchase. It is good practice to check all flow cells upon receipt and again prior to sequencing.*

# Preparing your SeqAfrica Sample Sheet

For the library preparation you will need 100 ng of genomic DNA as input per sample. This can be calculated using the formula below.

$$\textit{Sample volume}\ (\mu l) = {}^{100\ ng}/_{sample\ DNA\ concentration\ (ng/\mu l)}$$

The **total** starting volume in each tube must be 10 µl, and nuclease-free water is used to adjust the volume up to 10 µl.

SeqAfrica has incorporated this formula in a spreadsheet for you to easily calculate for all your samples: SeqAfrica_lab_sheet.xlsx

- ☐ Ensure you have measured the concentration of all your samples using the "Measuring DNA concentration using Qubit" protocol.
- ☐ Use the "Calculating sample volumes" tab in the SeqAfrica_lab_sheet.xlsx to calculate **sample volume** and **water volume** needed for each your DNA extractions.

| | A | B | C | D | E |
|---|---|---|---|---|---|
| 1 | **Sample name** | **Sample DNA conc. (ng/ul)** | **Sample volume for library (ul)** | **Water volume (ul)** | **Barcode** |
| 2 | isolate_123 | Concentration measured by Qubit | =100/B2 | =10-C2 | Individual barcode chosen for this sample |
| 3 | 1 | 22.8 | 4.4 | 5.6 | barcode01 |
| 4 | 2 | 11.1 | 9.0 | 1.0 | barcode02 |
| 5 | 3 | 10.0 | 10.0 | 0.0 | barcode03 |
| 6 | 4 | 61.8 | 1.6 | 8.4 | barcode04 |
| 7 | 5 | 29.5 | 3.4 | 6.6 | barcode05 |
| 8 | 6 | | #DIV/0! | #DIV/0! | barcode06 |
| 9 | 7 | | #DIV/0! | #DIV/0! | barcode07 |
| 10 | 8 | | #DIV/0! | #DIV/0! | barcode08 |
| 11 | 9 | | #DIV/0! | #DIV/0! | barcode09 |
| 12 | 10 | | #DIV/0! | #DIV/0! | barcode10 |
| 13 | 11 | | #DIV/0! | #DIV/0! | barcode11 |
| 14 | 12 | | #DIV/0! | #DIV/0! | barcode12 |
| 15 | 13 | | #DIV/0! | #DIV/0! | barcode13 |
| 16 | 14 | | #DIV/0! | #DIV/0! | barcode14 |
| 17 | 15 | | #DIV/0! | #DIV/0! | barcode15 |
| 18 | 16 | | #DIV/0! | #DIV/0! | barcode16 |
| 19 | 17 | | #DIV/0! | #DIV/0! | barcode17 |
| 20 | 18 | | #DIV/0! | #DIV/0! | barcode18 |
| 21 | 19 | | #DIV/0! | #DIV/0! | barcode19 |
| 22 | 20 | | #DIV/0! | #DIV/0! | barcode20 |
| 23 | 21 | | #DIV/0! | #DIV/0! | barcode21 |
| 24 | 22 | | #DIV/0! | #DIV/0! | barcode22 |
| 25 | 23 | | #DIV/0! | #DIV/0! | barcode23 |
| 26 | 24 | | #DIV/0! | #DIV/0! | barcode24 |

# Before you begin library prep and sequencing

☐ Set one heating block to 30ºC and another heating block to 80ºC.

*OR*

☐ Program the thermal cycler:

- 30ºC for 2 minutes, then 80ºC for 2 minutes, finish with 0-4 ºC for 5 minutes.

Some of the library kit components are frozen and must be thawed. Additionally, some components require gentle mixing by pipetting up and down, while others can be vortexed. The table below lists all library kit components and how they should be stored while performing the library prep and how they should be mixed.

| Reagent | Thaw at room temperature (22-28ºC) | Keep on ice throughout library prep workflow | Mix well by pipetting* |
|---|---|---|---|
| AMPure XP Beads (AXP) | ✓ | ✓ | Mix by pipetting or vortexing *immediately before use* |
| Elution Buffer (EB) | ✓ | ✓ | ✓ |
| Adapter Buffer (ADB) | ✓ | ✓ | Mix by vortexing |
| Rapid Barcodes (RB01-24) or Rapid Barcode Plate (RB01-96) | Not frozen | ✓ | ✓ |
| Rapid Adapter (RA) | Not frozen | ✓ | ✓ |

*This information will be repeated in the step-by-step instructions to avoid any confusion.

NOTE

! *Place ALL kit components (AXP, EB, ADB, RB, RA) back in freezer for long time storage once you are done using them in your library prep.*

NOTE

! *You should not vortex DNA samples when sequencing with ONT. Vortexing can shear or break DNA strands resulting in fragmented sequencing reads (shorter reads).*

Instead, gently mix samples up and down with a pipette or gently flick the tube.

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Step 2

# Barcoding your samples

# 2.A. Barcoding using 1.5 ml tubes

- ☐ Transfer the calculated amount of genomic DNA per sample (**sample volume**) into individual 1.5 ml LoBind tubes.
- ☐ Adjust the volume of each sample to 10 µl with the calculated amount of **nuclease-free water**.
- ☐ Mix gently by pipetting up and down 10-15 times.

  NOTE
  ! If the diluted samples are not at the bottom of the tubes, spin down briefly (4-6 seconds) in a benchtop centrifuge at 200-400 rpm.

- ☐ Mix the Rapid Barcodes well by pipetting up and down and add 1.5 µl Rapid Barcodes to each tube containing your diluted genomic DNA from the previous step. Place the Rapid Barcodes back on ice.

  NOTE
  ! You should always use different barcodes for each sample you run on a given flow cell.

- ☐ Ensure all components are thoroughly mixed by pipetting up and down.
- ☐ If you can see droplets on the side of the tubes, spin down tubes briefly (4-6 seconds) in a benchtop centrifuge at 200-400 rpm.

  Spinning ensures all liquid (i.e. reagents) collects at the bottom of the tube.

- ☐ Put tubes on heating blocks:
  - ☐ First 30ºC for 2 minutes.
  - ☐ Then 80ºC for 2 minutes.
- ☐ Take tubes out of heating block and put on ice to cool.
- ☐ Spin down briefly (4-6 seconds) in a benchtop centrifuge at 200-400 rpm.
- ☐ Pool all barcoded samples in a clean 1.5 ml or 2 ml LoBind tube. **This is your library** (total volume when multiplexing 24 samples: 24x10 µl + 24x1.5µl = **276 µl**).
- ☐ Continue to Step 3. Completing your library prep (**page 14**).

# 2.B. Barcoding using PCR tubes/plates

- ☐ Transfer the calculated amount of genomic DNA per sample (**sample volume**) into 0.2 ml thin-walled PCR tubes/PCR plate 96.
- ☐ Adjust the volume of each sample to 10 µl with the calculated amount of **nuclease-free water**.
- ☐ Mix gently by pipetting up and down 10-15 times.

  NOTE
  ! If the diluted samples are not at the bottom of the tubes/wells, spin down briefly (4-6 seconds) in a benchtop centrifuge/microplate centrifuge at 200-400 rpm.

- ☐ Mix the Rapid Barcodes well by pipetting up and down and add 1.5 µl Rapid Barcodes to each tube/well containing your diluted genomic DNA from the previous step. Place the Rapid Barcodes back on ice.

  NOTE
  ! You should always use different barcodes for each sample you run on a given flow cell.

- ☐ Ensure all components are thoroughly mixed by pipetting up and down.
- ☐ Spin down briefly (4-6 seconds) in a benchtop centrifuge/microplate centrifuge at 200-400 rpm.

  Spinning ensures all liquid (i.e. reagents) collects at the bottom of the tube/well.

- ☐ Put tubes/plate in thermal cycler and run program (30ºC for 2 minutes, then 80ºC for 2 minutes and cooling at 0-4 ºC for 5 minutes).

OR

- ☐ Put tubes/plate on heating block at 30ºC for 2 minutes, transfer tubes/plate to second heating block at 80ºC for 2 minutes.
- ☐ Take tubes/plate out of thermal cycler/heating block.

  If you used a heating block, put tubes/plate on ice to cool.

- ☐ Spin down briefly (4-6 seconds) in a benchtop centrifuge/microplate centrifuge at 200-400 rpm.
- ☐ Pool all barcoded samples in a clean 1.5 ml or 2 ml LoBind tube. **This is your library** (total volume: 24x10 µl + 24x1.5µl = **276 µl**).
- ☐ Continue to Step 3. Completing your library (**page 14**).

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Step 3

# Completing your library preparation

# 3. Completing your library preparation

- ☐ Resuspend the AMPure XP Beads (AXP) by vortexing or mixing vigorously immediately before use.
- ☐ Add **276 µl** resuspended AMPure XP Beads (AXP) to the LoBind tube containing your pooled library.
- ☐ **Mix gently** by flicking the LoBind tube or pipetting up and down.
- ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.

  If you don't have a Hula mixer; rotate gently by hand.

- ☐ Prepare at least 2 ml (2,000 µl) of fresh 80% ethanol in nuclease free water by mixing:
  - ☐ 1600 µl ethanol absolute (100%)
  - ☐ 400 µl nuclease-free water
- ☐ Spin down the sample (the tube containing your pooled library).
- ☐ Place sample on magnetic rack to "pellet" the magnetic beads (containing your DNA library). Link to video tutorial here or QR code:
- ☐ Keep tube on the magnet and pipette off/discard the supernatant.
- ☐ Keep the tube on the magnet and wash the beads by:
  - ☐ Adding 1 ml (1,000 µl) 80% ethanol.
  - ☐ Rotating the tube 180º, making the beads move through the ethanol to the opposite side of the tube.
- ☐ Remove and discard the ethanol using a pipette.
- ☐ Repeat the two previous steps (washing with ethanol).
- ☐ Briefly spin down and place the tube back on the magnet. Pipette off any residual ethanol.

  NOTE
  ! If you still see droplets of ethanol on the side of the tube, spin down again and pipette off the residual ethanol.

- ☐ Allow to dry for 30 seconds, but do not dry the pellet to the point of cracking.

  The pellet should look matte black when dry.

- ☐ Remove the tube from the magnetic rack.
- ☐ Mix the Elution Buffer (EB) well by pipetting up and down.
- ☐ Resuspend the pellet in your sample by adding 15 µl Elution Buffer (EB) to your sample tube. Place the Elution Buffer (EB) back on ice.
- ☐ Incubate for 10 minutes at room temperature.

- [ ] Pellet the beads on the magnetic rack for at least 1 minute until the eluate is clear and colourless.
- [ ] Transfer 11 µl of the eluate into a clean 1.5 ml LoBind tube (tube A).

  The eluate contains the barcoded DNA library.
- [ ] Set the magnetic rack with the tube containing the leftover library aside (tube Z).

  This will be used to measure the DNA concentration after completing the library preparation.
- [ ] Mix the Rapid Adapter (RA) well by pipetting up and down.
- [ ] Mix the Adapter Buffer (ADB) well by vortexing.
- [ ] In a clean 1.5 ml LoBind tube (tube B), **dilute the Rapid Adapter** (RA) as follows:
  - [ ] 1.5 µl Rapid Adapter (RA)
  - [ ] 3.5 µl Adapter Buffer (ADB)
- [ ] Place the Rapid Adapter (RA) and Adapter Buffer (ADB) back on ice.
- [ ] Add 1 µl of the **diluted Rapid Adapter** (tube B) to the barcoded DNA (tube A).
- [ ] Mix gently by flicking the tube, and spin down briefly (4-6 seconds) in a benchtop centrifuge at 200-400 rpm.
- [ ] Incubate the reaction for 5 minutes at room temperature.

  This is your prepared library (total volume 12 µl).
- [ ] Keep the **library on ice** until ready for loading into the flow cell.

  The library can also be stored in the freezer until ready for loading into the flow cell. You can store the library for up to approximately 24 hours.

## Measure DNA concentration of your library

- [ ] Use the “Measuring DNA concentration using Qubit” protocol to check the DNA concentration of your eluted DNA library from the leftover library on the magnet (tube Z).

NOTE

! If you do not have 2 µl of library left in tube Z on the magnet, only use 1 µl of sample and adjust the protocol accordingly.

! This step is part of quality control to make sure the library preparation was successful. If it was not successful, this step prevents you from proceeding to sequencing, hence avoiding sequencing in vain.

! The DNA concentration of your library should be at least 20 ng/µl for you to proceed with WGS.

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Step 4

# Priming & loading the MinION flow cell

# Step 4. Priming and loading the MinION flow cell

☐ Before priming and loading the flow cell for the first time we recommend watching this video tutorial: via this link or QR code ⟶

NOTE

! We recommend keeping the Flow cell in the MinION device after flow cell check and loading the flow cell while still inserted in the MinION.

☐ Thaw frozen sequencing kit components at room temperature.

- ☐ Sequencing Buffer (SB)
- ☐ Library Beads (LIB)
- ☐ Flow Cell Tether (FCT)
- ☐ Flow Cell Flush (FCF)

☐ Vortex or mix by pipetting the sequencing kit components as described in the table below.

| Reagent | Thaw at room temperature (22-28°C) | Keep on ice throughout library prep workflow | Mix well by vortexing |
|---|---|---|---|
| Sequencing Buffer (SB) | ✓ | ✓ | ✓ |
| Library Beads (LIB) | ✓ | ✓ | Mix by pipetting or vortexing *immediately before use* |
| Flow Cell Tether (FCT) | ✓ | ✓ | ✓ |
| Flow Cell Flush (FCF) | Not frozen | ✓ | ✓ |

☐ Briefly spin down sequencing kit components using microcentrifuge.

☐ Store sequencing kit components **on ice**.

NOTE

! If the reagents are already at the bottom of the tube, you do not need to spin the tubes down unless there is not enough reagent otherwise.

☐ To prepare the flow cell priming mix, combine the following reagents in a fresh 1.5 ml LoBind tube:

- ☐ 1,170 µl Flow Cell Flush (FCF)
- ☐ 30 µl Flow Cell Tether (FCT)

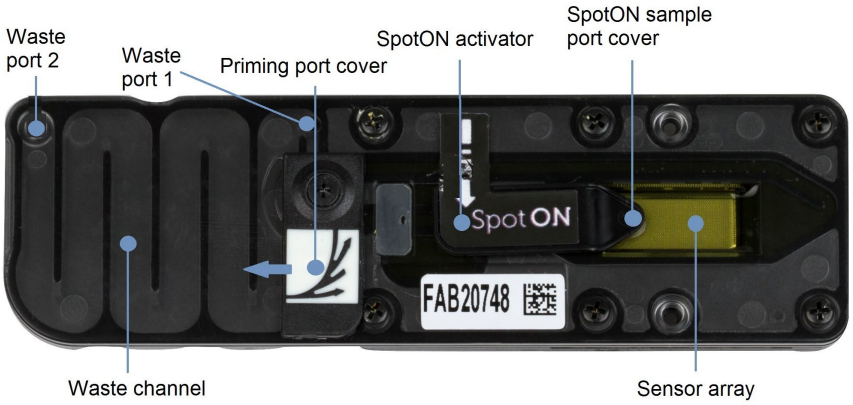



*Figure from Oxford Nanopore.*

- ☐ Open the MinION device lid and slide the flow cell under the clip.
- ☐ Press down firmly on the flow cell to ensure correct thermal and electrical contact.
- ☐ Slide the flow cell priming port cover clockwise to open the priming port.

  NOTE

  ! It is important to slide the priming port cover all the way open (approx. 90°).

- ☐ After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles by following these steps:
  - ☐ Set a P1000 pipette to 200 µl.
  - ☐ Insert the pipette tip into the priming port.
  - ☐ Turn the wheel until the dial shows 220-230 µl, to draw back 20-30 µl of buffer, or until you can see a small volume of buffer entering the pipette tip.

  NOTE

  ! Visually check that there is continuous buffer from the priming port across the sensor array. Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the pore is always covered by buffer. Introducing air bubbles into the array can irreversibly damage pores.

- ☐ Load 800 µl of the priming mix into the flow cell via the **priming port** using a P1000 pipette:
  - ☐ Place pipette tip in priming port.
  - ☐ Turn the wheel of the pipette until you cannot turn further or there is only a small volume of liquid left in the tip of the pipette.
  - ☐ Discard any remaining liquid in the tip along with the tip, to ensure no air bubbles are introduced.
- ☐ Wait for five minutes.

  NOTE

  ! During the five-minute waiting time, prepare the library for loading by following the next step.

- ☐ In the 1.5 ml LoBind tube containing your library (tube A) add the following:
  - ☐ 37.5 µl Sequencing Buffer (SB)
  - ☐ 25.5 µl Library Beads (LIB) mixed immediately before use by pipetting

    NOTE

    ! Flick the tube with Library Beads (LIB) hard to dislodge the beads from the bottom before mixing by pipetting.
- ☐ Once the five minutes have passed, complete the flow cell priming:
  - ☐ Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
  - ☐ Load 200 µl of the priming mix into the flow cell **priming port** (**NOT** the SpotON sample port), avoiding the introduction of air bubbles by turning the wheel of the pipette as described above.

    NOTE

    ! It is important to leave the priming port fully open while loading the library.
- ☐ Mix the prepared library gently by pipetting up and down just prior to loading.
- ☐ Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion without touching the port with the pipette.

  NOTE

  ! Touching the SpotON port with your pipette tip can cause irreversible damage. To prevent this, guide the pipette by placing a finger above the tip.

  ! Ensure each drop flows into the port before adding the next.
- ☐ Gently replace the SpotON sample port cover, making sure the bung enters the SpotON sample port, then close the priming port.
- ☐ Place the light shield onto the flow cell, as follows:
  - ☐ Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.

    NOTE

    ! Do not force the light shield underneath the clip. The bung is part of the SpotON sample cover which goes into the SpotOn port.
- ☐ Close the lid of the MinION device and set up a sequencing run on MinKNOW.

If you encounter any issues, please look at the ONT troubleshooting guide found here: via this link and QR code (below)

# Protocol

## Data analysis using EPI2ME's Bacterial Genomes wf

for Sentinel Sites in SeqAfrica

V. 1.0

| Version | Date | Author | Edits | Contact |
|---|---|---|---|---|
| 1.0 | 04.11.2024 | Niamh Lacy-Roberts, Natasia R. Thornval and Pernille Nilsson (design) | | nlac@food.dtu.dk; nareth@food.dtu.dk; pnil@food.dtu.dk |

# Table of contents

# Summary

This protocol is built around sequence data analysis using the open-analysis platform EPI2ME created by Oxford Nanopore Technologies to analyse ONT sequencing data. The analysis platform is compatible with macOS, Windows, and Linux operating systems and can be installed on laptops, desktop computers, cluster or cloud services and can run locally (no internet needed) or in the cloud (internet required).

The EPI2ME website contains a quick start guide as well as troubleshooting guide and other resources which you can access through this and this link, respectively or the QR codes below.

This protocol covers the Bacterial Genomes Workflow in EPI2ME. The Bacterial Genomes Workflow performs *de novo* or reference-based assembly of bacterial genomes, annotation of regions of interest within the assembly, species identification and sequence typing, and identification of AMR genes.

Quick start guide

Troubleshooting guide

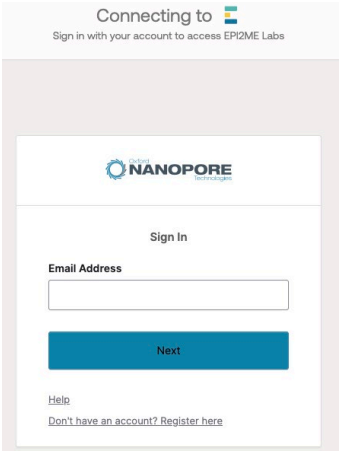
# Preparing for analysis

Before you can start analysing you data you need to

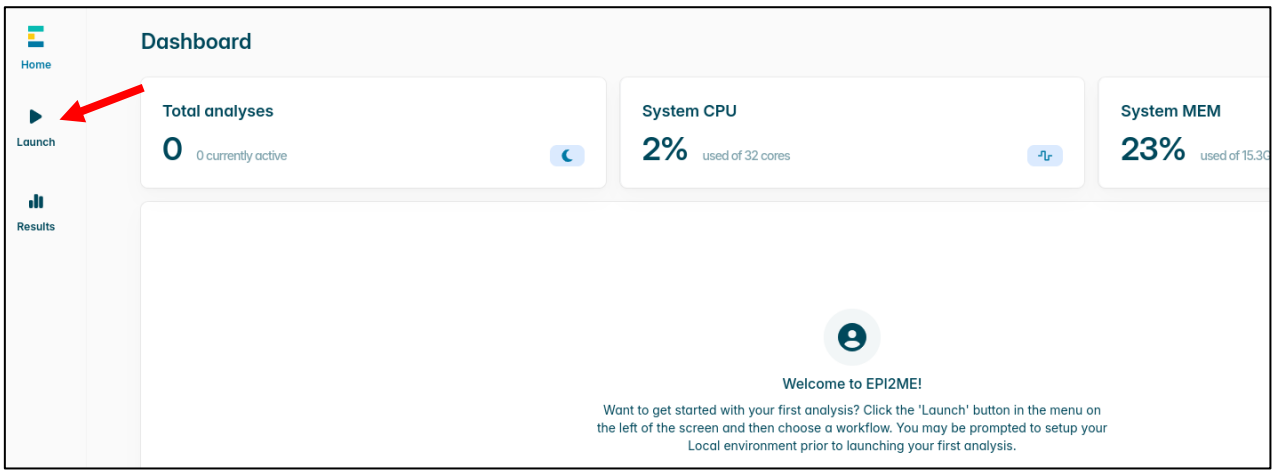
- Make sure that EPI2ME is installed and working on your laptop.
- You have created an account with login credentials that can be accessed by any team member performing the analysis.

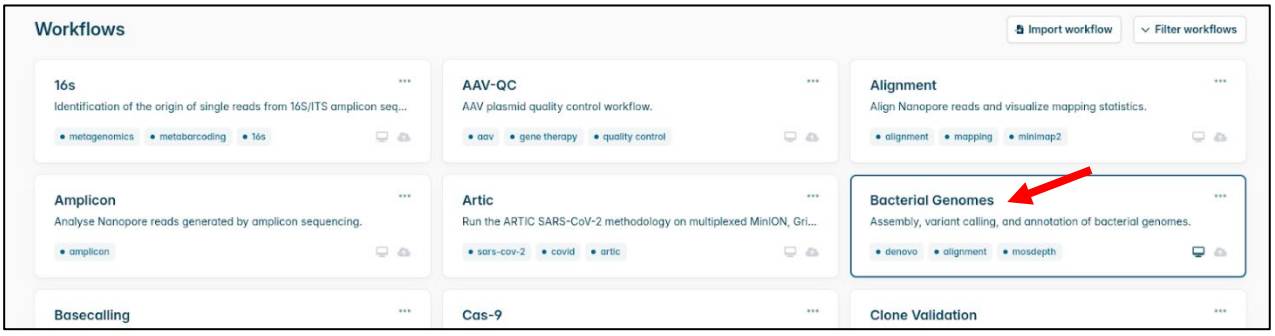
# Running the analysis

1. **Open the Epi2me Desktop App**
   - Launch the Epi2me app on your laptop/computer.
   - Ensure that you are logged in with your user credentials.



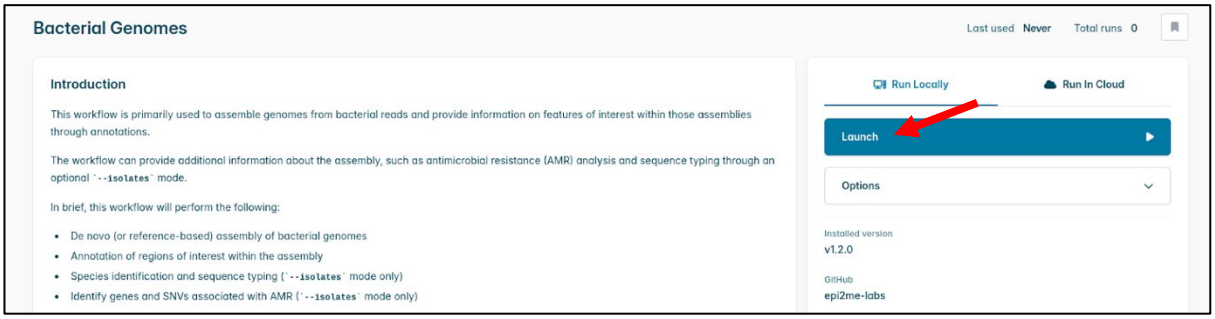

2. **Select the Bacterial Genomes Workflow**
   - From the dashboard click on **“Launch”** in the left side bar.
   - Then select the **"Bacterial Genomes"** workflow.

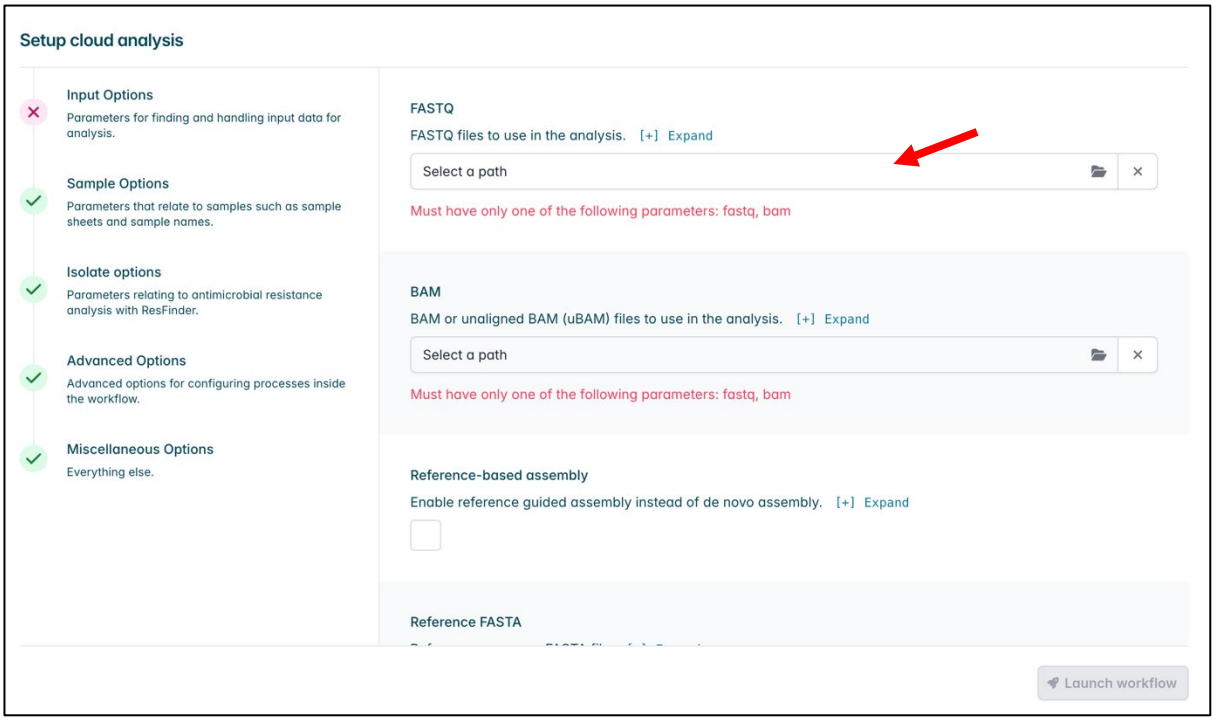


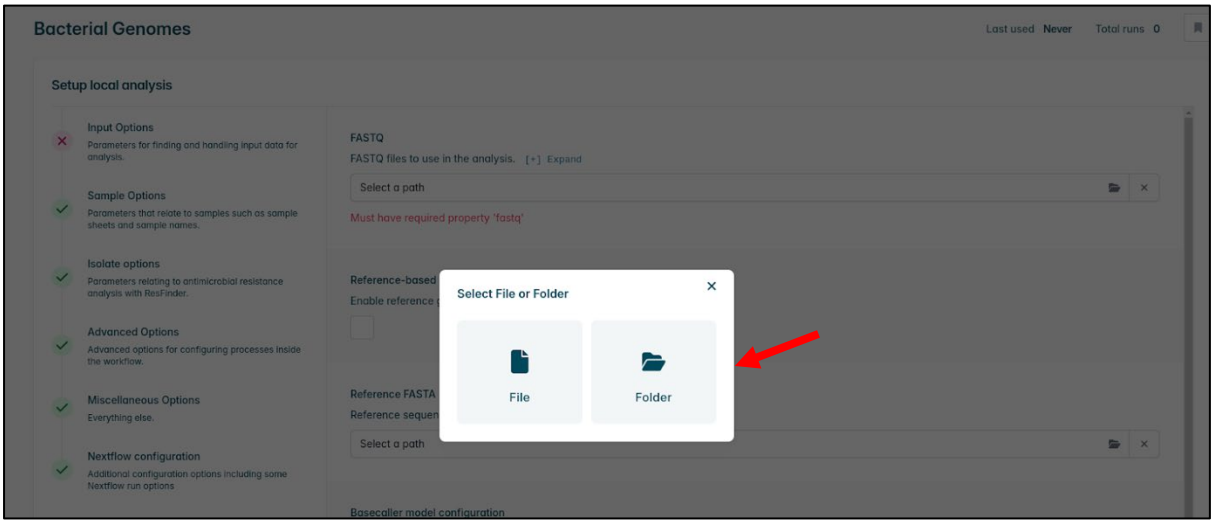



### 3. Upload Sequence Data Files

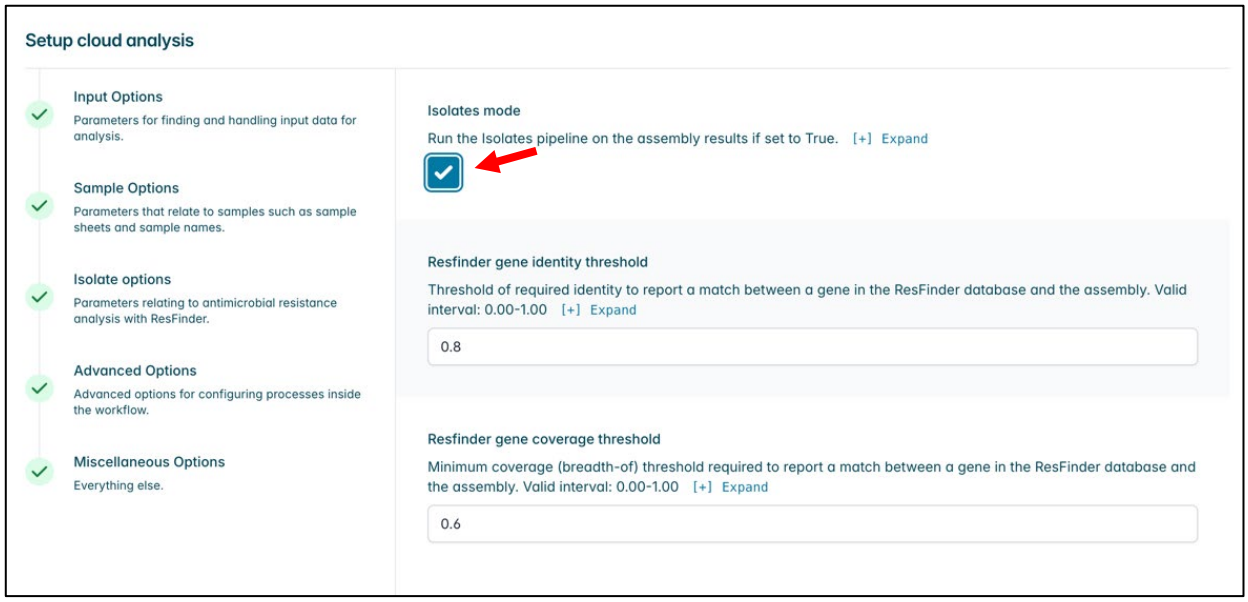
- In the workflow, click **“Launch”** under **“Run Locally”**. Alternatively, you can run the workflow in the cloud.
- In **“Input Options”**, click on **"Select a path"**. Then you can either upload a single fastq file, or a folder containing multiple fastq files.



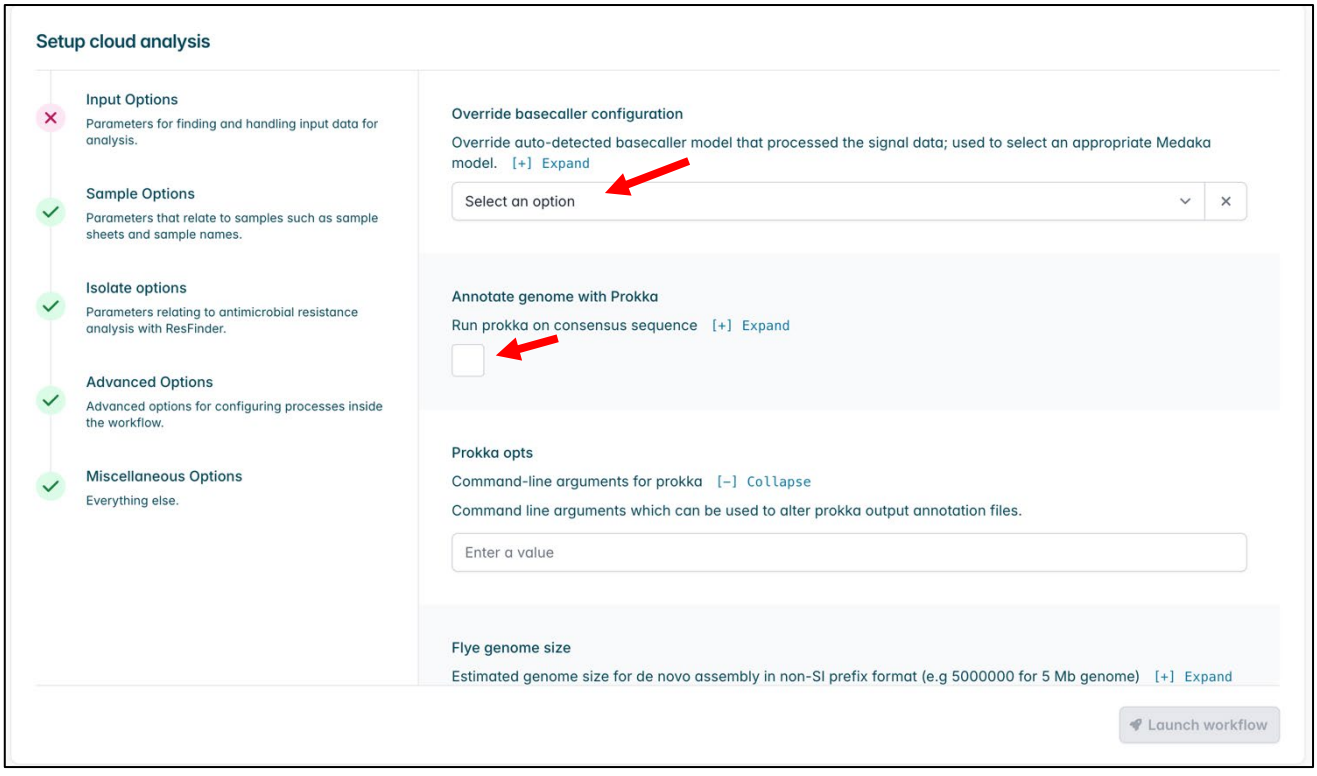



4. **Check and Enable Isolate Mode**
   - Click on **"Isolate Options"**.
   - Check the box for **"Isolate Mode"**. This mode enables MLST and AMR calling.

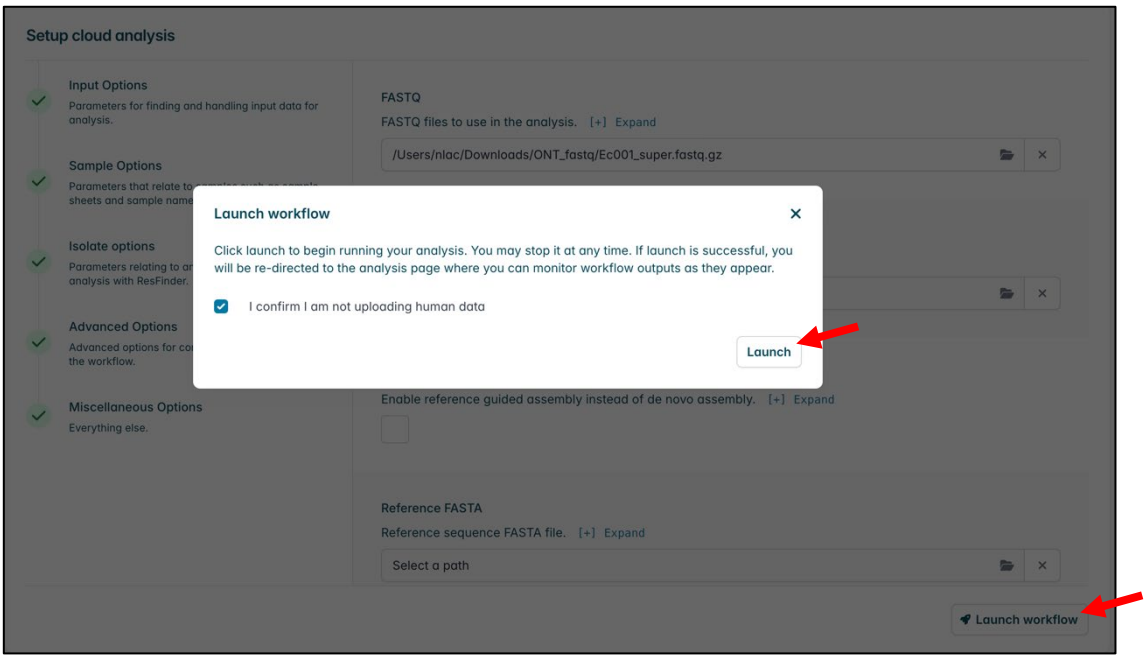


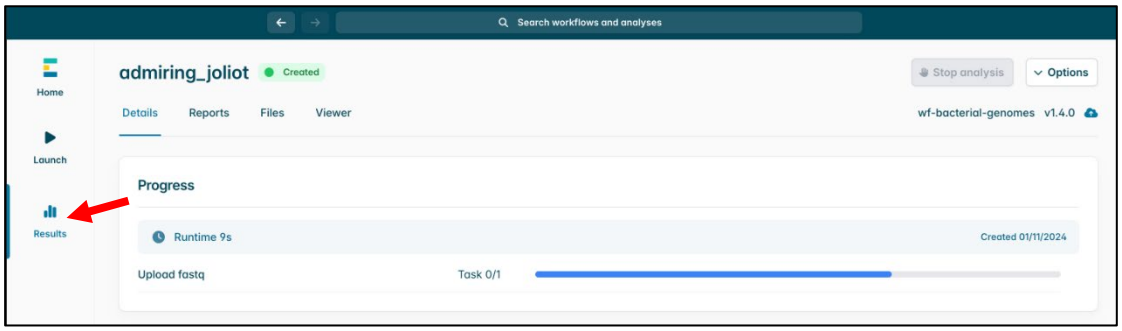

5. **Adjust Advanced Options (Optional)**
   - To speed up the analysis, you can choose to disable the **"Annotate Genome with Prokka"** option in the Advanced Options section.
   - The basecalller configuration is automatically determined, but you can choose to select the model used during your sequencing run instead.
   - You can also change the minimum read length at the bottom of the **“Advanced Options page”**. The default is 1000, and any reads below this threshold will be removed from analysis.



## 6. Start the Workflow

- After configuring options, click on **"Launch Workflow"** in the lower right hand corner.
- After you have launched the workflow, you will automatically be redirected to the **“Results”** page, and you can see the workflow progress under **“Details”**.



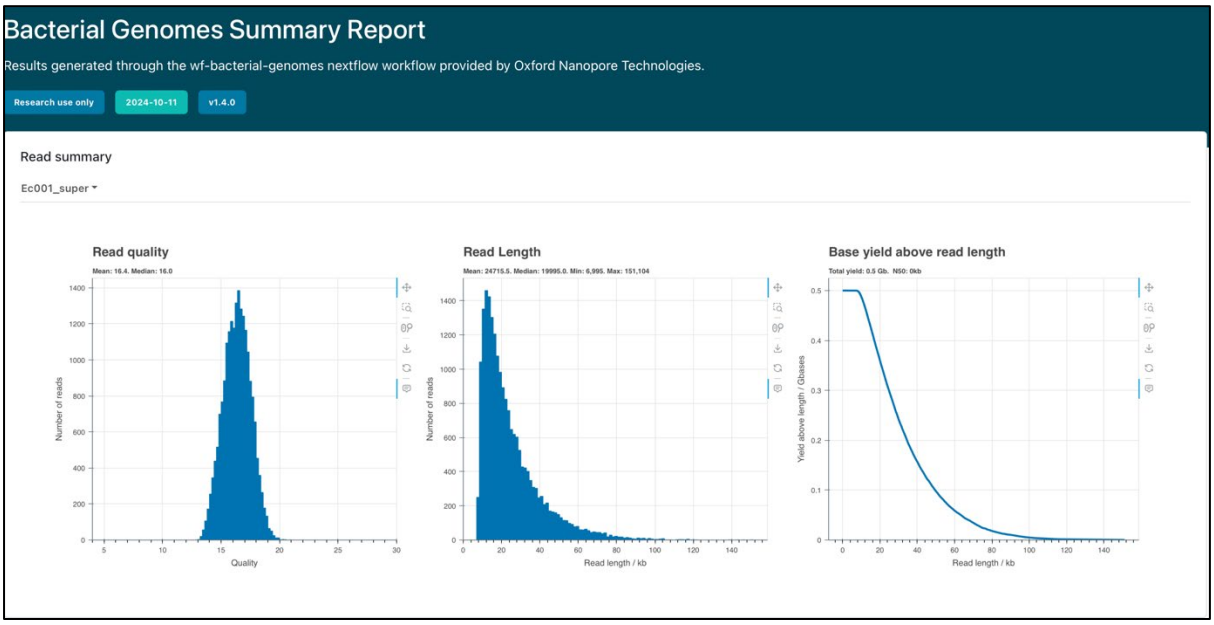




## 7. Review the Results

- Once the workflow is complete, you can click on **“Reports”** to view the analysis results.
- You can also click on **“Files”** to download the assembled FASTA file.

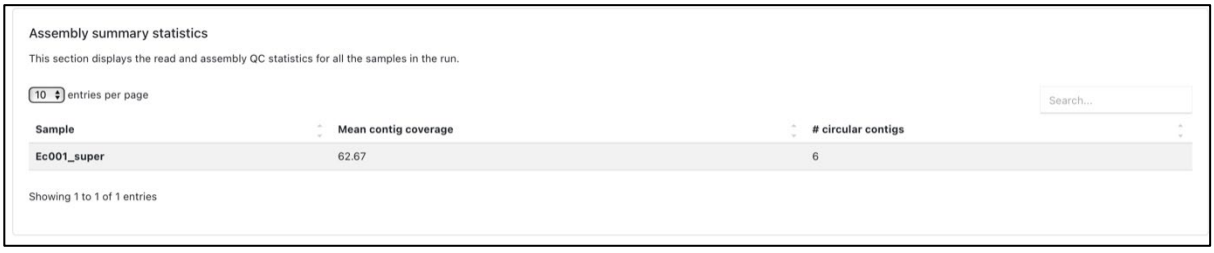
# Report Interpretation

Below are screenshots of sections from an exmaple report and explainations of the figures.



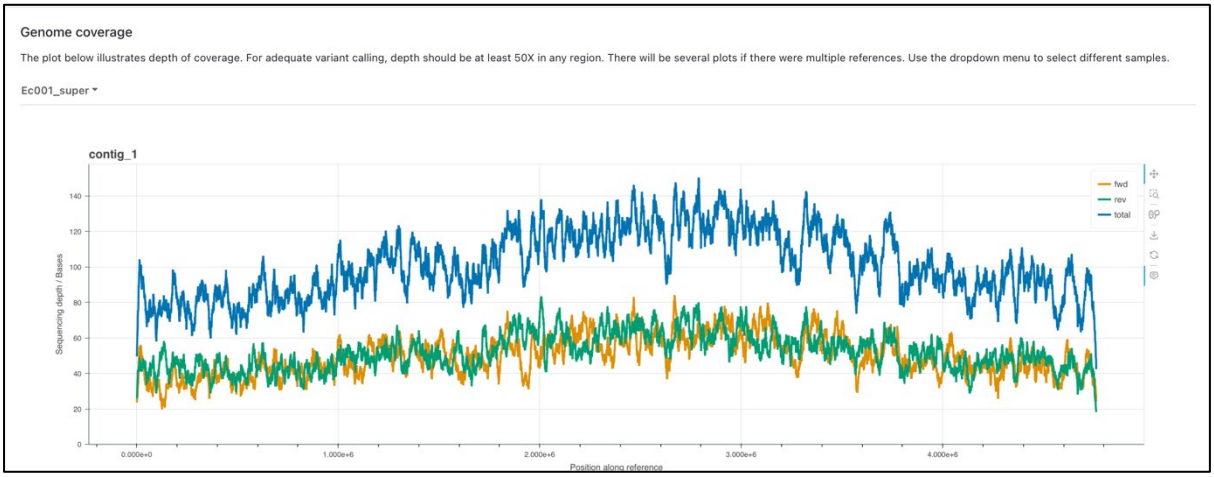

## A. Read Quality, Read Length, and Base Yield

- **Read Quality Plot:** Shows the distribution of read quality scores. A high mean and median quality score indicate reliable data.
- **Read Length Plot:** Displays the distribution of read lengths. Longer reads are generally more useful for genome assembly and also indicate higher quality data.
- **Base Yield Above Read Length Plot:** Indicates cumulative base yield across different read lengths. High yield at longer lengths suggests robust sequencing output.



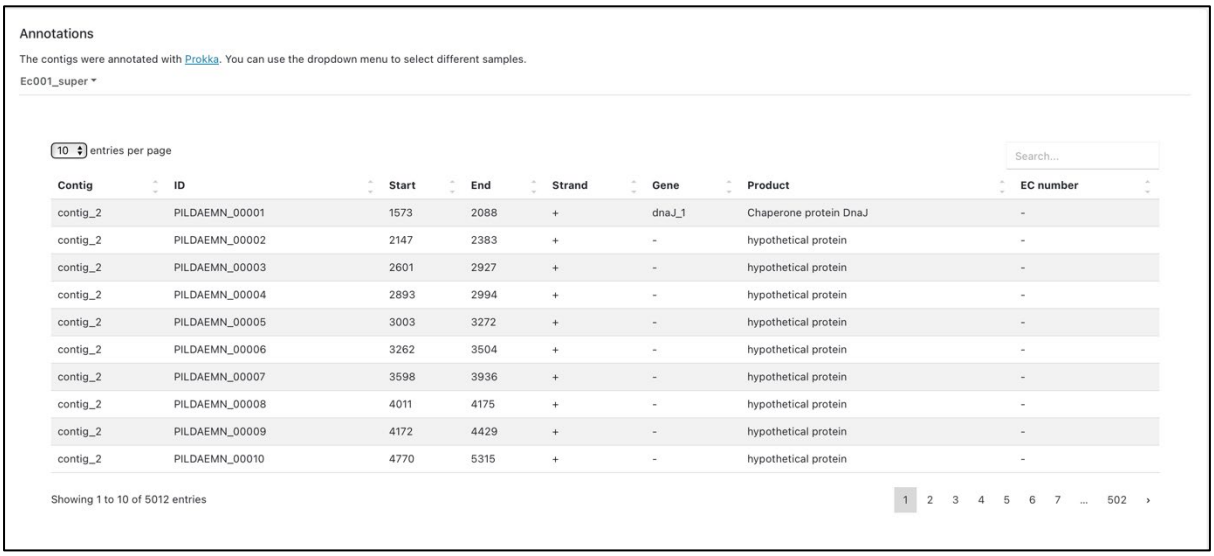

## B. Assembly Summary Statistics

- **Mean Contig Coverage:** The average depth of coverage across contigs; higher coverage suggests more reliable data.
- **# Circular Contigs:** Number of circular contigs detected, which may indicate plasmids that usually harbour AMR genes.




## C. Genome Coverage

- **Depth of Coverage Plot:** Illustrates sequencing depth across the genome. For reliable variant calling, depth should be at least 30x. A uniform distribution indicates good data quality.

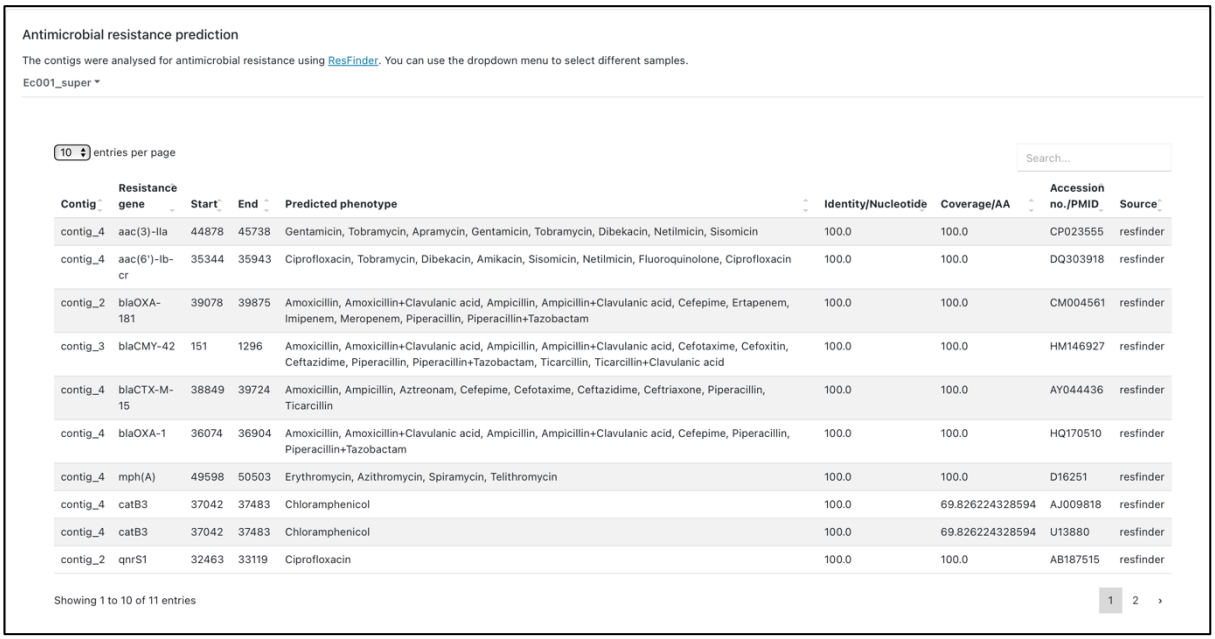



| Contig | ID | Start | End | Strand | Gene | Product | EC number |
|---|---|---|---|---|---|---|---|
| contig_2 | PILDAEMN_00001 | 1573 | 2088 | + | dnaJ_1 | Chaperone protein DnaJ | - |
| contig_2 | PILDAEMN_00002 | 2147 | 2383 | + | - | hypothetical protein | - |
| contig_2 | PILDAEMN_00003 | 2601 | 2927 | + | - | hypothetical protein | - |
| contig_2 | PILDAEMN_00004 | 2893 | 2994 | + | - | hypothetical protein | - |
| contig_2 | PILDAEMN_00005 | 3003 | 3272 | + | - | hypothetical protein | - |
| contig_2 | PILDAEMN_00006 | 3262 | 3504 | + | - | hypothetical protein | - |
| contig_2 | PILDAEMN_00007 | 3598 | 3936 | + | - | hypothetical protein | - |
| contig_2 | PILDAEMN_00008 | 4011 | 4175 | + | - | hypothetical protein | - |
| contig_2 | PILDAEMN_00009 | 4172 | 4429 | + | - | hypothetical protein | - |
| contig_2 | PILDAEMN_00010 | 4770 | 5315 | + | - | hypothetical protein | - |

1 2 3 4 5 6 7 ... 502 ›

## D. Annotations (Optional if Prokka is Enabled)

- **Gene Annotations:** Lists identified genes with details like start and end positions, product descriptions, and enzyme commission (EC) numbers. This section provides insights into the gene and functions.
- **Note:** If you turned off Prokka annotation in Advanced Options, this section will not be generated, which speeds up the analysis.



Antimicrobial resistance prediction

The contigs were analysed for antimicrobial resistance using ResFinder. You can use the dropdown menu to select different samples.

Ec001_super

10 entries per page

Search...

| Contig | Resistance gene | Start | End | Predicted phenotype | Identity/Nucleotide | Coverage/AA | Accession no./PMID | Source |
|---|---|---|---|---|---|---|---|---|
| contig_4 | aac(3)-IIa | 44878 | 45738 | Gentamicin, Tobramycin, Apramycin, Gentamicin, Tobramycin, Dibekacin, Netilmicin, Sisomicin | 100.0 | 100.0 | CP023555 | resfinder |
| contig_4 | aac(6')-Ib-cr | 35344 | 35943 | Ciprofloxacin, Tobramycin, Dibekacin, Amikacin, Sisomicin, Netilmicin, Fluoroquinolone, Ciprofloxacin | 100.0 | 100.0 | DQ303918 | resfinder |
| contig_2 | blaOXA-181 | 39078 | 39875 | Amoxicillin, Amoxicillin+Clavulanic acid, Ampicillin, Ampicillin+Clavulanic acid, Cefepime, Ertapenem, Imipenem, Meropenem, Piperacillin, Piperacillin+Tazobactam | 100.0 | 100.0 | CM004561 | resfinder |
| contig_3 | blaCMY-42 | 151 | 1296 | Amoxicillin, Amoxicillin+Clavulanic acid, Ampicillin, Ampicillin+Clavulanic acid, Cefotaxime, Cefoxitin, Ceftazidime, Piperacillin, Piperacillin+Tazobactam, Ticarcillin, Ticarcillin+Clavulanic acid | 100.0 | 100.0 | HM146927 | resfinder |
| contig_4 | blaCTX-M-15 | 38849 | 39724 | Amoxicillin, Ampicillin, Aztreonam, Cefepime, Cefotaxime, Ceftazidime, Ceftriaxone, Piperacillin, Ticarcillin | 100.0 | 100.0 | AY044436 | resfinder |
| contig_4 | blaOXA-1 | 36074 | 36904 | Amoxicillin, Amoxicillin+Clavulanic acid, Ampicillin, Ampicillin+Clavulanic acid, Cefepime, Piperacillin, Piperacillin+Tazobactam | 100.0 | 100.0 | HQ170510 | resfinder |
| contig_4 | mph(A) | 49598 | 50503 | Erythromycin, Azithromycin, Spiramycin, Telithromycin | 100.0 | 100.0 | D16251 | resfinder |
| contig_4 | catB3 | 37042 | 37483 | Chloramphenicol | 100.0 | 69.826224328594 | AJ009818 | resfinder |
| contig_4 | catB3 | 37042 | 37483 | Chloramphenicol | 100.0 | 69.826224328594 | U13880 | resfinder |
| contig_2 | qnrS1 | 32463 | 33119 | Ciprofloxacin | 100.0 | 100.0 | AB187515 | resfinder |

Showing 1 to 10 of 11 entries

1 2 ›

## E. Antimicrobial Resistance Prediction

- **AMR Gene Detection:** Lists resistance genes detected by ResFinder, with details on the predicted resistance phenotype, identity, and coverage.
- High identity and coverage percentages suggest a confident detection.
- The predicted phenotypes indicate potential resistance profiles, useful for clinical applications and surveillance.

Multilocus sequence typing

Multilocus sequencing typing was performed on contigs using MLST. Typing scheme information is available at PubMLST. You can use the dropdown menu to select different samples.

Ec001_super

10 entries per page

Search...

| Id | Scheme | Sequence type | Adk | FumC | GyrB | Icd | Mdh | PurA | RecA |
|---|---|---|---|---|---|---|---|---|---|
| Ec001_super | ecoli_achtman_4 | 410 | 6 | 4 | 12 | 1 | 20 | 18 | 7 |

Showing 1 to 1 of 1 entries

## F. Multilocus Sequence Typing (MLST)

- **Sequence Type (ST):** Provides a standardized sequence type based on allelic profiles for housekeeping genes. This is useful for strain tracking in epidemiology.
- **Allelic Profile:** Lists the specific alleles identified in the typing scheme, which collectively determine the sequence type.