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Sample collection, preparation, and sequencing protocols for developing metagenomic approaches in skin and wound microbiome analysis

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Merideth Freiheit¹, Dave H Lunt¹, Graham S Sellers¹

¹University of Hull, UK



Merideth Freiheit

Student

 Sample collection, preparation, and sequencing protocols for developing metagenomic approaches in skin and wound microbiome analysis

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We use this protocol and it's working

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Abstract

This protocol outlines the collection, preparation, and sequencing of skin and wound microbiome samples for metagenomic analysis, including swab-based sampling, optional culturing, DNA extraction and quantification, mock-community construction, and preparation of low-biomass clinical specimens. It further details library preparation, nanopore sequencing setup, and the configuration of adaptive sequencing workflows using ReadFish, including recommended playback runs for initial optimisation.

Using these procedures, high-quality DNA can be obtained from skin, wound, and mock samples despite low microbial biomass and high host content. The expected outcomes include consistent recovery of microbial DNA suitable for metagenomic sequencing, stable community profiles across replicates, and effective implementation of adaptive sequencing strategies that enable real-time host depletion or targeted enrichment when computational and reference conditions allow.

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Guidelines

- This protocol integrates multiple workflows (sample collection, DNA extraction, quality control, library preparation, and sequencing). Users should review all sections before beginning to ensure correct planning and reagent availability.
- Maintain strict sterile technique throughout to prevent contamination, especially when working with low-biomass samples such as skin swabs.
- Keep all samples and DNA preparations consistently labelled, and record volumes, dilutions, and barcodes carefully to avoid downstream misidentification.
- Use low-retention plasticware and high-sensitivity quantification methods to minimize loss of DNA and ensure accurate measurements.
- Follow institutional biosafety procedures for handling human materials, clinical isolates, and BSL-2 organisms.
- Ensure that all equipment (pipettes, centrifuges, thermocyclers, nanopore devices) is calibrated and functioning before starting long workflows.
- Many steps in the protocol allow for pause points; plan timing accordingly, especially for multi-day experiments or when batching samples.
- Store all extracted DNA, intermediate products, and final libraries under appropriate conditions to maintain integrity and avoid repeated freeze–thaw cycles.
- Before sequencing, confirm that required software, drivers, and configuration files are installed and compatible with your instrument and operating system.
- Maintain detailed notes on any deviations from the protocol in case troubleshooting or repeatability assessments are needed later.

Materials

A. GENERAL MATERIALS & EQUIPMENT

(Used across most sections; not repeated in section-specific lists)

General Reagents

- Nuclease-free water
- 70% ethanol (for workspace cleaning)
- 10% bleach or equivalent disinfectant (for workspace cleaning)
- Ice (crushed or flaked)

General Consumables

- Low-retention pipette tips (P2, P10, P20, P100, P200, P1000; sterile filter tips strongly recommended)
- DNA LoBind microcentrifuge tubes (1.5 mL)
- Standard 1.5 mL and 2.0 mL microcentrifuge tubes (sterile)
- 0.2 mL PCR tubes (low-retention recommended)
- Parafilm
- Gloves (powder-free, nitrile)
- Lab coat and appropriate PPE
- Kimwipes or lint-free tissues
- Permanent marker for tube labeling
- Tube racks for PCR tubes and microcentrifuge tubes
- Waste container for used tips and tubes

General Equipment

- Calibrated pipettes (P2, P10, P20, P100, P200, P1000)
- Benchtop microcentrifuge
- Mini-centrifuge for quick spins
- Vortex mixer
- Bench timer
- Class II biological safety cabinet (required for cell culture steps)
- Benchtop heating block / thermomixer (up to 55 °C)
- Ice bucket
- Standard laboratory workspace with sterile working area

B. SECTION-SPECIFIC MATERIALS

Section 1: Skin sample collection

Reagents

- Sterile 0.9% NaCl (normal saline)

Consumables

- Sterile FLOQSwabs® (Copan)
- Sterile 1.5 mL microcentrifuge tubes
- Ruler or template for marking ~3.8 cm² area (optional)



Equipment

- Ice box or cold block for transport/storage

Section 2: Culturing & human cell harvesting

Reagents

- Nutrient agar plates (sterile)
- PBS (sterile)
- Non-enzymatic dissociation buffer (HBSS + EDTA; "HBSE")
- Cell culture medium (complete, supplied by maintaining lab)

Consumables

- Cryobead bacterial stocks (−80 °C)
- Sterile inoculating loops (plastic)
- Sterile FLOQSwabs®
- 1.5 mL sterile microcentrifuge tubes
- 15 mL conical tubes

Equipment

- Incubator (typically 37 °C)
- Bunsen burner or sterile workspace
- Class II biosafety cabinet (for cell harvesting)
- Centrifuge capable of 300 × g

Section 3: DNA extraction (muDNA protocol)

(All reagent compositions included as part of the protocol's stock/working solution recipes)

Reagents

Stock solutions (for preparation of MU-DNA protocol):

- 5 M NaOH
- 1 M Tris-HCl pH 8
- 0.5 M EDTA pH 8
- 5 M ammonium acetate
- 180 mM aluminium ammonium sulfate
- 3% CaCl₂
- 5 M NaCl
- 50% PEG-8000
- 10% Tween-20

Working solutions:

- Proteinase K dilution: Add 5 µL of Proteinase K (20 mg/mL) to 995 µL ddH₂O. Invert to mix thoroughly.
- Lysis Solution: Add 8.7 g trisodium phosphate dodecahydrate and 0.2 g sodium chloride to bottle. Add 70 ml ddH₂O, 6.7 ml 1 M Tris HCl (pH 8) and 5.3 ml 0.5 M EDTA (pH 8). Place on Hula Mixer until all solids dissolve. Add 2.5 ml 5 M HCl to adjust to pH 9. Bring to 100 ml final volume with ddH₂O. Invert to mix.
- Water Lysis Additive: To 93.75 mL (15 volumes) ddH₂O add 6.25 mL (1 volume) 20% SDS. Vortex briefly to mix.
- Flocculant Solution: To 50 ml 5 M Ammonium acetate add 25 ml 180 mM Aluminium etc.. Invert to mix before adding 25 ml 3% Calcium chloride. Invert to mix.
- Wash Solution (80% ethanol): To 80 ml 100% ethanol add 20 ml ddH₂O. Invert to mix.



- Elution Buffer: To 1 ml 1 M Tris HCl (pH 8) and 0.2 ml 0.5 M EDTA (pH 8). Bring to 100 ml final volume with ddH₂O. Invert to mix.
- DNA Extraction Bead Solution: Mix 100 µl 1 M Tris HCl (pH 8), 20 µl 0.5 M EDTA (pH 8) and 3.2 ml 5 M NaCl. Add 4 ml 50% PEG 8000 and invert to mix. Add 2.53 ml ddH₂O. Invert to mix thoroughly. Add 50 µl of 10% Tween 20 then add 100ul prepared Bead suspension (see below). Place on Hula Mixer until mixed thoroughly.
- Bead suspension preparation (Sera-Mag SpeedBeads):
 1. Allow Sera-Mag SpeedBeads aliquot to reach room temperature.
 2. Vortex thoroughly to resuspend beads. Centrifuge briefly to remove droplets from tube lid.
 3. Place on magnetic stand until supernatant is completely clear and beads are bound towards magnet. This should take approximately ten minutes but can take longer.
 4. While on the stand carefully remove and discard supernatant without disturbing beads.
 5. Add 500 µl ddH₂O. Vortex tube to resuspend beads. Centrifuge briefly to remove droplets from tube lid.
 6. Place on magnetic stand until supernatant is completely clear and beads are bound towards magnet. This should take approximately ten minutes but can take longer.
 7. While on the stand carefully remove and discard supernatant without disturbing beads.
 8. Repeat steps 5 to 7 three more times.
 9. Add Elution Buffer to match the starting volume of aliquot. Vortex tube to resuspend beads. Centrifuge briefly to remove droplets from tube lid.
- 10. Bead suspension can now be added to the bead solution

Consumables

- Sera-Mag SpeedBeads aliquots
- Sterile garnet grit

Equipment

- Magnetic stand
- HulaMixer or similar for bead mixing
- Heating block (for dissolving solids)

Section 4: DNA quality control

Reagents

- Agarose powder
- 10× sodium borate buffer (diluted for electrophoresis)
- GelRed™ nucleic acid stain
- Thermo Scientific GeneRuler DNA Ladder (1 kb or equivalent)
- Loading dye
- Qubit dsDNA HS Assay Kit
- Nuclease-free water

Consumables

- Gel casting tray & comb
- Electrophoresis tank
- Qubit assay tubes
- Parafilm

Equipment

- Microwave



- Electrophoresis power supply
- UV or blue-light transilluminator
- Qubit 3.0 or Qubit 4 fluorometer
- NanoDrop spectrophotometer

Section 5: Mock community samples

Reagents

- Oral Microbiome Genomic Mix (ATCC MSA-1004)
- Normalized DNA from: *E. coli* NCTC 13351, Clinical *E. coli* isolate, *S. aureus* NCTC 13373, Clinical *S. aureus* isolate, Clinical *S. epidermidis* isolate
- Normalized human genomic DNA (A549 pellet)
- Elution buffer (from extraction)

Consumables

- Low DNA-binding 1.5 mL tubes

Equipment

- Mini-centrifuge
- Vortex mixer

Section 6: Skin microbiome samples

Reagents

- Extracted skin DNA (Section 1 & 3)
- Normalized DNA from: *E. coli* NCTC 13351, Clinical *E. coli* isolate, *S. aureus* NCTC 13373, Clinical *S. aureus* isolate
- Elution buffer

Consumables

- Low DNA-binding 1.5 mL tubes

Equipment

- Mini-centrifuge
- Ice bucket

Section 7: Library preparation (SQK-RPB004)

Reagents

- ONT Rapid PCR Barcoding Kit (SQK-RPB004): Fragmentation Mix (FRM), Rapid Barcodes (RLB01–RLB12a)
- LongAmp Taq 2× Master Mix
- Library Prep Bead Solution: Mix 100 µl 1 M Tris HCl (pH 8), 20 µl 0.5 M EDTA (pH 8) and 3.2 ml 5 M NaCl. Add 4 ml 50% PEG 8000 and invert to mix. Add 2.43 ml ddH₂O. Invert to mix thoroughly. Add 50 µl of 10% Tween 20 then add 200ul prepared Bead suspension (see above). Place on Hula Mixer until mixed thoroughly.
- Lysis Solution: Add 8.7 g trisodium phosphate dodecahydrate and 0.2 g sodium chloride to bottle. Add 70 ml ddH₂O, 6.7 ml 1 M Tris HCl (pH 8) and 5.3 ml 0.5 M EDTA (pH 8). Place on Hula Mixer until all solids dissolve. Add 2.5 ml 5 M HCl to adjust to pH 9. Bring to 100 ml final volume with ddH₂O. Invert to mix.
- Tissue Lysis Additive: To 600 ml (2 volumes) ddH₂O add 300 ml (1 volume) 20% SDS. Invert to mix.
- Proteinase K
- Wash Solution: To 80 ml 100% ethanol add 20 ml ddH₂O. Invert to mix.



- Library Buffer: To 1 ml 1 M Tris HCl (pH 8) and 1 ml 5 M NaCl. Bring to 100 ml final volume with ddH₂O. Invert to mix.
- Gel electrophoresis reagents (as per Section 4)
- Qubit dsDNA HS reagents
- Nuclease-free water

Consumables

- DNA LoBind 1.5 mL tubes
- 0.2 mL PCR tubes
- Magnetic-stand compatible tubes

Equipment

- Thermal cycler
- Magnetic stand
- Qubit fluorometer
- Gel electrophoresis system + transilluminator
- Bench centrifuge & mini-spin
- Vortex mixer
- 55 °C heating block

Section 8: Flow cell priming and loading

Reagents

- ONT Rapid PCR Barcoding Kit (SQK-RPB004): Sequencing Buffer (SQB), Loading Beads (LB), Flush Buffer (FB), Flush Tether (FLT), Rapid Adapter (RAP)
- Library Buffer: To 1 ml 1 M Tris HCl (pH 8) and 1 ml 5 M NaCl. Bring to 100 ml final volume with ddH₂O. Invert to mix.

Consumables

- DNA LoBind microcentrifuge tubes
- 0.2 mL tubes (for RAP ligation)
- Low-retention pipette tips
- Parafilm (optional)
- Kimwipes
- Ice bucket

Equipment

- Oxford Nanopore MinION or GridION device
- Computer running MinKNOW
- Vortex mixer
- Microcentrifuge
- Pipettes
- Flongle Flow Cell (FLO-FLG001 or FLO-FLG114)
- Flongle Adapter
- MinION Flow Cell (FLO-MIN106 R9.4.1 or R10.4) (if used)

Section 9: Sequencing & adaptive runs

Hardware



- MinION Mk1B
- Flongle Adapter
- Flongle flow cells
- MinION flow cells (if required)
- GridION Mk1 (for ONT in-house adaptive sequencing)
- GPU-enabled computer (for Guppy server)
- External SSD or sufficient high-speed storage

Software

- MinKNOW (v5.x)
- Guppy basecaller
- ReadFish
- ReadBouncer
- Minimap2
- Python + required environments
- Bash shell

Files / Configurations

- ".toml" ReadFish configuration files
- MinKNOW configuration "sequencing_MIN106_DNA.toml"
- Guppy configuration "dna_r9.4.1_450bps_fast.cfg"
- Reference FASTA files and ".mmi" indexes
- Log file paths

Protocol materials

☒ Sterile Saline (0.9% NaCl)

☒ PBS Invitrogen - Thermo Fisher Catalog #2610807

☒ Cell Dissociation Buffer enzyme-free Hanks Balanced Salt Solution **Thermo Fisher Scientific Catalog #13150016**

☒ GeneRuler 1 kb DNA Ladder **Thermo Scientific Catalog #SM0312**

☒ LongAmp Taq 2X Master Mix - 500 rxns **New England Biolabs Catalog #M0287L**



Troubleshooting

Problem

Low DNA yield from skin or mock samples

Solution

Ensure lysis is complete and pellets are fully resuspended at each step, use low-retention tubes and tips to minimise DNA loss, elute in pre-warmed buffer (around 37–55 °C), concentrate the end volume, and avoid repeated freeze–thaw cycles by aliquoting DNA immediately after extraction.

Problem

DNA concentration too low for tagmentation

Solution

Increase the input volume to 5 µL for the SQK-RPB004 kit when DNA is below 1 ng/µL, concentrate DNA using a bead cleanup or a speed-vac if possible, and always re-quantify with a Qubit HS assay rather than relying on NanoDrop at low concentrations.

Problem

Sequencing output drops rapidly

Solution

Check for bubbles or debris in the flow cell and consider reloading with fresh sequencing mix, confirm that the loaded library concentration is close to 2 fmol/µL, verify that no air was introduced during priming or loading, and ensure the flow cell is within expiry and has been stored at 2–8 °C.

Problem

ReadFish appears inactive

Solution

Confirm that the Guppy basecalling server is running and reachable before starting ReadFish, check that `break_reads_after_seconds` in the MinKNOW configuration is set to 0.4, verify that the paths to the ReadFish .toml file and the .mmi reference index are correct, ensure the Guppy configuration file matches the flow cell chemistry, and, if this is the first adaptive experiment, run a playback test to confirm communication between MinKNOW, Guppy, and ReadFish.

Problem

Adaptive sequencing stops mid-run

Solution

Check whether the Guppy server has crashed or lost GPU access and restart it if needed, reduce GPU load by lowering the number of runners or callers, confirm that log and output directories exist and are writable, and close other GPU-intensive applications that might be competing for resources.

Safety warnings

- ! ■ Handle all human-derived samples (skin swabs, wound isolates, human cells) under approved BSL-2 procedures and ethical approvals.
- Clinical bacterial isolates (e.g., MRSA, *E. coli*) require BSL-2 containment and strict PPE use.
- DNA extraction reagents such as guanidine thiocyanate, SDS, EDTA, and NaOH are hazardous; avoid skin/eye contact and do not mix guanidine salts with bleach.
- Flow cells are fragile, avoid air bubbles, physical shock, and aggressive pipetting when priming or loading.
- This workflow uses low-biomass samples; contamination risk is high. Use sterile consumables, filtered tips, and clean work areas.
- Use caution with heating blocks, microwaves, and thermal cyclers to avoid burns or tube rupture.
- For adaptive sequencing, verify all Guppy and MinKNOW settings before sequencing to prevent data loss.
- Dispose of chemical and biological waste according to institutional safety regulations.

Ethics statement

Ethical approval was provided by the Hull Royal Tissue ethics committee (REC: 19/NE/0150) and the Faculty Ethics Committee for the Faculty of Science and Engineering at the University of Hull.

Before start

- Review the full workflow and plan timing, equipment access, and any pause points.
- Identify which steps allow pause points and plan your timing accordingly.
- Confirm all reagents, consumables, and flow cells are available, within expiry, and stored correctly. Some require thawing or preparation in advance.
- Ensure access to required equipment: biosafety cabinet (for human materials), PCR machine, Qubit/NanoDrop, gel system, magnetic stand, nanopore device, and calibrated pipettes.
- Prepare clean, sterile workspaces. Use PPE, low-retention tubes, and filter tips to prevent contamination and DNA loss, especially with low-biomass samples.
- Verify that all samples, controls, and barcodes are clearly labelled and that a tracking system is in place.
- Ensure the computational environment is ready: updated MinKNOW, available Guppy installation, GPU functionality, and accessible configuration files.
- Check that all necessary approvals, biosafety procedures, and risk assessments for human or clinical samples are completed.

Section 1: Skin sample collection

30s

1 Collect forearm skin microbiome samples.

- 1.1 Open one FLOQSwab® package without touching the swab head.
- 1.2 Immerse the swab head in  Sterile Saline (0.9% NaCl) and gently press the swab against the inside wall.
- 1.3 Locate the designated sampling region (Position 1 or Position 2) and estimate or outline a 3.8 cm² area using a sterile ruler or template.
- 1.4 Press the pre-moistened swab onto the skin and rub firmly across the 3.8 cm² area for  00:00:30 , using both vertical and horizontal strokes while rotating the swab slowly.
- 1.5 Immediately insert the swab into a labeled 1.5 mL tube and snap or cut the shaft to allow the lid to close securely.
- 1.6 Use a new sterile FLOQSwab® and repeat Steps 1.1-1.5 for Position 1 and 2 of both forearms.
- 1.7 If proceeding directly to DNA extraction (Section 3): place tubes  On ice immediately after sampling.
If processing will occur later: store samples at  4 °C until extraction.

30s

For long-term storage before extraction, transfer samples to  -20 °C or  -80 °C .

2 Collect peri-wound microbiome samples.

Note

Peri-wound swabs used in this study were not collected by the authors. Collection was performed by trained staff at Hull University Teaching Hospitals NHS Trust according to established wound sampling procedures.

- 2.1 Peri-wound swabs were collected by trained research staff at Hull University Teaching Hospitals NHS Trust.
- 2.2 Sampling followed the standard wound swab collection procedure under ethical approval REC: 19/NE/0150 (Hull Royal Tissue Biobank).
- 2.3 Swabs were placed into clinical transport medium and processed by the clinical laboratory.
- 2.4 DNA extraction for these samples was performed independently by the clinical laboratory using the DNeasy PowerSoil Kit (Qiagen 2013-24) prior to receipt for downstream quantification and sequencing.

Equipment

DNeasy PowerLyzer PowerSoil Kit	NAME
DNA Extraction Kit	TYPE
Qiagen	BRAND
12855-50	SKU
https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/microbial-dna/dneasy-powerlyzer-powersoil-kit/	LINK

Section 2: Culturing & human cell harvesting

2d 0h 10m

3 **Revive and culture bacterial wound isolate strains.**

Note

These isolates were originally obtained from chronic antibiotic-resistant wound samples as described in Step 2 and preliminarily identified using VITEK 2 by the clinical microbiology team. Only revival and subculture steps described above were performed by the authors; initial isolation and storage were performed externally.



- 3.1 Remove the cryobead storage vial from -80 °C and keep On ice briefly to minimize thawing.
- 3.2 Quickly transfer one bead to the surface of a sterile nutrient agar plate using sterile forceps.
- 3.3 Roll the bead gently across a small central region of the agar surface to deposit bacteria.
- 3.4 Remove the bead and discard it into appropriate biohazard waste.
- 3.5 Using a sterile plastic inoculating loop, streak the deposited bacterial region using a quadrant streak method.
- 3.6 Change loops between quadrants or flame if using reusable loops.
- 3.7 Invert plates and incubate at 37 °C for 24:00:00 or until single colonies appear.
- 3.8 Visually inspect for well-isolated colonies.

Note

If growth is inadequate, which is common for some species in cryobead storage, re-streak from an isolated colony onto a fresh plate and incubate an additional 24 hours.
- 3.9 Using a sterile FLOQSwab[®], gently sweep across multiple well-isolated colonies.
- 3.10 Transfer the swab into a labeled 1.5 mL tube as described in Section 1.
- 3.11 If proceeding directly to DNA extraction (Section 3): place tubes On ice immediately after sampling.
If processing will occur later: store samples at 4 °C until extraction.
- 4 **Culture reference strains (NCTC13351 and NCTC13373).**

1d

**Note**

Reference and clinical isolates should be cultured in parallel to ensure comparable growth conditions and downstream performance.

- 4.1 Open the NCTC vial following **UKHSA instructions**.
- 4.2 Using a sterile loop, transfer a small amount of material onto a sterile nutrient agar plate.
- 4.3 Perform quadrant streaking to obtain single colonies.
- 4.4 Invert and incubate at  37 °C for  24:00:00 or until colonies are well defined.
- 4.5 Use a sterile FLOQSwab® to collect colonies and transfer to a labeled 1.5 mL tube.
- 4.6 If proceeding directly to DNA extraction (Section 3): place tubes  On ice immediately after sampling.
If processing will occur later: store samples at  4 °C until extraction.

1d

5 Collect human alveolar epithelial cells (ATCC CCL-185).**Note**

Human alveolar epithelial cells (A549; ATCC CCL-185) were maintained and passaged by a collaborating laboratory according to **Thermo Fisher Scientific protocols**. The author performed the cell-harvesting procedure described below to obtain cell pellets for DNA extraction.

Safety information

Perform all steps inside a Class II biosafety cabinet.

- 5.1 Gently pour spent medium from the flask into a Virkon/Chemsgene waste container.



- 5.2 Add  3 mL sterile  PBS Invitrogen - Thermo Fisher Catalog #2610807 to the flask.
- 5.3 Tilt and rock the flask to wash the cell monolayer.
- 5.4 Pour  PBS Invitrogen - Thermo Fisher Catalog #2610807 into the Virkon waste container.
- 5.5 Add  3 mL  Cell Dissociation Buffer enzyme-free Hanks Balanced Salt Solution Thermo Fisher Scientific Catalog #13150016 directly to the cell monolayer and replace the cap securely.
- 5.6 Tilt to ensure complete coverage.
- 5.7 Place the flask in a  37 °C incubator for  00:03:00 (up to 5 minutes). 3m
- 5.8 Check the flask under the microscope if possible. Look for: cells rounding, edges lifting, loss of adherence. *
- 5.9 Remove the flask from the incubator. Gently rock back and forth to encourage cell lifting.
- 5.10 Tap firmly but carefully on the sides to detach remaining adherent cells.
- 5.11 The flask bottom should appear clear rather than cloudy.
- 5.12 If cells remain attached, return to incubator for an additional  00:02:00 . 2m
- 5.13 Add  3 mL of complete cell culture medium to neutralize the non-enzymatic dissociation buffer.



- 5.14 Pour or pipette the cell suspension into a 15 mL conical tube.
- 5.15 Centrifuge  300 x g, Room temperature, 00:05:00 5m
- 5.16 Carefully aspirate and discard the supernatant without disturbing the pellet.
- 5.17 Resuspend the pellet in  200 μ L
 PBS Invitrogen - Thermo Fisher Catalog #2610807 if required for handling.
- 5.18 Transfer the resuspended pellet into a labeled 1.5 mL tube.
- 5.19 Proceed directly to DNA extraction (Section 3) or store the pellet at  -20 °C or  -80 °C .

Section 3: DNA extraction (muDNA protocol)

6h 51m 30s

6 Perform cell lysis.

Note

Before starting: Take DNA Extraction Bead Solution from the fridge and allow it to reach room temperature. Vortex to mix thoroughly. Incubate Elution Buffer at 55°C until required.

- 6.1 Add  0.5 g (2 X sample weight) of  -1.4 mm diameter sterile garnet beads and  0.5 g (2 X sample weight) of sterile fine grit to a 2 ml screw cap tube

- 6.2 Add sample to tube.

Note

At this point the skin swabs, wound isolates, reference strains, and human cell pellets samples are extracted for DNA.



- 6.3 Add  550 μL of Lysis Solution and vortex briefly.
- 6.4 Add  200 μL of Water Lysis Additive.
- 6.5 Add  60 μL of Proteinase K Dilution.
- 6.6 Place in TissueLyser II (or similar horizontal beating apparatus) at  30 Hz for  00:10:00 10m
- 6.7 Place in Thermomixer  400 rpm, 56°C, 04:00:00 4h
- 6.8 Place in TissueLyser II (or similar horizontal beating apparatus) at  30 Hz for  00:10:00 10m
- 6.9 Centrifuge  10000 x g, Room temperature, 00:01:00 1m
- 6.10 Transfer  700 μL supernatant to a 1.5 ml tube.
- 7 Remove inhibitors with flocculant solution.**
- 7.1 Add  200 μL (0.3 X volume) of Flocculant Solution.
- 7.2 Invert several times to mix and incubate  On ice for a minimum of  00:10:00 10m
- 7.3 Centrifuge  10000 x g, Room temperature, 02:00:00 2h
- 7.4 Without disturbing the pellet, transfer  600 μL supernatant to a fresh 1.5 ml LoBind tube.
- 8 Carry out SPRI DNA binding.**



8.1 Add  600 μL (1 X volume) of DNA Extraction Bead Solution.

8.2 Place on HulaMixer (continual rotation, 30 01 orbital rpm, reciprocal and vibro off) for

 00:10:00

10m

8.3 Place on magnetic stand until supernatant is clear and beads are bound towards magnet.

8.4 While on the stand carefully remove and discard supernatant without disturbing beads.

9 Wash beads with 80% ethanol.

9.1 Add  1000 μL Wash Solution.

9.2 Incubate at  Room temperature for  00:00:30

30s

9.3 While on the stand carefully remove and discard supernatant without disturbing beads.

9.4 Repeat Steps 9.1-9.3 a further time.

9.5 Very briefly spin down tube and place back on magnetic stand ensuring beads are bound towards magnet.

9.6 Remove all remaining Wash Solution with a 10 μL pipette.

9.7 Air dry tube with cap open until beads are completely dry (i.e. dull and no longer shiny), but do not allow the beads to crack.

10 Suspend DNA in elution buffer.



- 10.1 Add  100 μL Elution Buffer ( 55 $^{\circ}\text{C}$) and gently flick to resuspend beads (ensure all beads are resuspended with no clumps). Spin down.

Note

For samples expected to have low DNA yield it is highly recommended to initially concentrate with a lower amount of elute, ideally  25 μL of Elution Buffer for skin microbiome swabs.

- 10.2 Thermomixer  550 rpm, 55 $^{\circ}\text{C}$, 00:10:00 then spin down.

10m

- 10.3 Place on magnetic stand until supernatant is clear and beads are bound towards magnet.

- 10.4 Carefully transfer eluate to a fresh 1.5 mL LoBind tube without disturbing beads.

Section 4: DNA quality control

17m

11 Agarose gel electrophoresis for fragment-size estimation.

Note

At this point all DNA should go through QC: skin swabs, wound isolates, reference strains, human cell pellets, mock community DNA standard, and peri-wound swabs.

- 11.1 Add  1.125 g agarose to  75 mL sodium borate buffer in an Erlenmeyer flask (for a medium size 1.5% gel).

- 11.2 Heat in a microwave until the solution becomes completely clear.

Safety information

Use short bursts to avoid boiling over. Wear heat-resistant insulated laboratory gloves when handling hot agarose melted in the microwave.



- 11.3 Add  75 μ L GelRed™ and swirl gently to mix.
- 11.4 Seal the ends of the gel tray with tape.
- 11.5 Pour the agarose solution slowly into the tray, removing bubbles with a sterile tool or folded paper towel.
- 11.6 Insert a comb and allow the gel to solidify for  00:15:00
- 11.7 Remove tape and place gel into the tank.
- 11.8 Add sodium borate running buffer until the gel is fully submerged.
- 11.9 Place a strip of Parafilm on the bench.
- 11.10 Pipette  1 μ L loading dye onto the Parafilm for each sample.
- 11.11 Add  2 μ L DNA to each dye droplet and gently pipette to mix.
- 11.12 Pipette  2 μ L  GeneRuler 1 kb DNA Ladder Thermo Scientific Catalog #SM0312 directly into an empty well.
- 11.13 Leave one empty well between the ladder and the first sample.
- 11.14 Using  2 μ L per sample, carefully load each mixture into the gel wells.
- 11.15 Run the gel at 200 V for 15–20 minutes, depending on gel size and required resolution.

15m



11.16 Image the gel using a UV or blue-light transilluminator.

11.17 Compare sample bands to the GeneRuler ladder to assess approximate fragment length and degree of shearing.

12 DNA quantification using the Qubit dsDNA HS assay.

Equipment

Qubit 4

NAME

Fluorometer

TYPE

Invitrogen

BRAND

Q33238

SKU

<https://www.thermofisher.com/order/catalog/product/Q33238>^{LINK}



12.1 Allow Qubit reagents to equilibrate to room temperature.

12.2 Prepare working solution by combining Qubit reagent with buffer as described in the kit instructions.

12.3 Prepare standards (Standard #1 and #2) according to kit instructions.

12.4 Add  198 μ L working solution +  2 μ L DNA sample to each assay tube.

12.5 Mix by gentle vortexing or pipetting.

12.6 Incubate for  00:02:00 at  Room temperature

2m

12.7 Insert tubes into the Qubit 4 Fluorometer and read concentrations.

12.8 Record DNA concentrations in ng/μL.

13 Purity assessment using NanoDrop spectrophotometry.

Note

DNA purity ratios (A260/280 and A260/230) were assessed using a NanoDrop spectrophotometer (Thermo Scientific, 2006-24). NanoDrop quantification was not used for concentration estimates due to low sample yield.

Equipment

NanoDrop Spectrophotometer

NAME

2000/2000c Spectrophotometers

TYPE

NanoDrop

BRAND

ND2000CLAPTOP

SKU

<https://www.thermofisher.com/order/catalog/product/ND2000CLAPTOP>^{LINK}



13.1 Launch the NanoDrop software and select the dsDNA measurement mode.

13.2 Blank the instrument using  1 μL nuclease-free water.



- 13.3 Load  1 μL DNA directly onto the pedestal.
- 13.4 Measure absorbance spectra and record: A260/280 ratio (protein contamination) and A260/230 ratio (salt/solvent contamination).

Note

Acceptable purity ratios for high-quality DNA:

A260/280 $\approx$ 1.8

A260/230 $\approx$ 2.0–2.2

Due to low biomass and extraction from complex matrices (e.g., skin swabs), purity ratios may deviate; samples were still carried forward if concentrations were sufficient and gels showed usable fragment sizes.

- 13.5 Clean the pedestal thoroughly between samples using a lint-free tissue.

Section 5: Mock community samples

- 14 **Compare the Section 4 results of microbiome DNA standard, Oral Microbiome Genomic Mix (ATCC MSA-1004), to extracted DNA.**
- 14.1 Thaw the mock community, bacterial strain DNAs, and human DNA on ice. Mix gently by flicking or pipetting.
- 14.2 For each sample type (mock community, each bacterial strain, and human DNA), dilute with Elution Buffer to 6 ng/ μL .
- 14.3 Repeat Step 11 to confirm that fragment size distributions are broadly similar.
- 14.4 Repeat Step 12 to verify they are all within an acceptable range around 6 ng/ μL .

Note

Ensure that the mock community, each bacterial strain of interest, and human genomic DNA are all at the same DNA concentration ($\approx$ 6 ng/ μL) and fragment size before mixing.

- 15 **Spike mock community with each strain of interest (*E. coli* NCTC, clinical *E. coli*, *S. aureus* NCTC, clinical *S. aureus*, clinical *S. epidermidis*).**



- 15.1 Label tube with strain, e.g., "Mock + E. coli NCTC", etc.
- 15.2 Combine 1 volume normalized strain DNA with 6 volumes normalized ATCC mock community DNA.
Example for a  35 μL total volume:
-  5 μL strain DNA (6 ng/ μL)
 -  30 μL mock community DNA (6 ng/ μL)
- 15.3 Flick or gently pipette to mix. Avoid vigorous vortexing to minimise shearing. Spin down.
- 15.4 Repeat Steps 15.1-15.3 for each of the five pathogen isolates to create 5 different spiked mock communities.

16 For each strain-specific spiked mock community, prepare four human diluted conditions.

Note

All components are normalised to the same concentration, so relative abundance is controlled by volume ratios alone.

- 16.1 Choose a convenient final volume V for each sample (e.g.,  20 μL). The exact volume is flexible as long as ratios are preserved and volume is sufficient for downstream steps (approx.  10 μL).
- 16.2 0% human: Add V μL of the pathogen-spiked mock community. No human DNA added. Mix by gentle pipetting. Spin down.
- 16.3 10% human: Add 0.1V human DNA + 0.9V spiked mock community. Mix by gentle pipetting. Spin down.
Example for  20 μL :  2 μL human DNA +  18 μL spiked mock.
- 16.4 75% human: Add 0.75V human DNA + 0.25V spiked mock community. Mix by gentle pipetting. Spin down.
Example for  20 μL :  15 μL human DNA +  5 μL spiked mock.



16.5 90% human: Add 0.9V human DNA + 0.1V spiked mock community. Mix by gentle pipetting. Spin down.

Example for  20 μL :  18 μL human DNA +  2 μL spiked mock.

16.6 Repeat Steps 16.1–16.5 for each of the five pathogen-spiked mock stocks.

16.7 If proceeding directly to Library preparation (Section 7): place tubes  On ice immediately after.

If processing will occur later: store samples at  4 °C until preparation.

Section 6: Skin microbiome samples

17 **Compare the Section 4 results of skin microbiome DNA to each strain of interest.**

Note

Ensure that the skin microbiome DNA and each strain of interest are at comparable concentrations (~1–2 ng/ μL) and fragment size before mixing.

17.1 Thaw skin microbiome DNA and strain DNA stocks on ice. Mix gently by flicking or pipetting.

17.2 If necessary, dilute each skin DNA sample with Elution Buffer to approximately 1–2 ng/ μL .

17.3 Dilute each of the four strains (*E. coli* NCTC, clinical *E. coli*, *S. aureus* NCTC, clinical *S. aureus*) with Elution Buffer to have approximately 1–2 ng/ μL DNA.

17.4 Repeat Step 11 to confirm that fragment size distributions are broadly similar.

17.5 Repeat Step 12 to verify they are all within an acceptable range around 1–2 ng/ μL .

18 **For each of the five bacterial strains, perform an additional dilution.**

Note

Previous sequencing of these unspiked forearm skin microbiome samples showed that:

- Each individual bacterial species in the native community represented roughly 0.01% of total DNA.

To mimic this:

- Each strain of interest was spiked into a skin microbiome DNA sample to achieve a target relative abundance $\approx$ 0.01% of total DNA.

- 18.1 For each strain, dilute again with Elution Buffer (e.g., 1:10 or 1:20), such that adding 0.5–1 μL of this diluted strain stock to a typical skin sample aliquot yields the desired $\sim$ 0.01% relative abundance.

Example (conceptual):

- If a skin sample aliquot is  20 μL at 1 ng/ μL (20 ng total), and you want 0.002 ng spike-in (0.01%), then: Prepare a diluted strain stock at 0.004 ng/ μL so that

 0.5 μL gives $\sim$ 0.002 ng.

- 18.2 Label diluted strain stocks clearly. Include strain name, dilution factor, and date.

19 **Spike skin microbiome with each strain of interest (*E. coli* NCTC, clinical *E. coli*, *S. aureus* NCTC, clinical *S. aureus*).**

- 19.1 Mix the skin DNA sample gently.

- 19.2 Divide it into four equal portions in separate LoBind tubes.

- 19.3 Assign one strain per aliquot.

- 19.4 Decide on a convenient working volume per aliquot (e.g.,  20 μL), ensuring there is enough DNA for downstream library preparation (minimum  6 μL).

- 19.5 To each aliquot, add the calculated volume (e.g., 0.5–1 μL) of the corresponding secondary diluted strain stock.

- 19.6 Mix by gentle pipetting to avoid shearing. Avoid vigorous vortexing. Spin down.



- 19.7 If proceeding directly to Library preparation (Section 7): place tubes On ice immediately after.
If processing will occur later: store samples at 4 °C until preparation.

Section 7: Library preparation (SQK-RPB004)

27m

20 DNA fragmentation/tagmentation and PCR barcoding.

Note

Before starting:

- Thaw all extracted DNA samples on ice.
- Thaw Rapid Barcodes (RLB01–RLB12a) at room temperature.
- Place Fragmentation Mix (FRM) on ice.
- Briefly vortex and quick-spin all kit reagents before use.
- Keep all reagents chilled unless otherwise indicated.

- 20.1 Repeat Step 12 and record concentrations to determine appropriate input volume for tagmentation.
Important:
- If sample DNA concentration is < 1 ng/μL (common for skin and peri-wound samples): use 5 μL DNA.
 - If sample DNA concentration is ≥ 1 ng/μL: use 3 μL DNA.
 - Maximum DNA input is 5 ng as per SQK-RPB004 specifications.
- 20.2 Add 3 μL or 5 μL DNA (see above) and 1 μL Fragmentation Mix (FRM) in a 0.2 mL PCR tube.
- 20.3 Mix by flicking the tube gently. Spin down.
- 20.4 Incubate in a thermal cycler for 00:01:00 at 30 °C then 00:01:00 at 80 °C and hold at 4 °C .
- 20.5 Keep tagmented DNA at 4 °C or On ice until PCR.
- 20.6 Prepare a PCR master mix, for each sample (add ~5% overage for pipetting error):

1m



- 20 µL nuclease-free water

- 25 µL

LongAmp Taq 2X Master Mix - 500 rxns **New England Biolabs Catalog #M0287L**

20.7 Add 45 µL of PCR master mix to each tagged sample.

20.8 Add 1 µL of the appropriate Rapid Barcode (RLB).

20.9 Flick to mix gently. Spin down.

20.10 Thermal cycler conditions:

- 00:03:00 at 95 °C

- 20 cycles of:

- 00:00:15 at 95 °C

- 00:00:15 at 56 °C

- 00:06:00 at 65 °C

- Final extension: 00:06:00 at 65 °C

- Hold at 4 °C

15m 30s



Note

Stopping point: PCR-barcoded samples may be stored at 4 °C for up to 1 week.

21 **Post-PCR sample concentration (bead cleanup).**

Note

Before starting:

- A magnetic stand is required. If the stand cannot accommodate 0.2 mL tubes, transfer samples to DNA LoBind 1.5 mL tubes.
- Remove Library Prep Bead Solution from  4 °C and bring to room temperature.

Vortex thoroughly.

- Prepare Lysis Master Mix:

-  730 µL Lysis Solution
-  250 µL Tissue Lysis Additive
-  60 µL Proteinase K

Vortex and incubate at  55 °C until needed.

- Incubate Library Buffer at  55 °C until needed.

21.1 Add  50 µL (1× volume) of Library Prep Bead Solution to each PCR-barcoded sample.

21.2 Mix thoroughly by pipetting.

21.3 Incubate  00:10:00 at  Room temperature .

10m

21.4 Place tubes on a magnetic stand until beads are fully pelleted and supernatant is clear.

21.5 Remove and discard supernatant carefully without disturbing beads.

21.6 Add  150 µL Wash Solution.

21.7 Incubate  00:00:30 at  Room temperature

30s

21.8 Remove supernatant while on the magnetic stand.

21.9 Repeat Steps 21.6-21.8 once more.



- 21.10 Very briefly spin down, return to the magnetic stand, and remove any residual wash solution with a 10 µL pipette.
- 21.11 Air dry tube with cap open until beads are completely dry (i.e. dull and no longer shiny), but do not allow the beads to crack.
- 21.12 Add  15 µL Library Buffer ( 55 °C) and flick gently to fully resuspend beads. Spin down.
- 21.13 Incubate  00:10:00 at  Room temperature
- 21.14 Place on magnetic stand until the eluate is fully clear.
- 21.15 Transfer the eluate to a fresh 0.2 mL PCR tube, do not touch or disturb the beads.

Note

Stopping point: Concentrated libraries may be stored at  4 °C for up to 1 week.

22 DNA quantification and molarity calculation for pooling.

- 22.1 Repeat Step 11 to estimate the average fragment size in kilobases (kb).
- 22.2 Repeat Step 12 and record concentrations in ng/µL.

- 22.3 Calculate the molarity in each sample (fmol/µL) using:

$$fmol/\mu L = (DNAconcentration(ng/\mu L) / (Fragmentlength(kb) \times 1000 \times 617.96) + 36.04) \times 10^6$$

Where:

- 617.96 g/mol = average molecular weight of a base pair
- 36.04 = molecular weight of DNA ends
- Fragment length is input in kb



- 22.4 Pool samples equimolarly whenever possible for optimal read balance.
- 22.5 Aim for a final pooled concentration of ~2 fmol/ μ L, yielding ~5–10 fmol DNA in a standard  5 μ L library loading volume, ideal for Flongle flow cells.
- 22.6 If needed concentrate the pooled library using bead cleanup (Step 21) or dilute using Library Buffer.
- 22.7 Repeat Step 12 of pooled samples and recalculate molarity if adjusted.

Section 8: Flow cell priming and loading

10m

23 Sequencing adapter ligation (SQK-RPB004).

Note

Before starting:

- Thaw Rapid Adapter (RAP) at room temperature.
- Keep RAP at room temperature while thawing; once thawed, place on ice.
- Vortex RAP briefly and spin down.

- 23.1 Transfer  5 μ L of the final pooled library into a DNA LoBind 1.5 mL tube.
- 23.2 Add  0.5 μ L Rapid Adapter (RAP).
- 23.3 Flick gently to mix. Spin down.
- 23.4 Incubate  00:10:00 at  Room temperature
- 23.5 Place the completed library preparation on ice until loading.
- 24 **Check Flongle flow cell.**

10m



Before priming or loading the flow cell:

1. Insert the Flongle adapter into the sequencing device (MinION/GridION).
2. Insert the Flongle flow cell into the adapter until you hear a distinct click.
3. Open MinKNOW.
4. Run a Flow Cell Check following on-screen instructions.

Proceed only if pore count is acceptable.

Equipment

Flongle Flow Cell (R10.4.1)

NAME

Flow cell

TYPE

Oxford Nanopore Technologies

BRAND

FLO-FLG114

SKU

<https://store.nanoporetech.com/flongle-flow-cell-pack-r10-4-1.html>^{LINK}

If using a standard MinION R9.4.1 or R10.4 flow cell, follow **the ONT steps for priming and loading full-sized flow cells**.

25 Prime and load a Flongle flow cell.

Note

Thaw the following sequencing reagents at room temperature, then transfer to ice:

- SQB (Sequencing Buffer)
- LB (Loading Beads)
- FB (Flush Buffer)
- FLT (Flush Tether)

Mixing:

- Vortex SQB, FB, and FLT briefly, spin down, and return to ice.
- Vortex LB immediately before use (loading beads settle extremely quickly).

25.1 Add  1117 μL FB and  3 μL FLT to a fresh 1.5 mL LoBind tube. Mix by gentle pipetting. Keep  On ice

25.2 Ensure the Flongle flow cell is firmly seated in the adapter.



- 25.3 Peel back the seal tab just enough to expose the sample port. *Do not remove the adhesive tab completely.*
- 25.4 Load a pipette with the FB + FLT priming mix.
- 25.5 Place the tip gently inside the sample port, ensuring there are no air bubbles in either.
- 25.6 Slowly dispense the priming mix until the port is full.
Recommended: twist the pipette plunger down for a controlled dispense OR use multiple dispenses with a P20.

Note

Important: Avoid introducing air into the sample port. Because these steps are visually sensitive and require controlled hand movements, ONT strongly recommends following [the step-by-step diagrams or videos on their website](#).

- 25.7 Allow the priming mix to sit for ≥ 30 seconds.
- 25.8 Add  13.5 μL SQB,  11 μL LB (*vortex immediately before use*), and  5.5 μL adapter-ligated DNA library to new LoBind tube. Mix gently by pipetting. Spin down.
- 25.9 Ensure no air is in the pipette tip or the sample port.
- 25.10 Load the sequencing mixture with a P100 pipette by inserting the tip gently into the sample port. Slowly dispense the entire sequencing mix using twisting plunger pressure for control.
- 25.11 Close the sample port by pressing down the adhesive seal tab.
- 25.12 Close the MinION/GridION device lid.

Section 9: Sequencing & adaptive runs

26 Initiate standard sequencing in MinKNOW.





Software

MinKNOW™

NAME

Oxford Nanopore

DEVELOPER

https://community.nanoporetech.com/technical_documents/minknow-tech-doc/v/mitd_5000_v1_revaj_16may2016/introduction-to-the-minkno

SOURCE
LINK

- 26.1 Select the appropriate kit configuration (e.g., *Rapid PCR Barcoding* — SQK-RPB004).
- 26.2 Select the appropriate flow cell type (e.g., FLO-MIN106 (R9.4.1)).
- 26.3 Set Run parameters: Minimum read length (20 bp), basecalling (disabled), output format (FAST5).
- 26.4 Confirm run metadata (sample ID, experiment name, flow cell ID).
- 26.5 Start the sequencing run.

Note

Although MinKNOW allows longer runs (e.g., 48 h), using this protocol we observed that data yield typically plateaus around ~24 hours.

27 Configure MinKNOW for adaptive sequencing.



- 27.1 Modify the main configuration file for DNA sequencing on MinION "sequencing_MIN106_DNA.toml" (typically found in /opt/ont/minknow/conf/package/sequencing/).
- 27.2 Default settings are suitable for adaptive sequencing; however break_reads_after_seconds must be set to:

```
break_reads_after_seconds = 0.4
```

⚠ After performing playback runs (see below) for adaptive or non-adaptive runs, ensure any temporary config changes are reverted as recommended in the [ReadFish documentation](#).

28 Perform control runs prior to adaptive sequencing.

28.1 Prior to every adaptive sequencing experiment, a control run should be performed using the setup:

- Same final library
- Same MinION device and computer
- Same MinKNOW configuration and settings
- Same Flongle adapter
- A fresh flow cell was used for each run.
- Runs occurred on different days.
- Adaptive software *not* activated during control runs.

29 Run ReadFish-based adaptive sequencing.

Note

Before the first experimental adaptive run, a playback run is strongly recommended:

- Follow the instructions in the [ReadFish documentation](#).
- Use a previously collected dataset to simulate real-time sequencing.
- Adjust the MinKNOW configuration as required for playback mode and restore settings before real runs.

This step ensures all components (MinKNOW, Guppy, ReadFish) communicate correctly.

29.1 Set up ReadFish configuration ".toml" file to arrange experiment and define:

- Address (host/port) of the Guppy basecalling server
- Mapping parameters (e.g. Minimap2 options)
- Path to the reference index (".mmi" file)
- Conditions for enrichment or depletion
- List of contig/sequence IDs to target

29.2 Prior to initiating the sequencing run, launch a Guppy basecalling server. Example command (optimized for bioinformatics workstation):



```
sudo guppy_basecall_server \  
  --log_path guppy.log \  
  --config dna_r9.4.1_450bps_fast.cfg \  
  --port /tmp/.guppy/5557 \  
  --device cuda:0 \  
  --gpu_runners_per_device 8 \  
  --num_callers 4 \  
  --chunks_per_runner 1024 \  
  --chunk_size 1000
```

Guppy configuration must match the flow cell chemistry and desired speed.

29.3 Start the sequencing run in MinKNOW as described in Step 26.

29.4 Allow a few minutes of stable sequencing.

29.5 Launch ReadFish:

```
sudo /path/to/anaconda3/envs/readfish_api/bin/readfish targets \  
  --device MinIONID \  
  --experiment-name "Descriptive_experiment_name" \  
  --toml /path/to/readfish/config/file/for/experiment.toml \  
  --log-file Descriptive_experiment_name.log \  
  --channels 1 126
```

30 **Perform adaptive sequencing with ReadBouncer.**



30.1 Repeat steps 27-29.3, configuring the ReadBouncer config file and follow [the usage and configuration instructions](#).

30.2 Start the sequencing run in MinKNOW as described in Step 26.

30.3 Allow a few minutes of stable sequencing before activating ReadBouncer.

**Note**

Because ReadBouncer is under active development and its command-line interface may change, users should follow the most recent instructions in its official [documentation/GitHub page](#) for detailed usage.

31 ONT in-house adaptive sequencing on GridION Mk1.**Note**

In addition to external adaptive tools, in-house ONT adaptive sequencing was tested on a GridION Mk1 using MinKNOW v5.2.2.

31.1 Repeat Steps 23-26, but in Section 8 use a MinION flow cell in the GridION Mk1 and in Step 26 activate adaptive sampling with:

- Reference genome: [GRCh38.p14](#).
- Reads mapping to the reference: unblocked (rejected).
- No Minimapp2 index or BED file was used.

Protocol references

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