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GETCampy Sequencing protocols

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GETcampy

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Disclaimer

Protocols for research purposes only

Abstract

As part of the GETCampy-Africa project, this protocol provides a modular workflow for extracting, sequencing, and analysing enteric pathogens from stool and environmental samples. It includes short-read (Illumina MiSeq) and long-read (Oxford Nanopore) sequencing methods, enabling hybrid metagenomic approaches for high-resolution genome assembly. DNA extraction protocols support both total community DNA and high molecular weight recovery. Library preparation workflows include Nextera XT (Illumina), rapid barcoding and ligation kits (ONT), and full-length 16S rRNA sequencing for taxonomic profiling. The protocol also outlines hybrid assembly, binning, and quality control steps for metagenome-assembled genomes (MAGs). Designed for field-lab compatibility and resource-limited settings, these methods support genomic surveillance of *Campylobacter* and other enteric pathogens across Africa.

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DNA extraction

1 🧬 **QIAamp DNA Extraction Protocol for *Campylobacter***
Kit: QIAGEN QIAamp DNA Mini Kit
Adapted from: QIAamp DNA Mini Handbook, Appendix D (page 55) and Step 3 (page 33)
Organism: *Campylobacter spp.*

1.1 🧫 **Sample Preparation**
1. Grow *Campylobacter* isolates:
- **Medium**: Chocolate, blood, or Brucella agar
- **Conditions**: 42°C for **48 hours**

Using a sterile loop, harvest **at least one loop-full** of bacterial growth.
Resuspend cells in **180 µL Buffer ATL**.
Incubate at **37°C for 30 minutes**.

1.2 🧪 **Cell Lysis and Protein Digestion**
Add **20 µL Proteinase K** (from the kit).
Mix thoroughly by vortexing.
Incubate at **56°C for 30–60 minutes**.
Briefly spin down to remove condensation from the tube lid.

1.3 🔬 **RNase Treatment and Lysis Enhancement**
Add:
- **4 µL RNase** (100 mg/mL)
- **200 µL Buffer AL** (from the kit)

Vortex to mix completely.
Incubate at **70°C for 10 minutes**.

1.4 💧 **DNA Binding**
Add **200 µL of 95–100% ethanol** to the lysate.
Vortex to mix.
Transfer the full volume (~600 µL) into a **QIAamp spin column** in a **2 mL collection tube**.
Centrifuge at **8000 rpm for 1 minute** at **room temperature (RT)**.
Discard the flow-through and move the column to a clean 2 mL collection tube.

1.5 🚿 **Wash Steps**
Add **500 µL Buffer AW1** to the column.
Centrifuge at **8000 rpm for 1 minute** at RT.
Discard flow-through.
Add **500 µL Buffer AW2**.
Centrifuge at **14,000 rpm for 3 minutes** to dry the membrane.

1.6 💦 **DNA Elution**
Place the QIAamp column into a **clean 1.5 mL microcentrifuge tube**.
Add **50 µL Buffer AE** (or nuclease-free water) directly to the column membrane.

Let sit at **room temperature for 1 minute**.
Centrifuge at **8000 rpm for 1 minute** to elute DNA.

1.7 📦 **Storage**

- **Short-term**: Store at **4°C**
- **Long-term**: Store at **-20°C**

1.8 📝 **Notes**

- Ensure all reagents from the kit are at working temperature.
- Eluted DNA is suitable for downstream applications including PCR and sequencing.

Library preparation for short read sequencingUntitled section

2 🧬 **Illumina Custom Protocol (Nextera XT on MiSeq)**
Instrument: MiSeq
Library Prep: Nextera XT DNA
Reagent Kit: MiSeq v3
Indexing: Dual Indexing
Software: BaseSpace Sequence Hub (FASTQ generation)

2.1 📋 **Generate Sample Sheet with IEM**
Start IEM Software

- Start → All Programs → Illumina → Illumina Experiment Manager
  or click IEM icon.

Create Sample Plate
Select **Create Sample Plate** → Choose **Nextera XT**.
Set:

- Unique plate name
- Index reads = 2

Enter sample details per well (ID, i7/i5 index, etc.)
Save as *.nexxt28.plt

2.2 **Create Sample Sheet**
Select **Create Sample Sheet** → Instrument: **MiSeq**.
Application: **FASTQ Only** → Click **Next**.
Set:

- Reagent barcode
- Prep kit: Nextera XT
- Index reads: 2
- Experiment name, date, paired/single-end, cycles

Load plate, select samples, and save sample sheet.

2.3 🧪 **Nextera XT Library Prep**
Tagment Genomic DNA

- Thermal cycler: **55°C for 5 min**, hold at **10°C**
- Mix in PCR plate:
- 10 µL TD + 5 µL gDNA (0.2 ng/µL)
- Add 5 µL ATM, mix
- Spin 280 × g, 1 min → Run tagmentation
- Add 5 µL NT, mix → Spin → RT 5 min

2.4 **Amplify Libraries**

- Thermal cycler:
- 72°C 3 min → 95°C 30s
- 12 cycles: 95°C 10s → 55°C 30s → 72°C 30s
- Final: 72°C 5 min → hold at 10°C
- Add i7 and i5 indices (5 µL each), then 15 µL NPM
- Spin → Run PCR

Safe stop: 2–8°C for 2 days or overnight on thermal cycler

2.5 🧼 **Clean Up Libraries**

Transfer 50 µL PCR product to new plate.
Add 30 µL AMPure XP beads → Shake 1800 rpm 2 min → RT 5 min
Magnetic stand → remove supernatant.
Wash ×2 with 200 µL 80% EtOH → Remove residual EtOH
Air dry 15 min.
Elute in 52.5 µL RSB → Shake, incubate, magnet, transfer 50 µL to new plate
Safe stop: -25°C to -15°C for 7 days

2.6 🔍 **Check Libraries (Optional)**

- Run 1 µL on Agilent Bioanalyzer (HS DNA chip)

2.7 ⚖️ **Normalize Libraries**

Transfer 20 µL to new plate.
Mix 4.4 mL LNA1 + 800 µL LNB1 → Add 45 µL per well
Shake 1800 rpm 30 min → Magnet → Remove supernatant
Wash ×2 with 45 µL LNW1
Add 30 µL 0.1 N NaOH → Shake 5 min
Add 30 µL LNS1 to new plate (SGP)
Resuspend beads → Shake 5 min → Magnet → Transfer to SGP plate
Spin at 1000 × g for 1 min
Safe stop: -25°C to -15°C for 7 days

2.8 🧬 **Pool Libraries**

Spin SGP plate.
Transfer 5 µL per sample to PCR tube strip → Pool into tube (PAL)
Dilute to loading concentration.
Store PAL and SGP: -25°C to -15°C (7 days)

2.9 🧊 **Denature and Dilute Libraries**

Prepare HT1

- Thaw at RT, store at 2–8°C.

2.10 **Dilute Library**

	Library Volume	HT1 Volume
	6 µL	594 µL
	24 µL (XT)	576 µL

- Vortex → Spin → Incubate 98°C 2 min → Ice 5 min

Illumina short read sequencing

3 🧬 **C-SOP-601: Illumina MiSeq Operation for Whole Genome Sequencing (WGS)**

🧤 **Preparation Before Starting**

Clean workspace with 80% ethanol.
Wear PPE and follow local GLP guidelines.
Prepare ice bucket for temporary reagent/sample storage.
Thaw HT1 buffer at room temperature, then store at 4°C until use.
Prepare 0.2N NaOH:
- Mix 200 µL 1.0N NaOH + 800 µL nuclease-free water.
- Use within 8 hours.

❄️ **Reagent Cartridge Thawing (2 Hours Prior to Run)**

Remove cartridge from -20°C.
Submerge base only in room-temp water bath (max line indicated).
Thaw time:
- v3 kits: ~1.5 hours
- v2 kits: ~1 hour

Dry base and invert 10x to mix reagents.
Check positions 1, 2, and 4 for full thawing (no precipitates).
Store at 4°C until ready to load.

⚡ **MiSeq Instrument Initialisation**

Power on MiSeq (switch on back).
Log in to BaseSpace, if networked.
Wait for MiSeq Control Software (MCS) to initialise.

🧪 **Denature and Dilute DNA Library**

Mix:

- 5 µL 4nM DNA library
- 5 µL 0.2N NaOH

Vortex 10 sec, spin 1 min.
Incubate 5 min at RT.
Add 990 µL chilled HT1 → final 20pM denatured library.
Dilute to final loading concentration (e.g., 10pM).

🧬 Denature and Dilute PhiX (Optional Spike-in)
Dilute 2 µL 10nM PhiX + 3 µL Tris-Cl/Tween → 4nM.
Mix:

- 5 µL 4nM PhiX
- 5 µL 0.2N NaOH

Vortex 10 sec, spin 1 min, incubate 5 min.
Add 990 µL HT1 → final 20pM PhiX.
For v2 kits: mix 375 µL PhiX + 225 µL HT1 → 12.5pM.
Add 1–5% PhiX to final sample mix depending on library complexity.

💻 MiSeq Run Setup
Ensure ≥100 GB free disk space.
Enable local analysis replication:

- MCS → Run Options → check "Replicate analysis locally".

Prepare and load Sample Sheet via Illumina Experiment Manager (IEM).

🧫 Prepare and Load Flow Cell
Retrieve Flow Cell + PR2 buffer from 4°C.
Rinse flow cell with lab-grade water → dry with lint-free lens paper.
Clean glass with ethanol-wetted lens tissue.
Install flow cell into clamp → wait for RFID recognition.

💧 Load Incorporation Buffer (PR2) and Empty Waste
Raise sipper handle.
Load mixed PR2 buffer bottle → confirm RFID.
Empty and replace waste bottle.
Lower sipper into PR2 + waste bottle.

👄 Load DNA Library into Cartridge
Tap cartridge to gather contents.
Pierce foil at "Load Sample".
Pipette 600 µL denatured library into well.
Tap gently to collect sample at base.

🔄 Load Cartridge into MiSeq
Wait for “Load Reagent Cartridge” prompt.

Remove wash tray, dry chiller base if needed.
Insert cartridge (Illumina label facing out) until fully seated.
Confirm cartridge RFID is read.

📝 **Sample Sheet Confirmation**
MCS looks for matching barcode sample sheet.
If not found, manually browse and select.
Click “Save and Continue” → “Next”.

▶️ **Start Sequencing Run**
Review:
- Experiment Name
- Workflow
- Read Length

Perform Pre-run Check.
When all checks pass, click “Start Run”.

🧼 **Post-Run Wash (Required)**
Follow on-screen prompts to load:
- Wash tray
- Wash bottle
- Optional: MiSeq wash tube (0.01% NaOCl for bleach wash)

Start Wash.
Do not remove flow cell/wash tray during wash.
Leave wash solution in tray/bottle after wash completes.

📈 **Monitor Run**
- Use Sequencing screen (view-only) or BaseSpace/SAV.
- Monitor: cycles, cluster intensities, quality scores.

📂 **Export Data**
- Navigate to:
`D:\Illumina\MiSeq Output\Run Folder\Data\Intensities\BaseCalls`
- Download `fastq.gz` files from R1 and R2.
- Or use BaseSpace/local network share.

🔁 **Instrument Shutdown or Reboot**
- Weekly reboot recommended
- MCS → Manage Instrument → Reboot
- For shutdown:
- MCS → Manage Instrument → Shutdown
- Power switch off → wait 10 min → power on

🧽 **Monthly Maintenance**

Week	Action
1	PTL (post-run bleach)
2	PTL
3	PTL
4	PTL or Maintenance

- See MiSeq System Guide for detailed cleaning steps.

🆘 **Troubleshooting Resources**

- MiSeq System Guide
- Sample Sheet Quick Guide
- MiSeq Reporter / SAV Software Guide
- Re-Queue Tutorials

High molecular weight DNA extraction

4 🧬 **C-SOP-1001: HMW DNA Extraction from Bacteria Using NEB Monarch HMW DNA Extraction Kit**

🔧 **Materials Required (Not Supplied)**

- Microcentrifuge
- Thermal mixer (1.5 mL + 2 mL blocks)
- Vertical rotating mixer (e.g., HulaMixer)
- Ethanol ≥95%
- Cold PBS or TE/Tris
- Isopropanol (550 µL/sample or 275 µL low input)
- 1.5 mL DNase-free low DNA binding tubes
- Wide-bore pipette tips
- Lysozyme (Gram-neg: 25 mg/mL; Gram-pos: 10 mg/mL in STET)
- Additional lysis agents (e.g., lysostaphin if needed)

🧤 **Preparation**

Clean bench with 80% ethanol. Wear PPE.
Preheat thermal mixers to 37°C and 56°C.
Add ethanol to gDNA Wash Buffer as per label.
Chill PBS/TE/Tris buffer.
Label tubes. Store Proteinase K and RNase A at -20°C.

🧫 **Step 1: Cell Pellet**

- Centrifuge bacterial culture at **12,000 × g for 1 min** in a Monarch Pestle Tube.

🦠 **Step 2: Lysis**

A. Gram-negative Bacteria

Resuspend pellet in **300 µL cold PBS** (150 µL for low input).
Add **10 µL lysozyme (25 mg/mL)**, vortex briefly.
Add **300 µL HMW Lysis Buffer**, invert 5–10×.
Incubate at **37°C** with agitation:

- **2000 rpm** (faster lysis, shorter fragments)
- **500 rpm** (slower lysis, longer fragments)
- Until lysate turns clear (~5 min)

B. Gram-positive Bacteria
Resuspend in **STET buffer + lysozyme (10 mg/mL)**.
Incubate **30 min at 37°C (no agitation)**.
Add **300 µL HMW Lysis Buffer**, invert 5–10×.

🔥 **Step 3: Proteinase K Digestion**
Increase thermal mixer to **56°C** if needed.
Add **20 µL Proteinase K** (10 µL for low input), invert 10–20×.

Choose Homogenization:

- **Thermal Mixer:** Incubate **30 min at 56°C**, 2000 rpm.
- **Rotor-stator:** Homogenize 5–15 sec, then incubate **30 min at 56°C**.

💧 **Step 4: RNA Removal**
Add **10 µL RNase A** (5 µL for low input), invert 5–10×.
Incubate **10 min at 56°C**, same speed as lysis.

🧪 **Step 5: Protein Separation**
Add **300 µL Protein Separation Solution**, invert for 1 min or mix at 20 rpm.
Centrifuge **10 min at 16,000 × g**.
Carefully transfer **upper DNA phase (~800 µL)** to new 2 mL tube.

🧬 **Step 6: DNA Binding to Beads**
Add **2 DNA Capture Beads** to tube.
Add **550 µL isopropanol** (275 µL low input).
Mix gently:

- Rotate at 10 rpm for 5 min OR
- Invert 25–30× slowly (5–6 sec per inversion)
- Watch for visible DNA wrapping around beads

🚿 **Step 7: Wash Steps**
Remove liquid carefully (avoid touching beads).
Wash with **500 µL gDNA Wash Buffer**, invert 2–3×, then remove.
Repeat wash.

🧲 **Step 8: Bead Transfer**
Transfer beads to **Monarch Collection Tube II + bead retainer**.
Pulse spin (≤1 sec).
Move beads to a fresh **2 mL tube**.
Insert bead retainer into **1.5 mL DNA low-bind tube**.

💦 **Step 9: Elution**
Add **100 µL Elution Buffer II** (≥5 min at 56°C, 300 rpm).
- Optional: use **50 µL** with more frequent gentle shaking

Pour eluate + beads into bead retainer in 1.5 mL tube.

🌀 **Step 10: Final Handling**
Centrifuge **30 sec at 12,000 × g**.
Discard beads and retainer.
Pipette eluate 5–10× with wide-bore tips to disperse aggregates.
Dissolve DNA:
- **1 h at 37°C**, or
- **24 h at RT**, or
- **Overnight at 4°C**

Pipette again before use.

📦 **Storage**
- Short-term: **4°C**
- Long-term: **-20°C** (in Elution Buffer: 10 mM Tris, pH 9.0, 0.5 mM EDTA)

📚 **Additional Resources**
- UHMW DNA Extraction for Ultra-Long Read Nanopore
- UHMW DNA Cleanup Protocol

Rapid barcoding for long read sequencing

5 🧬 **Rapid Barcoding Workflow**
Kit: ONT Rapid Barcoding Kit 96 (SQK-RBK114.96)
Platform: MinION / GridION / PromethION
Purpose: For barcoded, multiplexed sequencing of DNA using ONT's transposase-based tagmentation

5.1 🧪 **DNA Input Requirements**
- **Input amount**: 10–50 ng per sample (in ≤2.5 µL nuclease-free water)
- **Volume of RB reagent per sample**: 2.5 µL
- **Volume of barcode (BCXX) per sample**: 0.5 µL
- **Multiplexing**: Up to 96 samples

5.2 🔬 **Barcoding Reaction**

Prepare Reaction Mix

In a PCR strip tube or plate (per sample):

	Reagent	Volume (µL)
	Genomic DNA	≤2.5
	RB (Fragmentation Mix)	2.5
	Barcode (BCXX)	0.5
	Total	5.5 max

5.3 **Mix and Incubate**

- Gently flick or pipette to mix.
- Incubate at **30°C for 1 minute**, then **80°C for 1 minute**.
- Immediately place on ice.

5.4 🧹 **Pooling and Cleanup**

Pool 2 µL from each barcoded sample into a single tube.

Total volume should not exceed 10 µL.

Proceed to magnetic bead cleanup:

- Add 1.8× volume of AMPure XP beads.
- Incubate 5 minutes at RT.
- Place on magnet for 5 minutes.
- Wash twice with 70% ethanol.
- Elute in 10 µL Elution Buffer or nuclease-free water.

5.5 🧪 **Adapter Ligation**

	Reagent	Volume (µL)
	Barcoded DNA	10.0
	RAP (Rapid Adapter)	1.0
	Total	11.0

- Mix by flicking or pipetting.
- Incubate at **room temperature for 5 minutes**.
- Proceed immediately to loading.

5.6 💉 **Loading onto Flow Cell**

- Load the library onto a primed flow cell following the Flow Cell Priming protocol.

- Use appropriate loading buffer and loading volume as per the platform used (MinION/GridION/PromethION).
- Avoid bubbles when loading.

5.7 🧊 **Storage and Stability**

- **Barcoded samples**: Use immediately or store at -20°C (short-term).
- **Final library**: Load immediately. Do not freeze post-adapter ligation.

Long read sequencing using ONT

6 🧬 **Long Read Sequencing Protocol using Oxford Nanopore Technologies**

Workflow: Ligation Sequencing Kit (LSK114)
Platform: MinION / GridION / PromethION
Purpose: For high-yield, long-read sequencing using ONT flow cells

6.1 🧪 **Input DNA Requirements**

- **Amount**: ≥1 µg high molecular weight DNA
- **Concentration**: ≥50 ng/µL in ≥20 µL volume
- **Buffer**: Nuclease-free water or low-salt TE (avoid EDTA >0.1 mM)
- **Fragmentation**: Avoid shearing to preserve read length

6.2 🔬 **DNA Repair & End-Prep**

	Reagent	Volume (µL)
	DNA sample	Up to 48.0
	NEBNext FFPE DNA Repair Buffer	7.0
	NEBNext FFPE DNA Repair Mix	3.0
	NEBNext Ultra II End Prep Reaction Buffer	3.5
	NEBNext Ultra II End Prep Enzyme Mix	3.0
	Total	~64.5

Mix gently, spin briefly.
Incubate:

- **20°C for 5 min**
- **65°C for 5 min**

Place on ice.

6.3 🧲 **AMPure XP Bead Cleanup**

Add 1× volume (e.g., 65 µL) of AMPure XP beads.
Incubate 5 min at RT.
Magnet for 5 min → remove supernatant.

Wash twice with 200 µL 70% ethanol.
Air dry beads for 2 min (avoid over-drying).
Elute DNA in 61 µL nuclease-free water.

6.4 🔗 **Adapter Ligation**

	Reagent	Volume (µL)
	Eluted DNA	60.0
	NEBNext Quick T4 DNA Ligase Buffer (5X)	25.0
	Adapter Mix (AMX)	10.0
	Quick T4 DNA Ligase	5.0
	Total	100.0

Mix by gentle flicking.
Incubate **room temperature for 10–15 minutes**.

6.5 🧲 **Post-Ligation Cleanup**
Add 0.4× volume (~40 µL) of AMPure XP beads.
Incubate 5 min at RT.
Magnet for 5 min → discard supernatant.
Wash twice with 250 µL Long Fragment Buffer (LFB).
Air dry beads (≤2 min).
Elute final library in **15 µL Elution Buffer (EB)**.

6.6 💧 **Flow Cell Priming (MinION/GridION)**
Thaw and mix:
- Flush Buffer (FB)
- Flush Tether (FLT) → mix with FB to make priming mix

Open SpotON port and priming port.
Remove ~20 µL from priming port to release air.
Slowly inject **800 µL priming mix** via priming port.

6.7 💉 **Load Library**
Prepare loading mix:
- 37.5 µL Sequencing Buffer (SQB)
- 25.5 µL Loading Beads (LB, mix just before use)
- 12 µL Final library (from step 5)

Mix gently by pipetting.
Load **75 µL** into the SpotON port drop-by-drop.
Close ports and lid.

6.8 ▶️ **Start Sequencing Run**

Use **MinKNOW** software.
Choose appropriate flow cell and kit settings (e.g., LSK114).
Set run time (e.g., 24–72 hours depending on goal).
Start sequencing and monitor real-time QC metrics.

6.9 🧼 **After the Run**

- Optionally wash and reuse flow cells using **Flow Cell Wash Kit** (up to 2–3 times).
- Store remaining library at **-20°C** for short-term re-use (avoid freeze-thaw).

6.10 ✅ **Optional Tips**

- For ultra-long reads, minimize pipetting and vortexing.
- Use wide-bore or cut tips during extraction and library prep.
- Consider using Qubit and Femto Pulse for DNA QC and sizing.

16S sequencing for sample taxonomy profiling

7 🧬 **16S rRNA Gene Amplicon Sequencing (ONT Workflow)**
Goal: Taxonomic profiling via full-length 16S rRNA gene sequencing
Platform: MinION / GridION / PromethION
Kit: 16S Barcoding Kit (SQK-16S024 or similar)
Read Type: Long-read, full-length 16S (~1.5 kb)

7.1 🧫 **Sample Preparation**

- Extract total DNA from microbial or environmental samples.
- Use a high-fidelity, contamination-free method (e.g., Qiagen PowerSoil for stool).
- Quantify DNA using Qubit (dsDNA HS).
- Normalize DNA to **10–50 ng per PCR reaction**.

7.2 🔁 **16S rRNA Gene PCR Amplification**

	Reagent	Volume (µL)
	Template DNA (10–50 ng)	~2–5
	Barcoded 16S primers (forward + reverse)	1.0
	LongAmp Taq 2X Master Mix (NEB)	25.0
	Nuclease-free water	to 50.0

Cycling Conditions (for full-length 16S):

- 95°C for 3 min
- 25 cycles of:
- 95°C for 20 sec
- 55°C for 30 sec
- 65°C for 2 min

- Final: 65°C for 5 min → hold at 4°C

✅ Tip: Use primers covering full-length 16S gene (e.g., 27F/1492R).

7.3 🧲 **Clean Up PCR Products**

- Use **AMPure XP beads (1× ratio)**:
- Mix, incubate 5 min RT
- Magnet, wash ×2 with 70% EtOH
- Elute in 10 µL nuclease-free water

7.4 🧬 **Pooling and Quantification**

- Pool 10 µL of each barcoded sample.
- Quantify final pool via Qubit.
- Aim for **50–200 fmol total DNA** (up to 1 µg).

7.5 🔗 **Adapter Ligation (if not pre-tagged)**

	Reagent	Volume (µL)
	Pooled amplicons	~10
	Rapid Adapter (RAP) or AMX	1.0
	Incubate	5 min at RT

7.6 💉 **Load Library onto Flow Cell**

Prime the flow cell (see ONT Flow Cell Priming SOP).

Prepare the loading mix:

- ~12 µL DNA library
- 25.5 µL Loading Beads (LB)
- 37.5 µL Sequencing Buffer (SQB)

Mix gently and load **75 µL** dropwise onto SpotON port.

7.7 ▶️ **Start Sequencing**

- Use **MinKNOW** and select:
- Kit: SQK-16S024 (or latest)
- Flow Cell: FLO-MIN106D (R10.4.1 recommended)
- Duration: 12–48 hours depending on depth

7.8 🧠 **Data Analysis**

- Basecalling via **Guppy** or **MinKNOW**
- Taxonomic classification:
- Use **EPI2ME Fastq 16S Workflow**, or
- Tools like **QIIME 2**, **Kraken2**, or **NanoCLUST**
- Generate bar plot of relative abundances per sample

7.9 📦 **Storage**

- Store final amplicon libraries at **-20°C** short-term.
- Flow cells can be washed and reused (1–2 times).

7.10 ✅ **Notes**

- Full-length 16S sequencing provides species-level resolution.
- Minimize chimera formation by using high-fidelity polymerases.
- Always run negative controls to detect contamination.

Hybrid sequencing for metagenomes

8 🧬 **Hybrid Metagenomic Sequencing Protocol (Illumina + ONT)**

📦 **Sample Collection & DNA Extraction**

Collect samples (e.g. stool, soil, rumen, etc.) under sterile conditions.

Store samples at −80°C if not processing within 1 hour.

Extract high molecular weight (HMW) DNA using a suitable kit (e.g. QIAGEN MagAttract HMW DNA Kit).

- Minimise mechanical shearing.
- Aim for fragment sizes >20 kb for ONT compatibility.

Quantify DNA with Qubit and assess quality on TapeStation or agarose gel.

8.1 🧪 **Library Preparation**

Illumina (Short-Read) Library Prep

- Input: ~100 ng DNA.

Recommended kits:

- Illumina Nextera XT, or
- NEBNext Ultra II FS.

Fragmentation: ~300–500 bp.

Add adapters and indices.

Clean up with AMPure XP beads.

Validate size distribution with Bioanalyzer or similar.

8.2 **ONT (Long-Read) Library Prep**

- Input: ~1–2 µg of high-quality HMW DNA.

Recommended kits:

- Ligation Sequencing Kit (SQK-LSK114) – highest accuracy.
- OR Rapid Sequencing Kit (SQK-RBK114.96) – faster prep.

Perform end-repair/dA-tailing if needed.

Ligate adapters.

Clean up and elute DNA for sequencing.

8.3 🧬 **Sequencing**

Illumina

- Platform: HiSeq, NovaSeq or NextSeq.
- Paired-end 150 bp reads.
- Target depth: ~20–40 Gbp per sample.

ONT

- Platform: MinION, GridION, or PromethION.
- Flowcell: R10.4.1 or latest.
- Target depth: ~5–10 Gbp per sample.
- Monitor in real-time, reload flow cell if necessary.

8.4 🧱 **D. Data Processing & Hybrid Assembly**

Quality Control

- **ONT**: Basecall using Guppy (super accuracy), filter reads < Q10.
- **Illumina**: Trim adapters and low-quality bases using fastp or TrimGalore

Assembly

Short-read only:

- metaSPAdes
- MEGAHIT

Long-read only:

- Flye
- Raven

Hybrid assembly:

- metaFlye
- HybridSPAdes
- OPREA-MS

Polishing

Use

- Racon (long reads)
- Pilon (short-read) for iterative polishing.

8.5 🧱 **Binning & Genome Reconstruction**

Contig Binning

Tools:

- MetaBAT2
- MaxBin2
- VAMB
- SemiBin

Inputs: k-mer composition and coverage profiles.

Bin Refinement

Merge/refine bins with

- DAS Tool
- MetaWRAP

MAG Quality Assessment

Use to assess completeness and contamination.

- CheckM
- BUSCO

Taxonomic Assignment

Annotate bins using

- GTDB-Tk

8.6 🧬 **Downstream Analysis (Optional)**

Functional annotation:

- PROKKA / BAKTA
- eggNOG-mapper

Phylogenomics:

- PhyloPhlAn

AMR/virulence:

- ABRicate
- CARD
- VfDB
- AMRfinder Plus

8.7 💡 **Best Practices & Notes**

- Aim for **>40 Gbp** of Illumina data per sample for deep profiling.
- **ONT long reads** help resolve repeats, rRNA operons, plasmids, and MGEs.
- For low-complexity samples, less sequencing depth may suffice.
- Optional: Use **Hi-C** or read cloud technologies for improved binning.