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FLEX(-) Bacterial DNA Extraction Protocol for Nanopore Sequencing V.1

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We use this protocol and it's working

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Disclaimer

Please note: although this workflow may be broadly applicable to Gram-negative bacteria, it has been validated only for *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Acinetobacter* spp., *Stenotrophomonas maltophilia*, and *Serratia marcescens*.



Abstract

This protocol provides a **rapid, high-throughput, and cost-effective** method to extract **high-quality, long-fragment genomic DNA** from **Gram-negative bacteria**, optimized for producing DNA with **sufficient purity, concentration, and fragment length** for downstream molecular applications, with a primary focus on **whole-genome sequencing using Oxford Nanopore R10 chemistry** (including library preparation requirements). This workflow has been validated for *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Acinetobacter* spp., *Stenotrophomonas maltophilia*, and *Serratia marcescens*.

In addition to Nanopore sequencing, the extracted DNA is suitable for common downstream workflows such as **qPCR** and other **library preparation** applications. The protocol can also be used for **Illumina short-read sequencing**; however, faster Illumina-specific extraction options may be available. In settings where **both Nanopore and Illumina sequencing** are performed in parallel, this protocol enables a **single extraction** to support both workflows, avoiding duplicate extraction steps.

This method has been **validated and implemented** by the **Department of Clinical Microbiology at Mount Sinai Hospital**, and is currently used routinely to support sequencing workflows.

Guidelines

Definitions and Abbreviations:

- **FLEX(+)**: Fast, Long-fragment, EXtraction for Gram + bacteria.
- **SPRI**: Solid-Phase Reversible Immobilization paramagnetic bead chemistry.
- **RT**: Room temperature (20–25 °C).



Materials

Reagents

- **TE buffer (pH 8.00, filtered):** 10 mM Tris, 1 mM EDTA
- **Sarkosyl (N-lauroylsarcosine; stock):** 10% (prepared in ddH₂O or TE buffer)
- **Lysozyme (stock):** 100 mg/mL (prepared in ddH₂O or TE buffer)
- **Proteinase K (stock):** 20 mg/mL (prepared in ddH₂O or TE buffer)
- **SPRI-Select paramagnetic beads:** Beckman Coulter
- **Molecular-grade water:** ddH₂O
- **Ethanol (fresh):** 80% (v/v) for bead washes

Consumables

- **0.5 mm glass bead tubes:** Qiagen PowerBead tubes with screw caps (or equivalent)
- **Inoculation loops:** sterile 1 µL loop (or 10 µL loop)

Collection vessels (choose one):

- 1.5 mL low-bind microcentrifuge tubes, **or**
- 0.8 mL “midi” or PCR 96-well plate
- **Pipette tips:** aerosol-resistant filter tips (20–1000 µL range)

Equipment

- **Vortex mixer:** compatible with a Qiagen PowerBead vortex adapter top
- Qiagen PowerBead vortex adapter top
- **Heating:** calibrated temperature blocks or water baths at **37 °C** and **56 °C**
- **Magnetic separation (choose one):**
- Thermo **DynaMag** rack (for 1.5 mL tubes), **or**
- Illumina-style **96-well magnetic rack** (for midi plates)
- **DNA quantification:** Qubit fluorometer (HS dsDNA assay) and NanoDrop (or equivalent spectrophotometer)
- **Fragment analysis (optional/as available):** TapeStation, Femto Pulse, or equivalent system



Troubleshooting

Problem

If concentration QC fails: Too low

Solution

Discard the eluate and rerun the protocol with more biomass than initial run.

Problem

If concentration QC fails: Too high

Solution

Simply dilute the final eluate with more ddH₂O until appropriate

Problem

If contamination QC fails:

Solution

Perform another SPRI Cleanup on the final eluate, but use 100 μ L of SPRI beads instead of original 225 μ L.

Problem

If fragment analyses QC fails: Too short

Solution

Discard eluate, repeat full FLEX protocol with 3 min of bead beating instead of 5 min.

Problem

If fragment analyses QC fails: Too long

Solution

Discard eluate, repeat full FLEX protocol with 8 min of bead beating instead of 5 min.

Safety warnings

⚠ Throughout the protocol, particularly the section that utilizes magnets, it is recommended that you review the actual mechanism behind DNA binding, and understand the chemistry of the SPRI select beads. This makes troubleshooting, as well as appropriate removal from the magnet, much more intuitive.

Warning: Sarkosyl (N-lauroylsarcosine) is an irritant and can be harmful if inhaled or if it contacts skin/eyes—handle it in a fume hood when possible, wear appropriate PPE (lab coat, nitrile gloves, eye protection), avoid generating aerosols, and clean spills immediately according to your institution's chemical safety procedures.



Before start

Specimen Collection

- **Specimen:** Single pure isolate from a confluent purity plate (18–24 h typical, organism-appropriate conditions).
- **Transport/Storage:** Room temperature (RT) bench-top during setup
- **Rejection criteria:** Mixed culture, plate age under 72 h (discretionary), or visible contamination.
- **Recommendations:**
 1. Confirm species ID using MALDI-TOF or other equivalent identification mechanism.
 2. Use an ethanol safe marker/pen when labelling tubes to prevent washing of labels



FLEX(-) Extraction Preparation

- 1 Label a **1:1 matched set** of **PowerBead tubes** and **LoBind tubes** for each isolate accession (use the same sample ID format across all tubes). Then label the **collection vessels** using the same IDs—either:
 - **1.5 mL Eppendorf tubes** (recommended for ≤ 48 samples), **or**
 - a **0.8 mL 96-well PCR plate** (recommended for > 48 samples).

- Note: Each requires a different magnet type; ensure you have the appropriate magnet.
- 2 Pre-equilibrate a temperature block or water bath to **56 °C** (either may be used based on personal preference/equipment availability).
- 3 Attach the **Qiagen Vortex Adapter top** to a **Vortex-Genie** (or equivalent vortex mixer).

Note: The adapter top holds **up to 24 samples**; use multiple adapter tops in parallel if processing more than 24 samples at once.

- 4 Calculate how much of each enzyme is required for given batch size (See Table 1).

- 5 Table 1:

	A	B
Reagent:		Proteinase K 20mg/mL
Volume/Sample:		5 μ L

- 6 Calculate the amount of each reagent required for a given batch size (See Table 2).

- 7 Table 2:



	A	B	C	D	E	F
	Reagent:	TE Buffer (10 mM Tris, 1 mM EDTA), pH 8.00)	SPRI Select Beads (Beckman Coulter)	Fresh 80% Ethanol	Molecular Grade Water (ddH ₂ O)	10% Sarkosyl Solution
	Volume/ Sample:	300μL	225μL	1200μL	100μL	8μL

- 8 Remove the enzyme (Proteinase K) from the freezer and allow it to thaw. Keep at 4 °C until ready for use.
- 9 Aliquot **300 μL** of TE Buffer into each 0.5mm Glass Powerbead Tube.
- 10 Inoculate each PowerBead tube with **isolated bacterial colonies** using either a **heaping 1 μL loop** or **half of a 10 μL loop**, while **minimizing agar carryover**.

Note: After inoculation, gently **spin/rotate the loop in the tube** to help disperse and homogenize the biomass before proceeding.

Membrane Solubilization

- 11 Pipette 8μL Sarkosyl (10% sol), (Using a P10) into each Powerbead tube, directly into the solution



- 12 Slowly and **gently** pipette mix up and down 2-3 times.

Over-pipetting may lead to foam that makes later steps far more difficult. In the case this occurs, allow foam to settle, gently tapping the tube to speed up the process. Some foam is expected regardless

Mechanical Lysis

- 13 Insert Powerbead tubes into a Qiagen Vortex Adapter Top, with the cap side facing inward.

- 14 Place the top onto a Vortex Genie.

- 15 Set the vortex **speed to 9** (or the max speed on an equivalent Vortex), and allow the top to spin for **5 minutes**.

Note: If using an alternative Vortex, samples may spin faster than expected, causing over fragmentation of DNA, this can be corrected for by lowering the speed until fragment analysis returns optimal results)

- 16 Turn off the vortex and remove the adapter top.

- 17 Place the tubes back onto a rack, maintaining the original format.

Denaturation of Interfering Proteins

- 18 Pipette **5 µL** of Proteinase K (20 mg/ml) (Using a P10) into each Powerbead tube, directly into solution.

- 19 Slowly and **gently** pipette mix up and down 2-3 times.

- 20 Place Powerbead tube into a 56 °C dry incubator or a 56 °C water bath, maintaining the original layout of the tubes on their respective racks.



- 21 Incubate for **15 min.**
- 22 Remove from incubator.

Removal of Contaminants/DNA Binding

- 23 Transfer **225 µL** of the solution from Powerbead tubes into either a 0.8 ml, 96 well MIDI/PCR Plate, or a 1.5 ml Eppendorf tube (see note in step 1)

Note: You may find that the powerbeads are sucked up into the pipette. This can be solved by tilting the tube sideways and drawing solution from the side beads are not sitting on.
- 24 Add **225 µL** of SPRI Select Beads into each well/tube.

Note: a 1:1 ratio of SPRI select beads to solution ensures you select for fragments of all sizes.
- 25 Mix by pipetting up and down 3-5 times.

- Homogeneity is critical for this step as too little mixing will prevent complete DNA binding.
- 26 Place onto appropriate magnet (96 well Illumina Magnet for MIDI plates, Thermo DYNAMag Magnet for Eppendorfs).
- 27 Leave on magnet until lysate appears clear (3-5 min), and a pellet has formed on the well/tube side wall.
- 28 With the plate/tube STILL on the magnet, carefully remove the supernatant with a P1000 set to 1000 µL. Avoid touching the pellet. If any pellet is sucked into the pipette, put it back into the well/tube, towards the magnet side, and leave for another 2 minutes before removal of supernatant.

It is highly recommended that you review the mechanism behind SPRI select beads to best understand and proactively troubleshoot proper lysate removal.

Ethanol Washes x 2

- 29 With the following steps, you must put on and take off the plate/tube from the magnet as stated. Failure to do so will result in the complete loss of eluate.

- 30 **Remove** the tube/plate from the magnet.
- 31 Pipette 600 μ L of 80% ethanol into each well/tube directly onto the pellet.
- 32 Resuspend the pellet in the ethanol by pipetting up and down until homogeneous.
Note: Some pellets may require scraping with the tip of the pipette to help them better homogenize.
- 33 Place tube/plate **back onto the magnet**.
- 34 Allow pellet to reform (2-3 min).
- 35 Remove supernatant with a P1000 set to 1000 μ L.
- 36 **Remove tube/plate** from magnet again.
- 37 Pipette 600 μ L of 80% ethanol into each well/tube directly onto the pellet.
- 38 This time, **do not** resuspend the pellet fully by mixing, simply allow the ethanol to sit on the pellet.
- 39 Place the tube/plate **back onto the magnet**.
- 40 Wait 2-3 min.
- 41 Remove and discard the supernatant with a P1000 set to 1000 μ L, carefully avoiding the pellet.
- 42 Allow pellet to air dry for **10 min**, monitoring and wicking away any residual pooling; do not over-dry to the point of visible cracking. That said, it is better to over-dry than under-

dry, as any remaining ethanol will act as a contaminant in your final eluate.

- 43 Once dry, take the tube/plate **off the magnet**.

Final DNA Elution

- 44 Add **100 µL** of Molecular Grade Water (ddH₂O) directly onto the pellet.

Note: if you are finding that your yield is too low using 100µL, you may decrease the volume accordingly.

- 45 Resuspend pellet **fully**, by mixing up and down with a pipette.

- 46 Place tube/plate **back onto the magnet**.

- 47 Wait 2-3 min for pellet to reform.

- 48 Transfer all 100 µL of the supernatant into a labelled Lo-Bind Eppendorf tube, this is your final DNA solution, which can now be stored at -80 °C for up to 10 years.

- 49 You may now discard the tubes/plate with the pellet (DNA is now in the supernatant you just realiquoted)

Eluate Quality Control (Optional, but highly recommended)

- 50 Quantify DNA with a Qubit Fluorometer, using an eluate input of 4 µL, and a High Sensitivity Reagent input of 196 µL. (See Oxford Nanopore Technologies NO-MISS Protocol for acceptable eluate concentrations)

- 51 Check for contamination using a Nanodrop or equivalent device. (See Oxford Nanopore Technologies NO-MISS Protocol for acceptable 260/280 and 260/230 ratios)

- 52 Optionally: Check for high fragment length retention using a Bioanalyzer or Fragment Analyzer (See Oxford Nanopore Technologies NO-MISS Protocol for acceptable fragment lengths)

- 53 See Troubleshooting Guide for related fixes

