Apr 23, 2024

Sequencing Bacterial Isolates from Contaminated Food Samples with ONT

DOI
dx.doi.org/10.17504/protocols.io.81wgbz7zogpk/v1

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DOI: dx.doi.org/10.17504/protocols.io.81wgbz7zogpk/v1

Protocol Citation: Sarah Fletcher 2024. Sequencing Bacterial Isolates from Contaminated Food Samples with ONT. **protocols.io** https://dx.doi.org/10.17504/protocols.io.81wgbz7zogpk/v1

Protocol status: In development
We are still developing and optimizing this protocol

Created: April 23, 2024

Last Modified: April 23, 2024

Protocol Integer ID: 98681

Abstract

In this protocol, we aim to sequence multiple bacterial isolates from 1 contaminated food sample. The goal is to obtain long-read sequences for food-borne pathogens to provide information regarding their virulence factors. We will use Oxford Nanopore Technologies devices, kits, and protocols to prepare DNA libraries, sequence our bacterial samples, and analyze our sequencing data.

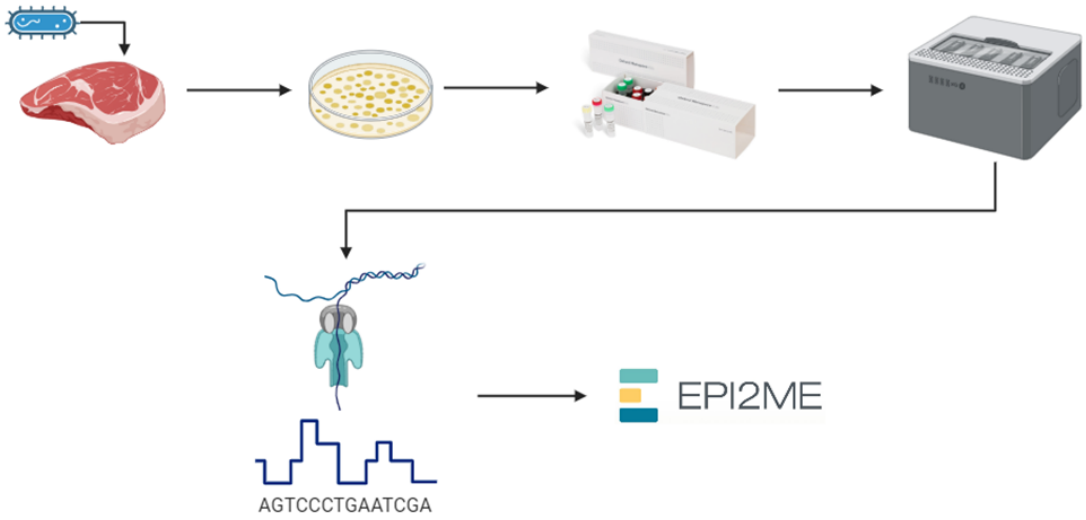


Figure 1. Overview of entire protocol workflow. Obtain a culture of the bacteria from contaminated food samples of interest. Perform colony PCR-based DNA extraction and measure quantity and quality using a nanospectrophotometer or Qubit device. Prepare your DNA library using the Rapid Barcoding Kit from Oxford Nanopore Technologies (ONT). Load your library into ONT MinION flow cells and sequence using the GridION device. Enable high-accuracy real-time basecalling. Analyze your sequence data using the NextFlow wf-metagenomics and What's in My Pot (WIMP) workflows with EPI2ME Labs.

Part 1: Colony PCR DNA Extraction

1h

1 Because we are interested in genomic DNA for this protocol, obtain 1 colony from the culture plate using a sterile toothpick, needle, or inoculation loop and swirl it in 50µL of 10mM Tris-HCl (pH: 8.0) for 10 seconds.

1.1 **Enriching for plasmid DNA only:** If you are interested in the genomic DNA, proceed straight to step 2.3. If you are interested in the plasmid DNA, transfer the 50 µl cell suspension to a 0.2 ml thin-walled PCR tube and incubate at 95°C for 5 minutes.
Note: It is observed that pre-treating the colony by heating leads to an enrichment in the observation of plasmid reads in the downstream sequencing, compared with non-heat treated libraries.

1.2 **Enriching for plasmid DNA only:** Add 1 µl of Thermolabile Proteinase K and incubate at 37°C for 15 minutes, then 55°C for
10 minutes.

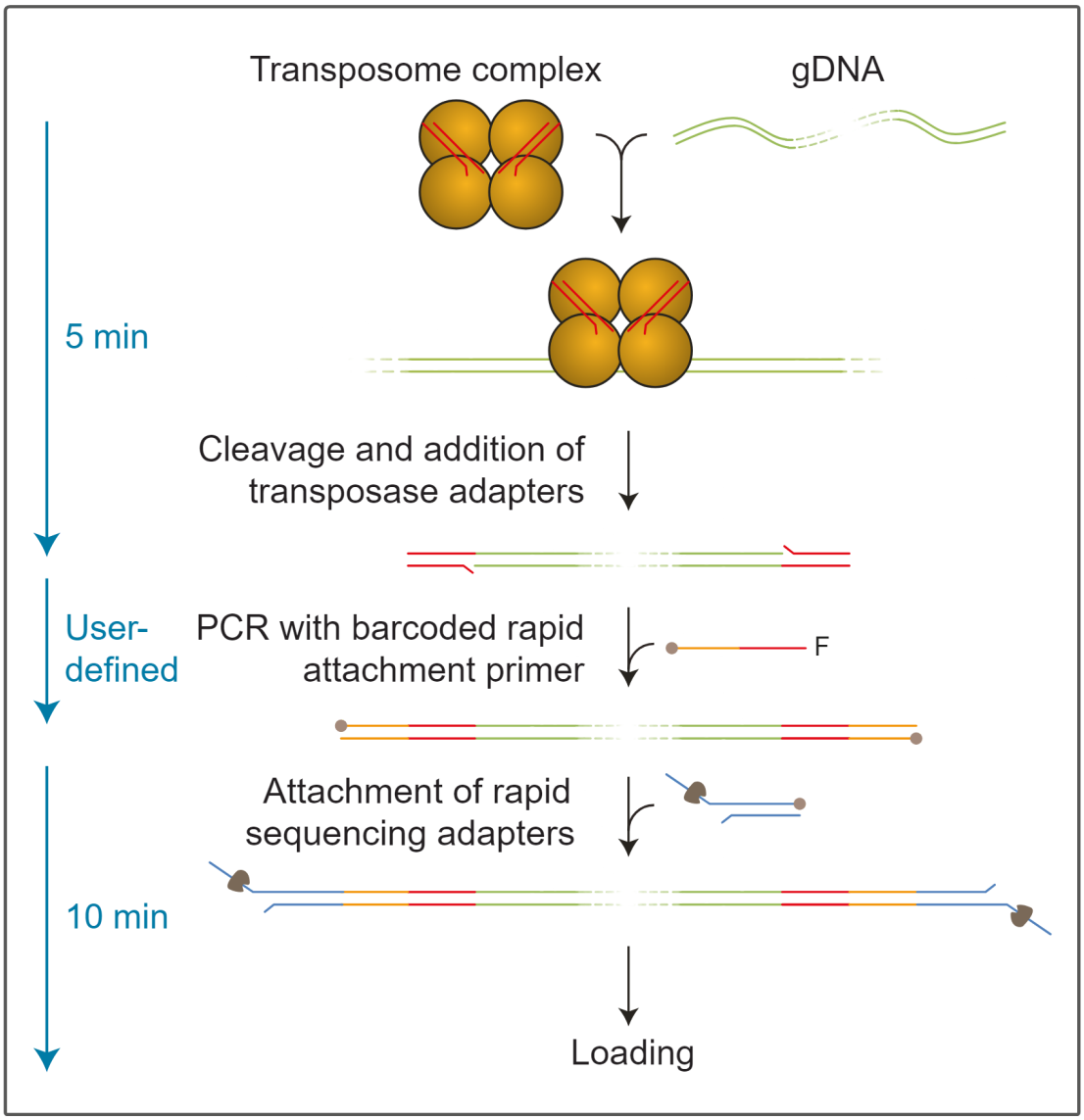
2 Libraries can be prepared for sequencing using the Rapid PCR Barcoding Kit, using 3 µl of the treated cell suspension as the template. If using cells without the heat treatment in step two, it is recommended to PCR for 25 cycles, and if using heat-treated cells, it is recommended to PCR for 30 cycles.

3 Repeat for however many individual colonies (i.e., bacterial samples) you wish to run (up to 24).

Part 2: DNA Library Prep using ONT Rapid PCR Barcoding Kit

3h

4 .



Rapid PCR Barcoding Kit Workflow from ONT.

5 Transfer 1-5ng of each sample to a 1.5mL Eppendorf DNA LoBind tube.

6 Adjust the volume to 3µL

7 Flick to mix and briefly spin down

8 Mix the following in a 0.2mL thin-walled PCR tube: 1-5ng template DNA (3µL), Fragmentation Mix (FRM) (1µL)

9 Flick to mix and briefly spin down

10 Incubate PCR tube in a thermal cycler at 30°C for 2 minutes, then 80°C for 2 minutes. a. Afterward, put the tube on ice briefly to cool down.

11 For each sample, set up a PCR reaction in a 0.2mL thin-walled PCR tube as follows: a. Nuclease-free water (20µL) b. Tagmented DNA (4 µL) c. RLB Barcode (1-24, 1 µL) d. LongAmp Taq 2X Master Mix (25 µL)

12 Flick to mix and briefly spin down.

13 Again in the thermal cycler, amplify DNA under the following conditions:

14 SS

	A	B	C	D
	Cycle Step	Temperature (°C)	Time	# Cycles
	Initial Denaturation	95	3 min	1
	Denaturation	95	15 sec	14
	Annealing	56	15 sec	14
	Extension	65	6 min	14
	Final Extension	65	6 min	1

PCR Settings for Step 14.

14.1 After PCR, hold at 4°C or continue

15 Add 4µL EDTA to each barcoded sample.

16 Pipet to mix and briefly spin down.

17 Incubate for 5 minutes at room temperature.

18 Quantify 1 µL of each barcoded sample using a Qubit 4 Fluorometer.

19 Pool all barcoded samples in equimolar ratios for a final concentration of 200-400fmol (~400-800ng) for pooled samples.

20 Resuspend AMPure XP Beads (AXP) by vortexing.

21 To the pool of barcoded samples, add a 0.6X volume ratio of resuspended AMPure XP Beads (AXP) and mix by pipetting.

22 Incubate on a Hula mixer for 5 minutes at room temperature.

23 Prepare 2 mL of 80% ethanol in nuclease-free water.

24 Briefly spin down.

25 Pellet on magnet until supernatant is clear & colorless.

26 Keeping tube on the magnetic rack, pipet off supernatant and discard

27 Keeping tube on magnetic rack, wash beads with 1 mL 80% ethanol without disturbing pellet.

27.1 Remove the ethanol using a pipet and discard.

27.2 Repeat step 28.

28 Spin down and place the tube back on the magnet. Pipet any residual ethanol and allow to dry for 30 seconds.

29 Remove tube from magnetic rack and resuspend pellet in 15 µL elution buffer (EB).

30 Spin down and incubate for 5 minutes at room temperature.

31 Pellet the beads on a magnet until supernatant is clear and colorless.

31.1 . At least 1 minute.

32 Remove and retain eluate into a sterile 1.5mL Eppendorf DNA LoBind tube.

33 Quantify 1 µL using Qubit.

34 Transfer 10–50 fmol of your eluted samples into a clean 1.5 mL Eppendorf DNA LoBind tube. Make up the volume to 11 µl with Elution Buffer (EB).

35 In a fresh 1.5 mL Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix: Rapid Adapter (RA, 1.5 µL), Adapter buffer (ADB, 3.5 µL)

36 Add 1 µL of the diluted Rapid Adapter (RA) to the barcoded DNA.

37 Flick to mix and briefly spin down.

38 Incubate for 5 minutes at room temperature.

Part 3: Load into MinION Flow Cell and Sequence Using GridION Sequencing Device

30m

39 Load following manufacturer's instructions, adding sample to SpotOn port in a drip-wise fashion.

40 Insert the MinION flow cell into the GridION device and sequence using MinKNOW with the correct DNA Library Prep Kit selected (Rapid PCR Barcoding Kit).

Part 4: Analyze using EPI2ME Labs

10m

41 Analyze the sequence data using the wf-metagenomics (NextFlow) and What's in My Pot (WIMP, EPI2ME) workflows on the EPI2ME Labs software.

42 Upload sample sheet with barcode IDs.

43 Follow default settings.

43.1 Decide if you want to gather antimicrobial resistance (AMR) data.

Protocol references

Part 1:
https://community.nanoporetech.com/extraction_methods/colony-pcr-dna

Part 2 & 3: https://community.nanoporetech.com/docs/prepare/library_prep_protocols/rapidsequencing-dna-v14-pcr-barcoding-sqk-rpb114-24/v/rpb_9191_v114_revb_28jun2023?devices=minion

Part 4:
https://github.com/epi2me-labs/wf-metagenomics
https://nanoporetech.com/resource-centre/epi2me-wimp-workflow-quantitative-realtime-species-identification-metagenomic