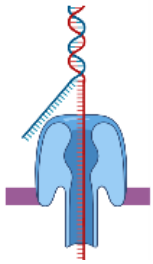
Sep 24, 2025 Version 2

Shotgun Metagenomic Sequencing of Bacterial Enrichments on the Oxford Nanopore Platform Using the R10 flow cell and Ligation sequencing Kit V.2



Version 1 is forked from Nanopore Library Preparation for R10 Ligation Sequencing Kit

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We use this protocol and it's working

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Abstract

This protocol produces multiplexed amplicon libraries from DNA extracted from general and selective bacterial enrichments using Nanopore's Ligation sequencing kit, suitable for sequencing on Oxford Nanopore Technologies ® (ONT) MinION/Gridion systems using ONT V14 chemistry (SQK-NBD114-96).

Guidelines

Overview
This protocol provides guidelines for multiplexing up to 16 bacterial enrichments on a single R10 flow cell, or up to 48 single-colony isolate DNA libraries on one flow cell. The sequencing depth generated is insufficient to close isolate genomes or to recover high-coverage metagenome-assembled genomes. However, the reads obtained are suitable for applications such as serotyping using genome indexing approaches.

Materials

	A	B	C	D	E
	Component	Acronym	Quantity	Color	Volume per vial
	Native barcode plate	NB01-96	3	-	8 µl per well
	DNA control sample	DCS	3	Yellow	35 µl
	Native adapter	NA	2	Green	40 µl
	Sequencing buffer	SB	2	Red	700 µl
	Library beads	LIB	2	Pink	600 µl
	Library solution	LIS	2	White	600 µl
	Elution buffer	EB	1	Black	1500 µl
	AMPure XP beads	AXP	1	Amber	6000 µl
	Long fragment buffer	LFB	1	Orange	7500 µl
	Short fragment buffer	SFB	1	Clear	7500 µl
	EDTA	EDTA	1	Clear	700 µl
	Flow cell flush	FCF	1	Blue	15500 µl
	Flow cell tether	FCT	2	Purple	200 µl

Required Materials Not Included

- Nuclease-free water, molecular biology grade
- AMPure® XP beads (Beckman Coulter A63880) or equivalent
- 80% Ethanol (freshly prepared, molecular biology grade)
- DNA LoBind Tubes (Eppendorf® #022431021)
- Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Inc.® Q32851)

- Magnetic rack/stand (NEB #S1515, Alpaqua®, cat. #A001322 or equivalent)
- Thermal cycler
- Vortex Mixer
- Microcentrifuge
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables (#4150 or #4200 TapeStation System)
- DNase RNase free PCR strip tubes (USA Scientific 1402-1708)
- 1.5 ml tube magnet stand (NEB #S1506)
- NEB Blunt/TA Ligase Master Mix (NEB #M0367)
- NEBNext UltraII End repair/dA-tailing Module (NEB #E7546)
- NEBNext Quick Ligation Module (NEB #E6056)
- Bovine Serum Albumin (BSA)

Troubleshooting

Safety warnings

Please refer to Safety Data Sheets (SDS) for health and environmental hazards.

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

Before start

- Ensure you have sufficient DNA for your planned sequencing run.
- Decide on the number of samples to multiplex on a single flow cell.
- Thaw all NEB reagents and the Ligation Sequencing Kit components in advance, and confirm they are ready for use before starting the protocol.
- The library preparation here has some modifications from the original protocol in the barcoding step. Please read the step carefully before proceeding with barcoding.

Step 1: End-prep

1h

1 This section will take you from extracted DNA with a known concentration to a set of samples ready to be barcoded.
Before you start:
1. Start thawing on ice the reagents listed in step 3
2. You will need a significant amount of nuclease free water so make sure to have a bottle of it accessible.

2 Dilute DNA to 650 ng total, at 12 µL per sample
* If the concentration of DNA is too low, i.e. a negative number in column B, just use 12 µL of DNA.
The equation for this calculation is 650/DNA concentration.

The table will populate after values are entered! See attached Excel file!

DNA Dilution Tool.xlsx

3 For each sample, calculate a master mix of the following reagents per sample.
* Calculate for 2 extra samples to account for pipetting error. See attached Excel doc!

End Repair Master Mix Tool.xlsx

Add 3 µL of the above prepared master mix to each of your 650 ng/µL samples, pipetting up and down 10-20 times after each addition. Cover and spin down once all reagents are added.

4 Place the plate in a thermal cycler at 20 °C for 5 minutes, then 65 °C for 5 minutes.

Step 2: Barcoding and Cleanup

1h

5 On ice thaw:
- NEB Blunt/TA Ligase Master Mix
- EDTA blue cap tube(0.5M)
- Native Barcode (NB01-96)

At room Temperature thaw:
AMPure XP Beads (AXP). Vortex to mix.

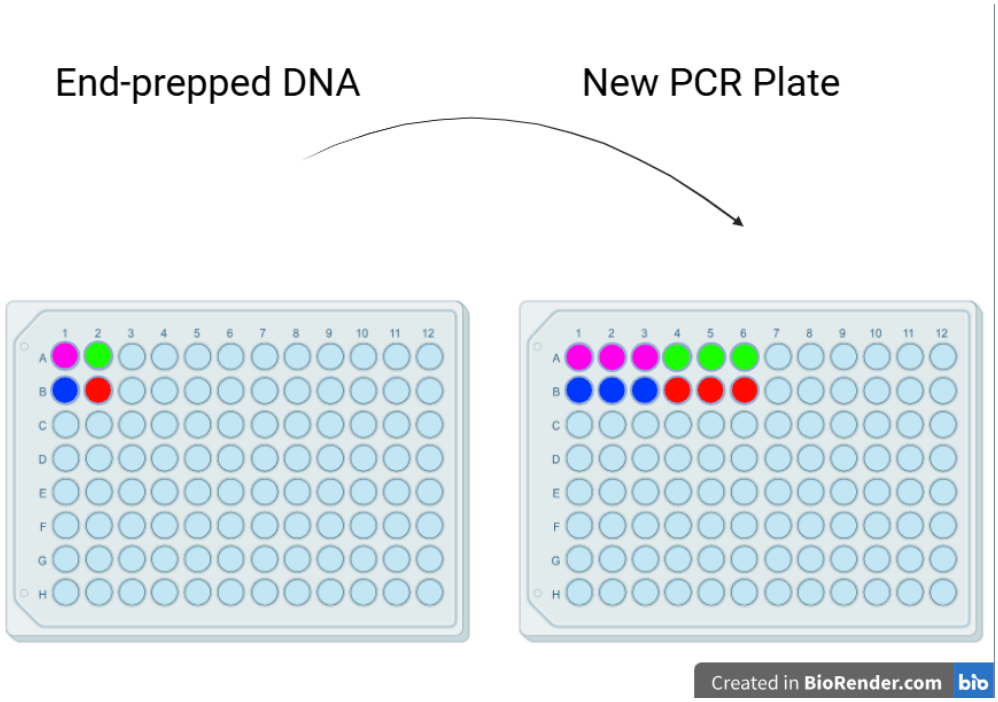
Note: It is important to add all reagents in the order listed. Adding the Ligase Master Mix and the Native Barcode without end prepped DNA will cause the Barcode to bind to itself.

Do not vortex the sample at all during barcoding!

6 Barcoding happens in triplicate to maximize the number of quality read DNA fragments.
To do this:
1. Get a new and separate 96 well PCR plate. (Unless you have 2 columns or less).

7

2. Thrice, take 3.75 µL from one sample and put it into a well on the separate plate. It should look like this:
Note: Do not use the first well as a sample for end prep and barcoding! Only use the 3 new ones from this step.



8 For each set of 3 end-prepped DNA samples, take from 1 well from a Native Barcode (NB01-96) plate.
Add 1.25 µL of barcode to each well, making sure each set of triplicates get the same barcode, and only one barcode goes to each set of triplicates.

9 Lastly, add 5 µL of Blunt/TA Ligase Master Mix to each well. Then cover and spin down the plate.

10 Incubate the plate for 20 minutes at room temperature.

11 Add 2 µL EDTA (blue cap tube) to each well to stop the reaction.

12 **Combine all samples into one 1.5 mL LoBind Eppendorf tube. There are 13 µL of sample in each well.**
For example: In an 8-sample run, there should be 24 total wells with 13 µL of sample in each well, with a total pooled volume of 312 µL

13 Resuspend AXP by vortexing and add 0.4X of pooled sample volume to the pooled reaction, mix by pipetting.

14 Incubate on a hula mixer for 10 minutes at room temperature.

15 Make 5 mL 80% ethanol in in nuclease free water per sample tube.

16 Spin down the sample and pellet on a magnet for 5 minutes until the supernatant is clear and colorless. Pipette off the supernatant and discard.

17 Wash the bead with 700 µL of 80% ethanol by placing the pipette tip against the side of the Eppendorf tube above the pellet and slowly dispensing the ethanol. Remove the ethanol and discard.

18 Repeat Step 16.

19 Spin down and place the tube back on the magnetic rack. Pipette off any residual ethanol, allow the pellet to dry for ~30 seconds, but do not let the pellet dry to the point of cracking.

20 Remove the tube from the magnet and resuspend the pellet in 35 µL of nuclease free water.

21 Incubate for 10 minutes at 37 °C on a hula mixer on the lowest setting, rocking for 10 seconds every 2 minutes.

22 Pellet the beads on a magnet until the supernatant is clear and colorless. Remove the 35 µL of supernatant and place it into a 1.5 mL LoBind Eppendorf tube.

23 Use a Qubit fluorometer to quantify 1 µL of barcoded sample.

Step 3: Native Adapter Ligation and Cleanup

30m

24 Thaw all reagents in the following table then place on ice. They are found in the NEBNext Quick Ligation Reaction Module.

	Reagent
	Native Adatper (NA)
	NEBNext Quick Ligation Buffer (5X)
	Quick T4 DNA Ligase
	Elution Buffer (EB)

Do not vortex the Quick T4 DNA Ligase
Thaw all reagents, then spin them down for 5 seconds. Mix all reagents by performing 10 full volume pipette mixes.
If the Buffer has precipitate in it, vortex it until the precipitate dissolves fully.

25 Mix the following reagents in a 1.5 mL LoBind tube in the following order, pipetting 10-20 times between addition of reagents:

	Reagent	Volume
	Pooled Barcode Sample	30 µL
	Native Adapter (NA)	5 µL
	NEBNext Quick Ligation Buffer (5X)	10 µL
	Quick T4 DNA Ligase	5 µL
	Total	50 µL

Mix the reaction by gently pipetting up and down and briefly spinning down.

26 Incubate the reaction for 20 mins at room temperature

27 Resuspend the AMPure XP beads by vortexing.

28 Add 20 µL well vortexed AMPure XP Beads (AXP) to the tube and mix by flicking the tube.

29 Incubate for 10 minutes on a Hula mixer at room temperature (25°C).

30 Spin down the sample and pellet on a magnet until the eluate is clear and colorless, for at least 1 minute. Keep the tube on the magnet and pipette off the supernatant.

31 Wash the beads by adding 125 µL Long Fragment Buffer (LFB). Flick the beads to resuspend, spin down, and pellet the beads on a magnet until the eluate is clear and colorless, for at least 1 minute.

31.1 Remove the supernatant using a pipette and discard.

32 Repeat the previous step (step 31 &31.1).

33 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for 5~30 seconds. Do not dry the pellet to the point of cracking.

34 Remove the tube from the magnetic rack and resuspend the pellet in 15 µL Elution Buffer (EB). Spin down and incubate for 10 minutes at 37°C.

35 Spin down the sample and pellet on a magnet until the eluate is clear and colorless, for at least 1 minute. Keep the tube on the magnet and retain 15 µl of eluate containing the DNA library into a clean 1.5 mL microtubes.

36 Use a Qubit fluorometer to quantify 1 µL of final library.

37 For short-term storage or reloading flow cells between washes, you can keep the prepared library at 4°C. While for long-term storage of more than 3 months, storing libraries at -80°C is recommended.

If you are sequencing this library within an hour, continue on to the next section where you will prime the nanopore flow cell.

Step 5: Flow cell check, Priming and loading the SpotON flow cell

38 Before priming the flow cell, load the flow cell onto the Gridion or minion, and complete the flow cell check. Assess the number of available pores. You need above 800 available pores for a successful run.

39 Thaw the flow cell, Sequencing Buffer (SQB), Library beads (LIB), BSA (not included in the kit), Flow cell flush, Flow cell Tether (FCT) at room temperature on cooling block.

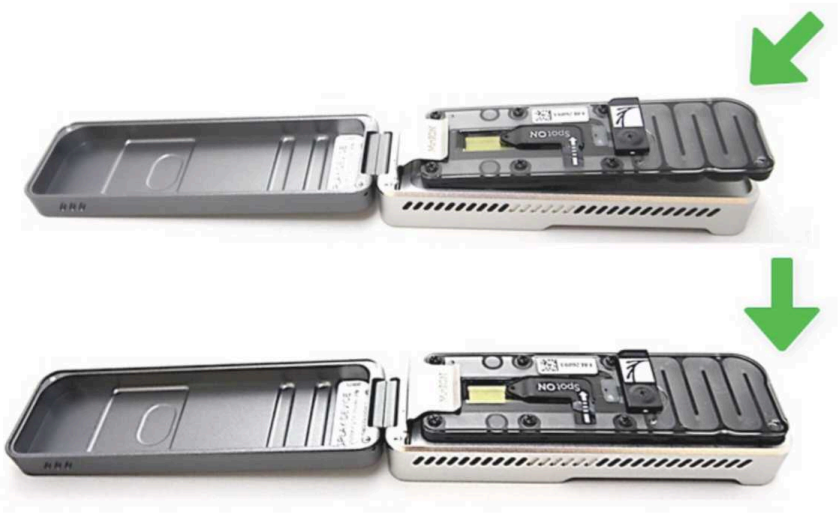
40 Mix the flow cell priming mix with BSA in accordance with the table below:

	Reagent	Volume / flow cell
	Flow Cell Flush (FCF)	1,170 µL
	50 mg/mL Bovine Serum Albumin (BSA)	5 µL
	Flow Cell Tether (FCT)	30 µL
	Final Total Volume	1,205 µL

41 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.

Note

Make sure there are no bubbles in the flow cell.



42 Slide the flow cell priming port cover clockwise to open the priming port.

43 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:
1. Set a P1000 pipette to 200 µl.
2. Insert the tip into the priming port.
3. Turn the wheel and aspirate fluid until the dial shows 220–230 µl, 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.

44 Load 800 µl of the flow cell priming mix into the flow cell via the priming port by turning the pipet wheel, avoiding the introduction of air bubbles. Wait for 5 minutes. During this time, prepare the library for loading by following the steps from step 43 and 44 below.

45

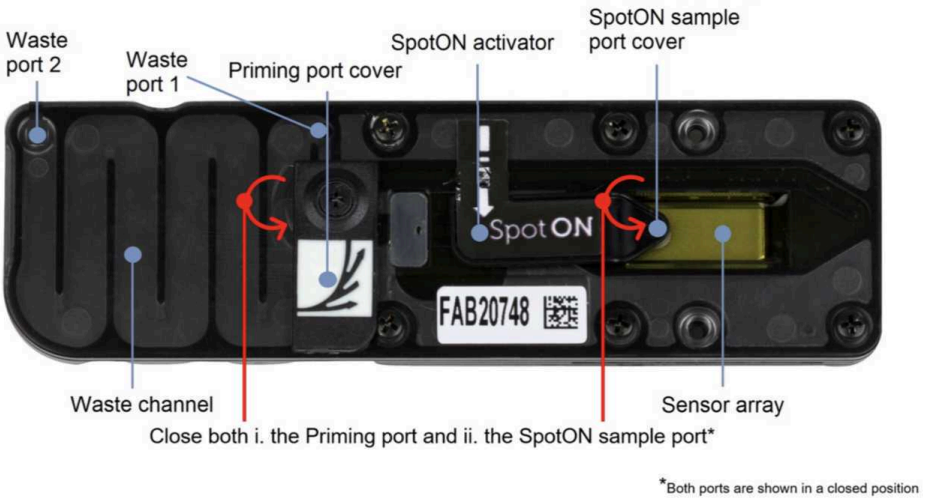
	Reagent	Volume
	Sequencing Buffer (SB)	37.5 µl
	Library Beads (LIB) Mixed immediatly before use	25.5 µl
	DNA Library	12 µl
	Total	75 µl

46 Complete the flow cell priming:
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load 200 µl of the priming mix into the flow cell via the priming port (**not the SpotON sample port**), avoiding the introduction of air bubbles.

47 Mix the prepared library gently by pipetting up and down in the tip using a P200 pipet just prior to loading.

48 Add the library to the flow cell via the **SpotON sample port** in a dropwise fashion. Ensure each drop flows into the port before adding the next.

49 Gently close the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port, cover the sensor array with the sensory cover, and close the MinION device lid.



Setting up parameters of sequencing

50 Name the experiment, sample id and library preparation kit (Native barcoding kit 114-96) and choose 48 hrs of sequencing while collecting fastqs every hour. Hit start sequencing.