Oct 09, 2025 Version 4

Nanopore Library Preparation for R10 NativeBarcoding/Ligation Kit V.4

DOI
dx.doi.org/10.17504/protocols.io.3byl4wpm2vo5/v4

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Protocol Citation: Yin-Tse Huang 2025. Nanopore Library Preparation for R10 NativeBarcoding/Ligation Kit. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.3byl4wpm2vo5/v4**Version created by **Yin-Tse Huang**

Protocol status: Working
We use this protocol and it's working

Created: September 04, 2025

Last Modified: October 09, 2025

Protocol Integer ID: 226414

Keywords: nanopore, library construction, nanopore library preparation for r10 nativebarcoding, nanopore library preparation, r10 nativebarcoding, flow cell

Abstract

Nanopore R10 flow cell Native Barcoding library construction.

Troubleshooting

Step1. End-prep

1h

1 Make sure you have high concentration of DNA (>100 ng/µL, quantified by Qubit).
Use the "**Volume_original**" by default

2 In 200 µL tube(s), combine the following chemicals per tube:

	A	B	C
	Reagent	**Volume_down**	**Volume_original**
	DNA	6.4 uL	11.5 uL
	KAPA HyperPrep End Repair & A-tailing Buffer	0.77 uL	1.38 uL
	KAPA HyperPrep End Repair & A-tailing Enzyme	0.33 uL	0.59 uL
	TOTAL	**7.5 uL**	**13.47 uL**

Note

Make sure the E&A buffer does not contain white precipitates before aspirate it.

Note

Mix the chemicals by flicking the tube with your finger. Spin down the tube.

2.1 Incubate in a thermocycler at 20 °C for 00:30:00 and 65 °C for 00:30:00 . Hold at 4 °C

1h

Step3. Native barcode ligation

3 Thaw the Native Barcodes (NB) required for your number of samples at room temperature on a cooling block.

Note

Individually mix the barodes by vortexing, spin down, and place them on ice.

4 In a clean 200 µL tube, add the reagents in the following order per tube:

	A	B	C
	Reagent	**Volume_down**	**Volume_original**
	End-prep Product	7.5 µL	13.47 uL
	Native Barcode	2.5 µL	4.49 uL
	ddH2O	1.1 µL	1.97 uL
	Ligation Buffer	6.6 µL	11.85 uL
	Ligase	2.2 µL	3.95 uL
	TOTAL	**19.9 uL**	**35.73 uL**

Note

Ensure the reaction is thoroughly mixed by flicking the tube with your finger. Spin down the tube brieftly.

5 Incubate for 20 minutes at Room temperature .

6 Thaw EDTA at Room temperature on a cooling block, mix by vortexing, spin down, and place on ice.

7 Add **2 µL (Volume_down)** or **3.5 uL (Volume_original)** of EDTA to each tube and mix thoroughly by flicking the tube and spin down briefly.

Note

This step is to terminate the end-repair process

8 Pool all the barcoded samples in a 1.5 mL microtube.

Note

Per sample, it contains
22 uL for Volume_down
39.23 for Volume_original

9 Prepare fresh 80% ethanol in ddH_2O.

10 Resuspend the AMPure XP beads by vortexing.

11 Add 0.45X AMPure XP Beads (AXP) to the tube and mix by flicking the tube.

	A	B	C	D	E	F	G
		Volume_down			**Volume_original**		
	Number of sample	2	3	4	2	3	4
	AMPure	19.8 uL	29.7 uL	39.6 uL	35.3 uL	52.9 uL	70.6 uL

12 Incubate for 10 minutes at Room temperature.

13 Spin down the sample and pellet on a magnet for 5 mintues.
Keep the tube on the magnet until the elute is clear and colorless, and pipette off the supernatant.

14 Keep the tube on the magnetic rack and wash with 700 µL of freshly prepared 80% ethanol without disturbing the pellet.

Flip the magnetic rack for 30 seconds. Remove the ethanol using a pipet and discard.

15 Repeat previous step .

16 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for 5~30 seconds.

Note

Do not dry the pellet to the point of cracking.

17 Remove the tube from the magnetic rack and resuspend the pellet in 35 µL ddH_2O by gently flicking.

18 Incubate for 10 minutes at 37 °C on an incubator .

19 Pellet the beads on a magnet until the eluate is clear and colourless.

20 Remove and retain 35 µL of elute into a clean 200 uL microtube.

21 **Qubit to quatify the resulting DNA concentration before Adaptor ligation**

Note

If too low (e.g. < 10 uL/ng), then stop here and start all over again

Step2. Adapter ligation

30m

22 Thaw the Native Adapter (LA) and KAPA HyperPrep Ligation Buffer. Vortex and spin down the tubes, and place on ice immediately afterwards.

Note

Although the recommended 3rd party ligase is supplied with its own buffer, the ligation efficiency of Adapter Mix (AMX) is higher when using Ligation Buffer supplied within the Ligation Sequencing Kit.

Note

Ensure the Ligation Buffer is thoroughly vortexing until the droplets or any precipitations were dissolved.

22.1 Spin down the KAPA HyperPrep Ligase and place on ice immediately.

22.2 In a 1.5 mL tube, add the following chemicals:

	Reagent	Volume
	Pool Barcoded Sample	30 µL
	Native Adapter (NA)	5 µL
	ddH2O	1.67 µL
	Ligation Buffer	9.99 µL
	Ligase	3.33 µL
	TOTAL	**49.99 uL**

Note

Mix the chemicals by flicking the tube with your finger. Spin down the tube.

22.3 Incubate overnight at Room temperature

Note

in ONT manual, it says at least 20 min, but it can be as longer as overnight, which may improve the adaptor ligation so the sequencing efficiency

Step3. Cleanup

30m

23 Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at room temperature on a cooling block, mix by vortexing, spin down and place on ice.

24 Resuspend the AMPure XP beads by vortexing.

25 Add 0.45X AMPure XP Beads (AXP) to the tube and mix by flicking the tube.

Note

49.99 uL * 0.45X = 22.5 uL AMPure XP Beads (AXP)

26 Incubate for 10 minutes at Room temperature .

27 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

Note

Pellet the beads on a magnet until the eluate is clear and colorless, for at least 1 minute.

28 Wash the beads by adding either 125 µL Long Fragment Buffer (LFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet.

Note

Pellet the beads on a magnet until the eluate is clear and colorless, for at least 1 minute.

28.1 Remove the supernatant using a pipette and discard.

29 Repeat previous step .

30 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for 5~30 seconds.

Note

Do not dry the pellet to the point of cracking.

31 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 on an incubator .

32 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.

33 Leave the tube aside, and go to the priming steps.

Note

If you are not going to sequence the prepared library in an hour later, remove and retain 14 µl of eluate containing the DNA library into a clean 1.5 mL microtubes.
For short-term storage or reloading flow cells between washes, you can keep the prepared libray at 4°C. While for long-sterm storage of more than 3 months, storing libraries at -80°C is recommended.

Step3. Cleanup 30m

34 **Qubit to quatify the resulting DNA concentration before Adaptor ligation**

Note

If too low (e.g. < 1 uL/ng), then stop here and start all over again

Step4. Priming and loading the SpotON flow cell

35 Thaw the Flow cell, Sequencing Buffer (SB), Loading Solution (LIS) or Library Bead (LIB, if using) at room
temperature before mixing by vortexing

36 Combine Flow Cell Flush (FCF) and Flow Cell Tether (FCT), mix by pipetting at room temperature.

	A	B
	Reagent	**Volum per flow cell**
	Flow Cell Flush (FCF)	1,170 µL
	Flow Cell Tether (FCT)	30 µL
	(OPTIONAL) Bovine Serum Albumin (BSA) at 50 mg/ml	5 µL
	TOTAL	**1200 uL**

37 Priming and loading your flow cell

Note

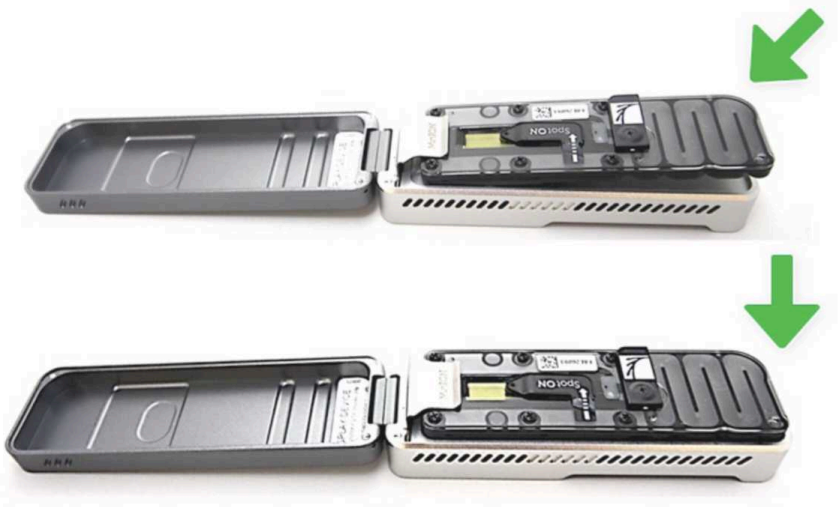
Watching the priming and loading flow cell video if unsure about the process

https://community.nanoporetech.com/nanopore_learning/lessons/priming-and-loading-your-flow-cell

38 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 is no air ibubbles in the flow cell.



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

40 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 800 µl.
2. Insert the tip into the priming port.
3. Turn the wheel until the dial shows 820–830 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.

41 Load 800 µl of the 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 below.

42 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.

Note

Load the library as soon as possible after this step.

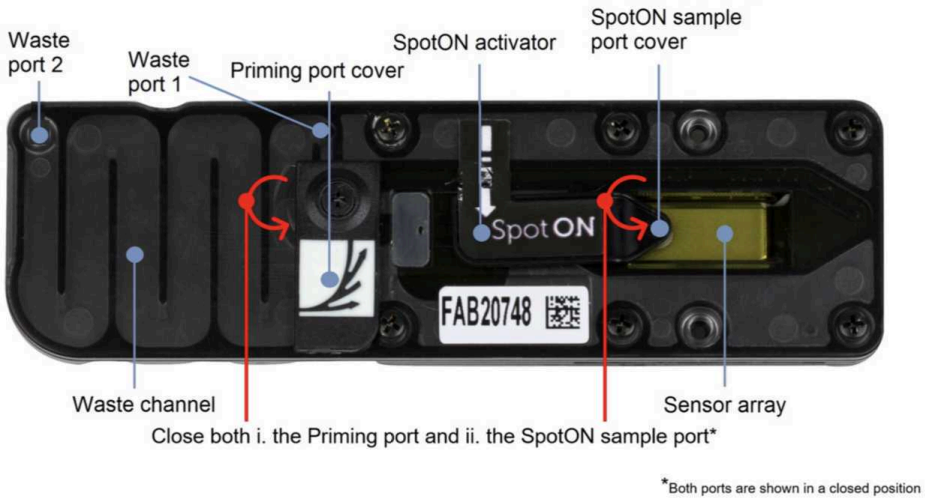
43 In the eluted original DNA library tube, add Sequencing Buffer (SB) and Library Solution (LIS) or Library Bead (LIB, if using):

	A	B
	Sequencing Buffer (SB)	37.5 µL
	Library Solution (LIS) or Library Bead (LIB, if using)	25.5 µL
	DNA library	12 µL
	TOTAL	**75 µL**

44 Gently flipping the prepared library just prior to loading.

45 Keep the priming port cover open.
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.

46 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.



Step5. Flow Cell Wash and Storage

30m

47 Stop or pause the sequencing experiment in MinKNOW, and leave the flow cell in the device.

Note

If you are not going to wash it immediately. Keep in 4°C.

48 Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube.
Thaw one tube of Wash Diluent (DIL) at room temperature on a cooling block.

Note

Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, spin down briefly and place on ice.

48.1 In a clean 1.5 mL microtube, prepare the following Flow Cell Wash Mix:

	Reagent	Volume
	Wash Mix (WMX)	1 µL
	Wash Diluent (DIL)	199 µL

Note

Mix well by pipetting, and place on ice. Do not vortex the tube.

48.2 Lift the sensory cover.

Note

Ensure that the priming port cover and SpotON sample port cover are in the positions indicated in the figure below.

48.3 Rotate the flow cell priming port cover clockwise so that the priming port is visible.

48.4 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 800 µl.
2. Insert the tip into the priming port.
3. Turn the wheel until the dial shows 820–830 µl, or until you can see a small volume of liquid entering the pipette tip.

Note

Visually check that there is continuous buffer from the priming port across the sensor array.

48.5 Load 200 µl of the prepared Flow Cell Wash Mix into the flow cell via the priming port, avoiding the introduction of air.

48.6 Close the priming port and keep into 4°C for 2 days.

48.7 go to step #48 Repeat this step.

49 Take out the MinION device from 4°C fridge.

50 Thaw one tube of Storage Buffer (S) at room temperature on a cooling block.

50.1 Mix contents thoroughly by pipetting and spin down briefly.

51 Rotate the flow cell priming port cover clockwise so that the priming port is visible. Open the device lid.

52 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 800 µl.
2. Insert the tip into the priming port.
3. Turn the wheel until the dial shows 820–830 µl, or until you can see a small volume of liquid entering the pipette tip.

Note

Visually check that there is continuous buffer from the priming port across the sensor array.

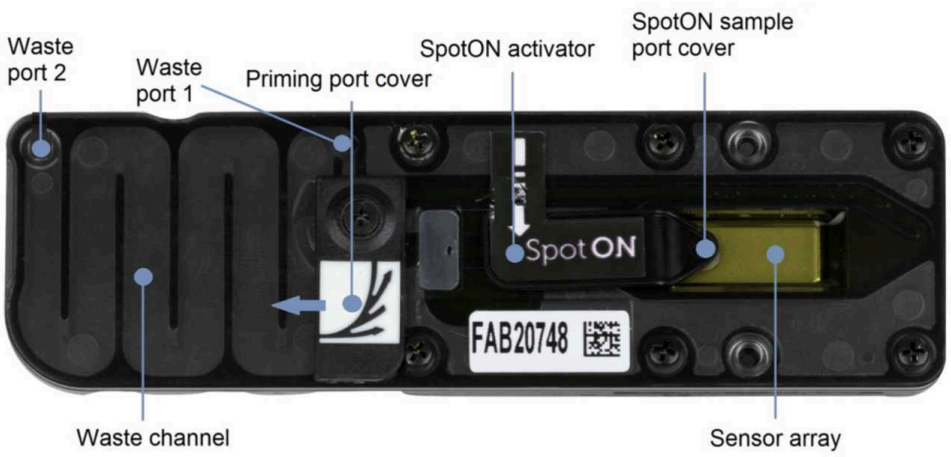
53 Add 500 µl of Storage Buffer (S) through the priming port of the flow cell by turning the pipet wheel.

53.1 Close the priming port.

54 Using a P1000, remove all fluids from the waste channel through Waste port 2. As both the priming port and SpotON sample port are closed, no fluid should leave the sensor array area.

Note

It is vital that the flow cell priming port and SpotON sample port are closed to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.



55 Perform a Flow Cell Check.

56 The flow cell can now be stored at 4°C.