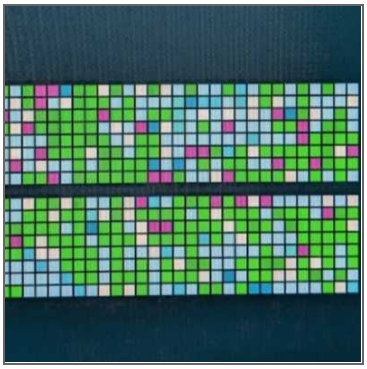

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Strategic Savings in Ligation Sequencing: A Practical Nanopore Library Preparation Workflow

Jie Hao Ou[1], Yin-Tse Huang[2]

[1]National Chung Hsing University, Taichung;
[2]Kaohsiung Medical University

Mycology Lab Jho

Jie Hao Ou

ABSTRACT

This protocol introduces a cost-effective alternative for end repair and dA-tailing in DNA library preparation, particularly tailored for samples with an N50 of 3 kb. By employing a homebrew end repair reagent solution, this method replaces the use of the NEBNext® Ultra™ II End Repair/dA-Tailing Module recommended by Nanopore. Optimization of reagent quantities and a bead-free purification method are combined to achieve efficient adapter ligation while minimizing costs. Notably, an extended incubation time during adapter ligation enhances efficiency. This resource provides a strategic approach for researchers aiming to customize their sequencing workflows while achieving optimal results and substantial savings.

GUIDELINES

1. **Optimized Homebrew Approach:** This protocol features a unique homebrew end repair reagent solution as a cost-effective alternative to the Nanopore-recommended NEBNext® Ultra™ II End Repair/dA-Tailing Module. Adhere to the specified volumes meticulously to achieve successful results with your customized reagent.
2. **Bead-Free Purification:** The protocol employs a bead-free PEG/NaCl precipitation method for efficient purification. Ensure precise centrifugation to maintain DNA pellet integrity and maximize recovery rates.
3. **Strategic Ligation Enhancement:** An extended incubation period during adapter ligation enhances efficiency. Dedicate adequate time to this step to optimize adapter ligation results.

Keywords: DNA library preparation, Nanopore, Cost-effective ligation, Bead-free purification

MATERIALS

1. 4X Quick Ligase Buffer

	A	B	C
		A	B
	1	H2O	70 ml
	2	Tris-Base	2.42 g (200 mM)
	3	MgCl2・6H2O	0.8 g (40 mM)
	4	DTT	0.6 g (40 mM)
	5	PEG 8000	20 g (20% w/w)
	6	HCl	Adjust pH to 7.6
	7	H2O	bring the volume up to 100 mL

Add the materials in the following table sequentially

2. 33.5% (w/v) PEG 8000

3. NP (5M NaCl + 6.7% PEG8000)

	A	B	C
	1	PEG 8000	3.35 g (6.7%)
	2	NaCl	14.61 g (5M)

4. 80% (v/v) ethanol

5. 99.5% ethanol

6. Elution buffer (1X TE, pH=8.0)

7. LFB wash buffer

	A	B	C
	1	33.5% (w/v) PEG 8000	80 uL
	2	NP	40 uL
	3	H2O	320 uL

SAFETY WARNINGS

1. Ethanol Flammability: Ethanol is highly flammable. Use caution when handling and storing ethanol solutions. Work in well-ventilated areas away from open flames, sparks, or heat sources.

Homebrew End Repair/dA-Tailing

50m

1 Add the materials in the following table sequentially.

	Materials	Quantity
	DNA	1600 ng (for samples with N50 of 3 kb)
	H2O	Bring up to a volume of 68 ul
	4X Quick Ligase Buffer	25 ul
	ATP (25 mM)	1 ul
	dNTP (10 mM)	5 ul
	Taq polymerase	0.5 ul (2.5 U)
	T4 PNK	0.5 ul (5 U)

Note

1. Due to the high concentration of PEG in the solution, it may be slightly viscous. Gently invert several times to ensure thorough and uniform mixing.

2 Incubate at 37 °C for 00:30:00

30m

Note

1. T4 PNK will add phosphate to the 5' ends of both strands of DNA

3 Incubate at 65 °C for 00:20:00

20m

Note

T4 PNK will be inactivated, and Taq polymerase will begin repairing DNA ends and adding A-tails.

Purification

30m

4 For the subsequent purification, commercially available **spin-column** or **magnetic bead-based methods** can be used.
Here, to save costs, we will use the **PEG/NaCl precipitation method.**

5 Add 25 µL of **33.5% PEG 8000**

6 Add 15 µL of **NP**

Note

1. Due to the high concentration of PEG in the solution, it may be slightly viscous. Gently invert several times to ensure thorough and uniform mixing.

7 Centrifuge at maximum speed (at least 8000 rpm) for 00:30:00 . 30m

8 Carefully remove the supernatant using a pipette.

Note

Most of the time, the pellet is not visible to the naked eye, so pay attention to the orientation during centrifugation

9 Add 200 µL of 80% ethanol.

Note

Sometimes the pellet becomes more visible after adding ethanol.

10 Centrifuge at maximum speed (at least 8000 rpm) for 00:02:00 2m

11 Carefully remove the supernatant using a pipette.

12 Add 200 µL of 99.5% ethanol.

13 Centrifuge at maximum speed (at least 8000 rpm) for 2 minutes. 2m

14 Carefully remove the supernatant using a pipette.

15 Wait for approximately 00:10:00 to ensure complete ethanol evaporation. 10m

16 Add 25 µL of **elution buffer.**

Note

Optional: Measure the DNA concentration.
Normally, the DNA concentration **should be >30 ng/µL.**

Adapter ligation

30m

17 **When using LNB provided by Nanopore**

	Reagent	Volume
	DNA	400 ng (for samples with N50 of 3 kb)
	H2O	Bring up to a volume of 15.25 µl
	Ligation Buffer (LNB)	6.25 µl
	T4 Ligase	2.5 µl
	Ligation Adapter	1 µl

When using homemade 4X Quick Ligase Buffer

	A	B
	Reagent	Volume
	DNA	400 ng (for samples with N50 of 3 kb)
	H2O	Bring up to a volume of 14.25 µl
	4X Quick Ligase Buffer	6.25 µl
	ATP (25 mM)	1 ul
	T4 Ligase	2.5 µl
	Ligation Adapter	1 µl

Note

Note that the reagent amounts used in this step are about **1/4** of the manufacturer's recommended volume. This means that the ligation sequencing kit, which was originally designed for 6 uses, **can now be utilized for 24 uses.**
This adjustment is primarily due to our utilization of a **bead-free purification** method later on, which significantly enhances the recovery rate.

18 Incubate at room temperature for 01:00:00 .

1h

Note

Despite the official recommendation of a 5-minute reaction, based on our own experience, **a 60-minute reaction** significantly improves adapter ligation efficiency.

19 Add 3 µL of **NP**

Note

1. Due to the high concentration of PEG in the solution, it may be slightly viscous. Gently invert several times to ensure thorough and uniform mixing.
2. Normally, PEG and NaCl buffer is required for DNA precipitation. However, it's evident that LNB buffer already contains a high concentration of PEG, so adding NP alone is sufficient for DNA precipitation. For more information, refer to: https://dx.doi.org/10.17504/protocols.io.7erhjd6.

20 Centrifuge at maximum speed (at least 8000 rpm) for at least 00:30:00 (If a higher recovery rat 30m needed, centrifuging for up to one hour is also possible).

Note

To prevent overheating, it is recommended to use a refrigerated centrifuge.
If a refrigerated centrifuge is unavailable, it is advised to perform centrifugation in two steps of 15 minutes each, with a 10-minute interval in between.

21 Carefully remove the supernatant using a pipette.

Note

Most of the time, the pellet is not visible to the naked eye, so pay attention to the orientation during centrifugation

22 Add 200 uL of **LFB wash buffer**

Note

Note that in this step, use the **LFB wash buffer** instead of alcohol.
This is because the DNA now has motor proteins attached, and using alcohol could disrupt the motor proteins.

23 Centrifuge at maximum speed (at least 8000 rpm) for 00:02:00 2m

24 Carefully remove the supernatant using a pipette.

25 Repeat the above washing step twice.

26 Add 20 µL of **EB buffer** (provided by the Ligation Sequencing Kit).

Note

Optional: Measure the DNA concentration.
Normally, the DNA concentration **should be >15 ng/µL.**