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Date last updated: 20th June 2024	Total Pages: 9	Written by: Jeanie Wu

Oxford Nanopore Library Preparation, Flow cell preparation and sequencing

1. PURPOSE

To prepare 24 different barcoded amplicon libraries from cDNA using Nanopore's workflow, to be pooled together in one sequencing experiment. Then to set up ONT flow cells and run the sequencing.

2. MATERIALS & EQUIPMENTS

Samples

- Dengue or Zika cDNA that was PCR amplified

Reagents

- Nanopore Native Barcoding kit 24 v14 (Nanopore, SQK-NBD114.24)
- MinION GridION Flow Cell R10.4.1 version (Nanopore, FLO-MIN114)
- NEB Blunt/TA Ligase Master Mix (NEB, M0367)
- NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- NEBNext Quick Ligation Module (NEB, E6056)
- Qubit 1X dsDNA HS Assay kit (ThermoFisher, cat #Q33231)
- Qubit Assay Tubes (Invitrogen, Q32856)
- Mag-Bind TotalPure NGS magnetic beads (SciMed Asia Pte Ltd, M1378-01)
- Absolute (100%) Ethanol
    - Freshly prepared 80% Ethanol in nuclease – free water from 100% Ethanol
- Nuclease – free ultrapure water

Consumables

- 0.2ml PCR tubes
- 1.5ml Eppendorf DNA Lo-Bind tubes
- Qubit 0.5ml assay tubes (ThermoFisher Scientific, Q32856)

Equipments

- Thermal cycler
- Nanopore GridION sequencer
- Hula Mixer (Biolabs, H5600-230V-UK))
- Qubit 4 Fluorometer (ThermoFisher, Q33238)
- DynaMag-2 Magnetic Stand (Invitrogen, 12321D)

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3. PROCEDURE

Step 1

End-Prep:

1. Thaw magnetic beads at room temperature and mix by vortexing. Keep the beads at room temperature.
2. Thaw NEBNext Ultra II End repair/dA-tailing Module reagents on ice. Flick mix and spin down.

   Note: Do not vortex the Ultra II End Prep Enzyme Mix.
   Important to mix NEBNext Ultra II End Prep Reaction Buffer well by vortexing.

3. Prepare 200 fmol (130ng for 1 kb amplicons) of DNA per sample in a PCR tube.

   ng = (fmol) x (size bp) x (660 fg/fmol) x (1 ng/10^6)

Sample name	Conc. (ng/ul)	Vol. DNA (ng) (ul)	H_20 (ul)

4. Make up each sample to 12.5 ul using nuclease-free water and mix gently by pipetting.
5. Combine the following components in a tube:
   a. 12.5 ul 200 fmol amplicon DNA
   b. 1.75 ul Ultra II End-Prep Reaction Buffer
   c. 0.75 ul Ultra II END-prep Enzyme Mix
6. Pipette mix 10 times and quick spin
7. Place tubes in thermal cycler, incubate at 20°C for 5 mins and 65°C for 5 mins
8. Transfer each sample to clean 1.5 ml Eppendorf DNA LoBind tube
9. Resuspend magnetic beads by vortexing and add 15 ul to each end-prep reaction
10. Flick mix and incubate on Hula mixer (rotator mixer) for 5 mins at RT
11. Prepare 500 ul of fresh 80% ethanol in nuclease-free water

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12. Spin down samples and place on magnetic stand
13. Pipette off supernatant when eluate is clear and colorless
14. Keep tube on magnet and wash beads with 200 ul of freshly prepared 80% ethanol without disturbing the beads
15. Remove ethanol using pipette and discard
16. Repeat steps 14 and 15
17. Briefly spin down and place tubes on magnetic stand
18. Remove residual ethanol and allow beads to dry for 30s – 1min. Do not dry the beads to point of cracking
19. Remove tube and resuspend beads in 12 ul nuclease-free water
20. Flick mix and quick spin. Incubate for 2 mins at RT
21. Place on magnetic stand until eluate is clear and colorless
22. Remove and retain 10 ul eluate into a new PCR tube. Discard the pelleted beads
23. Quantify 1 ul of each eluted sample using Qubit fluorometer

Sample name	Conc. (ng/ul)	Vol. DNA ( ) (ul)	H_20 (ul)

24. Take forward equimolar mass of each sample to be barcoded into next step

Note: Safe stopping point. Samples may be stored at 4^0C overnight.

Step 2

Native barcode ligation:

1. Thaw NEB Blunt/TA Ligase Master Mix at room temperature, mix well by pipetting, spin down and place on ice
2. Thaw EDTA at room temperature and mix by vortexing. Spin down and place on ice

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3. Thaw Native Barcodes (NB01 – 24) required for your number of samples at room temperature. Individually mix barcodes by pipetting, spin down and place on ice

Note: select a unique barcode for each sample to be run together on same flow cell. Up to 24 samples can be barcoded and combined in one experiment.

4. In a clean PCR tube, add reagents in the following order:
    a. 7.5 ul End-prepped DNA (calculated to equimolar mass for each tube)
    b. 2.5 ul Native Barcode (NB01-24)
    c. 10 ul Blunt/TA Ligase Master Mix

5. Pipette mix gently and quick spin
6. Incubate 20 mins at room temperature
7. Add 2 ul of EDTA to each tube, mix thoroughly by pipetting and spin down
8. Pool all barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube
9. Resuspend magnetic beads by vortexing
10. Add 0.4x magnetic beads to pooled reaction and mix by pipetting
11. Incubate on Hula mixer (rotator mixer) for 10 mins at room temperature
12. Prepare 2 ml of fresh 80% ethanol in nuclease-free water
13. Spin down samples and place on magnet for 5 mins. When eluate is clear and colorless, pipette and discard supernatant
14. Keep tube on magnetic stand and wash beads with 700 ul of freshly prepared 80% ethanol without disturbing the beads
15. Remove ethanol using pipette and discard
16. Repeat steps 14 and 15
17. Spin down and place tube back on magnetic stand. Remove any residual ethanol
18. Allow beads to dry for 30s – 1min but do not over-dry to the point of cracking
19. Remove tube and resuspend beads in 36 ul of nuclease-free water
20. Flick mix gently and incubate for 10 mins at 37^{0}C. Every 2 mins, agitate sample gently by flicking
21. Place on magnetic stand until eluate is clear and colorless
22. Remove and retain 35 ul of eluate in a clean 1.5 ml Eppendorf DNA LoBind tube
23. Quantify 1 ul of eluted sample using Qubit fluorometer

Note: Safe stopping point. Store at 4^{o}C overnight.

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Step 3

Adapter ligation and clean-up:

Note: Depending on wash buffer (LFB or SFB) used, the clean-up step after adapter ligation is designed to either enrich for DNA fragments of > 3 kb, or purify all fragments equally.

- **To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB)**
- **To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)**

1. Thaw Native Adapter (NA) at room temperature. Pipette mix thoroughly and spin down. Place on ice
2. Thaw NEBNext Quick Ligation Reaction Module reagents at room temperature. Pipette mix reagents thoroughly and spin down. Place on ice

Note: Do not vortex the Quick T4 DNA ligase.

3. Thaw Elution Buffer (EB) at room temperature and mix by vortexing. Spin down and place on ice
4. Mix the following in a 1.5 ml Eppendorf DNA LoBind tube:
    a. 30 ul pooled barcoded sample
    b. 5 ul Native Adapter (NA)
    c. 10 ul NEBNext Quick Ligation Reaction Buffer (5X)
    d. 5 ul Quick T4 DNA Ligase
5. Pipette mix gently and quick spin
6. Incubate reaction for 20 mins at room temperature
7. Resuspend magnetic beads by vortexing
8. Add 20 ul of resuspended magnetic beads to the reaction and mix by pipetting
9. Incubate on Hula mixer (rotator mixer) for 10 mins at room temperature
10. Spin down samples and place on magnetic stand. Once eluate is clear and colorless, pipette off supernatant

Note: The following clean-up step uses Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) rather than 80% ethanol to wash the beads. The use of ethanol will be detrimental to the sequencing reaction.

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11. Wash beads by adding either 125 ul Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB). Flick beads to resuspend and spin down
12. Return tube to magnetic stand and allow beads to pellet.
13. Remove supernatant using a pipette and discard
14. Repeat steps 11, 12 and 13
15. Quick spin down and place tube back on magnetic stand. Pipette off any residual supernatant
16. Remove tube from magnetic stand and resuspend beads in 16 ul Elution Buffer (EB)
17. Spin down and incubate for 10 mins at 37^{0}C. Every 2 mins agitate sample by gently flicking to encourage DNA elution
18. Place tube on magnetic stand until eluate is clear and colorless (at least 1 min)
19. Remove and retain 15 ul of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube
20. Quantify 1 ul of eluted sample using Qubit fluorometer
21. Prepare 10 – 20 fmol of your final library in 12 ul of Elution Buffer (EB)

Sample name	Conc. (ng/ul)	Vol. DNA (ng) (ul)	H20 (ul)

Prepared library is used for loading onto flow cell. Store library on ice until ready to load.

Step 4

Priming and loading the SpotON flow cell:

Note: This kit is only compatible with R10.4.1 flow cells (FLO-MIN114).
It is recommended to check the GridION's flow cells upon receiving the shipment to assess the number of active nanopores (minimum 800 nanopores for a flowcell) that are available for sequencing. Flow cells can be exchanged if they do not meet the minimum requirement.

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1. Thaw Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Spin down and store on ice
2. To prepare the flow cell priming mix with BSA, combine Flow Cell Flush (FCF) and Flow Cell Tether (FCT), as follows. Mix by pipetting at room temperature
    a. 1170 ul Flow Cell Flush (FCF)
    b. 5 ul Bovine Serum Albumin (BSA) at 50 mg/ml
    c. 30 ul Flow Cell Tether (FCT)

3. Open the GridION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact
4. Slide the flow cell priming port cover clockwise to open the priming port
5. After opening the priming port, draw back a small volume to remove any bubbles:
    a. Set a P1000 pipette to 200 ul
    b. Insert the tip into priming port
    c. Turn the wheel until the dial shows 220 – 230 ul, to draw back 20 – 30 ul, or until you can see a small volume of buffer entering the pipette tip

Note: Take care when drawing back buffer from the flow cell. Do not remove more than 20 – 30 ul, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

6. Load 800 ul of priming mix into flow cell via the priming port, avoid introduction of air bubbles. Wait for 5 mins. During this time, prepare the library for loading by following the steps below

7. Thoroughly mix the contents of the Library Beads (LIB) by pipetting

Note: The Library Beads (LIB) tube contains a suspension of beads that settle very quickly. It is vital that they are mixed immediately before use.

8. In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:
    a. 37.5 ul Sequencing Buffer (SB)
    b. 25.5 ul Library Beads (LIB) mixed immediately before use
    c. 12 ul DNA library

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9. Complete the flow cell priming:
    a. Gently lift the SpotON sample port cover to make the SpotON sample port accessible
    b. Load 200 ul of the priming mix into flow cell priming port (not the SpotON sample port), avoiding introduction of bubbles

10. Mix the prepared library gently by pipetting up and down just prior to loading
11. Add 75 ul of the prepared 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.**
12. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port
13. Place the light shield onto flow cell as follows:
    a. Carefully place the leading edge of light shield against the clip. Do not force the light shield underneath the clip.
    b. Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of flow cell.

14. Close the device lid and set up the sequencing run on MinKNOW

Step 5

Flow cell reuse and returns:

1. After sequencing experiment is complete, if re-using the flow cell, please follow the Flow Cell Wash Kit protocol and store washed flow cell at 2 – 8^0C
2. Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore

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Process Map/ workflow chart

- Prepare the DNA ends for adapter attachment
- Ligate Native barcodes supplied in the kit to the DNA ends
- Ligate sequencing adapters supplied in the kit to the DNA ends
- Prime the flow cell, and load your DNA library into the flow cell