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Nanopore Sequencing with Flongle Flow Cells

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We use this protocol and it's working

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Abstract

The 2023 NUS-Singapore iGEM Team followed this protocol to sequence their STAR oligo variants. The original protocol was released by Oxford Nanopore Technologies, but due to the use of a new type of flow cell in the experiment, some minor modifications to the steps were made to tailor it to our project.

Protocol materials

- AMPure XP Beads
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- NEB Blunt/TA Ligase Master Mix **Catalog #**M0367
- Quick T4 DNA Ligase **New England Biolabs Catalog #**E7180S
- Quick T4 DNA Ligase **New England Biolabs Catalog #**E7180S
- AMPure XP Beads
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- AMPure XP Beads
- NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #**E7546S
- NEBNext Ultra II End Prep Enzyme Mix **New England Biolabs Catalog #**E7646
- NEBNext Ultra II End Prep Reaction Buffer **New England Biolabs Catalog #**E7647

Troubleshooting

Safety warnings

Proper lab PPE must be worn at all times.

End-prep

18m

1 Mix AMPure XP Beads and DNA Control Sample (DCS) at room temperature by vortexing. Keep the beads at room temperature and store the DNA Control Sample (DCS) on ice.

2 Prepare the NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #**E7546S in accordance with the manufacturer's instructions, and place on ice. (NEBNext Ultra II End Prep Enzyme Mix **New England Biolabs Catalog #**E7646 do not vortex, NEBNext Ultra II End Prep Reaction Buffer **New England Biolabs Catalog #**E7647 vortex for at least 00:00:30 until no precipitate is visible.) 30s

3 Dilute the DNA Control Sample (DCS) by adding 105 µL Elution Buffer directly to one DCS tube. Mix gently by pipetting and spin down. (excess storage at -20 °C)

4 In clean 0.2 ml thin-walled PCR tubes (or a clean 96-well plate), aliquot 200 fmol (130 ng for 1 kb amplicons) of DNA per sample.

5 Make up each sample to 11.5 µL using nuclease-free water. Mix gently by pipetting and spin down.

6 Combine the following components per tube/well:
Between each addition, pipette mix 10 - 20 times.
Recommend to prepare a master mix and add 2.5 µL to each well.

	Item	Volume
	200 fmol (130 ng for 1 kb amplicons) amplicon DNA	11.5µL
	Diluted DNA Control Sample (DCS)	1µL
	Ultra II End-prep Reaction Buffer	1.75µL

	Item	Volume
	Ultra II End-prep Enzyme Mix	0.75µL

7 Ensure the components are thoroughly mixed by pipetting and spin down in a centrifuge.

8 Using a thermal cycler, incubate at 20 °C for 00:05:00 and 65 °C for 00:05:00 . 10m

9 Transfer each sample into a clean 1.5 ml Eppendorf DNA LoBind tube.

10 Resuspend AMPure XP Beads by vortexing.

11 Add 15 µL of resuspended AMPure XP Beads to each end-prep reaction and mix by flicking the tube.

12 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. 5m

13 Prepare 500 µL of fresh 80% ethanol in nuclease-free water.

14 Spin down the samples and pellet the beads on a magnet until the eluate is clear and colourless. Keep the tubes on the magnet and pipette off the supernatant.

15 Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

16 Repeat step 15.

17 Briefly spin down and place the tubes back on the magnet for the beads to pellet. Pipette off any residual ethanol. Allow to dry for 00:00:30, but do not dry the pellets to the point
of cracking. 30s

18 Remove the tubes from the magnetic rack and resuspend the pellet in 10 µl nuclease-free water. Spin down and incubate for 00:02:00 at room temperature. 2m

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

20 Remove and retain 10 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. Sample storage at 4 °C.

Native Barcode Ligation 47m 40s

21 Prepare NEB Blunt/TA Ligase Master Mix **Catalog #**M0367 according to the manufacturer's instructions, and place on ice.

22 Thaw the EDTA at room temperature and mix by vortexing. Then spin down and place on ice.

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

24 Select a unique barcode for each sample to be run together on the same flow cell. Up to 24 samples can be barcoded and combined in one experiment. (only use one barcode per sample)

25 In clean 0.2 ml PCR-tubes or a 96-well plate, add the reagents in the following order per well:

	Item	Volume
	End-prepped DNA	7.5µL
	Native Barcode (NB01-24)	2.5µL

	Item	Volume
	Blunt/TA Ligase Master Mix	10µL

26 Ensure the reaction is thoroughly mixed by gently pipetting and spin down briefly.

27 Incubate for 00:20:00 at room temperature. 20m

28 Add the 2 µL of clear cap EDTA to each well and mix thoroughly by pipetting and spin down briefly.

29 Pool all the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube; 22 µL per sample

30 Resuspend AMPure XP Beads by vortexing.

31 Add 9 µL AMPure XP Beads per sample to the pooled reaction, and mix by pipetting for a 0.4X clean.

32 Incubate on a Hula mixer (rotator mixer) for 00:10:00 at room temperature. 10m

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

34 Spin down the sample and pellet on a magnet for 00:05:00. Keep the plate on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant. 5m

35 Keep the tube on the magnetic rack and wash the beads with 700 µL of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

36 Repeat step 35.

37 Spin down and place the tube back on the magnetic rack. Pipette off any residual ethanol. 30s
Allow the pellet to dry for 00:00:30, but do not dry the pellet to the point of cracking.

38 Remove the tube from the magnetic rack and resuspend the pellet in 35 µL nuclease-free water by gently flicking.

39 Incubate for 00:10:00 at 37 °C. Every 00:02:00, agitate the sample by gently flicking for 00:00:10 to encourage DNA elution. 12m 10s

40 Pellet the beads on a magnetic rack until the eluate is clear and colourless. Remove and retain 35 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

Adaptor Ligation and Clean-up 43m 10s

41 Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice. (Do NOT vortex Quick T4 DNA Ligase **New England Biolabs Catalog #**E7180S.)

42 Spin down the Native Adapter (NA) and Quick T4 DNA Ligase **New England Biolabs Catalog #**E7180S, pipette mix and place on ice.

43 Thaw Elution Buffer at room temperature and mix by vortexing. Then spin down and place on ice.

44 Thaw either Long Fragment Buffer (LFB) >3kb or Short Fragment Buffer (SFB) <3kb at room temperature and mix by vortexing. Then spin down and place on ice.

45 In a 1.5 ml Eppendorf LoBind tube, mix in the following order:

	Item	Volume
	Pooled barcoded sample	30µL
	Native Adapter (NA)	5µL
	NEBNext Quick Ligation Reaction Buffer(5X)	10µL
	Quick T4 DNA Ligase	5µL

46 Ensure the reaction is thoroughly mixed by gently pipetting and spin down briefly.

47 Incubate the reaction for 00:20:00 at room temperature. 20m

48 Resuspend AMPure XP Beads by vortexing.

49 Add 20 µL of resuspended AMPure XP Beads to the reaction and mix by pipetting.

50 Incubate on a Hula mixer (rotator mixer) for 00:10:00 at room temperature. 10m

51 Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant.

52 Wash the beads by adding either 125 µL Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

53 Repeat Step 52.

54 Spin down and place the tube back on the magnet. Pipette off any residual supernatant.

55 Remove the tube from the magnetic rack and resuspend pellet in 7 µL Elution Buffer (EB).

56 Spin down and incubate for 00:10:00 at 37 °C. Every 00:02:00, agitate the sample by gently flicking for 00:00:10 to encourage DNA elution. 12m 10s

57 Pellet the beads on a magnet until the eluate is clear and colorless, for at least 00:01:00. 1m

58 Remove and retain 7 µL of EB containing the DNA library into a clean 1.5 ml Eppendorf SNA LoBind tubes.

59 Quantify 1 µL of eluted sample using Nanodrop.

60 Make up the library to 5 µL at 5-10 fmol.

Loading the Flongle Flow Cell

61 Thaw the Sequencing Buffer(SB), Library Beads(LIB), Flow Cell Tether(FCT) and one tube of Flow Cell Flush(FCF) at room temperature before mixing by vortexing. Then spin down and
store on ice.

62 In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix 117 µL of Flow Cell Flush(FCF) with 3 µL of Flow Cell Tether(FCT) and mix by pipetting.

63 Place the Flongle adaptor into the MinION or one of the five GridION positions.

64 Place the flow cell into Flonge adapter, and press the flow cell down until hearing a click.

65 Peel back the seal tab from the Flongle flow cell, up to a point where the sample port is exposed. Lift up the seal tab, pull the seal tab to open access to the sample port, then hold the seal tab open by using adhesive on the tab to stick to the MinION Mk1B lid.

66 To prime the flow cell with the mix of Flow Cell Flush(FSF) and Flow Cell Tether(FCT) that was prepared earlier, ensure that there is no air gap in the sample port or the pipette tip. Place the P200 pipette tip inside the sample port and slowly dispense the priming fluid into the Flonge flow cell by slowly pipetting down. Recommend twisting the pipette plunger down to avoid flushing the flow cell too vigorously.

67 Vortex the vial of Library Beads(LIB). Immediately prepare the sequencing Mix in a fresh 1.5 ml Eppendorf DNA LoBind tube for loading the Flongle as follows:

	Item	Volume
	Sequencing Buffer(SB)	15µL
	Library Beads(LIB) mixed immediately before use	10µL
	DNA Library	5µL

68 To add the sequencing mix to the flow cell, ensure that there is no air gap in the sample port or the
pipette tip.
Place the P200 tip inside the sample port and slowly dispense the sequencing mix into the flow cell by slowly pipetting down. Recommend twisting the pipette plunger down to avoid flushing the flow cell too vigorously.

69 Seal the Flonge flow cell using the adhesive on the seal tab: stick the transparent adhesive tape to the sample port, replace the top(wheel icon section) of the seal tab to its original position.

70 Replace the sequencing platform lid and start sequencing.

Protocol references

Oxford Nanopore Technologies. (n.d.). *Ligation sequencing amplicons - Native Barcoding Kit 24 V14 (SQK-NBD114.24)*. Retrieved October 12, 2023, from https://community.nanoporetech.com/docs/prepare/library_prep_protocols/ligation-sequencing-amplicons-native-barcoding-v14-sqk-nbd114-24/v/nba_9168_v114_revk_15sep2022