Ligation sequencing DNA V14 - automated Hamilton NGS STAR 96 (SQK-LSK114-XL)

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Last update: 04/10/2023

Flow Cell Number: ..... DNA Samples: .....

Before start checklist

Materials

- [ ] 1 µg (or 100-200 fmol) high molecular weight genomic DNA
- [ ] OR 100+ ng high molecular weight genomic DNA if performing DNA fragmentation
- [ ] Ligation Sequencing Kit XL V14 (SQK-LSK114-XL)

Consumables

- [ ] NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L). Alternatively, you can use the NEBNext® products below:
- [ ] NEBNext FFPE Repair Mix (NEB, M6630)
- [ ] NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- [ ] NEBNext Quick Ligation Module (NEB, E6056)
- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 80% ethanol in nuclease-free water
- [ ] Hamilton 50 µl CO-RE tips with filter (Cat# 235948)
- [ ] Hamilton 300 µl CO-RE tips with filter (Cat# 235903)
- [ ] Hamilton 1000 µl CO-RE tips with filter (Cat# 235905)
- [ ] Hamilton 60 ml Reagent Reservoir, Self-Standing with Lid (Cat# 56694-01)
- [ ] Hamilton PCR ComfortLid (Cat# 814300)
- [ ] Bio-Rad Hard-Shell® 96-Well PCR Plates (Cat# HSP9601)
- [ ] Hamilton 20 ml Reagent Reservoirs (Cat# 96424-02)
- [ ] Sarstedt Inc Screw Cap Micro tube 2 ml, PP 1000/case (e.g. FisherScientific, Cat# NC0418367)
- [ ] Thermo Scientific™ Abgene™ 96 Well 0.8 ml

Equipment

- [ ] Ice bucket with ice
- [ ] Vortex mixer
- [ ] Microplate centrifuge, e.g. Fisherbrand™ Mini Plate Spinner Centrifuge (Fisher Scientific, 11766427)
- [ ] Hamilton NGS STAR 96 (NGS STAR with Multi-Probe Head 96)
- [ ] Hamilton On-Deck Thermal Cycler (ODTC)

Polypropylene Deepwell Storage Plate

INSTRUCTIONS	NOTES/OBSERVATIONS

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INSTRUCTIONS	NOTES/OBSERVATIONS
DNA repair and end-prep	

Reagents quantities:

Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice.

- ☐ Thaw all reagents on ice.
- ☐ Flick and/or invert the reagent tubes to ensure they are well mixed.
  Note: Do not vortex the FFPE DNA Repair Mix or Ultra II End Prep Enzyme Mix.
- ☐ Always spin down tubes before opening for the first time each day.
- ☐ The Ultra II End Prep Buffer and FFPE DNA Repair Buffer may have a little precipitate. Allow the mixture to come to RT and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for 30 seconds to solubilise any precipitate.
  Note: It is important the buffers are mixed well by vortexing.
- ☐ The FFPE DNA Repair Buffer may have a yellow tinge and is fine to use if yellow.

Prepare each DNA sample per well with Nuclease-free water in the input plate.

- ☐ Per sample, transfer 1 µg (or 100-200 fmol) of input DNA into a well of the input plate
- ☐ Adjust the volume to 48 µl with Nuclease-free water
- ☐ Mix thoroughly by pipetting
- ☐ Spin down briefly in a microfuge

☐ Quantify 1 µl of each eluted sample using a Qubit fluorometer plate reader off deck.

☐ Switch on the Hamilton NGS STAR 96 robot and open 'Hamilton Run Control' on the computer by clicking the icon:

☐ Click 'File' and 'Open' to choose the method to run on the liquid handling robot.

☐ Click 'Process01: DNA repair and end-prep' to start.

☐ Click 'Process02: DNA repair and end-prep clean-up' to stop the automated library preparation and quantify the samples before the adapter ligation step.

IMPORTANT

☐ It is mandatory for users to have an MPH module installed and we recommend the use of an ODTC module.

Click 'Browse' to choose the Input File Worklist for the specific number of samples in the run and click 'OK'.

- ☐ Sample_01
  - Source_Well: A1
  - Target_Well: A1
- ☐ Sample_02
  - Source_Well: B1
  - Target_Well: B1
- ☐ Sample_03
  - Source_Well: C1
  - Target_Well: C1

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INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare the End Prep Mastermix with the following reagents according to the Hamilton user interface. Click either 'Yes' or 'No' to continue. ☐ NEBNext FFPE DNA Repair Buffer 213.3 µl @ 106.6 µl (414.8 µl cycles) ☐ NEBNext FFPE DNA Repair Mix 121.8 µl @ 60.9 µl (237 µl cycles) ☐ Ultra II End-prep Reaction Buffer 213.3 µl @ 106.6 µl (414.8 µl cycles) ☐ Ultra II End-prep Enzyme Mix 182.8 µl @ 91.4 µl (355.6 µl cycles) ☐ Insert the ComfortLid position as displayed on screen. Click 'Ok' to continue. ☐ Insert plates to their corresponding positions. Click 'Ok' to continue. ☐ Load a full deck of 50 µl tips into the positions on screen. Click 'Ok' to continue. ☐ Highlight the 50 µl tips available to use on the 'Edit Tip Count' window and click 'Ok' to continue. ☐ Load a full deck of 300 µl tips in the positions on screen. Click 'Ok' to continue. ☐ Highlight the 300 µl tips available to use on the 'Edit Tip Count' window and click 'Ok' to continue. Freshly prepare 80% ethanol in Nuclease-free water in a trough. ☐ 80% ethanol 28 ml @ 16.5 ml (51 ml cycles) ☐ Insert the trough of 80% ethanol in the position on screen and click 'Ok' to continue. Prepare the AMPure XP beads by vortexing and load the 20 ml trough with the volume required: ☐ Beads 5.4 ml @ 3.7 ml (8.9 ml cycles)	
IMPORTANT ☐ Ensure the AMPure XP beads are well mixed before use by vortexing.	
☐ Insert the trough of AMPure XP beads and Nuclease-free water in their positions on screen. Click 'Ok' to continue. ☐ Load 1000 µl tips and insert the input plate of DNA samples into the position on screen. Click 'Ok' to continue. ☐ Highlight the 1000 µl tips available to use on the 'Edit Tip Count' window and click 'Ok' to continue. ☐ Mix and insert the prepared End Prep Mastermix into the positions on screen. ☐ Click 'Ok' to start the DNA repair and end-prep automation process. ☐ Once the automation process has finished, there will be an on screen prompt to unload the plate. Click 'Ok' to continue. ☐ Quantify 1 µl of each eluted sample using a Qubit fluorometer plate reader off deck.	
Take forward the repaired and end repaired DNA into the adapter ligation and clean-up step.	

Flow Cell Number: ..... DNA Samples: .....

INSTRUCTIONS	NOTES/OBSERVATIONS
Adapter ligation and clean-up	
IMPORTANT ☐ Although the recommended third-party ligase is supplied with its own buffer, the ligation efficiency of the Ligation Adapter (LA) is higher when using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit.	
Reagents quantities: ☐ Spin down the Ligation Adapter (LA) and Quick T4 Ligase, and place on ice. ☐ Thaw the Ligation Buffer (LNB) at RT, spin down and combine all the required tubes. Place on ice immediately after thawing and mixing. ☐ Thaw a bottle of Elution Buffer (EB) at RT, mix by vortexing and place on ice.	
IMPORTANT Depending on the 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)	
☐ To enrich for DNA fragments of 3 kb or longer, thaw the Long Fragment Buffer (LFB) at RT, mix by vortexing and combine all the required bottles before storing on ice. ☐ To retain DNA fragments of all sizes, thaw the Short Fragment Buffer (SFB) at RT, mix by vortexing and combine all the required bottles before storing on ice. ☐ Click 'Process03: Adapter ligation' to start. ☐ Click 'Process04: Adapter ligation and clean-up' to stop the automated library preparation and quantify the samples before sequencing.	
IMPORTANT ☐ It is mandatory for the MPH module to be installed on the liquid handling robot. Select 'Yes' to use the MPH (96 Head) module.	
☐ Click 'Browse' to choose the Input File Worklist used during DNA repair and end-prep. Prepare the Adapter Ligation Mastermix with the following reagents according to the Hamilton user interface. Select either 'Yes' or 'No' to continue. ☐ Ligation Adapter (LA) 281 µl @ 140.5 µl (559.5 µl cycles) ☐ Ligation Buffer (LNB) 1405 µl @ 702.5 µl (2797.5 µl cycles) ☐ Quick T4 DNA Ligase 562 µl @ 281 µl (1119 µl cycles) ☐ Insert plates to their corresponding positions on screen. Click 'Ok' to continue. ☐ Load a full deck of 50 µl tips into the positions on screen. Click 'Ok' to continue. ☐ Highlight the 50 µl tips available to use on the 'Edit Tip Count' window. Click 'Ok' to continue.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Load a full deck of 300 µl tips in the positions on screen. Click 'Ok' to continue. ☐ Highlight the 300 µl tips available to use on the 'Edit Tip Count' window. Click 'Ok' to continue. Prepare the AMPure XP beads by vortexing and load the 20 ml trough with the volume required: ☐ Beads 4.3 ml @ 3.1 ml (6.6 ml cycles)	
IMPORTANT ☐ Ensure the AMPure XP beads are well mixed before use by vortexing.	
Insert troughs of AMPure XP beads, LFB/SFB and EB in the positions on screen. Click 'Ok' to continue. ☐ Long/Short Fragment Buffer 4 bottles @ 2 bottles (8 bottles cycles) ☐ Elution Buffer 1 bottle @ 1 bottle (1 bottle cycle) ☐ Insert 1000 µl tips and the Clean End Prep Plate to the correct positions on screen. Click 'Ok' to continue. ☐ Highlight the 1000 µl tips available to use on the 'Edit Tip Count' window. Click 'Ok' to continue. ☐ Insert the prepared Adapter Ligation Mastermix into the positions on screen. Click 'Ok' to continue. ☐ Once the automation process has finished, there will be an on screen prompt to unload the plate. Click 'Ok' to continue. ☐ Quantify 1 µl of each eluted sample using a Qubit fluorometer plate reader off deck.	
Seal the plate once the library is prepared and store on ice until ready to load onto the flow cell.	
☐ Depending on your required data output, prepare your final library to 35-50 fmol for high output of simplex data, or 10-20 fmol for duplex data, in 32 µl of Elution Buffer (EB).	
Priming and loading the PromethION flow cell	
IMPORTANT ☐ This kit is only compatible with R10.4.1 flow cells (FLO-PRO114M).	
☐ Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at RT before mixing by vortexing. Then spin down and store on ice. Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing. ☐ 30 µl Flow Cell Tether (FCT) ☐ 1170 µl Flow Cell Flush (FCF)	

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INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT ☐ After taking flow cells out of the fridge, wait 20 minutes before inserting the flow cell into the PromethION for the flow cell to come to RT. Condensation can form on the flow cell in humid environments. Inspect the gold connector pins on the top and underside of the flow cell for condensation and wipe off with a lint-free wipe if any is observed. Ensure the heat pad (black pad) is present on the underside of the flow cell.	
For PromethION 2 Solo, load the flow cell(s) as follows: ☐ Place the flow cell flat on the metal plate. ☐ Slide the flow cell into the docking port until the gold pins or green board cannot be seen. For the PromethION 24/48, load the flow cell(s) into the docking ports: ☐ Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position. ☐ Press down firmly onto the flow cell and ensure the latch engages and clicks into place.	
IMPORTANT ☐ Insertion of the flow cells at the wrong angle can cause damage to the pins on the PromethION and affect your sequencing results. If you find the pins on a PromethION position are damaged, please contact support@nanoporetech.com for assistance.	
☐ Slide the inlet port cover clockwise to open.	
IMPORTANT ☐ Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, 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.	
After opening the inlet port, draw back a small volume to remove any air bubbles: ☐ Set a P1000 pipette tip to 200 µl. ☐ Insert the tip into the inlet port. ☐ Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. ☐ Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. During this time, prepare the library for loading using the next steps in the protocol. ☐ Thoroughly mix the contents of the Library Beads (LIB) by pipetting.	
IMPORTANT ☐ The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.	
In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows: ☐ 100 µl Sequencing Buffer (SB) ☐ 68 µl Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS) ☐ 32 µl DNA library ☐ Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ Load 200 µl of library into the inlet port using a P1000 pipette.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Close the valve to seal the inlet port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
If the light shield has been removed from the flow cell, install the light shield as follows: ☐ Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID. ☐ Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.	
Close the PromethION lid when ready to start a sequencing run on MinKNOW.	
Flow cell reuse and returns	
☐ After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Flow Cell Wash Kit protocol and store the washed flow cell at 2-8°C. ☐ Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore.	
IMPORTANT ☐ If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Guide that can be found in the online version of this protocol.	