Ligation sequencing gDNA - automated Hamilton NGS STAR 96 with Multiplex Ligation Sequencing Kit XL (SQK-MLK111.96-XL)

Version: MLKH_9145_v111_revM_15Dec2021
Last update: 06/12/2023

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

Before start checklist

Materials

- [ ] Multiplex Ligation Sequencing Kit XL (SQK-MLK111.96-XL)
- [ ] 1200 ng gDNA per sample

Consumables

- [ ] NEB Blunt/TA Ligase Master Mix (NEB, M0367)
- [ ] NEBNext® Quick Ligation Reaction Buffer (NEB, B6058)
- [ ] NEBNext FFPE Repair Mix (NEB, M6630)
- [ ] NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- [ ] NEBNext Quick Ligation Module (NEB, E6056)
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 80% ethanol in nuclease-free water
- [ ] Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 2.0 ml Eppendorf DNA LoBind tubes
- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] Qubit™ Assay Tubes (Invitrogen, Q32856)
- [ ] 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 PCR ComfortLid (Cat# 814300)
- [ ] Bio-Rad Hard-Shell® 96-Well PCR Plates (Cat# HSP9601)
- [ ] Hamilton 60 ml Reagent Reservoir, Self-Standing with Lid (Cat# 56694-01)

Equipment

- [ ] P200 pipette and tips
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Qubit fluorometer (or equivalent)
- [ ] Hamilton NGS STAR 96 (NGS STAR with Multi-Probe Head 96)
- [ ] Hamilton On-Deck Thermal Cycler (ODTC)
- [ ] Hamilton MTP landscape carrier (cat# 182365)
- [ ] Hamilton Ambion magnet adapter (cat# 10107866)
- [ ] Pipettes and pipette tips P20, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Prepare the deck	
IMPORTANT Extra equipment required for the deck layout ☐ Hamilton MTP landscape carrier (cat # 182365) ☐ Hamilton Ambion magnet adapter (cat # 10107866)	
Remove the plate stacker and two tube racks. ☐ Plate stacker ☐ Two tube racks ☐ Place both tubes racks in positions 1 and 2. ☐ Shift the four tip carriers to the left, starting from position 3. ☐ Place the two trough carriers next to the 300 µl tips and insert the new carrier in the remaining slot on deck. ☐ Place the Ambion magnet adapter on the Ambion magnet.	
Once the deck is correctly set up, the robot can be prepared to run the automation protocol.	
Pre-processes	
Users have the option to use pre-process to complete automated upstream methods to prepare their samples. ☐ Click "Pre-processes" to open the following dialogue and select the method you would like to complete and click "Ok". ☐ Enter the number of samples to process and click "Ok". ☐ Enter the input volume of your samples to process and click "Ok". ☐ Dialogue boxes will follow on the UI to illustrate how to correctly load the deck.	
Once the process is complete, you will be returned to the method selection page, enabling the user to either start the library preparation process or another pre-process.	
Complete automated library preparation	
IMPORTANT ☐ It is required to use full decks of tips to run this protocol for all conditions. Partially used tip decks will cause an error with the liquid handling robot.	
IMPORTANT ☐ Do not vortex the NEBNext FFPE DNA Repair Mix, NEBNext Ultra II End Prep Enzyme Mix or NEBNext Quick T4 DNA Ligase.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
In a clean hard shell PCR plate, prepare the sample input plate as follows: ☐ Dispense 1200 ng DNA into each sample well. Note: We suggest aliquoting your DNA at 80 ng/µl per sample. ☐ Make up the volume of each well containing DNA samples to at least 15 µl. ☐ Switch on the Hamilton NGS STAR 96 robot and open the method from the desktop shortcut. ☐ When the method is loaded, click 'Start'. ☐ Click 'MLK111.96-XL' to proceed to the method parameter selection. Choose the number of samples to process from the drop-down menu, your multiplexing method and the file directory to the input workfile. Click 'Ok' to continue. ☐ 2 samples on 1 flow cell ☐ 3 samples on 2 flow cells	
IMPORTANT ☐ An error message will appear if an invalid number of samples is selected for your choosen multiplexing method.	
☐ Enter the barcode of the input plate containing the samples and the output plate which will contain the prepared DNA libraries.	
IMPORTANT ☐ If the entered barcodes do not match what is stored in the workfile, the correct barcodes will need to be re-entered.	
☐ Click "Full method" to run the entire automated protocol. Once settings for the run have been selected, there will be a series of dialogues illustrating how to load the deck depending on steps selected.	
IMPORTANT ☐ Ensure all seals are removed from plates before loading the deck.	
☐ Place the Hamilton Comfort PCR lid on the PCR lid position.	
IMPORTANT ☐ Ensure the tip decks are full before running the protocol.	
☐ Load 50 µl tips as indicated on screen. ☐ Load 300 µl tips as indicated on screen.	
IMPORTANT ☐ Ensure to use the correct volume of AMPure XP beads and they are well mixed before use by vortexing.	

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☐ Load the reagent troughs as indicated on screen. ☐ Ensure the foil seal is removed from the native barcode plate foil seal. ☐ Load the native barcode plate, 3 fresh PCR plates and the input plate containing the DNA samples. ☐ Load 1000 µl tips as indicated on screen and ensure the magnet is in place with the adapter for PCR plates. ☐ Load the CPAC module as indicated on screen with the reagent tubes before loading on deck. ☐ Once the deck is correctly loaded, click 'Begin method' to start with the parameters selected before loading. ☐ During the thermal cycle step for Step 1: End repair and adenylation thermal cycling reaction, the user will be prompted to remove the input plate with the input samples and to load 3 fresh PCR plates as indicated on screen.	
IMPORTANT ☐ We recommend loading >10 fmols of this final prepared library onto the flow cell for R9.4.1 flow cells.	
The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C until ready to load.	
Select steps in the automated library preparation	
Users have the option to run select steps in the protocol. We recommend quantification after End Repair for barcode balancing.	
IMPORTANT ☐ It is required to use full decks of tips to run this protocol for all conditions. Partially used tip decks will cause an error with the liquid handling robot.	
IMPORTANT ☐ Do not vortex the NEBNext FFPE DNA Repair Mix, NEBNext Ultra II End Prep Enzyme Mix or NEBNext Quick T4 DNA Ligase.	
In a clean hard shell PCR plate, prepare the sample input plate as follows: ☐ Dispense 1200 ng DNA into each sample well. Note: We suggest aliquoting your DNA at 80 ng/µl per sample. ☐ Make up the volume of each well containing DNA samples to at least 15 µl. ☐ Switch on the Hamilton NGS STAR 96 robot and open the method from the desktop shortcut. ☐ When the method is loaded, click 'Start'. ☐ Click 'MLK111.96-XL' to proceed to the method parameter selection.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Choose the number of samples to process from the drop-down menu, your multiplexing method and the file directory to the input workfile. Click 'Ok' to continue. ☐ 2 samples on 1 flow cell ☐ 3 samples on 2 flow cells	
IMPORTANT ☐ An error message will appear if an invalid number of samples is selected for your choosen multiplexing method.	
☐ Enter the barcode of the input plate containing the samples and the output plate which will contain the prepared DNA libraries.	
IMPORTANT ☐ If the entered barcodes do not match what is stored in the workfile, the correct barcodes will need to be re-entered.	
☐ Click "Select steps" and click "Ok". ☐ Choose a specific starting point from the drop-down menu on the protocol step selection dialogue. ☐ To perform a singular step, check "Perform only selected step" and click "Ok". ☐ To perform multiple steps, leave the check box unselected and click "Ok". Once settings and the steps for the run have been selected, there will be a series of dialogues illustrating how to load the deck depending on the steps selected.	
The library can be either stored or loaded onto a flow cell once adapter ligation has been completed.	
IMPORTANT ☐ We recommend loading >10 fmols of this final prepared library onto the flow cell for R9.4.1 flow cells.	
Priming and loading multiple flow cells on a PromethION	
☐ Thaw the Flush Tether (FLT) and Flush Buffer (FB) at RT before mixing the reagents by vortexing and spin down at RT.	
IMPORTANT ☐ Scale up reagent volumes as needed.	
Prepare the flow cell priming mix in a suitable vial for the number of flow cells to flush. Once combined, mix well by briefly vortexing. ☐ 30 µl Flush Tether (FLT) ☐ 1,170 µl Flush Buffer (FB)	

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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.	
☐ If not already completed, perform a flow cell check on all flow cells. ☐ 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. ☐ 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. ☐ Using a P1000, insert the pipette tip into the inlet port and add 150 µl of library. ☐ 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.	

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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.	
☐ For multiple flow cell washing, use the same experiment name and identifying sample IDs for all runs to enable all flow cells to be paused simultaneously.	
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.	