

Ligation sequencing gDNA - automated Hamilton NGS STAR 96 (SQK-LSK109-XL)

Version: GDA_9107_v109_revM_30Oct2020
Last update: 12/07/2023



Flow Cell Number:

DNA Samples:

Before start checklist

Materials

- 1 µg (or 100-200 fmol) gDNA
- 1.5-3 µg (or 150-300 fmol) high molecular weight genomic DNA for R10.3 flow cells
- Ligation Sequencing Kit XL (SQK-LSK109-XL)
- Flow Cell Priming Kit XL (EXP-FLP002-XL)

Consumables

- Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- 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)
- 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)
- Roche Diagnostics MagNA Pure LC Medium Reagent Tubs 20 (Cat# 03004058001)
- 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

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INSTRUCTIONS	NOTES/OBSERVATIONS
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<p>DNA repair and end-prep</p> <p>Reagents quantities:</p> <p>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.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Thaw all reagents on ice. <input type="checkbox"/> 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. <input type="checkbox"/> Always spin down tubes before opening for the first time each day. <input type="checkbox"/> 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. <input type="checkbox"/> The FFPE DNA Repair Buffer may have a yellow tinge and is fine to use if yellow. <p>Prepare each DNA sample per well with Nuclease-free water in the input plate.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Per sample, transfer 1 µg (or 100-200 fmol) of genomic DNA into a well of the input plate <input type="checkbox"/> Adjust the volume to 48 µl with Nuclease-free water <input type="checkbox"/> Mix thoroughly by pipetting <input type="checkbox"/> Spin down briefly in a microfuge <ul style="list-style-type: none"> <input type="checkbox"/> Quantify 1 µl of each eluted sample using a Qubit fluorometer plate reader off deck. <input type="checkbox"/> Switch on the Hamilton NGS STAR 96 robot and open 'Hamilton Run Control' on the computer by clicking the icon: <input type="checkbox"/> Click 'File' and 'Open' to choose the method to run on the liquid handling robot. <input type="checkbox"/> Click 'Process01: DNA repair and end-prep' to start. <input type="checkbox"/> Click 'Process02: DNA repair and end-prep clean-up' to stop the automated library preparation and quantify the samples before the adapter ligation step. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> It is mandatory for users to have an MPH module installed and we recommend the use of an ODTG module. 	
<p>Click 'Browse' to choose the Input File Worklist for the specific number of samples in the run and click 'OK'.</p> <ul style="list-style-type: none"> <input type="checkbox"/> Sample_01 - Source_Well: A1 - Target_Well: A1 <input type="checkbox"/> Sample_02 - Source_Well: B1 - Target_Well: B1 <input type="checkbox"/> Sample_03 - Source_Well: C1 - Target_Well: C1 	

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<p>Prepare the End Prep Mastermix with the following reagents according to the Hamilton user interface. Click either 'Yes' or 'No' to continue.</p> <ul style="list-style-type: none"> <input type="checkbox"/> NEBNext FFPE DNA Repair Buffer 213.3 µl @ 106.6 µl (414.8 µl cycles) <input type="checkbox"/> NEBNext FFPE DNA Repair Mix 121.8 µl @ 60.9 µl (237 µl cycles) <input type="checkbox"/> Ultra II End-prep Reaction Buffer 213.3 µl @ 106.6 µl (414.8 µl cycles) <input type="checkbox"/> Ultra II End-prep Enzyme Mix 182.8 µl @ 91.4 µl (355.6 µl cycles) <p><input type="checkbox"/> Insert the ComfortLid position as displayed on screen. Click 'Ok' to continue.</p> <p><input type="checkbox"/> Insert plates to their corresponding positions. Click 'Ok' to continue.</p> <p><input type="checkbox"/> Load a full deck of 50 µl tips into the positions on screen. Click 'Ok' to continue.</p> <p><input type="checkbox"/> Highlight the 50 µl tips available to use on the 'Edit Tip Count' window and click 'Ok' to continue.</p> <p><input type="checkbox"/> Load a full deck of 300 µl tips in the positions on screen. Click 'Ok' to continue.</p> <p><input type="checkbox"/> Highlight the 300 µl tips available to use on the 'Edit Tip Count' window and click 'Ok' to continue.</p> <p>Freshly prepare 80% ethanol in Nuclease-free water in a trough.</p> <ul style="list-style-type: none"> <input type="checkbox"/> 80% ethanol 28 ml @ 16.5 ml (51 ml cycles) <p><input type="checkbox"/> Insert the trough of 80% ethanol in the position on screen and click 'Ok' to continue.</p> <p>Prepare the AMPure XP beads by vortexing and load the 20 ml trough with the volume required:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Beads 5.4 ml @ 3.7 ml (8.9 ml cycles) 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> Ensure the AMPure XP beads are well mixed before use by vortexing. 	
<ul style="list-style-type: none"> <input type="checkbox"/> Insert the trough of AMPure XP beads and Nuclease-free water in their positions on screen. Click 'Ok' to continue. <input type="checkbox"/> Load 1000 µl tips and insert the input plate of DNA samples into the position on screen. Click 'Ok' to continue. <input type="checkbox"/> Highlight the 1000 µl tips available to use on the 'Edit Tip Count' window and click 'Ok' to continue. <input type="checkbox"/> Mix and insert the prepared End Prep Mastermix into the positions on screen. <input type="checkbox"/> Click 'Ok' to start the DNA repair and end-prep automation process. <input type="checkbox"/> Once the automation process has finished, there will be an on screen prompt to unload the plate. Click 'Ok' to continue. <input type="checkbox"/> Quantify 1 µl of each eluted sample using a Qubit fluorometer plate reader off deck. 	
<p>Take forward the repaired and end repaired DNA into the adapter ligation and clean-up step.</p>	

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

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<p><input type="checkbox"/> Highlight the 50 µl tips available to use on the 'Edit Tip Count' window. Click 'Ok' to continue.</p> <p><input type="checkbox"/> Load a full deck of 300 µl tips in the positions on screen. Click 'Ok' to continue.</p> <p><input type="checkbox"/> Highlight the 300 µl tips available to use on the 'Edit Tip Count' window. Click 'Ok' to continue.</p> <p>Prepare the AMPure XP beads by vortexing and load the 20 ml trough with the volume required:</p> <p><input type="checkbox"/> Beads 4.3 ml @ 3.1 ml (6.6 ml cycles)</p>	
<p>IMPORTANT</p> <p><input type="checkbox"/> Ensure the AMPure XP beads are well mixed before use by vortexing.</p> <p>Insert troughs of AMPure XP beads, LFB/SFB and EB in the positions on screen. Click 'Ok' to continue.</p> <p><input type="checkbox"/> Long/Short Fragment Buffer 4 bottles @ 2 bottles (8 bottles cycles)</p> <p><input type="checkbox"/> Elution Buffer 1 bottle @ 1 bottle (1 bottle cycle)</p> <p><input type="checkbox"/> Insert 1000 µl tips and the Clean End Prep Plate to the correct positions on screen. Click 'Ok' to continue.</p> <p><input type="checkbox"/> Highlight the 1000 µl tips available to use on the 'Edit Tip Count' window. Click 'Ok' to continue.</p> <p><input type="checkbox"/> Insert the prepared Adapter Ligation Mastermix into the positions on screen. Click 'Ok' to continue.</p> <p><input type="checkbox"/> Once the automation process has finished, there will be an on screen prompt to unload the plate. Click 'Ok' to continue.</p> <p><input type="checkbox"/> Quantify 1 µl of each eluted sample using a Qubit fluorometer plate reader off deck.</p>	
<p>Seal the plate once the library is prepared and store on ice until ready to load onto the flow cell.</p>	
<p>IMPORTANT</p> <p><input type="checkbox"/> We recommend loading 5-50 fmol of final prepared library onto a flow cell.</p>	
<p>Priming and loading multiple flow cells on a PromethION</p>	
<p><input type="checkbox"/> Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and Flush Buffer (FB) at RT before mixing the reagents by vortexing and spin down.</p>	
<p>IMPORTANT</p> <p><input type="checkbox"/> Scale up reagent volumes as needed.</p>	
<p>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.</p> <p><input type="checkbox"/> 30 µl Flush Tether (FLT)</p> <p><input type="checkbox"/> 1,170 µl Flush Buffer (FB)</p>	

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<p>IMPORTANT</p> <p><input type="checkbox"/> 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.</p>	
<p>For PromethION 2 Solo, load the flow cell(s) as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Place the flow cell flat on the metal plate. <input type="checkbox"/> Slide the flow cell into the docking port until the gold pins or green board cannot be seen. <p>For the PromethION 24/48, load the flow cell(s) into the docking ports:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position. <input type="checkbox"/> Press down firmly onto the flow cell and ensure the latch engages and clicks into place. 	
<p>IMPORTANT</p> <p><input type="checkbox"/> 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.</p>	
<ul style="list-style-type: none"> <input type="checkbox"/> If not already completed, perform a flow cell check on all flow cells. <input type="checkbox"/> Slide the inlet port cover clockwise to open. 	
<p>IMPORTANT</p> <p><input type="checkbox"/> 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.</p>	
<p>After opening the inlet port, draw back a small volume to remove any air bubbles:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Set a P1000 pipette tip to 200 µl. <input type="checkbox"/> Insert the tip into the inlet port. <input type="checkbox"/> Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip. <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Meanwhile, thoroughly mix the contents of the thawed Sequencing Buffer (SQB) and Loading Beads (LB) tube(s) by vortexing.</p>	
<p>IMPORTANT</p> <p><input type="checkbox"/> The Loading Beads (LB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.</p>	
<p>In a separate tube for each library, prepare for loading by adding the following reagents:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 75 µl SQB <input type="checkbox"/> 51 µl LB <input type="checkbox"/> 24 µl DNA library 	

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<ul style="list-style-type: none"> <input type="checkbox"/> Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port. <input type="checkbox"/> Mix the prepared library gently by pipetting up and down just prior to loading. <input type="checkbox"/> Using a P1000, insert the pipette tip into the inlet port and add 150 µl of library. <input type="checkbox"/> Close the valve to seal the inlet port and close the PromethION lid when ready. <input type="checkbox"/> 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. 	
<p>Flow cell reuse and returns</p>	
<ul style="list-style-type: none"> <input type="checkbox"/> 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. <input type="checkbox"/> Alternatively, follow the returns procedure to flush out the flow cell ready to send back to Oxford Nanopore. 	
<p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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. 	