1D PCR barcoding genomic DNA (SQK-LSK108)

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

Before start checklist

Materials

- [ ] PCR Barcoding Expansion 1-12 (EXP-PBC001)
- [ ] Ligation Sequencing Kit 1D (SQK-LSK108)
- [ ] Library Loading Bead Kit (EXP-LLB001)

Consumables

- [ ] NEBNext End repair / dA-tailing Module (E7546)
- [ ] NEB Blunt/TA Ligase Master Mix (M0367)
- [ ] Agencourt AMPure XP beads
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- [ ] LongAmp Taq 2X Master Mix (e.g. NEB M0287)
- [ ] Pipette tips P2, P10, P20, P100, P200, P1000

Equipment

- [ ] Thermal cycler
- [ ] Microfuge
- [ ] Vortex mixer
- [ ] Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- [ ] Heating block at 37° C capable of taking 1.5 ml tubes
- [ ] Pipettes P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Preparing input DNA	
Prepare the DNA in Nuclease-free water. ☐ Transfer 1-1.5 µg genomic DNA into a DNA LoBind tube ☐ Adjust the volume to 46 µl with Nuclease-free water ☐ Mix thoroughly by inversion avoiding unwanted shearing ☐ Spin down briefly in a microfuge ☐ Record the quality, quantity and size of the DNA.	
IMPORTANT Criteria for input DNA ☐ Purity as measured using Nanodrop - OD 260/280 of 1.8 and OD 260/230 of 2.0-2.2 ☐ Input mass, as measured by Qubit - <1 µg, or 100-200 fmol for short fragment libraries ☐ No detergents or surfactants in the buffer	
Check your flow cell	
☐ Set up the MinION, flow cell and host computer Once successfully plugged in, you will see a light and hear the fan.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Open the MinKNOW GUI from the desktop icon and establish a local or remote connection. ☐ If running a MinION on the same host computer, plug the MinION into the computer. ☐ If running a MinION on a remote computer, first enter the name or IP address of the remote host under Connect to a remote computer (if running from the Connection page), or Connections (if running from the homepage) and click Connect. ☐ Choose the flow cell type from the selector box. Then mark the flow cell as "Selected": Click "Check flow cells" at the bottom of the screen. ☐ R9.4.1 FLO-MIN106 ☐ R9.5.1 FLO-MIN107 ☐ Click "Start test". ☐ Check the number of active pores available for the experiment, reported in the System History panel when the check is complete.	
Flow cell check complete.	
DNA fragmentation	
OPTIONAL ☐ Transfer each sample of <1 µg genomic DNA in 46 µl to a Covaris g-TUBE. Spin the g-TUBE for 1 minute at RT at the speed for the fragment size required. ☐ Spin the g-TUBE for 1 minute ☐ Remove and check all the DNA has passed through the g-TUBE ☐ If DNA remains in the upper chamber, spin again for 1 minute at the same speed Invert the g-TUBE and spin again for 1 minute to collect the fragmented DNA. ☐ Remove g-TUBE, invert the tube and replace into the centrifuge ☐ Spin the g-TUBE for 1 minute ☐ Remove and check the DNA has passed into the lower chamber ☐ If DNA remains in the upper chamber, spin again for 1 minute ☐ Remove g-TUBE ☐ Transfer the 46 µl fragmented DNA to a clean 1.5 ml Eppendorf DNA LoBind tube.	
Analyse 1 µl of the fragmented DNA for fragment size, quantity and quality.	
<1 µg fragmented DNA in 45 µl from each sample is taken into the next step.	
End-prep	
Perform end-repair / dA-tailing of fragmented DNA as follows: ☐ 45 µl <1 µg end-repaired DNA ☐ 7 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ 5 µl Nuclease-free water	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Mix gently by flicking the tube, and spin down. ☐ Transfer the sample to a 0.2 ml PCR tube, and incubate for 5 minutes at 20° C and 5 minutes at 65° C using the thermal cycler. ☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube. ☐ Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. ☐ Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry. ☐ Remove the tube from the magnetic rack and resuspend pellet in 31 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove and retain 31 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer - recovery aim > 700 ng.	
Take forward approximately 700 ng of end-prepped DNA in 30 µl into adapter ligation.	
Ligation of Barcode Adapter	
Add the reagents in the order given below, mixing by flicking the tube between each sequential addition: ☐ 30 µl End prep DNA ☐ 20 µl Barcode Adapter ☐ 50 µl Blunt/TA Ligase Master Mix ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 10 minutes at RT. ☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Add 40 µl of resuspended AMPure XP beads to the reaction and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. ☐ Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry. ☐ Remove the tube from the magnetic rack and resuspend pellet in 25 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube ☐ Dispose of the pelleted beads ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer. ☐ Dilute the library to a concentration of 10 ng/µl with Nuclease-free water or 10 mM Tris-HCl pH 8.5	
Barcoding PCR	
Set up a barcoding PCR reaction as follows for each library: ☐ 2 µl PCR Barcode (one of BC01-BC12) ☐ 2 µl 10 ng/µl adapter ligated template ☐ 50 µl LongAmp Taq 2x master mix ☐ 46 µl Nuclease-free water ☐ Mix gently by flicking the tube, and spin down. Amplify using the following cycling conditions: ☐ Initial denaturation 3 mins @ 95 °C (1 cycle) ☐ Denaturation 15 secs @ 95 °C (12-15 (b) cycles) ☐ Annealing 15 secs (a) @ 62 °C (a) (12-15 (b) cycles) ☐ Extension dependent on length of target fragment (d) @ 65 °C (c) (12-15 (b) cycles) ☐ Final extension dependent on length of target fragment (d) @ 65 °C (1 cycle) ☐ Hold @ 4 °C ☐ Purify the barcoded DNA using standard methods which are suitable for the fragment size. ☐ Quantify the barcoded library using standard techniques, and pool all barcoded libraries in the desired ratios in a 1.5 ml DNA LoBind Eppendorf tube. ☐ Prepare 1 µg of pooled barcoded libraries in 45 µl Nuclease-free water.	
This pooled library is now ready to be adapted for nanopore sequencing.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
End-prep	
Perform end repair and dA-tailing of fragmented DNA as follows: ☐ 45 µl ~1 µg DNA (fragmented genomic DNA, amplicon or cDNA) ☐ 7 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ 5 µl DNA CS ☐ Mix gently by flicking the tube, and spin down. ☐ Transfer the sample to a 0.2 ml PCR tube, and incubate for 5 minutes at 20 °C and 5 minutes at 65 °C using the thermal cycler. ☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube. ☐ Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. ☐ Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry. ☐ Remove the tube from the magnetic rack and resuspend pellet in 31 µl Nuclease-free water. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. ☐ Remove and retain 31 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer - recovery aim > 700 ng.	
Take forward approximately 700 ng of end-prepped DNA in 30 µl into adapter ligation.	
Adapter ligation	
Thaw and prepare the kit reagents as follows: ☐ ABB Buffer (ABB) at RT ☐ Elution Buffer (ELB) at RT ☐ Adapter Mix 1D (AMX1D) on ice ☐ Running Buffer with Fuel Mix (RBF) on ice ☐ Blunt/TA Ligation Master Mix on ice	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Check the contents of each tube are clear of any precipitate and are thoroughly mixed before setting up the reaction. ☐ Mix the contents of each tube by flicking ☐ Check that there is no precipitate present (DTT in the Blunt/TA Master Mix can sometimes form a precipitate) ☐ Spin down briefly before accurately pipetting the contents in the reaction Taking the end-prepped DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition. ☐ 30 µl End-prepped DNA ☐ 20 µl Adapter Mix ☐ 50 µl Blunt/TA Ligation Master Mix ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 10 minutes at RT.	
AMPure XP bead binding	
☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Add 40 µl of resuspended AMPure XP beads to the adapter ligation reaction from the previous step and mix by pipetting. ☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ Place on magnetic rack, allow beads to pellet and pipette off supernatant. ☐ Add 140 µl of ABB Buffer (ABB) to the beads. Close the tube lid, and resuspend the beads by flicking the tube. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant. ☐ Repeat the previous step. ☐ Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer. Incubate for 10 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. Remove and retain 15 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Remove and retain the eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube ☐ Dispose of the pelleted beads	
The prepared library is used for loading into the flow cell. Store the library on ice until ready to load.	

Before sequencing checklist

☐ Prepared library on ice	☐ Computer set up to run MinKNOW	☐ Hardware check complete
☐ Sequencing device connected to computer with SpotON Flow Cell inserted	☐ Desktop Agent set up (if applicable)	☐ Flow cell check complete

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Priming and loading the SpotON flow cell	
☐ Thaw one tube of Running Buffer with Fuel Mix (RBF) and one tube of Library Loading Beads (LBB) at RT and mix thoroughly by vortexing. Place on ice. ☐ Flip back the MinION lid and slide the priming port cover clockwise so that the priming port is visible.	
IMPORTANT ☐ Care must be taken when drawing back buffer from the flow cell. The array of pores must be covered by buffer at all times. Removing more than 20-30 µl risks damaging the pores in the array.	
After opening the priming port, check for small bubble under the cover. Draw back a small volume to remove any bubble (a few µls): ☐ Set a P1000 pipette to 200 µl ☐ Insert the tip into the priming port ☐ Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip Prepare the flow cell priming mix in a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ 576 µl RBF ☐ 624 µl Nuclease-free water ☐ Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes. ☐ Thoroughly mix the contents of the RBF and LLB tubes by pipetting. Prepare the library for loading as follows: ☐ 35.0 µl RBF ☐ 25.5 µl LLB ☐ 2.5 µl Nuclease-free water ☐ 12 µl DNA library Complete the flow cell priming: ☐ Gently lift the SpotON sample port cover to make the SpotON sample port accessible. ☐ Load 200 µl of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles. ☐ Mix the prepared library gently by pipetting up and down just prior to loading. ☐ Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next. ☐ Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.	
Starting a sequencing run	
☐ Double–click the MinKNOW icon located on the desktop to open the MinKNOW GUI.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ If your MinION was disconnected from the computer, plug it back in. ☐ Choose the flow cell type from the selector box. Then mark the flow cell as "Selected". ☐ Click the "New Experiment" button at the bottom left of the GUI. On the New experiment popup screen, select the running parameters for your experiment from the individual tabs. ☐ Output settings - FASTQ: The number of basecalls that MinKNOW will write in a single file. By default this is set to 4000 ☐ Output settings - FAST5: The number of files that MinKNOW will write to a single folder. By default this is set to 4000 ☐ Click "Start run". Allow the script to run to completion. ☐ The MinKNOW Experiment page will indicate the progression of the script; this can be accessed through the "Experiment" tab that will appear at the top right of the screen ☐ Monitor messages in the Message panel in the MinKNOW GUI The basecalled read files are stored in :\data\reads	
Progression of MinKNOW protocol script	
The running experiment screen Experiment summary information Check the number of active pores reported in the MUX scan are similar (within 10-15%) to those reported at the end of the Flow Cell Check ☐ If there is a significant reduction in the numbers, restart MinKNOW. ☐ If the numbers are still significantly different, close down the host computer and reboot. ☐ When the numbers are similar to those reported at the end of the Flow Cell Check, restart the experiment on the Connection page. There is no need to load any additional library after restart. ☐ Stopping the experiment is achieved by clicking "Stop run" button at the top of the screen. Data acquisition will stop, but the software will continue basecalling unless the user clicks the "Stop basecalling" button. ☐ Check that the temperature has reached 34° C. Check pore occupancy in the channel panel at the top of the experimental view. ☐ A good library will be indicated by a higher proportion of light green channels in Sequencing than are in Pore. The combination of Sequencing and Pore indicates the number of active pores at any point in time. A low proportion of Sequencing channels will reduce the throughput of the run. ☐ Recovering indicates channels that may become available for sequencing again. A high proportion of this may indicate additional clean up steps are required during your library preparation. ☐ Inactive indicates channels that are no longer available for sequencing. A high proportion of these as soon as the run begins may indicate an osmotic imbalance. ☐ Unclassified are channels that have not yet been assigned one of the above classifications ☐ Monitor the pore occupancy	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Duty time plots ☐ Monitor the development of the read length histogram. Cumulative throughput ☐ the number of reads that have been sequenced and basecalled; and whether the reads have passed of failed the quality filters Trace viewer	
Onward analysis of MinKNOW basecalled data	
☐ Open the Desktop Agent using the desktop shortcut. ☐ Click on the New Workflow tab in the Desktop Agent and select the FASTQ barcoding workflow. Select the workflow parameters. ☐ Select the quality score cut-off (this defaults to 7 unless changed) ☐ Select "Yes" in answer to "Detect barcode?" ☐ If you are working with human data, please tick "Yes" in answer to "Is the data you are about to upload a whole or partial human genome?", and confirm that you have consent from the subject to upload the data. ☐ Check the correct settings are selected in the Desktop Agent. ☐ Click "Start Run" to start data analysis. ☐ Follow the progression of upload and download of read files in the Desktop Agent. Click on VIEW REPORT. ☐ Click on VIEW REPORT to navigate to the Metrichor website, this can be done at any point during data exchange ☐ Return to the Desktop Agent to see progression of the exchange	
Close down MinKNOW and the Desktop Agent	
☐ Quit Desktop Agent using the close x. ☐ Quit MinKNOW by closing down the web GUI. ☐ Disconnect the MinION.	
Prepare the flow cell for re-use or return to Oxford Nanopore.	
☐ If you would like to reuse the flow cell, follow the Wash Kit instructions and store the washed flow cell at 2-8 °C, OR ☐ Follow the returns procedure by washing out the MinION Flow Cell ready to send back to Oxford Nanopore.	