#3

1D Native barcoding genomic DNA (with EXP-NBD103 and SQK-LSK109)

Version: NBE_9065_v109_revA_23May2018
Last update: 09/08/2018

Flow Cell Number: ..... DNA Samples: A1 + G3

Before start checklist

Materials

- ☐ 1 µg (or 50-100 fmol) high molecular weight genomic DNA for every sample to be barcoded
- ☐ Native Barcoding Expansion 1-12 (EXP-NBD103)
- ☐ Ligation Sequencing Kit (SQK-LSK109)
- ☐ Flow Cell Priming Kit (EXP-FLP001)

Consumables

- ☐ Agencourt AMPure XP beads
- ☐ NEBNext FFPE Repair Mix (M6630)
- ☐ NEBNext End repair / dA-tailing Module (E7546)
- ☐ NEB Blunt/TA Ligase Master Mix (M0367)
- ☐ NEBNext Quick Ligation Module (E6056)
- ☐ 1.5 ml Eppendorf DNA LoBind tubes
- ☐ 0.2 ml thin-walled PCR tubes
- ☐ Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- ☐ Freshly prepared 70% ethanol in nuclease-free water

Equipment

- ☐ Hula mixer (gentle rotator mixer)
- ☐ Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- ☐ Microfuge
- ☐ Vortex mixer
- ☐ Thermal cycler
- ☐ Ice bucket with ice
- ☐ Timer
- ☐ Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Preparing input DNA	
☐ Record the quality, quantity and size of the DNA.	SAMPLES WERE SUBJECT TO ETHANOL PRECIP → 1000 ng A1 = 80 ng/µl = 12.5 µl + 35.5 H2O G3 = 18 ng/µl = USE ALL → ~ 900 ng
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 ☐ Average fragment size, as measured by pulse-field, or low percentage agarose gel analysis >30 kb ☐ Input mass, as measured by Qubit - 1 µg, or 100-200 fmol for short-fragment libraries ☐ No detergents or surfactants in the buffer	
Prepare the DNA in Nuclease-free water. ☐ Transfer 1 µg genomic DNA into a DNA LoBind tube ☐ Adjust the volume to 48 µl with Nuclease-free water ☐ Mix thoroughly by inversion avoiding unwanted shearing ☐ Spin down briefly in a microfuge	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
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. 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, for lower inputs)	
OPTIONAL Covaris g-TUBE ☐ Transfer the genomic DNA sample in 49 µl to the 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 49 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.	
48 µl of fragmented DNA is taken into the next step.	

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Oxford NANOPORE Technologies

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INSTRUCTIONS	NOTES/OBSERVATIONS
DNA repair and end-prep	
☐ Prepare the NEBNext FFPE DNA Repair Mix and NEBNext End repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice. In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 48 µl DNA ☐ 3.5 µl NEBNext FFPE DNA Repair Buffer ☐ 2 µl NEBNext FFPE DNA Repair Mix ☐ 3.5 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ Mix gently by flicking the tube, and spin down. ☐ Using a thermal cycler, incubate at 20° C for 5 minutes and 65° C for 5 mins. USED 10 MIN EACH	
IMPORTANT ☐ AMPure XP bead clean-up	
☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind 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 ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking. ☐ 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 25 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.	REMAINING STOCK = 114 ng/µl A1 = 82.6 ng/µl → 1,982 ng G3 = TOO HIGH → DILUTED W/ 6µl AND RE-RAN → DILUTED W/ ANOTHER 15µl
Take forward the repaired and end-prepped DNA into the native barcode ligation step.	DILUTED 15 µl OF THIS W/ 15 µl H_2O → 57.2 ng/µl → 1716 ng

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

INSTRUCTIONS	NOTES/OBSERVATIONS
Native barcode ligation	
☐ Thaw the Native Barcodes at RT, enough for one barcode per sample. Mix the barcodes by pipetting, and place them on ice. ☐ Select a unique barcode for every sample to be run together on the same flow cell, from the provided 12 barcodes. Up to 12 samples can be barcoded and combined in one experiment. ☐ Dilute 500 ng of each end-prepped sample to be barcoded to 22.5 µl in Nuclease-free water. → USED / u.s Add the reagents in the order given below, mixing by flicking the tube between each sequential addition: ☐ 22.5 µl 500 ng end-prepped DNA ☐ 2.5 µl Native Barcode — A1 = NB05 ☐ 25 µl Blunt/TA Ligase Master Mix — G3 = NB06 ☐ 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 50 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. ☐ 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 ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking. ☐ Remove the tube from the magnetic rack and resuspend pellet in 26 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 26 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	A1 = 82 ng/µl → 12 µl + 10.5 H_2O G3 = 57 ng/µl → 17.5 µl + 5 H_2O ↓ ~ 1000 ng each
Quantify 1 µl of eluted sample using a Qubit fluorometer.	A1 = 26.2 ng/µl = 665 ng G3 = 32.6 ng/µl = 815 ng
☐ Pool equimolar amounts of each barcoded sample into a DNA LoBind 1.5 ml Eppendorf tube, ensuring that sufficient sample is combined to produce a pooled sample of 700 ng total.	A1 = 26.2 ng/µl × 24 µl = 628 G3 = 32.6 ng/µl × 19.5 µl = 635

2000 ng

43.5 + 6.5 µl H_2O

7 × 200 = 1400 − 7 = 1393   NO GO YET

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Quantify 1 µl of pooled and barcoded DNA using a Qubit fluorometer. ☐ Dilute 700 ng pooled sample to 50 µl in Nuclease-free water. ~1263 ng	
Adapter ligation and clean-up	
IMPORTANT ☐ Depending on the wash buffer used in this section, the clean-up step after adapter ligation is designed to either enrich for long DNA fragments, or purify all fragments equally.	
☐ Thaw one tube of Barcode Adapter Mix (BAM 1D) at RT, spin down, mix by pipetting and place on ice. ☐ Thaw Elution Buffer (EB) and NEBNext Quick Ligation Reaction Buffer (5x) at RT, mix by vortexing, spin down and place on ice. Check the contents or each tube are clear of any precipitate. ☐ Spin down the T4 Ligase, and place on ice. ☐ To enrich for DNA fragments of 3 kb or longer, thaw one tube of L Fragment Buffer (LFB) at RT, mix by vortexing, spin down and place on ice. ☐ To retain DNA fragments shorter than 3 kb, thaw one tube of S Fragment Buffer (SFB) at RT, mix by vortexing, spin down and place on ice. Taking the pooled and barcoded DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition. ☐ 50 µl 700 ng pooled barcoded sample ☐ 20 µl Barcode Adapter Mix (BAM 1D) ☐ 20 µl NEBNext Quick Ligation Reaction Buffer (5X) ☐ 10 µl Quick T4 DNA Ligase ☐ 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 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. ☐ Wash the beads by adding either 250 µl L Fragment Buffer (LFB) or 250 µl S Fragment Buffer (SFB). Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard. ☐ Repeat the previous step. ☐ Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer (EB). 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 ☐ Quantify 1 µl of adapter ligated DNA using a Qubit fluorometer - recovery aim ~430 ng.	69.8 ng/µl × 14 = 980 ng
The prepared library is used for loading into the MinION flow cell. Store the library on ice until ready to load.	↳ 6.5 ul = 450 ng
Priming and loading the SpotON flow cell	
IMPORTANT ☐ Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.	
☐ Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FLB) at RT before placing the tubes on ice as soon as thawing is complete. ☐ Mix the Sequencing Buffer (SQB) and Flush Buffer (FLB) tubes by vortexing, spin down and return to ice. ☐ Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice. ☐ Open the lid of the nanopore sequencing device and slide the flow cell's 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: add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FLB), and mix by pipetting up and down. ☐ 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 LB tube by pipetting.	
IMPORTANT ☐ 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
In a new tube, prepare the library for loading as follows: ☐ 37.5 µl Sequencing Buffer (SQB) ☐ 25.5 µl Loading Beads (LB), mixed immediately before use ☐ 12 µl DNA library ~ 7ul + 5ul H_2O ~ 475 ng 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 50 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. ☐ 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 "Begin Experiment". 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	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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 experiment" button at the top of the screen. ☐ Check the temperature is approximately 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 Duty time plots ☐ Monitor the development of the read length histogram. 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.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
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.	