#3

cDNA-PCR Barcoding (SQK-PCS108 with SQK-PBK004)

10/22/18

Version: PCB_9037_v108_revK_30Jun2017
Last update: 03/09/2018

Oxford NANOPORE Technologies

Flow Cell Number: .....

DNA Samples: A1 + G4

Before start checklist

Materials

- [ ] 50 ng PolyA+ RNA
- [ ] cDNA-PCR Sequencing Kit (SQK-PCS108)
- [ ] PCR Barcoding Kit (SQK-PBK004)

Consumables

- [ ] Agencourt AMPure XP beads
- [ ] 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
- [ ] 10 mM dNTP solution (e.g. NEB N0447)
- [ ] LongAmp Taq 2X Master Mix (e.g. NEB M0287)
- [ ] SuperScript IV reverse transcriptase, 5x RT buffer and 100 mM DTT (ThermoFisher Scientific, 18090050)
- [ ] RNaseOUT™, 40 U/µl (Life Technologies, 10777019)
- [ ] Exonuclease I (NEB, M0293)
- [ ] Pipette tips P2, P10, P20, P100, P200, P1000

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
- [ ] Pre-chilled freezer block at -20° C for 200 µl tubes (e.g. Eppendorf 022510509)
- [ ] Qubit fluorometer (or equivalent for QC check)
- [ ] Pipettes P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Preparing input RNA	
Prepare the RNA in Nuclease-free water. ☐ Transfer 50 ng RNA into a DNA LoBind tube ☐ Adjust the volume to up to 9 µl with Nuclease-free water ☐ Mix by flicking the tube to avoid unwanted shearing ☐ Spin down briefly in a microfuge ☐ Record the quality, quantity and size of the input RNA.	A1 = 2 ng/ul G3 = 2 ng/ul

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

INSTRUCTIONS	NOTES/OBSERVATIONS
IMPORTANT Criteria for input RNA ☐ Average fragment size: ~2 kb ☐ Input mass, as measured by Qubit RNA HS assay: 50 ng ☐ A 260:280 ratio of ~2.0 ☐ A 260:230 ratio of 2.0-2.2 ☐ 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. 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.	
Reverse transcription and strand-switching	
Prepare the following reaction in a 0.2 ml PCR tube: ☐ x µl poly A+ RNA, 50 ng ☐ 1 µl VNP ☐ 1 µl 10 mM dNTPs ☐ 9-x µl RNase-free water ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate at 65° C for 5 minutes and then snap cool on a pre-chilled freezer block. In a separate tube, mix together the following: ☐ 4 µl Superscript IV buffer ☐ 1 µl RNaseOUT ☐ 1 µl 100 mM DTT ☐ 2 µl Strand-Switching Primer (SSP)	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Mix gently by flicking the tube, and spin down. ☐ Add the strand-switching buffer to the snap-cooled, annealed mRNA, mix by flicking the tube and spin down. ☐ Incubate at 42° C for 2 minutes. ☐ Add 1 µl of 200 U / µl SuperScript IV Reverse Transcriptase. The total volume is now 20 µl. ☐ Mix gently by flicking the tube, and spin down. Incubate using the following protocol: ☐ Reverse transcription 10 mins @ 50° C (1 cycle) ☐ Strand switching 10 mins @ 42° C (1 cycle) ☐ Heat inactivation 10 mins @ 80° C (1 cycle) ☐ Hold @ 4° C	
Selecting for full-length transcripts by PCR and barcoding samples	
The PCR step outlined below adds barcodes to each cDNA sample. The barcoded primers are provided in the PCR Barcoding Kit (SQK-PBK004), and can be used to multiplex up to 12 individual samples on a single flow cell. IMPORTANT ☐ Each PCR reaction uses 5 µl of reverse-transcribed RNA (out of a 20 µl reaction). Therefore, sufficient material is available to perform four PCR reactions per reverse transcription reaction. Do NOT use all 20 µl of the reverse transcription reaction in a single PCR reaction. In order to generate sufficient PCR product to make best use of the capacity of the flow cell, it is recommended that at least four PCR reactions (each of 50 µl) are performed. If four, or more, samples are being prepared for barcoded sequencing, it is recommended that one 50 µl PCR reaction is performed per sample. If two samples are being prepared, the recommendation is to perform two 50 µl PCR reactions per sample. It is recommended that any remaining reverse transcription reaction is retained to allow for further PCR reactions if greater yield is required. For each sample (up to 12), prepare the following reaction at RT: ☐ 25 µl 2x LongAmp Taq Master Mix ☐ 1.5 µl LWB 01-12 ☐ 18.5 µl Nuclease-free water ☐ 5 µl Reverse-transcribed RNA sample A1 = LWB05 G4 = LWB06 Amplify using the following cycling conditions: ☐ Initial denaturation 30 secs @ 95 °C (1 cycle) ☐ Denaturation 15 secs @ 95 °C (11-18* cycles) ☐ Annealing 15 secs @ 62 °C (11-18* cycles) ☐ Extension 50 secs per kb @ 65 °C (11-18* cycles) ☐ Final extension 6 mins @ 65 °C (1 cycle) ☐ Hold @ 4 °C	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Add 1 µl of NEB Exonuclease 1 (20 units) directly to each PCR tube.	
☐ Incubate the reaction at 37° C for 15 min, followed by 80° C for 15 min.	
☐ Pool the four PCR reactions (total 204 µl) in a clean 1.5 ml Eppendorf DNA LoBind tube.	
☐ Prepare the AMPure XP beads for use; resuspend by vortexing.	
☐ Add 160 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 70% ethanol. Briefly allow to dry.	
☐ Remove the tube from the magnetic rack and resuspend pellet in 21 µl of Rapid Annealing Buffer (RAB).	
☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.	
☐ Pellet beads on magnet until the eluate is clear and colourless.	
☐ Remove and retain 21 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	
☐ Analyse 1 µl of the amplified DNA for size, quantity and quality.	A# 2.62 ng/µl A5 3.44 A# 2.86 A# 2.14 G 3.22 G 2.62 G 2.74 G 2.24 ~300ng EACH ~1600ng TOTAL
IMPORTANT ☐ Sometimes a high-molecular weight product is visible in the wells of the gel when the PCR products are run, instead of the expected smear. These libraries are typically associated with poor sequencing performance. We have found that repeating the PCR with fewer cycles can remedy this.	
In a 1.5 ml Eppendorf DNA LoBind tube, pool together a total of 350-600 fmol of the amplified cDNA barcoded samples to a final volume of 23 µl in RAB. ☐ Please check the Mass to Molarity table in the protocol	7.5 ng/µl = ~150ng
Adapter addition	
☐ Add 2 µl of cDNA Adapter Mix (cAMX) to the amplified cDNA library.	
☐ Mix gently by flicking the tube, and spin down.	
☐ Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.	
☐ Spin down briefly.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
AMPure XP bead binding	
☐ Prepare the AMPure XP beads for use; resuspend by vortexing. ☐ Add 20 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube. ☐ 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 13 µl Elution Buffer (ELB). ☐ Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. Remove and retain 13 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Remove and retain the eluate which contains the cDNA library in a clean 1.5 ml Eppendorf DNA LoBind tube ☐ Dispose of the pelleted beads	9 ng/µl = 108 ng ~~ng/µl = 108 ng~~
Quantify 1 µl of eluted sample using a Qubit fluorometer.	
The prepared library is used for loading into the MinION 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

Priming and loading the SpotON flow cell	
IMPORTANT ☐ Thoroughly mix the contents of the RBF tube by vortexing or pipetting, and spin down briefly.	
☐ 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.	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
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. ☐ 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	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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 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	

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

INSTRUCTIONS	NOTES/OBSERVATIONS
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. ☐ 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.	