Rapid sequencing gDNA - low input PCR (SQK-PSK004)

Version: PSK_9072_v1_revP_14Aug2019
Last update: 18/05/2023

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Before start checklist

Materials

- [ ] 100 ng high molecular weight genomic DNA
- [ ] PCR Sequencing Kit (SQK-PSK004)
- [ ] Flow Cell Priming Kit (EXP-FLP002)

Consumables

- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- [ ] NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
- [ ] NEB Blunt/TA Ligase Master Mix (NEB, cat # M0367)
- [ ] Covaris g-TUBE
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, AM9937)
- [ ] Freshly prepared 70% ethanol in nuclease-free water
- [ ] LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S)
- [ ] 10 mM Tris-HCl pH 8.0 with 50 mM NaCl
- [ ] (optional) Exonuclease I (NEB, M0293)

Equipment

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

INSTRUCTIONS	NOTES/OBSERVATIONS
DNA fragmentation	

OPTIONAL

Prepare the DNA in Nuclease-free water.

- [ ] Transfer 100 ng genomic DNA into a 1.5 ml Eppendorf DNA LoBind tube
- [ ] Adjust the volume to 50 µl with Nuclease-free water
- [ ] Mix thoroughly by gently pipetting the entire volume up and down 10 times
- [ ] Spin down briefly in a microfuge

- [ ] Transfer 100 ng genomic DNA in 50 µl to the Covaris g-TUBE.

Spin the g-TUBE for 1 minute at RT (Eppendorf 5424; 6000 rpm for 8 kb fragments).

- [ ] 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

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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 50 µl fragmented DNA to a clean 1.5 ml Eppendorf DNA LoBind tube.	
100 ng fragmented DNA in 50 µl is taken into the next step.	
End-prep	
Prepare the NEBNext Ultra II End Repair/dA-Tailing Module reagents according to the manufacturer's instructions, and place on ice. ☐ Thaw all reagents on ice. ☐ Flick and/or invert reagent tube to ensure they are well mixed. ☐ Always spin down tubes before opening for the first time each day. ☐ 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 several seconds to ensure the reagent is thoroughly mixed. ☐ The FFPE DNA repair buffer may have a yellow tinge and is fine to use if yellow. Mix the following reagents in a 0.2 ml thin-walled PCR tube: ☐ 50 µl 100 ng fragmented DNA ☐ 7 µl Ultra II End-prep reaction buffer ☐ 3 µl Ultra II End-prep enzyme mix ☐ Mix well by gently pipetting the entire volume within the tube up and down 10 times. ☐ Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Transfer the 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 when clear and colourless. ☐ Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step.	

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☐ 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 16 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 16 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Quantify 1 µl of end-prepped DNA using a Qubit fluorometer.	
PCR adapter ligation and amplification	
☐ Thaw the Blunt/TA Ligase Master Mix, spin down and mix by pipetting the entire volume within the tube up and down 10 times. Check for any precipitate (if any is visible, continue to mix) and place on ice. Thaw and prepare the kit reagents as follows: ☐ PCR Adapter (PCA) at RT ☐ Whole Genome Primers (WGP) at RT ☐ Blunt/TA ligation Master Mix on ice ☐ LongAmp Taq 2x Master Mix on ice Check the contents of each tube are clear of any precipitate and are thoroughly mixed before setting up the reaction. ☐ Check that there is no precipitate present (DTT in the Blunt/TA Master Mix, if used, can sometimes form a precipitate) ☐ Spin down briefly before accurately pipetting the contents into the reaction Add the reagents in the order given below, mixing by flicking the tube between each sequential addition: ☐ 15 µl End-prepped DNA ☐ 10 µl PCR Adapters (PCA) ☐ 25 µl Blunt/TA Ligase Master Mix ☐ Mix well by gently pipetting the entire volume within the tube up and down 10 times. ☐ Incubate the reaction for 10 minutes at RT. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 20 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 when clear and colourless. ☐ Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. ☐ Repeat the previous step.	

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☐ 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 21 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 21 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 adapted DNA using a Qubit fluorometer. ☐ Calculate how much DNA to take forward into the PCR step for a final DNA concentration of 0.2 ng/µl in a 50 µl reaction. ☐ Thaw the LongAmp® Hot Start Taq 2X Master Mix at RT, spin down and mix by pipetting the entire volume within the tube up and down 10 times. Place on ice. ☐ Thaw the required Whole Genome Primers (WGP) at RT, spin down and mix by pipetting the entire volume within the tube up and down 10 times. Place on ice. Set up the adapted DNA PCR as follows: ☐ Adapter ligated DNA, diluted - Volume: x µl - Final concentration in 50 µl: 0.2 ng/µl ☐ Nuclease-free water - Volume: 24-x µl ☐ Whole Genome Primers (WGP, at 10 µM) - Volume: 1 µl ☐ LongAmp Hot Start Taq 2x Master Mix - Volume: 25 µl ☐ Mix well by gently pipetting the entire volume within the tube up and down 10 times. Amplify using the following cycling conditions: ☐ Initial denaturation 3 mins @ 95 °C (1 cycle) ☐ Denaturation 15 secs @ 95 °C (14 (b) cycles) ☐ Annealing 15 secs (a) @ 56 °C (a) (14 (b) cycles) ☐ Extension 50 secs/kb @ 65 °C (c) (14 (b) cycles) ☐ Final extension 6 mins @ 65 °C (1 cycle) ☐ Hold @ 4 °C ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 30 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.	

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☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless. ☐ Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 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 10 µl of 10 mM Tris.HCl pH 8.0 with 50 mM NaCl. Incubate for 2 minutes at RT. ☐ Pellet the beads on a magnet until the eluate is clear and colourless. Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ Dispose of the pelleted beads ☐ Quantify 1 µl of adapted DNA using a Qubit fluorometer. ☐ Make up 50-100 fmol of PCR product to 10 µl in 10 mM Tris-HCl pH 8.0 with 50 mM NaCl.	
Rapid Adapter ligation	
☐ Spin down the Rapid Adapter (RAP) tube, and place it on ice. ☐ Add 1 µl RAP to the 10 µl amplified DNA library. ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the reaction for 5 minutes at RT.	
The prepared library is used for loading into the flow cell. Store the library on ice until ready to load.	
Priming and loading the SpotON flow cell	
☐ Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before mixing the reagents by vortexing, and spin down at RT. ☐ To 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 (FB), and mix by vortexing at RT. ☐ Open the MinION device lid and slide the flow cell under the clip. ☐ Slide the priming port cover clockwise to open the priming port.	
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.	

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After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles: ☐ Set a P1000 pipette to 200 µl ☐ Insert the tip into the priming port ☐ Turn the wheel until the dial shows 220-230 µl, to draw back 20-30 µl, or until you can see a small volume of buffer entering the pipette tip Note: Visually check that there is continuous buffer from the priming port across the sensor array. ☐ Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading by following the steps below. ☐ Thoroughly mix the contents of the Loading Beads (LB) tubes by vortexing.	
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.	
In a new tube, prepare the library for loading as follows: ☐ 34 µl Sequencing Buffer (SQB) ☐ 25.5 µl Loading Beads (LB), mixed immediately before use ☐ 4.5 µl Nuclease-free water ☐ 11 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 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 the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.	
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.	