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

Before start checklist

Materials

- [ ] 5 µg high molecular weight genomic DNA (recommended); 1–10 µg (or 0.1–2 pmol) can be used accordingly.
- [ ] Cas9 Sequencing Kit (SQK-CS9109)
- [ ] Flow Cell Priming Kit (EXP-FLP002)

Consumables

- [ ] S. pyogenes Cas9 Alt-R™ crRNAs (resuspended at 100 µM crRNA in TE pH 7.5)
- [ ] S. pyogenes Cas9 tracrRNA (e.g., IDT Alt-R™, Cat # 1072532, 1072533 or 1072534) resuspended at 100 µM in TE pH 7.5
- [ ] Alt-R® S. pyogenes HiFi Cas9 nuclease V3, 100 µg or 500 µg (IDT, Cat # 1081060 or # 1081061)
- [ ] Nuclease-free duplex buffer (IDT Cat # 11-01-03-01)
- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- [ ] Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)

Equipment

- [ ] Microfuge
- [ ] Magnetic rack
- [ ] Vortex mixer
- [ ] Thermal cycler
- [ ] Ice bucket with ice
- [ ] Timer
- [ ] Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

INSTRUCTIONS	NOTES/OBSERVATIONS
Preparing the Cas9 ribonucleoprotein complexes (RNPs)	

IMPORTANT

- [ ] Here, the Cas9 is loaded with crRNA and tracrRNA to form ribonucleoprotein complexes (RNPs) in preparation for the cleavage reaction.

- [ ] Pre-heat a thermal cycler to 95ºC.
- [ ] Thaw an aliquot of Reaction Buffer (RB), mix by vortexing, and place on ice.

In an 1.5 ml Eppendorf DNA LoBind tube, pool the crRNA probes for each cleavage reaction by combining equal volumes of each crRNA probe, resuspended at 100 µM in TE (pH 7.5).

- [ ] A single crRNA or many crRNA probes (up to ~100) may be used in a single cleavage reaction.
- [ ] The crRNA probes may also be pre-mixed as an off-catalogue request from IDT.
- [ ] For example, probes for the HTT gene, found here, can be used as an individual experiment or in addition to other probes as an in-run control.
- [ ] Unused crRNA probe mix may be stored at -80ºC and minimal freeze thaw recommended.

Anneal the pooled crRNAs with tracrRNA in Duplex Buffer by assembling the following in a 0.2 ml thin-walled PCR tube, as follows:

- [ ] 8 µl Duplex buffer
- [ ] 1 µl crRNA pool (100 µM, equimolar)
- [ ] 1 µl tracrRNA (100 µM)

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Mix well by pipetting and spin down. Using a thermal cycler heat the above reaction mix at 95ºC for 5 mins, then remove the tube from the thermal cycler and allow it to cool to RT, then spin down the tube to collect any liquid in the bottom of the tube. ☐ Storage and reuse of the annealed mix is not recommended. To form Cas9 RNPs, assemble the components in the table in an 1.5 ml Eppendorf DNA LoBind tube; this will form the annealed crRNA•tracrRNA, through pooling in the stated order: ☐ 79.2 µl Nuclease-free water ☐ 10 µl Reaction Buffer (RB) ☐ 10 µl (Step 4, above) Annealed crRNA•tracrRNA pool (10 µM) ☐ 0.8 µl HiFi Cas9 (62 µM) ☐ Mix thoroughly by flicking the tube. ☐ Form the RNPs by incubating the tube at RT for 30 minutes, then return the RNPs on ice until required.	
Dephosphorylating genomic DNA	
This step reduces background reads by removing 5' phosphates from non-target DNA ends. ☐ Mix the Phosphatase (PHOS) in the tube by pipetting up and down. Ensure that it is at RT before use. Assemble the following components in a clean 0.2 ml thin-walled PCR tube: ☐ 3 µl Reaction Buffer (RB) ☐ 24 µl HMW genomic DNA (at ≥ 210 ng/µl)* ☐ Ensure the components are thoroughly mixed by pipetting, and spin down. ☐ Add 3 µl of PHOS to the tube. ☐ Mix gently by flicking the tube, and spin down. ☐ Using a thermal cycler, incubate at 37ºC for 10 minutes, 80ºC for 2 minutes then hold at 20ºC (RT).	
Cleaving and dA-tailing target DNA	
In this step, Cas9 RNPs (see 'Preparing the Cas9 ribonucleoprotein complexes' page) and Taq polymerase are added to the dephosphorylated genomic DNA sample. ☐ Thaw the dATP tube, vortex to mix thoroughly and place on ice. ☐ Spin down and place the tube of Taq Polymerase (TAQ) on ice. To the PCR tube containing 30 µl dephosphorylated DNA sample, add: ☐ 30 µl Dephosphorylated genomic DNA sample (Step 2) ☐ 10 µl Cas9 RNPs (Step 1) ☐ 1 µl dATP ☐ 1 µl Taq Polymerase (TAQ)	

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☐ Carefully mix the contents of the tube by gentle inversion, then spin down and place the tube in the thermal cycler. ☐ Using the thermal cycler, incubate at 37ºC for 15-60 minutes*, then 72ºC for 5 minutes and hold at 4ºC or return to tube to ice. IMPORTANT ☐ *The Cas9 enzyme is active at 37ºC, and denatured at 72ºC. We recommend a 15 minute cut time by default. Longer 37ºC incubations may increase the amount of off-target reads without increasing the yield of on-target reads, while shorter incubations may result in incomplete target cleavage. However, some regions may benefit from a longer incubation at 37ºC.	
Adapter ligation	
Here, AMX adapters from the Cas Sequencing Kit (SQK-CS9109) are ligated to the ends generated by Cas9 cleavage. ☐ Thaw the Ligation Buffer (LNB) at RT, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing. ☐ Carefully transfer the contents of the 0.2 ml thin-walled PCR tube to a fresh 1.5 ml Eppendorf DNA LoBind Tube using a wide-bore pipette tip. ☐ Thaw an aliquot of Adapter Mix (AMX), mix by flicking the tube, pulse-spin to collect the liquid in the bottom of the vial, then return the vial to ice. ☐ Place the AMPure XP beads at RT. Assemble the following at RT in a separate 1.5 ml Eppendorf DNA LoBind Tube, adding Adapter Mix (AMX) last, before you are ready to begin the ligation: ☐ 20 µl Ligation Buffer (LNB) ☐ 3 µl Nuclease-free water ☐ 10 µl T4 Ligase (LIG) ☐ 5 µl Adapter Mix (AMX)* ☐ Mix by pipetting the above ligation mix thoroughly. Ligation Buffer (LNB) is very viscous, so the adapter ligation mix needs to be well-mixed. ☐ IMPORTANT: Add 20 µl of the adapter ligation mix to the cleaved and dA-tailed sample. Mix gently by flicking the tube. Do not centrifuge the sample at this stage. Immediately after mixing, add the remainder of the adapter ligation mix to the cleaved and dA-tailed sample, to yield an 80 µl ligation mix. ☐ Ensure the components are thoroughly mixed by pipetting, and spin down. ☐ Incubate the reaction for 10 minutes at RT.	
AMPure XP bead purification	
This step removes excess unligated adapters and other short DNA fragments, and concentrates and buffer-exchanges the library in preparation for sequencing. IMPORTANT ☐ If using a 'tiling' approach	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ Thaw the Elution Buffer (EB) and SPRI Dilution Buffer (SDB) at RT, mix by vortexing, spin down and place on ice. ☐ To enrich for DNA fragments of 3 kb or longer, thaw one tube of Long Fragment Buffer (LFB) at RT, mix by vortexing, spin down and place on ice. ☐ To retain DNA fragments of all sizes, thaw one tube of Short Fragment Buffer (SFB) at RT, mix by vortexing, spin down and place on ice. ☐ Add 1 volume (80 µl) of the SPRI Dilution Buffer (SDB) to the ligation mix. Mix gently by flicking the tube. ☐ Resuspend the AMPure XP beads by vortexing. ☐ Add 0.3x volume (48 µl) of AMPure XP Beads to the ligation sample. The volume of beads is calculated based on the volume after the addition of SDB. Mix gently by inversion. If any sample ends up in the lid, spin down the tube very gently, keeping the beads suspended in liquid. ☐ Incubate the sample for 10 minutes at RT. Do not agitate or pipette the sample. ☐ Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless. ☐ Wash the beads by adding either 250 µl Long Fragment Buffer (LFB) or 250 µl Short Fragment Buffer (SFB), depending on the size of your target molecule. 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. ☐ Remove the tube from the magnetic rack and resuspend pellet in 13 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 12 µl of 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 onto 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 Mk1B lid and slide the flow cell under the clip.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
☐ 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.	
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) 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.	
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 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 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 and close the priming port.	
IMPORTANT ☐ Install the light shield on your flow cell as soon as library has been loaded for optimal sequencing output.	
Place the light shield onto the flow cell, as follows: ☐ Carefully place the leading edge of the light shield against the clip. Note: Do not force the light shield underneath the clip. ☐ Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.	

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INSTRUCTIONS	NOTES/OBSERVATIONS
Close the device lid and set up a sequencing run on MinKNOW.	
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.	