

Flow Cell Number:

DNA Samples:

| Before start checklist | | |
|---|--|--|
| Materials | Consumables | Equipment |
| <input type="checkbox"/> 5 µg high molecular weight genomic DNA (recommended); 1–10 µg (or 0.1–2 pmol) can be used accordingly. | <input type="checkbox"/> <i>S. pyogenes</i> Cas9 Alt-R™ crRNAs (resuspended at 100 µM crRNA in TE pH 7.5) | <input type="checkbox"/> Microfuge |
| <input type="checkbox"/> Cas9 Sequencing Kit (SQK-CS9109) | <input type="checkbox"/> <i>S. pyogenes</i> Cas9 tracrRNA (e.g., IDT Alt-R™, Cat # 1072532, 1072533 or 1072534) resuspended at 100 µM in TE pH 7.5 | <input type="checkbox"/> Magnetic rack |
| <input type="checkbox"/> Flow Cell Priming Kit (EXP-FLP002) | <input type="checkbox"/> Alt-R® <i>S. pyogenes</i> HiFi Cas9 nuclease V3, 100 µg or 500 µg (IDT, Cat # 1081060 or # 1081061) | <input type="checkbox"/> Vortex mixer |
| | <input type="checkbox"/> Nuclease-free duplex buffer (IDT Cat # 11-01-03-01) | <input type="checkbox"/> Thermal cycler |
| | <input type="checkbox"/> 1.5 ml Eppendorf DNA LoBind tubes | <input type="checkbox"/> Ice bucket with ice |
| | <input type="checkbox"/> 0.2 ml thin-walled PCR tubes | <input type="checkbox"/> Timer |
| | <input type="checkbox"/> Nuclease-free water (e.g. ThermoFisher, cat # AM9937) | <input type="checkbox"/> Pipettes and pipette tips P2, P10, P20, P100, P200, P1000 |
| | <input type="checkbox"/> Agencourt AMPure XP beads | |

| INSTRUCTIONS | NOTES/OBSERVATIONS |
|---|--------------------|
| <p>Preparing the Cas9 ribonucleoprotein complexes (RNPs)</p> <p style="background-color: #ffe4c4; margin: 5px 0;">IMPORTANT</p> <p><input type="checkbox"/> Here, the Cas9 is loaded with crRNA and tracrRNA to form ribonucleoprotein complexes (RNPs) in preparation for the cleavage reaction.</p> <p><input type="checkbox"/> Pre-heat a thermal cycler to 95°C.</p> <p><input type="checkbox"/> Thaw an aliquot of Reaction Buffer (RB), mix by vortexing, and place on ice.</p> <p>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).</p> <ul style="list-style-type: none"> <input type="checkbox"/> A single crRNA or many crRNA probes (up to ~100) may be used in a single cleavage reaction. <input type="checkbox"/> The crRNA probes may also be pre-mixed as an off-catalogue request from IDT. <input type="checkbox"/> 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. <input type="checkbox"/> Unused crRNA probe mix may be stored at -80°C and minimal freeze thaw recommended. <p>Anneal the pooled crRNAs with tracrRNA in Duplex Buffer by assembling the following in a 0.2 ml thin-walled PCR tube, as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 8 µl Duplex buffer <input type="checkbox"/> 1 µl crRNA pool (100 µM, equimolar) <input type="checkbox"/> 1 µl tracrRNA (100 µM) | |

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| <p><input type="checkbox"/> Mix by pipetting and spin down.</p> <p>Using a thermal cycler heat the above reaction mix at 95°C 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.</p> <p><input type="checkbox"/> Storage and reuse of the annealed mix is not recommended.</p> <p>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:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 79.2 µl Nuclease-free water <input type="checkbox"/> 10 µl Reaction Buffer (RB) <input type="checkbox"/> 10 µl (Step 4, above) Annealed crRNA•tracrRNA pool (10 µM) <input type="checkbox"/> 0.8 µl HiFi Cas9 (62 µM) <p><input type="checkbox"/> Mix thoroughly by flicking the tube.</p> <p><input type="checkbox"/> Form the RNPs by incubating the tube at RT for 30 minutes, then return the RNPs on ice until required.</p> | |
| <p>Dephosphorylating genomic DNA</p> | |
| <p>This step reduces background reads by removing 5' phosphates from non-target DNA ends.</p> <p><input type="checkbox"/> Mix the Phosphatase (PHOS) in the tube by pipetting up and down. Ensure that it is at RT before use.</p> <p>Assemble the following components in a clean 0.2 ml thin-walled PCR tube:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 3 µl Reaction Buffer (RB) <input type="checkbox"/> 24 µl HMW genomic DNA (at ≥ 210 ng/µl)* <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p><input type="checkbox"/> Add 3 µl of PHOS to the tube.</p> <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p><input type="checkbox"/> Using a thermal cycler, incubate at 37°C for 10 minutes, 80°C for 2 minutes then hold at 20°C (RT).</p> | |
| <p>Cleaving and dA-tailing target DNA</p> | |
| <p>In this step, Cas9 RNPs (see 'Preparing the Cas9 ribonucleoprotein complexes' page) and Taq polymerase are added to the dephosphorylated genomic DNA sample.</p> <p><input type="checkbox"/> Thaw the dATP tube, vortex to mix thoroughly and place on ice.</p> <p><input type="checkbox"/> Spin down and place the tube of Taq Polymerase (TAQ) on ice.</p> <p>To the PCR tube containing 30 µl dephosphorylated DNA sample, add:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 30 µl Dephosphorylated genomic DNA sample (Step 2) <input type="checkbox"/> 10 µl Cas9 RNPs (Step 1) <input type="checkbox"/> 1 µl dATP <input type="checkbox"/> 1 µl Taq Polymerase (TAQ) | |

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| <p><input type="checkbox"/> Carefully mix the contents of the tube by gentle inversion, then spin down and place the tube in the thermal cycler.</p> <p><input type="checkbox"/> Using the thermal cycler, incubate at 37°C for 15-60 minutes*, then 72°C for 5 minutes and hold at 4°C or return to tube to ice.</p> | |
| <p>IMPORTANT</p> <p><input type="checkbox"/> *The Cas9 enzyme is active at 37°C, and denatured at 72°C. We recommend a 15 minute cut time by default. Longer 37°C 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 37°C.</p> | |
| <p>Adapter ligation</p> <p>Here, AMX adapters from the Cas Sequencing Kit (SQK-CS9109) are ligated to the ends generated by Cas9 cleavage.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Place the AMPure XP beads at RT.</p> <p>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:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 20 µl Ligation Buffer (LNB) <input type="checkbox"/> 3 µl Nuclease-free water <input type="checkbox"/> 10 µl T4 Ligase (LIG) <input type="checkbox"/> 5 µl Adapter Mix (AMX)* <p><input type="checkbox"/> Mix by pipetting the above ligation mix thoroughly. Ligation Buffer (LNB) is very viscous, so the adapter ligation mix needs to be well-mixed.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Mix gently by flicking the tube, and spin down.</p> <p><input type="checkbox"/> Incubate the reaction for 10 minutes at RT.</p> | |
| <p>AMPure XP bead purification</p> | |
| <p>This step removes excess unligated adapters and other short DNA fragments, and concentrates and buffer-exchanges the library in preparation for sequencing.</p> | |
| <p>IMPORTANT</p> <p><input type="checkbox"/> If using a 'tiling' approach</p> | |

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| <p><input type="checkbox"/> Thaw the Elution Buffer (EB) and SPRI Dilution Buffer (SDB) at RT, mix by vortexing, spin down and place on ice.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Add 1 volume (80 µl) of the SPRI Dilution Buffer (SDB) to the ligation mix. Mix gently by flicking the tube.</p> <p><input type="checkbox"/> Resuspend the AMPure XP beads by vortexing.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Incubate the sample for 10 minutes at RT. Do not agitate or pipette the sample.</p> <p><input type="checkbox"/> Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Repeat the previous step.</p> <p><input type="checkbox"/> 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.</p> <p><input type="checkbox"/> Remove the tube from the magnetic rack and resuspend pellet in 13 µl Elution Buffer (EB). Incubate for 10 minutes at RT.</p> <p><input type="checkbox"/> Pellet the beads on a magnet until the eluate is clear and colourless.</p> <p>Remove and retain 12 µl of eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube.</p> <p><input type="checkbox"/> Dispose of the pelleted beads</p> | |
| <p>The prepared library is used for loading onto the flow cell. Store the library on ice until ready to load.</p> | |
| <p>Priming and loading the SpotON flow cell</p> | |
| <p>IMPORTANT</p> <p><input type="checkbox"/> 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.</p> | |
| <p><input type="checkbox"/> Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT.</p> <p><input type="checkbox"/> Mix the Sequencing Buffer (SQB), Flush Tether (FLT) and Flush Buffer (FB) tubes by vortexing and spin down at RT.</p> | |

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| <ul style="list-style-type: none"> <input type="checkbox"/> Open the MinION Mk1B lid and slide the flow cell under the clip. <input type="checkbox"/> Slide the priming port cover clockwise to open the priming port. | |
| <p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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. | |
| <p>After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few μl):</p> <ul style="list-style-type: none"> <input type="checkbox"/> Set a P1000 pipette to 200 μl <input type="checkbox"/> Insert the tip into the priming port <input type="checkbox"/> Turn the wheel until the dial shows 220-230 μl, or until you can see a small volume of buffer entering the pipette tip <ul style="list-style-type: none"> <input type="checkbox"/> 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. <input type="checkbox"/> 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. During this time, prepare the library for loading by following the steps below. <input type="checkbox"/> Thoroughly mix the contents of the Loading Beads (LB) by pipetting. | |
| <p>IMPORTANT</p> <ul style="list-style-type: none"> <input type="checkbox"/> 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. | |
| <p>In a new tube, prepare the library for loading as follows:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 37.5 μl Sequencing Buffer (SQB) <input type="checkbox"/> 25.5 μl Loading Beads (LB), mixed immediately before use <input type="checkbox"/> 12 μl DNA library <p>Complete the flow cell priming:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Gently lift the SpotON sample port cover to make the SpotON sample port accessible. <input type="checkbox"/> 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. <input type="checkbox"/> Mix the prepared library gently by pipetting up and down just prior to loading. <input type="checkbox"/> 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. <input type="checkbox"/> Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk1B lid. | |
| <p>Ending the experiment</p> | |
| <ul style="list-style-type: none"> <input type="checkbox"/> After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at 2-8°C, OR | |

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| <input type="checkbox"/> Follow the returns procedure by washing out the flow cell ready to send back to Oxford Nanopore. | |
| IMPORTANT <input type="checkbox"/> 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. | |