DOH Workshop Protocol Part 1: Manual Purification of High-Molecular Weight Genomic DNA from Gram-Negative Bacteria (MagAttract HMW DNA)

This protocol enables extraction of high-molecular-weight DNA from Gram-negative bacterial cultures and is an edited version of the Qiagen MagAttract HMW DNA Handbook protocol. Edited by Vesa Qarkaxhija and Bryan Wee

Starting Materials

- ❑ 1.5 ml of overnight bacterial cell culture.

Equipment

- ❑ P1000 pipette (Micropipette with 100 – 1000 µl range)
- ❑ P200 pipette (Micropipette with 20– 200 µl range)
- ❑ P20 pipette (Micropipette with 2 – 20 µl range)
- ❑ P10 pipette (Micropipette with 0.5 – 10 µl range)
- ❑ Thermomixer (for heating, cooling and mixing 1.5ml tubes)
- ❑ Benchtop centrifuge for 1.5 µl (up to 5000 Gs) OR Bento Lab
- ❑ Minicentrifuge (for brief spin downs of 1.5ml eppendorfs and 0.2ml PCR tubes) OR Bento lab with improvised adaptor made from 0.5ml eppendorf within a 1.5ml eppendorf with caps cut off.
- ❑ PCR thermoblock (21°C - 80°C required) OR Bento Lab
- ❑ DNA fluorometer (Promega Quantus or Themofisher Qubit)
- ❑ Vortex mixer

Consumables

- ❑ P1000 filter pipette tips (with 100 – 1000 µl range)
- ❑ P200 filter pipette tips (with 20– 200 µl range)
- ❑ P20 filter pipette tips (with 2 – 20 µl range)
- ❑ P10 filter pipette tip (with 0.5 – 10 µl range)
- ❑ 1.5 ml Eppendorf DNA LoBind tubes
- ❑ 0.2 ml thin-walled PCR tubes
- ❑ Absolute ethanol (>96%)
- ❑ Distilled water (1400 ml per sample)
- ❑ HMW Magattract Kit
    - Nuclease Free Water
    - Buffer MW1 (Ethanol must be added)
    - Buffer PE (Ethanol must be added)
    - MagAttract Suspension G
    - Proteinase K
    - RNAase A (100mg/ml)
    - Buffer ATL

Things to do before starting:

- If precipitate has formed in Buffer ATL, dissolve by incubating at 37°C with occasional shaking. The presence of precipitate can result in incomplete lysis.
- Prepare (but do not start) the thermomixer to 56°C at 900rpm for 30min for the lysis step.

Important points throughout protocol:

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Be gentle when pipetting, too much fast pipetting/ extended vortexing can shear the DNA which will result in poorer sequencing outcomes.
-  Do not let the beads dry after removal of supernatant. This can be avoided by keeping Eppendorf tubes closed if beads are not submerged. Note: When bead pellet is moist, it appears shiny. As they start to dry, the shine reduces, and cracks start to form.
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When working with beads ensure they are thoroughly mixed before using. This can be achieved by vortexing for at least 10 seconds before use and vortexing between use to prevent beads settling.

Start of protocol:

☐	1. Pellet ~1.5ml of the Gram negative bacterial cell culture by spinning at 5000 Gs / 5 kG for 3 min. **Note 1:** *When removing tube from centrifuge, be careful not to shake the tube too much which can dislodge the pellet.*	
☐	2. Remove and discard supernatant without disturbing the pellet. Use a P200/P20 pipette if there is a small amount of supernatant remaining. **Note:** *The cell pellet can be stored at –30 to –15°C or –90 to –60°C for future use, or can be used immediately*	
☐	3. Resuspend the bacterial pellet in 180 µl Buffer ATL, add 20 µl Proteinase K and mix by flicking/stirring the tube.	
☐	4. Incubate for 30 min on thermomixer at 56°C shaking at 900 rpm.	
	Things to do while waiting: • Ensure that Buffers MW1 and PE were prepared according to instructions on them (i.e. adding appropriate amount of Ethanol).	
☐	5. Add 4 µl RNase A to the sample, mix by pulse vortexing or by tapping the tube several times, and incubate for 2 min at room temperature.	
☐	6. Set the thermomixer to 21°C to give it time to cool for next steps	
	7. Fully mix MagAttract Suspension G. Vortex the MagAttract Suspension G vigorously until thoroughly mixed ~10 seconds **Note:** *It is important that beads are fully mixed and re-mixed between every few samples.*	

☐	8. Add 15 µl MagAttract Suspension G and 280 µl Buffer MB to the sample. Mix by pulse vortexing (*1-2 second quick presses x 3*).	
☐	9. Place the tube onto the thermomixer and incubate at 21°C for 3 min at 1400 rpm.	
☐	10. Place tubes onto magnetic rack and wait until beads pellet (~1min).	
☐	11. While leaving tubes on the magnet, carefully remove supernatant without disturbing the beads using a P1000.	
☐	12. Remove tube from magnetic rack and add 700 µl Buffer MW1 to the tube, flick the tube until bead pellet is unstuck from tube wall.	MW1 Wash 1
☐	13. Place the tube onto the thermomixer and incubate at 21°C for 3 min at 1400 rpm.	
☐	14. Place tubes onto magnetic rack and wait until beads pellet (~1min).	
☐	15. While leaving tubes on the magnet, carefully remove supernatant without disturbing the beads using a P1000.	
☐	16. For the second wash, remove tube from magnetic rack and add 700 µl Buffer MW1 to the tube, flick the tube until bead pellet is unstuck from tube wall.	MW1 Wash 2
☐	17. Place the tube onto the thermomixer and incubate at 21°C for **3 min** at 1400 rpm.	
☐	18. Place tubes onto magnetic rack and wait until beads pellet (~1min).	
☐	19. While leaving tubes on the magnet, carefully remove supernatant without disturbing the beads using a P1000.	
☐	20. Remove tube from magnetic rack and add 700 µl Buffer PE to the tube, flick the tube until bead pellet is unstuck from tube wall.	PE Wash 1
☐	21. Place the tube onto the thermomixer and incubate at 21°C for **3 min** at 1400 rpm.	
☐	22. Place tubes onto magnetic rack and wait until beads pellet (~1min).	
☐	23. While leaving tubes on the magnet, carefully remove supernatant without disturbing the beads using a P1000.	
☐	24. For the second wash, remove tube from magnetic rack and add 700 µl Buffer PE to the tube, flick the tube until bead pellet is unstuck from tube wall.	PE Wash 2
☐	25. Place the tube onto the thermomixer and incubate at 21°C for **3 min** at 1400 rpm.	
	26. Place tubes onto magnetic rack and wait until beads pellet (~1min).	
☐	27. While leaving tubes on the magnet, carefully remove supernatant without disturbing the beads using a P1000. Then use a P200 to remove any traces of Buffer PE.	
☐	28. While keeping the tube on the magnetic rack, slowly pipette 700µl distilled water by pipetting down the side of the tube opposite the pellet, without disturbing any of the beads/pellet. Leave for 30-60 seconds and then remove the supernatant without disturbing any of the beads/pellet.	
☐	29. Repeat step 28. **Note:** *If necessary, use a P20 to remove any remaining distilled water.*	
☐	30. Remove the tube from the magnetic rack and add 50µl of nuclease free water. Resuspend the beads by slowly pipetting or stirring with the pipette tip.	

	Note: *Be as gentle as possible while ensuring that pellet is resuspended.*	
☐	31. Place the tube onto the thermomixer and incubate at 21°C for **3 min** at 1400 rpm.	
☐	32. Pellet beads on magnet and slowly pipette DNA eluate into a new lo-bind tube. **Note:** *Save the tube with magnetic beads in case of incomplete DNA elution, so you can repeat.*	

Part 1.1: Promega Pronex protocol

This protocol is to purify extracted dsDNA, removing contaminants (e.g., buffers, proteins, salts, etc.) and low molecular weight DNA (e.g., dsDNA adapters, ssDNA oligonucleotides and nucleotides) and is an edited version of the Promega 6.A.Protocol. Edited by Vesa Qarkaxhija and Bryan Wee

Materials

- ❑ Extracted genomic DNA

Equipment

- ❑ P1000 pipette (Micropipette with 100 – 1000 µl range)
- ❑ P200 pipette (Micropipette with 20– 200 µl range)
- ❑ P20 pipette (Micropipette with 2 – 20 µl range)
- ❑ P10 pipette (Micropipette with 0.5 – 10 µl range)
- ❑ DNA fluorometer (Promega Quantus or Themofisher Qubit)
- ❑ Vortex mixer

Consumables

- ❑ P1000 filter pipette tips (with 100 – 1000 µl range)
- ❑ P200 filter pipette tips (with 20– 200 µl range)
- ❑ P20 filter pipette tips (with 2 – 20 µl range)
- ❑ P10 filter pipette tip (with 0.5 – 10 µl range)
- ❑ 1.5 ml Eppendorf DNA LoBind tubes
- ❑ Qubit dsDNA HS Assay Kit OR Promega QuantiFluor® ONE Dye
- ❑ Qubit™ Assay Tubes
- ❑ Absolute ethanol (>96%)
- ❑ ProNex® Size-Selective Purification System
    - Pronex beads
    - Wash buffer (Ethanol must be added)

Things to do before starting:

- Ensure that the Pronex Wash Buffer is prepared according to instructions on them (i.e. adding appropriate amount of Ethanol).

Important points throughout protocol:

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Be gentle when pipetting, too much fast pipetting/ extended vortexing can shear the DNA which will result in poorer sequencing outcomes.
-  Do not let the beads dry after removal of supernatant. This can be avoided by keeping Eppendorf tubes closed if beads are not submerged. Note: When bead pellet is moist, it appears shiny. As they start to dry, the shine reduces, and cracks start to form.
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When working with beads ensure they are thoroughly mixed before using. This can be achieved by vortexing for at least 10 seconds before use and vortexing between use to prevent beads settling.

Start of protocol:

☐	1. Resuspend the Pronex beads by vortexing for 10 seconds or longer	
☐	*2.* Into your extracted DNA tube pipette 80µl of Pronex beads and mix into the sample by slowly pipetting 10 times. **Note:** *If sticky clumps of bead-bound DNA form, be careful not to take any beads either in the pipette tip or on the outside of the pipette tip.*	
☐	3. Leave at room temperature for 10 min.	
☐	4. Place the sample on a magnetic rack for 2 min (or longer if necessary) until the solution becomes clear and the beads form a pellet.	
☐	5. While leaving tubes on the magnet, carefully remove and discard supernatant without disturbing the beads using a P1000.	
☐	6. While the tube is on the magnetic rack, add 200µl of Pronex Wash Buffer without flushing directly onto the pellet. If 200µl is not enough to submerge the pellet, use more Wash Buffer.	Wash Step 1
☐	7. Allow to incubate for 30-60 seconds	
☐	8. While leaving tubes on the magnet, carefully remove and discard Wash Buffer without disturbing the beads using a P1000.	
☐	9. For the second wash: Keeping the tube on the magnetic stand, carefully add 200µl of Pronex Wash Buffer without flushing directly onto the pellet. If 200µl is not enough to submerge the pellet, use more Wash Buffer.	Wash Step 2
☐	10. Allow to incubate for 30-60 seconds	
☐	11. While leaving tubes on the magnet, carefully remove and discard Wash Buffer without disturbing the beads using a P1000.	
☐	12. Allow the sample to air dry with lids open for 2-5 min watching it until the pellet is no longer shiny.	
☐	13. Remove the sample from the magnetic stand.	

☐	14. Remove the tube from the magnetic rack and add 32µl of nuclease free water. Resuspend the beads by slowly pipetting or stirring with the pipette tip. **Note:** *Be as gentle as possible while ensuring that pellet is resuspended.*	
☐	15. Leave for 5 min at room temperature to elute the DNA	
☐	16. Pellet beads on magnet for 1 minute until solution becomes clear and slowly pipette DNA eluate into a new LoBind tube. **Note:** *Save the tube with Pronex beads in case of incomplete DNA elution, so you can repeat.*	
☐	17. Quantify 1µl of DNA elute using the Qubit™ dsDNA HS Assay Kit or QuantiFluor® ONE dsDNA System.	

Troubleshooting:

1. My DNA yield is low, do I have to redo the extraction?

There are a few things you can do to increase yield before having to redo the extraction. Try these solutions:

- Try re-eluting the DNA from the remaining Pronex beads from step 16 of the **Pronex protocol**. Repeat steps 14 –16.
- Repeat **Pronex protocol**
- Try re-eluting the DNA from the remaining MagAttract Suspension G beads from step 32 of the **DNA extraction protocol**. Repeat steps 30, and re-elute by placing the tube onto the thermomixer and incubate at 21°C for **6 min** at 1400 rpm.

DOH Workshop Protocol Part 2: Library preparation for Rapid Sequencing DNA V14 Barcoding kit (SQK-RBK114.24) with Pronex modification

This protocol performs creation of Nanopore sequencing libraries for the MinION flow cell and is an edited version of the SQK-RBK114.24 protocol. Edited by Vesa Qarkaxhija and Bryan Wee

Material

- ❑ gDNA (~200ng- 300ng of DNA, maximum 10µl). If DNA is too concentrated, dilute with NFW to 10µl.

Equipment

- ❑ P1000 pipette (Micropipette with 100 – 1000 µl range)
- ❑ P200 pipette (Micropipette with 20– 200 µl range)
- ❑ P20 pipette (Micropipette with 2 – 20 µl range)
- ❑ P10 pipette (Micropipette with 0.5 – 10 µl range)
- ❑ PCR thermoblock (21°C - 80°C required) OR Bento Lab
- ❑ DNA fluorometer (Promega Quantus or Themofisher Qubit)

Consumables

- ❑ P1000 filter pipette tips (with 100 – 1000 µl range)
- ❑ P200 filter pipette tips (with 20– 200 µl range)
- ❑ P20 filter pipette tips (with 2 – 20 µl range)
- ❑ P10 filter pipette tip (with 0.5 – 10 µl range)
- ❑ 0.2 ml thin-walled PCR tubes x Number of Samples
- ❑ Nuclease free water (NFW)
- ❑ 1.5 ml Eppendorf DNA LoBind tubes
- ❑ ProNex® Size-Selective Purification System
    - Pronex beads
    - Wash buffer
- ❑ Nanopore Rapid sequencing V14 - Amplicon sequencing (SQK-RBK114.24)
    - Rapid Adapter (RA)
    - Adapter Buffer (ADB)
    - AMPure XP Beads (AXP) (not used in this protocol)
    - Elution Buffer (EB)
    - Sequencing Buffer (SB)
    - Library Beads (LIB)
    - Flow Cell Flush (FCF)
    - Flow Cell Tether (FCT)
    - Rapid Barcodes x 24 (RB01-24)
- ❑ Ice bucket with Ice

Important throughout the protocol:

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Be gentle when pipetting, too much fast pipetting/ extended vortexing can shear the DNA which will result in poorer sequencing outcomes.
-  Do not let the beads dry after removal of supernatant. This can be avoided by keeping Eppendorf tubes closed if beads are not submerged. Note: When bead pellet is moist, it appears shiny. As they start to dry, the shine reduces, and cracks start to form.
-  Be careful when pipetting into a Nanopore flow cell to avoid introducing bubbles into the channels. Bubbles can damage Nanopore array. Practice pipetting on a used flow cell.
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When working with beads ensure they are thoroughly mixed before using. This can be achieved by vortexing for at least 10 seconds before use and vortexing between use to prevent beads settling.

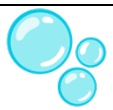
Things to do before starting:

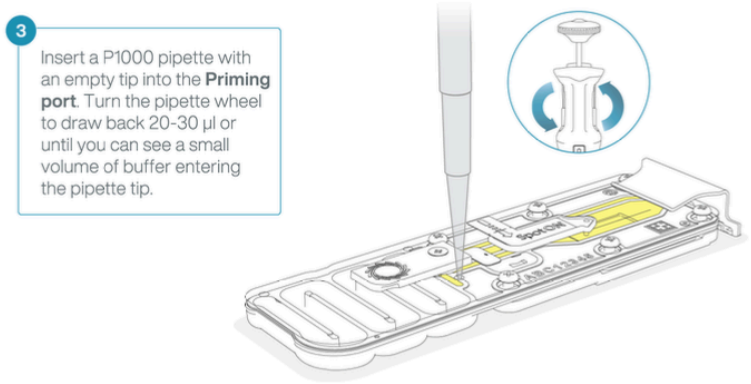
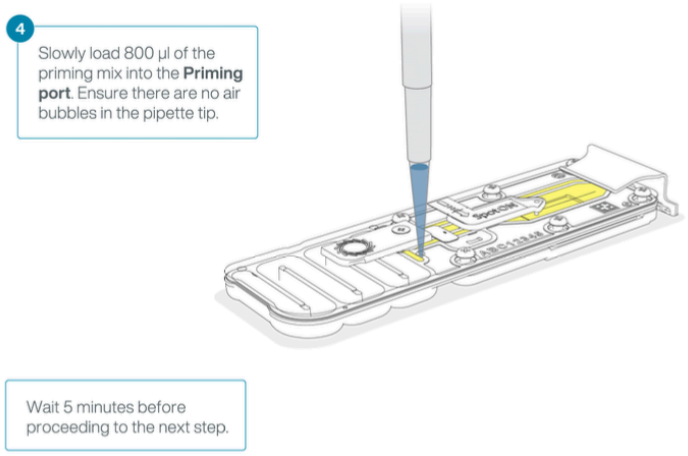
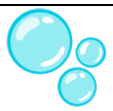
☐	1. Calculate the dilution of your starting genomic DNA from the extraction step to make up 10µl with a maximum concentration of 20 - 30ng/µl (200-300 ng total DNA). (For example, if your DNA from the previous protocol was 100ng/µl, you would only need 2µl of your sample, diluted in 8µl of NFW).	
☐	2. Assign each sample to a Barcode (1-24) and note this down	
☐	3. Program the thermal cycler to incubate at 30°C for 2 min then 80°C for another 2 min. Do not start it yet.	

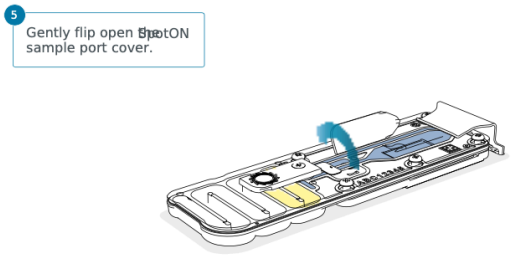
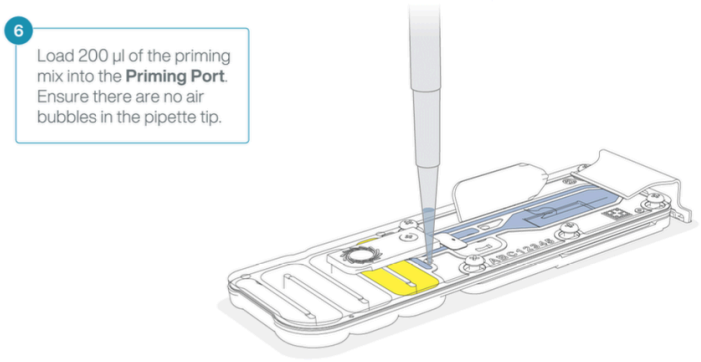
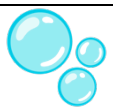
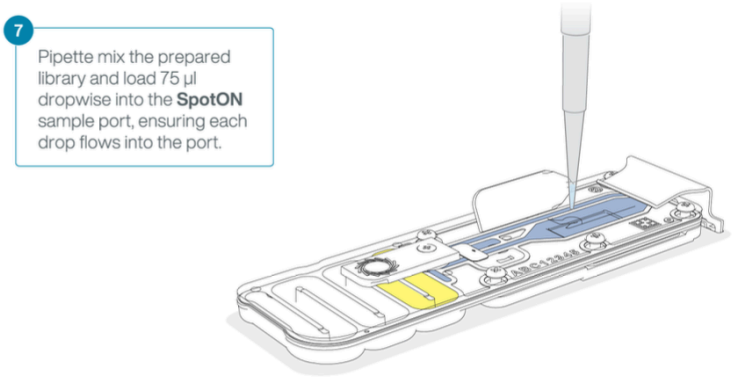
Start of protocol:

☐	4. Prepare one 0.2 ml thin-walled PCR tube for each sample from the previous step. Label the top of the tube with the barcode number.	
☐ ☐ ☐	5. In each 0.2 ml thin-walled PCR tubes: ○ Pipette an appropriate amount of your sample (1-10µl) and add NFW (1-10µl), if necessary, to get 200-300ng of DNA in a 10µl total volume. ○ 1.5 µl of your chosen Rapid Barcode (RB01-24). (1 barcode per sample) **Note**: *Spin barcodes down for 2-3 seconds before use*	
☐	6. Mix by gently by stirring or pipetting until thoroughly mixed. If there are any bubbles present, spin down briefly (2-3 seconds) to ensure all liquid is at the bottom. **Note:** *Barcodes will be a thicker liquid, visually check they have been mixed in.*	

☐	7. Incubate the tubes in the thermal cycler (PCR machine) at 30°C for 2 min then 80°C for another 2 min.	
☐	8. Briefly place the tubes on ice to cool.	
☐	9. Pool all your samples into a 1.5ml clean Eppendorf DNA LoBind tube. Note down the total volume after pooling. It should be 11.5µl multiplied by the number of samples.	
☐	**Only 1 person is required to carry out the following steps.**	
☐	10. Resuspend the Pronex beads by vortexing for 10 seconds or longer.	
☐	11. Use a 1:1 ratio of sample to Pronex beads and mix into the sample by slowly pipetting 10 times. **Note:** *If sticky clumps of bead-bound DNA form, be careful not to take any beads either in the pipette tip or on the outside of the pipette tip.*	
☐	12. Incubate the sample at room temperature for 10 min.	
	To do while waiting • Take out Elution Buffer (EB) to thaw on ice	
☐	13. Place the sample on a magnetic stand for 2 min until the solution becomes clear and the beads form a pellet on one side of the tube.	
☐	14. While leaving the tube on the magnet, carefully remove and discard supernatant without disturbing the beads.	
☐	15. Wash 1: While still on the magnetic stand, carefully add 200µl of Pronex Wash Buffer without flushing directly onto the pellet. If 200µl is not enough to submerge the pellet, use more Wash Buffer.	
☐	16. Allow to incubate for 30-60 seconds	
☐	17. While leaving the tube on the magnet, carefully remove and discard supernatant without disturbing the beads.	
☐	18. Wash 2: While still on the magnetic stand, carefully add 200µl of Pronex Wash Buffer without flushing directly onto the pellet. If 200µl is not enough to submerge the pellet, use more Wash Buffer.	
☐	19. Allow to incubate for 30-60 seconds	
☐	20. While leaving the tube on the magnet, carefully remove and discard supernatant without disturbing the beads.	
☐	21. Allow the sample to air dry with lids open for 2-5 min watching it until the pellet is no longer shiny.	
☐	22. Remove the tube from the magnetic rack and add 15ul of Elution Buffer (included in the Nanopore Rapid Barcoding kit). Resuspend the beads by slowly pipetting or stirring with the pipette tip. **Note:** *Be as gentle as possible while ensuring that pellet is resuspended.*	
☐	23. Incubate the samples at room temperature for 10 min to elute the DNA.	
☐	24. Return the tube to the magnetic stand for 1 minute until the solution becomes clear and the beads form a pellet.	
☐	25. Store the Pronex beads in the fridge.	

☐	26. Transfer 11µl of the eluate into a clean 1.5ml Eppendorf DNA LoBind tube.	
☐	27. Take another 1µl of the elute from the tube on the magnetic stand for quantification on a fluorometer (Qubit or Quantus). The remaining beads can be kept in a closed tube on ice, for re-elution, if necessary.	
☐ ☐ ☐	28. In a new 1.5 ml Eppendorf DNA LoBind tube, mix the following: ○ 1.5µl Rapid Adapter (RA) ○ 3.5µl Adapter Buffer (ADB)	
☐	29. Add 1µl of this RA + ADB mixture to the DNA	
☐	30. Mix gently by flicking and spin down briefly (2-3 seconds)	
☐	31. Incubate this for 30 min at room temperature.	
	Preparing the flowcell	
☐	32. Remove the following Nanopore Rapid Kit (RBK-114.24) items from the -20 °C freezer, spin down and store on ice. ○ SB (Sequencing Buffer) ○ LIB (Library Beads) ○ FCT (Flow Cell Tether) ○ FCF (Flow Cell Flush) ○ Bovine Serum Albumin (BSA) at 50mg/ml	
☐	33. Prepare the flow cell Priming Mix in a fresh 1.5ml Eppendorf DNA LoBind. Mix by inverting the tube. ○ 1,170µl FCF ○ 5 µl BSA (Bovine Serum Albumin) ○ 30 µl FCT	
☐	34. Remove the flow cell you want to use and slide it under the metal clip in the Mk1B or Mk1C MinION. Press down firmly to ensure correct contact with the thermal and electrical connections. ○ Mk1B: Plug in the MinION Mk1B to a laptop with Minknow software ○ Mk1C: Turn on the MinION Mk1C.	
☐	35. Complete a flow cell check to assess the number of pores available on the flow cell.	
☐	36. Rotate the flow cell priming port cover clockwise to open the priming port.	
☐ ☐ ☐ ☐	37. After opening the priming port there will be a small air bubble under the cover that needs to be removed. ○ Set a P1000 pipette to 200 µl ○ Insert the tip into the priming port ○ Turn the adjustment wheel slowly, pausing every few µls, until the pipette shows 220 µl - 230 µl to draw a total of 20-30 µl out of the priming port, or until you can see a small volume of liquid entering the pipette tip. **Note 1:** There may be a small delay before the liquid comes out of the port into the pipette tip. Do not draw out more than 30 µl. **Note 2:** Check that there is a continuous flow of buffer from the	

	priming port to the nanopore sensor array, and that no bubbles are present. 	
☐	38. Load slowly 800µl of the Priming Mix from Step 33 into the priming port, without introducing any bubbles. Leave for 5 min and while waiting, proceed to the next step. 	
☐	39. Mix the LIB (Library Beads) thoroughly by pipetting.	
☐ ☐ ☐ ☐	40. In a new 1.5 ml LoBind tube, mix the following to prepare your library: ○ 37.5 µl SB (Sequencing Buffer) ○ 25.5 µl LIB (Library Beads), mixed immediately before use ○ 12 µl of DNA library (your sample)	
☐	41. Gently open the SpotON sample port cover.	

☐	42. Load slowly 200 µl of the Priming Mix into the priming port (**not the SpotON sample port**). Again, avoid introducing any air bubbles.  	
☐	43. Mix the prepared library by gently pipetting just prior to loading drop by drop 75 µl of the prepared library from Step 40 onto the SpotON sample port. Let each drop flow into the port before adding the next drop. 	
☐	44. Replace the SpotON sample port cover and gently press down to ensure the bung is in the port.	
☐	45. Rotate the Priming port cover back to close the Priming port.	
☐	46. Quickly cover the lid of the MinION to protect from light.	
☐	47. Start the run on the Minknow software interface.	