May 16, 2024 Version 2

Amplicon Sequencing for Genotyping S. Typhi V.2

DOI
dx.doi.org/10.17504/protocols.io.36wgq31dylk5/v2

Jaspreet Mahindroo[1], Anton Spadar[2], Catherine Troman[1], Zoe Dyson[2], Kathryn Holt[2], Nick Grassly[1]

[1]Imperial College London; [2]London School of Hygiene and Tropical Medicine

Jaspreet Mahindroo: *Equal contribution;
Anton Spadar: *Equal contribution
Kathryn Holt: #Equal contribution
Nick Grassly: #Equal contribution

Typhoid Environmental Su…

Catherine Troman
Imperial College London

DOI: dx.doi.org/10.17504/protocols.io.36wgq31dylk5/v2

Protocol Citation: Jaspreet Mahindroo, Anton Spadar, Catherine Troman, Zoe Dyson, Kathryn Holt, Nick Grassly 2024. Amplicon Sequencing for Genotyping S. Typhi. **protocols.io https://dx.doi.org/10.17504/protocols.io.36wgq31dylk5/v2**Version created by **Catherine Troman**

Protocol status: Working
We use this protocol and it's working

Created: January 04, 2024

Last Modified: May 16, 2024

Protocol Integer ID: 99563

Keywords: Salmonella, Typhi, Paratyphi, wastewater, environmental surveillance, nanopore, AMR

Funders Acknowledgement:
Bill and Melinda Gates Foundation

Abstract

The following protocol is for amplifying and sequencing amplicons targeting *Salmonella* Typhi. It is primarily for use with samples that are already suspected to be positive for *S.* Typhi and has been designed for use with DNA extracted from environmental surveillance samples.

The resulting sequences can be analysed and used for genotyping (given that the genotype is targeted by the primer panel) and determining antimicrobial resistance. The genotypes targeted in the panel in this protocol are listed but primers can be designed for other *S.* typhi genotypes of interest.

The library preparation steps of this protocol are adapted from the Oxford nanopore protocol "Ligation Sequencing Amplicons - Native Barcoding Kit 24 V14 (SQK-NBD114.24)" which is available on the Nanopore community.

Materials

- NEBNext Ultra II End Repair/dA-Tailing Module - 96 rxns **New England Biolabs Catalog #**E7546L
- Blunt/TA Ligase Master Mix - 250 rxns **New England Biolabs Catalog #**M0367L
- Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
- Ultrapure BSA **Ambion Catalog #**AM2616
- LongAmp Taq 2X Master Mix - 100 rxns **New England Biolabs Catalog #**M0287S or
- LongAmp Taq 2X Master Mix - 500 rxns **New England Biolabs Catalog #**M0287L
- Nanopore Flow Cell R10.4.1 **Oxford Nanopore Technologies Catalog #**FLO-MIN114
- Native barcoding kit (96) **Oxford Nanopore Technologies Catalog #**SQK-NBD114.96 or
- ONT Native barcoding sequencing kit v14 (24) **Oxford Nanopore Technologies Catalog #**SQk-NBD114.24
- 80% Ethanol **Contributed by users**
- Nuclease-Free Water **Contributed by users**
- Qubit dsDNA Broad Range assay kit (500 assays) **Invitrogen - Thermo Fisher Catalog #**Q32853
- Qubit™ Assay Tubes **Invitrogen - Thermo Fisher Catalog #**Q32856

Equipment

Qubit Fluorometer	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33238	SKU
https://www.thermofisher.com/order/catalog/product/Q33238#/Q33238	LINK

Protocol materials

Agencourt AMPure XP **Beckman Coulter Catalog #**A63880	Step 9.1
Qubit™ dsDNA BR Assay Kit **Thermo Fisher Scientific Catalog #**Q32853	Step 10
Qubit assay tubes **Thermo Fisher Scientific Catalog #**Q32856	Step 10

Primer Panel

1 The following primers have been designed for identifying some *S.* Typhi genotypes and markers for AMR both in the chromosome and in a plasmid. These can be ordered lyophilised from your preferred oligo supplier.

1.1 Genotyping Panel_version1

	A	B	C	D	E
	Forward primer	Forward sequence	Reverse primer	Reverse sequence	Product size
	1_3_4.3.1_F	ACGATGGTACTGAACAACCCT	1_3_4.3.1_R	TACGCTGTTCAGCCCGATATC	1703
	2.2.2_2_F	AGCACAGTTCATCCGAGTGAT	2.2.2_2_R	AGCATCAGACTCTGCGACAC	2,126
	2.5_4.3.1.2.1_3.3_F	CGGTTCGTTGTCCATTTCGG	2.5_4.3.1.2.1_3.3_R	GGCGGCTTTCTTCAGTTTTTCA	1,155
	tviD_842_F	TGCAAGCTGCTTAGTGATCGA	tviD_842_R	TGAGTCCGGTAAAACGAGCTC	842
	4.3.1.1_F	TCTGGCCTGATACCTGGATGT	4.3.1.1_R	CGATCGGATATCCAGCACCA	702
	gyrA_F	TGACGCCTTCTTCGTACTCAC	gyrA_R	CTGAAGCTGATCGCCGATAAAC	2099
	4.3.1.2.1.1_4_F	GTCAGGCCTGGTTTGACAATC	4.3.1.2.1.1_4_R	CCTGTGAACTAACCCCTGCA	1626
	2.3.2_F	GACGATAAACCGCTTCCGTCA	2.3.2_R	AGCCGGGTACAGTAGTCCAA	711
	acrB_v2_F	ACACAGGAAGACGACGATTAGC	acrB_v2_R	AAAGTGCTGGATGAGGTCACG	897
	parC_v7_F	TGGCACAATCACTAAACGCG	parC_v7_R	GCGACGTACTGGGTAAGTAT	701
	3.3.1_1182_F	TCCGTTTTGCGAAATCGTTCC	3.3.1_1182_R	GGGCGCTCTGGTAGACATAC	1182
	4.3.1.2_3694947_v3_F	TGTTTCTGGCTTCGCTGCTGG	4.3.1.2_3694947_v3_R	TGATGTCTTTCCGGCAGTCC	734
	4.3.1.1.P1_F	TTAGGTCGACCAGCGCAAAT	4.3.1.1.P1_R	CCCCGTTAACCCAGGAGAAA	734
	3.1.1_v5_F	TGTATGGCTTCTGGTTGGCTT	3.1.1_v5_R	AAACAACACGCCATTCACGG	1155
	2.3.1_v12_F	ACTGCGCCCATTATTGATCTC	2.3.1_v12_R	GTAGTGTCCCTACCCCCTGT	859

1.2 MDR Panel_version1

	A	B	C	D	E
	Forward primer	Forward sequence	Reverse primer	Reverse sequence	Product size
	C19241A_F	ATTACTGGGCGAGCTGGATTC	C19241A_R	GACAGTCTTCTTCTGGGATCTCG	440
	chr_mdr_cyaA	CCATTGAGCGGAACAAGGTTT	mdr_R	CCATATCACCAGCTCACCGT	1,271
	chr_mdr_yidA	GAGGTGGGTTCTCACTTCCAC			1,290
	plasmid_2.2_none_LT904892.1_F	TCCCTACCATGGATTCCCACT			1,276
	plasmid_4.3.1.3_PST6_CP029957.1_F	AGCACTGCTGGCTCGATTATAT			1,160
	plasmid_4.3.1.1_PST6_CP029645.1_F	CCGTGAGCTCAGGAAAAAGC			1,280
	plasmid_4.3.1.3_PST6_CP029924.1_F	CATGCTACTCGTGCTGACCAT			1,341
	plasmid_4.3.1.1_PST6_LT904879.1_F	TCGCCAGTTTCTCAAACAACCT			1,328
	plasmid_3.2.1_non-PST6_AL513383.1_F	CAATGGATTATGCTCTCCCTCGA			1,301

	A	B	C	D	E
	plasmid_4.3.1.3_PST6_CP029879.1	GGGTCACTTCGGGCTGAAAA			1,309

PCR amplification

2 **Primer reconstitution**
The primers are received in lyophilized form and need to be reconstituted before use. Prepare a stock solution of 100µM primers.

2.1 To reconstitute the lyophilised primers, use the nmole information on the sheet received with the primers.
Convert the nmol to µmol and then divide by the 100 µmol/L.
For example: for a primer with 24 nmoles, to make 100µM stock solution:

24 nmol / 1000 = 0.024 µmol
0.024µmol / 100µmol/L =0.00024 L
0.00024 L x 1000 = 0.24 ml or 240 µl
So add 240 µl to make a 100 µM solution.

In simple words, multiply the nmol value by 10. Example: for 24 nmol x10 = 240 µl to make a 100 µM solution.

2.2 Add the required volume of nuclease free water to the primer vial, pulse vortex and spin down. This is the primer stock with 100µM concentration. Store at -20°C for long-term storage.

3 **Primer Dilution**
Using the 100µM stock, prepare a 10uM working stock of each primer

3.1 In a fresh tube add 5 µl of 100µM primer stock to 45 µl nuclease-free water to give 50 µl of 10µM primer.
Store at 4°C for frequent usage or -20°C for long-term storage.

4 **Primer pooling**
The PCR is currently performed with two reactions per sample with a different primer panel used in each reaction. Using the 10µM working stocks prepare the following primer panels:

4.1 **Genotyping panel**
Pool the primers listed in **1.1** into a single tube as follows:
Add 10 µl of each forward and reverse primer to a single tube, pulse vortex and spin down and use this pool for PCR reactions. Scale up the volume as required.

4.2 **MDR panel**
MDR panel has a common reverse primer for nine targets. To set the multiplex reaction pool the primers in **1.2** as follows:
Add 10 µl of each forward primer + 90 µl of common reverse primer + 10 µl of reverse primer for C19241A into a single tube. Pulse vortex, spin down and use this pool for PCR reactions. Scale up the volume as required.

5 PCR reaction

5.1 Thaw the primer panels and LongAmp taq 2x Mastermix on ice.

Pulse vortex and spin down the primer panels, then return to ice.
Do not vortex the LongAmp taq, mix by flicking or pipetting and spinning down before placing back on ice.

5.2 Prepare the master mix as follows for the number of samples, plus two controls, and one more extra reaction to account for the pipetting error.

	A	B
	Reagent	Volume for 1 reaction
	2x LongAmp Taq	12.5 µl
	Primer pool	2 µl
	Water	5.5 µl
	Total	20 µl

5.3 Dispense 20 µl of master mix per reaction into 0.2 ml PCR tubes.

Add 5 µl of sample DNA.

Mix well by pipetting and spin down.

6 PCR Controls

6.1 Positive control: *Salmonella* Typhi strain H58 DNA (or use appropriate control strain that is available)

Additionally, synthetic gene fragments such as G blocks (IDT) can be used as positive control. Use tviD amplicon sequence and the MDR amplicon sequences as positive control for genotyping and MDR panels respectively. Refer to the attached document for these sequences.

Positive control amplicon sequence.d… 16KB

G blocks are received lyophilized and need to be resuspended with Tris-EDTA pH 8.0 or nuclease free water (as recommended by the supplier). Centrifuge the tube briefly to bring down any contents of the tube sticking to the wall or cap.

For control strain DNA or the G blocks, a concentration of at least 10 ng/µL is recommended for a stock dilution. For example if the stock has 1000ng then resuspend in 100 µl of diluent to get 10 ng/µl concentration.

A working solution of **1 ng/µL** is used in PCR reactions. To make working solution dilute 10 µl of the 10ng/µl stock solution in 90 µl nuclease free water. Quantify using Qubit.
Make 6 µl aliquots of the working solution to avoid contamination and repeated free-thaw cycles then use **5 µl for each positive control reaction**.

6.2 Negative control: 5µl nuclease-free water for a no template control

7 **Thermocycler conditions/ program**

Set up the thermocycler conditions as follows:

	A	B	C	D
	Step	Temperature	Time	Cycles
	I	94°C	30 seconds	1 cycle
	II	94°C	30 seconds	40 cycles
		58°C	30 seconds	
		65°C	2 minutes 40 seconds	
	III	65°C	10 minutes	1 cycle
	IV	10°C	∞	

8 **Gel electrophoresis (Optional)**

You may check the amplification of PCR targets by running the PCR products on a 1% agarose gel or on a TapeStation using a D5000 DNA screen tape.

PCR clean-up and quantification

9 **PCR clean-up**

9.1 Prepare the Agencourt AMPure XP **Beckman Coulter Catalog #**A63880 for use; resuspend by vortexing.

9.2 Add the required volume of resuspended AMPure XP beads to the reaction and mix by pipetting or flicking the tube.
The volume is calculated as the 0.8x PCR reaction volume.
For example: For 25 ul of PCR reaction = 0.8 x 25 = 20 ul of beads

9.3 Incubate on a rotator for 5 minutes at room temperature.

9.4 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

9.5 Keep on the magnet, wash beads with 100 µL of freshly prepared 80% ethanol without disturbing the pellet

9.6 Remove the 80% ethanol using a pipette and discard.

9.7 Repeat steps 8.5 & 8.6.

9.8 Spin down briefly and place the tube back on the magnet.

9.9 Pipette off any residual 80% ethanol using a small tip (10-20µl).

9.10 Briefly allow to dry.

9.11 Remove the tube from the magnetic rack and resuspend the pellet in 20µl of nuclease free water, mix by gently flicking or pipetting.

9.12 Incubate for 2 minutes at room temperature.

9.13 Pellet beads on magnet until the eluate is clear and colourless.

9.14 Still on the magnet, remove 20µl eluate and store it in a clean tube.

Avoid disturbing the pelleted beads. If you find the beads keep getting drawn up the pipette tip, try removing 18µl instead.

10 **Quantification**

The PCR products are quantified using the

Qubit™ dsDNA BR Assay Kit **Thermo Fisher Scientific Catalog #**Q32853

Qubit assay tubes **Thermo Fisher Scientific Catalog #**Q32856

Equipment	
Qubit 4	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33238	SKU

10.1 Standards
The Qubit dsDNA kit requires 2 standards for calibration-- Standard #1 and Standard #2.

10.2 Label the tube lids. Do not label the side of the tube as this could interfere with the sample reading

10.3 Prepare Qubit working solution for the required number of samples and standards as follows

Each sample:
Qubit dsDNA BR Reagent 1 µL
Qubit dsDNA BR Buffer 199 µL

10.4 Aliquot Qubit working solution to each tube:
standard tubes require 190µL of Qubit working solution
sample tubes require 198µL of Qubit working solution

10.5 Add 10µL of the standard to the appropriate tube.

10.6 Add 2µL of each cleaned PCR product to the appropriate tube.

The final volume in each tube must be 200µL once the sample/standard has been added.

10.7 Mix each tube vigorously by vortexing for 3–5 seconds.

10.8 Allow all tubes to incubate at room temperature for 2 minutes, then proceed to “Read standards
and samples.

10.9 On the Home screen of the Qubit Fluorometer, press DNA, then select 1X dsDNA broad range as the assay type. The Read standards screen is displayed. Press Read Standards to proceed.

10.10 Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read standard. When the reading is complete (~3 seconds), remove Standard #1.

10.11 Insert the tube containing Standard #2 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard #2.

10.12 The instrument displays the results on the Read standard screen. Then press run samples.

10.13 On the assay screen, select the sample volume and units:

Press the + or – buttons on the wheel, or anywhere on the wheel itself, to select the sample volume added to the assay tube (2µL).

From the unit dropdown menu, select the units for the output sample concentration (in this case choose ng/µL).

10.14 Insert a sample tube into the sample chamber, close the lid, then press Read tube. When the reading is complete (~3 seconds), remove the sample tube and carefully record the calculated sample concentration.

10.15 The top value (in large font) is the calculated concentration of the original sample.

The bottom value is the dilution concentration. For information on interpreting the sample results, refer to the Qubit Fluorometer User Guide.

10.16 Repeat step 9.14 until all samples have been read.

10.17 All negative controls should ideally be ‘too low’ to read on the Qubit machine, but MUST be < 1ng
per ul. If your negative controls >1ng per ul, considerable contamination has occurred and you must redo previous steps.

11 200fmol calculation

The nanopore protocol recommends using 200fmol of your sample DNA in the first step of library preparation (End-preparation)

11.1 Based on the DNA concentration obtained from Qubit, transfer 200fmol of DNA into a fresh tube and add nuclease free water to total 12.5ul.

You can use the spreadsheet attached to help calculate the volume required of each sample. We have based the ng required of each sample using the average amplicon length for the primer panel to calculate the ng required for 200fmol product (1170ng for the genotyping panel and 1211ng for the MDR panel)

200fmol calculation.xlsx 10KB

11.2 Use this 12.5ul for the End-preparation reaction.

Preparation for sequencing using ONT Native barcodes

12 **End-preparation**

12.1 From the ONT kit (SQK-NBD114.24 or .96) thaw AMPure XP beads (AXP), mix by vortexing, then keep at room temperature.

Thaw the NEBnext Ultra II End Repair reagents on ice, flick or invert the tubes to mix, then spin down.

12.2 To the 12.5µl of prepared 200fmol amplicon DNA, add the following:

	A	B
	Reagent	Volume (µl)
	Ultra II End-prep Reaction Buffer	1.75
	Ultra II End-prep Enzyme Mix	0.75
	Total	2.5

You may make up a master mix of these reagents for the number of samples (+1 for pipetting error) and aliquot 2.5µl for each sample.

12.3 Gently mix by pipetting or flicking the tube then spin down.

12.4 Incubate in a thermal cycler at 20°C for 5 minutes then 65°C for 5 minutes

13 **Native barcode ligation**

13.1 From the ONT kit:
Thaw the EDTA at room temperature, mix by vortexing, spin down, then place on ice.
Thaw the required Native barcodes at room temperature (a different barcode for each sample), mix by flicking of pipetting, spin down, then place on ice.

Thaw the Blunt/TA ligase master mix at room temperature, mix by inverting and flicking well, spin down, then place on ice.

13.2 Select a different barcode for each sample and note this down in your sample spreadsheet (the same spreadsheet used to calculate sample volumes).

Add the following reagents to a clean 0.2ml tube:

A	B
Reagent	Volume
Native barcode	2.5µl
Blunt/TA Ligase Master Mix	10µl
End-prepped DNA	7.5ul

Mix by gently pipetting or flicking then spin down.

13.3 Incubate at room temperature for 20 minutes.

13.4 To each sample add the following volume of EDTA depending on the colour of its cap:

A	B
Clear cap EDTA	2µl
Blue cap EDTA	4µl

Pipette to mix on addition of the EDTA then spin each tube down.

13.5 Pool all samples into a single 1.5ml tube and note down the final volume.

You can estimate this by multiplying the volume of each sample (22µl if clear cap EDTA, 24µl if blue cap EDTA) by the total number of samples.

13.6 Add 0.4x AMPure XP beads to the pooled reaction and mix by flicking the tube.
For example if the pool volume is 200µl, add 80µl AMPure XP beads.

Incubate at room temperature for 10 minutes.

13.7 Spin down and pellet the beads on a magnet until the eluate is clear and colourless (3-5 minutes). Whilst still on the magnet remove and discard the supernatant.

13.8 Wash the pellet with 700µl 80% ethanol, without disturbing the pellet remove the ethanol and repeat this step.

13.9 Spin down, place the tube back on the magnet and remove any residual ethanol. Allow the pellet to air dry for 30 seconds then remove from the magnet and resuspend in 35µl nuclease free water.

13.10 Incubate at 37°C for 10minutes, gently flicking the tube every couple of minutes to encourage elution.

13.11 Pellet the beads on the magnet until clear and colourless then take 30µl into a clean 1.5ml tube.

14 Adapter ligation

14.1 Thaw NEBnext Quick ligation buffer, Short Fragment Buffer (SFB - ONT kit), and Elution Buffer (EB) at room temperature, vortex to mix, spin down and place on ice.

Flick gently to mix then spin down the NEBNext Quick T4 ligase enzyme and the Native Adapter (NA - from ONT kit) and place on ice.

14.2 To the tube with 30µl cleaned barcoded samples, add the following:

	A	B
	Reagent	Volume (µl)
	Native adapter (NA)	5
	NEBNext Quick Ligation buffer	10
	Quick T4 DNA ligase	5

Gently flick the tube to mix then spin down.

14.3 Incubate at room temperature for 20 minutes

Note: At this point, you can remove your flow cell (FLO-MIN114) from the fridge to allow it to come to room temperature.

14.4 Resuspend AMPure beads by vortexing then add 20µl to the reaction and mix by gently flicking the tube. Incubate at room temperature for 10 minutes.

Note: At this point you can start up MinKNOW and run the Flow Cell check.

14.5 Spin down and pellet on the magnet. Still on the magnet, remove and discard the supernatant.

14.6 Wash the pellet with 125µl Short Fragment Buffer, flick the tube to resuspend the beads, spin down, then place back on the magnet to pellet the beads.
Repeat this step.

14.7 Spin down and place back on the magnet then remove any residual buffer. Resuspend in 15µl of Elution Buffer.

14.8 Incubate at 37°C for 10 minutes, flicking every couple of minutes to aid elution.

During this time, thaw Flow Cell Flush (FCF), Flow Cell Tether (FCT), Sequencing Buffer (SB), and Library Beads (LB) at room temperature, mix by vortexing, then spin down and place on ice.

You can also perform the flow cell check. Insert the MinION device into your laptop, open MinKNOW, insert the room temperature flow cell into the MinION, then select Start, then Flow Cell Check.

14.9 Spin down then place back on the magnet to pellet the beads. Remove and retain 15µl in a clean 1.5ml tube.

15 Priming the flow cell and loading the library

15.1 Prepare the flow cell priming mix by adding the following to a clean 1.5ml tube:

	A	B
	Reagent	Volume (µl)
	Flow Cell Flush (FCF)	1170
	Bovine Serum Albumin (BSA; 50mg/ml)	5
	Flow Cell Tether (FCT)	30

15.2 Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover clockwise so that the priming port is visible. After opening the priming port, check for any bubbles under the cover. Draw back a small volume to remove any bubbles (a few µLs). Visually check that there is continuous buffer from the priming port across the sensor array.

15.3 Using a P1000 pipette, slowly load 800µL of the priming mix into the flow cell via the priming port.

Leave a small amount of liquid in the end of the pipette tip to ensure you do not introduce air into the flowcell.

Leave for 5 minutes.

15.4 Mix the contents of the LIB tube by pipetting just before adding to the following library mix in a 1.5ml tube:

	A	B
	Reagent	Volume (µL)
	DNA library	12
	Sequencing buffer (SB)	37.5
	Library beads (LIB)	25.5

15.5 Complete the flowcell priming by opening the SpotOn port cover and carefully loading 200µL of the priming mix into the priming port. As before, leave a small amount of liquid in the bottom of

the tip to avoid the introduction of air bubbles.

When adding the priming mix, you may see a small amount of liquid come up through the SpotOn port. If you do, pause and allow the liquid to flow back into the flowcell before continuing putting through the priming mix.

15.6 Mix the prepared library mix gently by pipetting.

Add the library mix to the flowcell via the SpotOn port in a dropwise fashion, allowing each drop to flow into the flowcell before adding the next.

15.7 Replace the SpotOn port cover and close the priming port, then replace the lid of your sequencing device.

16 **Starting the Sequencing Run**
Open the ONT MinKNOW software and follow the steps below to set up and start your sequencing run.

16.1 Click start, then start sequencing.
Create a name for you sequencing run, it is good practise to make this unique and identifiable for if you ever need to revisit the data. The date and an experiment name are recommended. In sample name you can put a number or repeat the experiment name - this is not as important as the run name. Then click continue.

16.2 Select the kit used - this is SQK-NBD114.24 or SQK-NBD114.96 depending on whether you have purchased the 24 or 96 barcode kit. Click continue.

16.3 In the run length options, set the run time to 8 hours. Click continue.

16.4 In the basecalling options, select high accuracy basecalling. In the barcoding options, make sure barcoding is enabled and toggle to use barcode at both ends. Click continue until you reach the run overview, where you can double check the selected options, then click start run.

Washing the flow-cell after sequencing

17 A nanopore flow cell can be used multiple times, so it must be washed to remove the library from the previous run.

17.1 Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube and thaw one tube of Wash Diluent (DIL) at room temperature.

17.2 In a clean 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix:

	A	B
	Reagent	Volume per cell (µl)
	Wash Mix (WMX)	2

	A	B
	Wash Diluent (DIL)	398
	Total	400

Mix well by pipetting, and place on ice. Do not vortex the tube.

17.3 After the sequencing run, close the ONT MinKNOW software. Detach the MinION device from the laptop and leave the flowcell in the device.

17.4 Before removing the waste fluid, ensure that the flow cell priming port cover and SpotON sample port cover are closed.

17.5 Using a P1000, remove all fluid from the waste channel through waste port 1. As both the flow cell priming port and SpotON sample port are closed, no fluid should leave the sensor array area.

17.6 Rotate the flow cell priming port cover clockwise so that the priming port is visible.

17.7 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles.

17.8 Load 400 µl of the prepared Flow Cell Wash Mix into the flow cell priming port, avoiding the introduction of air.

17.9 Close the flow cell priming port and wait for 60 minutes.

17.10 Before removing the waste fluid a second time, ensure that the flow cell priming port cover and SpotON sample port cover are closed.

17.11 Using a P1000, remove all fluid from the waste channel through waste port 1. As both the flow cell priming port and SpotON sample port are closed, no fluid should leave the sensor array area.

17.12 You can now either run a second library on the flow cell straight away or store the flow cell for later use.

17.13 To run a second library straight away, follow the flow cell priming instructions in step 14.

17.14 To store the flowcell for later use, thaw one tube of Storage Buffer (S) at room temperature, mix contents thoroughly by pipetting and spin down briefly.

17.15 Rotate the flow cell priming port cover clockwise so that the priming port is visible.

17.16 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles.

17.17 Slowly add 500 µl of Storage Buffer (S) through the flow cell priming port.

17.18 Close the priming port.

17.19 Using a P1000, remove all fluid from the waste channel through waste port 1. As both the flow cell priming port and SpotON sample port are closed, no fluid should leave the sensor array area.

17.20 The flow cell can now be stored at 4-8°C.