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Direct Detection of poliovirus and Nanopore Sequencing (DDNS) - Stool V.5

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Poliovirus Sequencing C...



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We use this protocol and it's working

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Abstract

This protocol is an update from the protocol described in the paper "Rapid and sensitive direct detection and identification of poliovirus from stool and environmental surveillance samples using nanopore sequencing" by Shaw et al in the Journal of Clinical Microbiology (2020), DOI: 10.1128/JCM.00920-20 and is commonly known as Direct Detection of Poliovirus by Nanopore Sequencing (DDNS).

The protocol aims to amplify the VP1 region of poliovirus through a semi-nested PCR using a pan-Enterovirus primer and polio specific primers followed by amplification of the VP1 region using a polio specific primer set. We use barcoded primers as this greatly simplifies the subsequent library preparation process. Within the protocol steps quality control checks are included. Following the QC steps as you go ensures the validity of your results and provides easier troubleshooting when needed.

This protocol is for use with Oxford Nanopore kit14 chemistry ligation sequencing reagents and can be used with the MinION Mk1B or GridION sequencer.

Guidelines

Steps 13 onwards are based on the Ligation Sequencing of Amplicons protocol from Oxford Nanopore Technologies.

If you have a Tapestation or similar in your lab, you may use this instead of running gels for the quality control steps.

Materials

 Equipment_Reagents_for_DDNS.xlsx 12.8KB

 SuperScript III One-Step RT-PCR System with Platinum Taq **Invitrogen - Thermo Fisher Catalog #12457-026**

 DreamTaq PCR Master Mix (2X) **Thermo Fisher Catalog #K1071**

 NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S**

 NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #E6056S**

 Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #SQK-LSK114**

 Ethanol, Absolute, Molecular Biology Grade **Thermo Fisher Scientific Catalog #BP2818500**

0.2ml PCR tubes

 1.5 mL LoBind tubes **Eppendorf Catalog #022431021**

 UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**

 Ultrapure BSA **Ambion Catalog #AM2616**

 Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

 Nanopore Flow Cell R10.4.1 **Oxford Nanopore Technologies Catalog #FLO-MIN114**

 ONT Flow Cell Wash Kit **Oxford Nanopore Technologies Catalog #EXP-WSH004**



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 SuperScript III One-Step RT-PCR System with Platinum Taq **Invitrogen - Thermo Fisher Catalog #12457-026**

Before start

This protocol describes the amplification of the VP1 region, sample barcoding and library preparation. We anticipate users will have performed an RNA extraction prior to this protocol to extract Poliovirus RNA. We recommend the MagMAX Viral RNA Isolation Kit for this process.

Barcoded VP1 Primers:

To allow a simplified protocol, we use a 96-well primer plate with 5 μ M barcoded Y7 primer and 5 μ M barcoded Q8 primer in each well to give a total primer concentration of 10 μ M.

Each well contains Q8 and Y7 primers with the same unique barcode e.g A1 = Y7 with barcode 1 and Q8 with barcode 1, A2 = Y7 with barcode 2 and Q8 with barcode 2, etc.

The full set of 96 barcoded primer sequences are shown in Dataset_S1 of Shaw et al, 2020 and in the attached spreadsheet.  BarcodedPrimers.xlsx 20KB

Sample Organisation

30m

- 1 Pairs of samples (with the same EPID) can have consecutive barcodes but try not to group samples from the same geographic area together. This helps detect any potential cross-contamination because identical sequences are then unlikely to be detected in samples with consecutive barcodes that are adjacent to one another on the 96-well plate.
- 1.1 Record sample data, and the order for the samples in a csv file. At this point you can also add any other metadata that you have such as the EPID, country of origin, and date of sample collection and receipt.

30m

Here is a template csv file:  samples.csv 771B

It is advised that you edit the name of the file so it is unique for each run you analyse. If you edit any column headers in the csv, do not use any spaces or special characters in the column header.

You should also include any positive and negative controls in your list of samples. A positive control (resuspended Coxsackievirus A20 provided by MHRA) and negative control (nuclease free water) should each be included on the first and last RNA extraction batches of the day at least. To be processed by PIRANHA, the positive control should just be called "positive" and the negative called "negative" in the sample column. If there are multiple positive or negative controls, these can be defined by adding an underscore and a number.

If any samples are repeats from a previous run, note this down in the "IsQCRetest" column and note down the original run number in the column "IfRetestOriginalRun."

If there has been a delay in the processing of the sample e.g. due to a lack of extraction kits or software updates preventing the run, note "Yes" in the column "DelaysInProcessingForDDNS" and enter the type of delay in the column "DetailsOfDelays."

Note

Do not include any personal information from the patients in your csv file, and avoid using special characters in any of the metadata columns, including the sample names (stick to - or _)

First Round PCR (semi-nest)

5h 40m

- 2 Prepare a master mix using the reaction volumes detailed in the table below for the number of samples you have plus negative controls. The reaction mix and SSIII enzyme are provided in

20m

 SuperScript III One-Step RT-PCR System with Platinum Taq **Invitrogen - Thermo Fisher Catalog #12457-026**

Forward primer: Y7 [GGGTTTGTGTCAGCCTGTAATGA]

Reverse Primers: Cre [TCAATACGGTGTTTGCTCTTGAAGT] (Arita et al. 2015)
nOPV-MM-R [TCGATACGGTGCTTGGATTAAATTG]

Note

The reverse primers include both a Pan-enterovirus primer and a primer which allows amplification of nOPV2. **These are mixed to make a working solution with each primer at 10 μ M .**

For example, 10 μ L of each 100 μ M reverse primer stock is added to 80 μ L of nuclease free water to create 100 μ L of a working solution with each primer at 10 μ M

	A	B
	Reagent	1 reaction (μ L)
	2x Reaction mix	12.5
	SSIII Platinum Taq mix	1
	Reverse primers	1
	Nuclease free water	4.5

Table1: Mastermix contents for a single first round PCR reaction. This can be multiplied up to fit the number of reactions you will be carrying out.

- 3 Vortex the mastermix for 3 seconds and spin down for 5 seconds to gather contents at the bottom of the tube. Aliquot 19 μ L to each PCR tube and add 5 μ L of sample RNA or nuclease free water for negative controls.
- 4 Incubate in a thermocycler for 30 minutes at 50°C.

10m

30m

5 Add 1µL of the forward primer to each reaction.

5m

6 Amplify using the following cycling conditions:

3h 20m

	A	B	C	D
	Cycle	Step	Temperature (°C)	Time
	1	Initial denaturation	94	2 minutes
	42	Denaturation	94	15 seconds
		Annealing	55	30 seconds
		Extension	68	2 minutes 30 seconds
	1	Final extension	68	5 minutes
	-	Hold	10	-

Table 2: Cycling conditions for the first round PCR

Note

The DDNS method was validated on Eppendorf Nexus and Applied Biosystems ProFlex thermal cyclers. Thermal cyclers of other models and manufacturers might need adjustments in their settings, including the temperature ramp rate, to achieve sufficient amplification.

6.1 Once the PCR is finished, check to see if any reactions have evaporated, if so note this down in the sample csv.

5m

Input into your csv the date that the first round PCR was carried out.

Second Round PCR (VP1 amplification)

5h 10m

7 VP1 amplification is performed using barcoded primers as described in Dataset_S1 in Shaw *et al* 2020. These should be ordered in a 96-well plate layout and the forward and

30m

reverse primers premixed to make an overall 10μM working stock.

Prepare a mastermix as described below using

 DreamTaq PCR Master Mix (2X) **Thermo Fisher Catalog #K1071** and multiplying up for the number of reactions required:

	A	B
	Reagent	1 Reaction (μL)
	DreamTaq 2x mastermix	12.5
	Nuclease free water	8.5

Table 3: Mastermix contents for second round PCR using barcoded primers.

- 8

Vortex the mastermix for 3 seconds and spin down for 5 seconds to gather contents at the bottom of the tube.

10s
- 9

Aliquot 21μL for each well of a 96-well PCR plate and add 2μL of 10uM barcoded primers (ensuring a different barcode is used for each sample) and 2μL of first round PCR product or nuclease free water for PCR negative controls.

10m
- 10

Amplify using the following cycling conditions:

2h

	A	B	C	D
	Cycle	Step	Temp (C)	Time
	1	Initial Denaturation	95	2 minutes
	35	Denaturation	95	30 seconds
		Annealing	55	30 seconds
		Extension	72	1 minute
	1	Final Extension	72	10 minutes
	-	Hold	10	

Table 4: Cycling conditions for VP1 PCR



- 11 Once the PCR is finished, check to see if any reactions have evaporated, if so note this down in the sample csv.

Input into your csv the date that the VP1 PCR was carried out.

- 12 Check all positive and negative controls from the VP1 reaction on a 1% agarose gel. The expected band for the positive control is around 1.2kb.

1h

All samples can be marked as "Pass" for the PositiveControlCheck in the barcodes csv if **all** positive controls extracted on the same day show a VP1 band on the gel.

All samples can be marked as "Pass" for the NegativeControlCheck if all negative controls extracted on the same day show no VP1 band on the gel.

If any positive controls fail, or any negative controls have a band, all samples extracted on the same day as the controls must be marked as fail.

- 12.1 If the positive control check is failed, run the positive control first round PCR product(s) on a 1% gel.

1h

If there is no band, repeat the VP1 reaction **for the control**.

If there is a band in the positive control, discard the VP1 amplicons and repeat the VP1 reactions **for all samples**.

If there is no band visible after repeating the VP1 reaction, repeat the RNA extractions after checking the RNA extraction kit is being used correctly and has not expired.

- 12.2 If the negative control check is failed, repeat both the first round PCR and the nested VP1.

30m

If the negative control still shows a band on a gel or tapestation:

1. Thoroughly clean the PCR and RNA extraction workstations.
2. Replace each of the First Round and VP1 reagents in turn whilst performing blank reactions to determine a contaminated reagent.
3. Perform an additional Negative RNA extraction to confirm that that RNA extraction kit is not contaminated.

Note

To avoid cross contamination due to contaminated lab surfaces/equipment, we recommend giving the PCR and RNA workstations a thorough clean every 5 runs.



Library Preparation for ONT MinION: Pooling, End-prep, and Adapter ligation

1h 45m

- 13 Pool 2µL of each VP1 PCR product into a 1.5mL tube and concentrate with AMPure beads

5m

 Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

- 13.1 Add a volume of AMPure beads equal to the volume of the pooled VP1 products and incubate at room temperature for 5 minutes. Flick gently after 2 minutes to aid binding.

6m

e.g. 50 samples, 2µl each pooled = 100µl pool, so add 100ul AMPure beads

- 13.2 Spin down the tube for 3 seconds then place on a magnetic rack until all the beads have formed a pellet and the solution is clear.

3m

- 13.3 Pipette off the solution, avoiding disturbing the bead pellet.

1m

- 13.4 Add 200µL of 80% Ethanol to the tube, leave for 30seconds, then remove and discard.

2m

Repeat.

- 13.5 Spin down the tube for 2 seconds, place back on the magnet, then remove any remaining Ethanol.

1m

Allow the pellet to air dry for 30 seconds or until dry but not cracking

- 13.6 Take the tube off the magnet and add 51µL of nuclease free water. Flick the tube to resuspend the beads and incubate at room temperature for 2 minutes.

3m

- 13.7 Spin down the tube for 3 seconds then place back on the magnet, allowing the beads to pellet completely.

30s

- 13.8 Remove 50µL of the eluted DNA and add to a clean 0.2mL PCR tube.

30s

- 14 End-preparation:
Add the following reagents from

2m

 NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S**

to the 0.2mL tube containing the cleaned DNA pool.

A	B
Component	Volume (μL)
Ultrall End-prep reaction buffer	7
Ultrall End-prep enzyme mix	3

Table 5: Reaction for end-prep of your pooled library

- 15 Mix gently by flicking the tube and spin down for 3 seconds. 1m
 - 16 In the thermocycler, incubate for 5 minutes at 20°C followed by 5 minutes at 65°C 10m
 - 17 Transfer to a 1.5mL tube and perform an AMPure bead clean. 15m
 - 17.1 Vortex the AMPure beads until all the beads are well mixed. 30s
 - 17.2 Add 60μl of resuspended beads to the tube and flick the tube to mix. 5m
Incubate at room temperature for 5 minutes. Flick gently after 2 minutes to aid binding.
 - 17.3 Spin down the tube for 3 seconds then place on a magnetic rack until all the beads have formed a pellet and the solution is clear. 1m
 - 17.4 Pipette off the solution, avoiding disturbing the bead pellet. 1m
 - 17.5 Add 200μL of 80% Ethanol to the tube, leave for 30seconds, then remove and discard. 2m
Repeat.
 - 17.6 Spin down the tube for 2 seconds, place back on the magnet, then remove any remaining Ethanol. 2m
- Allow the pellet to air dry for 1 minute or until dry but not cracking



- 17.7 Take the tube off the magnet and add 61µL of nuclease free water. Flick the tube to resuspend the beads and incubate at room temperature for 2 minutes. 3m
- 17.8 Spin down the tube for 3 seconds then place back on the magnet, allowing the beads to pellet completely. 1m
- 17.9 Remove 60µL of the eluted DNA and add to a clean 1.5mL tube. 30s

Note

At this point you can store the end-prepared library at 4°C. This can be stored up to one week before continuing to step 18, however it is advised to continue the library preparation protocol as soon as possible.

Ensure you label the tube clearly with the run name and what stage of the library preparation you were at e.g. 20240229 DDNSrun10 end-prep

18 From

 NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #E6056S**

:

Spin down the NEB Quick T4 Ligase and place on ice

From

 Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #SQK-LSK114**

:

Spin down and thaw Ligation Adapter (LA) on ice.

Thaw Ligation Buffer (LNB) at room temperature, spin down, mix by pipetting, then place on ice.

Thaw Elution Buffer (EB), and Short Fragment Buffer (SFB) at room temperature, mix by vortexing then place on ice.

If you plan to start the run on the same day, remove the Flush buffer, flush tether (FLT) and BSA from the freezer and thaw at room temperature. Once thawed, place on ice.

Remove your flow cell (FLO-MIN114, R10.4.1) from the fridge to allow it to get to room temperature.

- 19 Prepare the following reaction mix adding reagents to the 1.5mL tube with end-prepped DNA: 2m



	A	B
	Component	Volume (μL)
	End-prepped DNA	60
	Ligation buffer (LNB)	25
	Quick T4 Ligase	10
	Ligation Adapter (LA)	5

Table6: Reaction mix for sequencing adapter ligation

20 Mix gently by flicking the tube then spin down.

1m

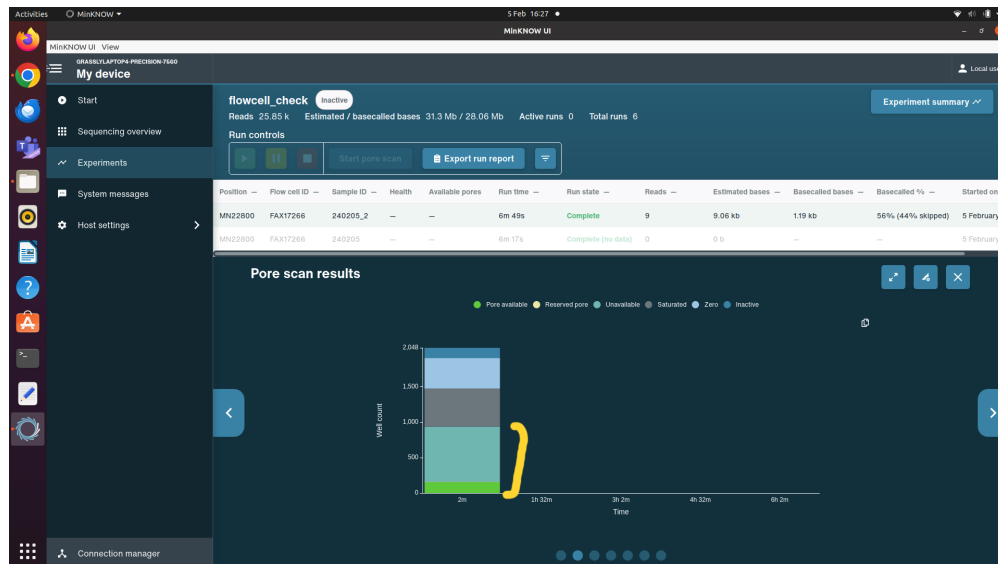
21 Incubate at room temperature for 10 minutes.

10m

21.1 During this time, you can run your flow cell check

Plug in your sequencing device, open the lid and insert your flowcell. In the MinKNOW software, navigate to the start panel then select flowcell check, then start. This will tell you how many pores are available for sequencing.

If your flow cell has been used before, instead of running a flow cell check, start a dummy sequencing run by selecting Start Sequencing, name the run "flowcell_check", select any kit, then set the time to 0.2 hours, then start the run. At the beginning of the run it will do a short flow cell check and give a more accurate number for the available pores (calculated by adding together the available and unavailable pores in the pore status graph (see example image below)).



If a flow cell has less than 700 pores, do not use it for a 96 sample DDNS run. Take out a different flow cell and perform a flow cell check.

The number of pores available in the flow cell you use for sequencing should be noted down in the sample csv in the column "PoresAvailableAtFlowCellCheck" and the flow cell ID should be recorded in the "FlowCellID" column. Also record the number of times the flow cell has been used previously in the column "FlowCellUses".

- 22 Carry out an AMPure bead purification using 40µL of resuspended AMPure XP beads.

Note: This clean-up is different to previous as it uses the ONT Short Fragment Buffer (SFB) and Elution buffer (EB) instead of 80% ethanol and water.

- 22.1 Vortex the AMPure beads until all the beads are well mixed.

30s

- 22.2 Add 40µL of resuspended beads to the 1.5mL tube and mix by flicking the tube.

6m

Incubate at room temperature for 5 minutes. Flick gently after 2 minutes to aid binding.

- 22.3 Spin down the tube for 3 seconds then place on a magnetic rack until all the beads have formed a pellet and the solution is clear.

1m

- 22.4 Pipette off the solution, avoiding disturbing the bead pellet.

30s

- 22.5 Add 250µL of Short Fragment Buffer (SFB). Remove the tube from the magnet and resuspend the beads in the SFB by flicking the tube.

4m

Spin down for 3 seconds then return the tube to the magnet.
Allow the beads to pellet, then remove and discard.

Repeat.

- 22.6 Spin down the tube for 2 seconds, place back on the magnet, then remove any remaining SFB. 1m

Allow the pellet to air dry for 1 minute or until dry but not cracked

- 22.7 Take the tube off the magnet and add 15µL of Elution buffer (EB). Flick gently to resuspend the beads and incubate at room temperature for 10 minutes. 10m

- 22.8 Spin down the tube for 3 seconds then place back on the magnet, allowing the beads to pellet completely. 1m

- 22.9 Remove 12µl of the eluted DNA and transfer to a clean 1.5ml tube. 30s

Note

At this point you can keep your prepared library at 4°C overnight with minimal loss of throughput. This can be kept for up to two weeks before sequencing, however we recommend that you continue to flow cell loading as soon as possible.

Ensure you label the tube clearly with the run name and what stage of the library preparation you were at e.g. 20240229 DDNSrun10 final library

Priming and Loading of the MinION Flowcell 30m

- 23 Thaw the Sequencing buffer (SB), Library beads (LIB), Flow Cell Tether (FCT) and one tube of Flow Cell Flush (FCF) at room temperature then place on ice. 10m

Mix the SQB, FCF, and FCT by vortexing, spin down, and return to ice. Spin down the LIB then place back on ice.

- 24 To create the priming mix the following reagents in a clean 1.5ml tube: 2m

	A	B
	Reagent	Volume (µL)
	Flow cell flush (FCF)	1,170



	A	B
	Flow cell tether (FCT)	30
	BSA (50mg/ml)	5

Mix by pipetting and spin down. Place on ice until ready to use.

- 25 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 μL s). Visually check that there is continuous buffer from the priming port across the sensor array. 3m

- 26 Using a P1000 pipette, slowly load 800 μL of the priming mix into the flow cell via the priming port. 2m

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.

- 27 Mix the contents of the LIB tube by pipetting just before adding to the following library mix in a 1.5ml tube: 2m

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

- 28 Complete the flowcell priming by opening the SpotOn port cover and carefully loading 200 μL 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. 2m



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.

- 29 Mix the prepared library mix gently by pipetting. 2m
- 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.
- 30 Replace the SpotOn port cover and close the priming port, then replace the lid of your sequencing device. 1m
- 31 In the MinKNOW software follow the steps below to set up and start your sequencing run. 30s
- 31.1 Click start, then start sequencing. 1m
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.
- 31.2 Select the kit used - this is SQK-LSK114. Once you click this the barcoding options will appear. Select EXP-PBC096, then click continue 30s
- 31.3 In the run length options, set the run time to 4 hours for a prospective DDNS run. 30s
- 31.4 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. Double check your selected options, then click start run. 1m
- 31.5 In your sample csv, record the run number in the column "RunNumber", the date in "DateSeqRunLoaded", and the run duration in "RunHoursDuration". 2m

Washing your flow cell

1h 23m

- 32 After the sequencing run is finished you can wash your flow cell to remove the remaining library and either prepare for another sequencing run or for storage at  4 °C . The wash uses the reagents supplied in the ONT wash kit:

 ONT Flow Cell Wash Kit **Oxford Nanopore Technologies Catalog #EXP-WSH004**



32.1 Thaw the Wash Diluent at room temperature and mix briefly by vortexing.

10m

Spin down the tube of wash mix (WMX) and place on ice.

32.2 Prepare the following wash solution in a clean 1.5ml tube

5m

	A	B
	Wash diluent	398 μ L
	Wash mix	2 μ L

33 Open the Priming port and using a P1000 pipette, carefully remove a small amount of liquid to remove any air bubbles under the port.

2m

34 Carefully add 200 μ L of the wash solution through the priming port, leaving a small amount of liquid in the tip towards the end to avoid introducing an air bubble. Close the priming port and incubate for 5 minutes at room temperature.

1m

34.1 Add the remaining 200 μ L of wash solution through the priming port. Close the priming port and incubate at room temperature for one hour.

1h

At this point you can remove all waste from the waste channel, ensuring that the Priming Port is closed before doing so.

You can also put a label on the packaging of the flow cell to detail the date it was run, what was run on it, and for how long e.g. 13/03/2024 DDNS-run30 4 hours

If you will be storing the flow cell for future reuse, take out the bottle of Storage Buffer from the Wash Kit to thaw at room temperature.

34.2 After incubation you can either follow the flow cell priming and loading steps starting from Step 23 to load a new run, or the following steps for storing the flow cell for future use.

5m

Briefly vortex the thawed Storage Buffer to mix, then add 500 μ L slowly through the Priming Port.

Close the priming port before removing all waste from the waste channel. Place the flow cell back in its plastic box and envelope, then store at 4°C