# PCR 2500 bp Tiling of Mpox Virus with ONT Rapid Barcoding

This protocol describes the procedure for PCR tiling of Mpox (MPXV) viral DNA samples. Multiplexing can occur for up to 48 samples in a single run. It is an adaptation of the protocol published by Oxford Nanopore Technologies using the Rapid Barcoding Kit 96 and Midnight RT PCR Expansion for barcoding and library preparation using ARTIC/INRB (Clinical) primers to generate MPXV amplicons (Quick Lab 2024). https://artic.network/mpxv/artic-mpxv-guide.html

## Equipment and Consumables

### Equipment

- Magnetic rack (1.5ml tubes)
- Microfuge and quick spin for tubes/PCR strip tubes
- Vortex mixer (optional)
- Thermal cycler
- Qubit® Fluorometer or equivalent
- Oxford Nanopore Technologies MinION Mk1B/Mk1D/GridION
- MinION R10.4.1 (FLO-MIN114) Flow Cells
- Timer

### Consumables

- Qubit® Assay tubes
- 1.5mL tubes
- 5mL Sarstedt Tubes: purchased from Sarstedt (for 80% ethanol)
- Interpath Individual Cap Strip Tube for RT-PCR
- Pipette and tips (P10, P100, P200, P1000)
- Markers (for labelling tubes)
- 1.5ml tube racks

- PCR 8 - strip tube holder
- Gloves (S,M,L)
- Safety Glasses
- Laboratory Gown
- Tip waste sharps container

**Samples**

- Extracted DNA from confirmed Mpox positive samples with cycle threshold values <30 Ct. Volume required for protocol 5 µL.

**Reagents**

- Q5® Hot Start HF 2x Master Mix: (can be taken from SARS-CoV-2 Midnight RT kit) or purchased from New England Biolabs separately, catalogue no. M0494.
- MPXV primers (INRB 2500 bp Amplicon scheme): purchased from Integrated DNA Technologies. Pooled as per V.1.0.1 scheme. (100 µM stock – diluted to 10 µM working stock) – CPG supplied.
- Rapid Barcoding Kit 96: purchased from Oxford Nanopore Technologies, catalogue no. SQK-RBK114.96. The kit contains:
  - Rapid Barcode Plate (RB96)
  - AMPure XP Beads (AXP)
  - Sequencing Buffer (SB)
  - Rapid Adapter (RA)
  - Adapter Dilution Buffer (ADB)
  - Elution Buffer (EB)
  - Library Beads (LB)
  - Flow Cell Tether (FCT)
  - Flow cell Flush (FCF)
- Qubit® dsDNA BR Assay kit (Q32853) 500 assays & Standards (Standard 1 & 2).
- Nuclease-free water.
- Freshly prepared 80% ethanol in nuclease-free water.

## Tiled amplicon PCR

**PCR Reaction**

This step takes approximately 235 minutes (3.5hrs)

**Reagents Required**

- Genomic Mpox DNA – 5 µL (Ct<30)
- Q5 HS Master Mix (Q5)
- MPXV 2500bp Primer Pool 1 - 10µM working stock
- MPXV 2500bp Primer Pool 2 - 10µM working stock
- Nuclease-free water (NFW)
- 1.5mL Eppendorf DNA LoBind tubes
- Interpath Individual Cap Strip Tube or 96 PCR plate + PCR plate seals

**Procedure**

- Prepare the following two master mixes (Pool 1 and Pool 2), based on the number of samples, in the 1.5 ml tubes and mix well.

**Note**: There are different volumes of primers and NFW in each pool.

- If available, always a positive control (previously positive sample Ct <19) and a NTC (NFW) on each PCR run.
    - For a single reaction:

| Reagent | Pool 1 (µL) | Pool 2 (µL) |
|---|---|---|
| Nuclease-free water | 7.1 | 7.3 |
| Monkeypox Primer pool 1 (10 µM) | 2.9 | - |
| Monkeypox Primer pool 2 (10 µM) | - | 2.7 |
| Q5 HF Master Mix (Q5) | 12.5 | 12.5 |
| **Total** | **22.5** | **22.5** |

    - For 8 reactions:

| Reagent | Pool 1 (µL) | Pool 2 (µL) |
|---|---|---|
| Nuclease-free water | 56.8 | 58.4 |
| Monkeypox Primer pool 1 (10 µM) | 23.2 | - |
| Monkeypox Primer pool 2 (10 µM) | - | 21.6 |
| Q5 HF Master Mix (Q5) | 100 | 100 |
| **Total** | **180** | **180** |

- Aliquot 22.5 µL of master mix to individual strip tubes labelled with the sample number and clearly labeled with primer pool number (1 or 2).
- Transfer 2.5 µL of DNA extract to the corresponding well for both Pool 1 and Pool 2 in the PCR strip tubes.

- There should be two PCR reactions per sample (one for Pool 1, one for Pool 2)
- Take care not to cross-contaminate different wells
- Mix by gently pipetting the contents of each well up and down

- Ensure each tube is capped and spin down briefly.

- Incubate the samples using the following program, with the heated lid set to 105°C.

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 98°C | 30 secs | 1 |
| Denaturation<br>Annealing and extension | 98°C<br>65°C | 15 secs<br>5 mins | 35 |
| Hold | 4°C | ∞ | - |

This program takes ~3.5 hours to complete.

## Amplicon QC

As a QC measure, quantify the DNA concentration of PCR products using the Qubit dsDNA BR Assay kit. The Qubit® dsDNA BR Assay kit is highly selective for double-stranded DNA and is accurate in the concentration range of 100 pg/µL to 1000 ng/µL.

Once the amplification reaction has finished, at the minimum quantify the NTC, high Ct and low Ct samples.

If there is a large discrepancy in the concentration of the PCR products between Pool 1 and Pool 2, do **NOT** proceed. If the discrepancy is >50%, repeat the PCR reaction and/or confirm primer pool quality.

The Qubit® dsDNA BR Assay kit is highly selective for double-stranded DNA over RNA and is accurate in the concentration range of 100 pg/µL to 1000 ng/µL.

- Do not hold the assay tubes in your hand before reading as this warms up the solution and may result in a lower reading

- After the incubation period the fluorescence signal is stable for 3 hours at room temperature

- Do not label the side of the tubes as this may interfere with the reading. Label the lid ONLY

**Reagents Required**

- Qubit® dsDNA BR Assay kit + Standards

- Qubit® Assay tubes

**Procedure**

- Set up the required number of assay tubes for standards and samples and label the lids.
    - There are two standards for the assay (Standard #1 and Standard #2)

- Prepare the Qubit® Working Solution by diluting the Qubit® dsDNA BR Reagent 1:200 in Qubit® dsDNA BR Buffer as per manufacturer instructions, mix well. The final solution will have a slight red tint.

  You will need at least 200 µL for each sample and standard you want to measure.

    - For example, if you have 16 samples (8 x pool 1, 8 x Pool 2) and 2 standards you will need to make up 3.6 mL of working solution.

    - Add 18 µL of the Qubit® dsDNA BR Reagent with 3.58 ml of Qubit® dsDNA BR Buffer to create Qubit® Working Solution.

- Label on the lid two Qubit® Assay tubes - S1 and S2. (standards).

- Label on the lid 16 tubes for Pool 1 and Pool 2 samples. (PCR product).

- Add 190 µL of the Qubit® Working Solution to the tubes for the standards (S1 & S2).

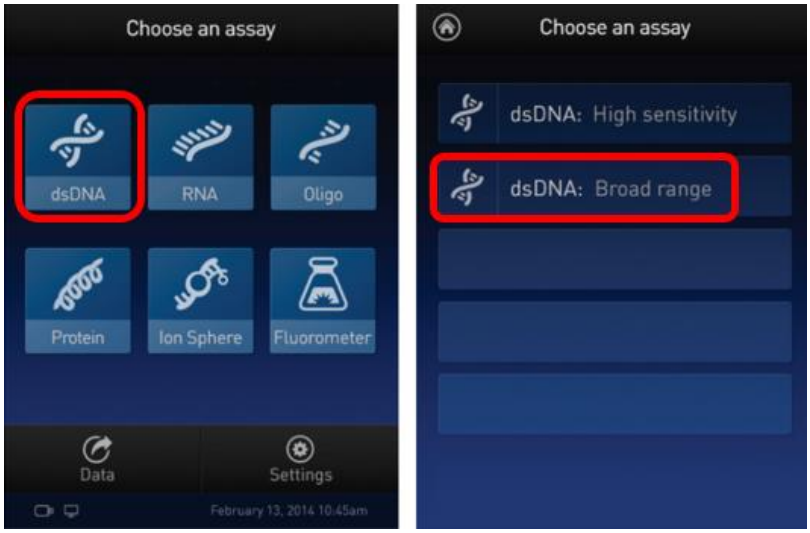
- Add 198 µL Qubit® Working Solution to the individual sample tubes (pool 1 and pool 2).

- Add 10 µL of each Qubit® Standard to the appropriate tube (S1 & S2) and then gently mix for 2-3 secs. Try not to create bubbles.

- Add 2 µL of the Pool 1 and Pool 2 PCR products to the individual sample tubes. So that the final volume of the assay is 200 µL. 198 µL and 2 µL sample.

- Allow all tubes to incubate at room temperature for 2 mins.

- On the Home screen of the Qubit® Fluorometer, select "DNA" and then "dsDNA Broad Range" as the assay type.




- The "Read Standards" screen will now be displayed – press "Read Standards" to proceed.


  - Insert the tube containing Standard #1 into the sample chamber, close the lid and press "Read Standard". Once the reading is complete, remove the tube.

  - Repeat with Standard #2


- The instrument will display the results on the Read Standard screen, record the result on worksheet.

- Press "Run Samples"

- On the assay screen, select the sample volume and units
  - Press the "+" or "-" buttons on the wheel to select the sample volume added to the assay tube (2µL)
  - From the dropdown menu, select the units for the output sample concentration (ng/ul)



- Insert a sample tube into the sample chamber, close the lid and then press "Read tube". Once the reading is complete, remove the sample tube.
  - The result is displayed on the assay screen

- The top value (in large font) is the concentration of the original sample
- The bottom value is the dilution concentration

- Repeat the above step for all test samples

The concentration of the PCR products for each primer pool should be approximately the same. If there is a large discrepancy between Pool 1 and Pool 2, do **NOT** proceed (>50%). In this event, repeat the PCR reaction and/or confirm primer pool quality. Expected Qubit results scale for this Mpox Scheme.

NTC: 5-10 ng/µl

High Ct samples (>25): 10-30 ng/µl

Low Ct samples (<25): 40-90+ ng/µl

## Library Preparation & Loading

## Library preparation workflow:

1. Prepare the reagents and amplicons for downstream Library preparation and flow cell loading
2. Combine Pool 1 and 2 amplicons into one tube
3. Add to Barcoding Strip (containing NFW)
4. Combine barcoded samples
5. Add Rapid Barcoding enzyme
6. Clean up library with Ampure beads
7. QC pooled Library
8. Load onto flowcell

**Reagents Required**

- Rapid Barcode Plate (RB96) – Stored frozen. Spin down before use.
- Nuclease-free water
- Thin-walled 0.2 mL PCR strip-tube

**Protocol**

- Aliquot 2.5 µL of NFW to fresh strip tubes and this is the "Barcode Attachment" strip.

- Spin down the Rapid Barcode reagent plate and the PCR reactions prior to opening to collect material to the bottom of the wells.

- Transfer the entire contents of Pool 2 (~22 µL) amplified PCR product into the corresponding tube of Pool 1 amplified PCR product. Mix well by pipetting.

**Note:** Take care when combining and mixing amplified PCR product and ensure that tubes are capped when not in use. Change tips between samples.

- Transfer 5 µL from each well of PCR Pool 1 strip (now containing the pooled PCR products of Pool 1 and Pool 2) to the corresponding well of the Barcode Attachment Strip and mix by pipetting.

**Note:** Record the barcodes used for each sample on your worksheet. Each sample MUST have a different Barcode.

- Transfer 2.5 µL from the Rapid Barcode Plate to the corresponding well of the Barcode Attachment strip, taking care to pierce the foil seal gently and to not cross-contaminate different wells. Mix gently by pipetting

- Final reaction volume for barcoding is 10 µL. (2.5 µL NFW, 5 µL of pooled PCR product, 2.5 µL of barcodes).

- Cap the tubes and briefly spin down.

- Immediately put the barcoding attachment strip into a thermocycler.

- Incubate the barcoding attachment strip samples using the following program in a thermal cycler: 30°C for 2 minutes and then at 80°C for 2 minutes, 4 °C hold.

**Note:** There will be surplus of pooled PCR product remaining. Freeze this PCR product until you have confirmed the ONT Library preparation and sequencing was successful.

## Pooling Samples and Clean Up

### Reagents Required

- AMPure XP beads (AXP) from the Rapid Barcoding Kit

- Elution Buffer (EB) from the Rapid Barcoding Kit

- Rapid Adapter (RA) from the Rapid Barcoding Kit

- Adapter Dilution Buffer (ADB) from the Rapid Barcoding Kit
- Ethanol
- Nuclease-free water.
- 1.5 mL DNA LoBind tubes
- 5 mL Sarstedt Tubes

**Procedure**

- Spin down the Barcode Attachment strip containing the barcoded samples to collect the liquid at the bottom of the wells prior to opening.

- Label a fresh 5ml tube "80% EtOH". Add 1.2 mL of 100% Ethanol (EtOH) and 300 µL of NFW to the 80% EtOH tube. Invert well to mix

- Pool the whole volume of each of the barcoded samples into a new single 1.5 mL tube labelled "pooled Library" (expecting ~10 µL per sample), total volume as indicated in the following table:

| | **x 8 samples** | **x24 samples** |
|---|---|---|
| Total volume | ~80 µL | ~240 µL |

- Mix pooled samples thoroughly by pipetting and briefly spin down the tube.

- Resuspend the AMPure XP beads by thorough vortexing.

- To the pooled barcoded samples add an equal volume (1:1) of resuspended AMPure XP beads and mix by pipetting. Volume to add as per the following table:

| **Example volume** | **x 8 samples** | **x24 samples** |
|---|---|---|
| Volume of AMPure XP | ~80 µL | ~240 µL |

- Incubate for 5 minutes at room temperature – gently mix.

- Spin down the sample and place tube on the magnet rack for 2-3 min.

- Once a pellet is formed, pipette off the supernatant. The DNA is now bound to the AMPure XP beads (pellet).

- Keep the tube on the magnet and wash the beads with 1 mL of 80% ethanol without disturbing the pellet.

- Remove the ethanol using a 1 ml pipette and discard.

- Repeat the 80% ethanol wash step of the pellet.

- Close the lid and remove tube from the magnet. Briefly spin down the tube and place it back on the magnet. Pipette off any residual ethanol with a 10 µL pipette, take care not to disturb the pellet.
- Allow the pellet to dry for 30 secs, taking care not to over dry the pellet to the point of cracking.

- Remove the tube from the magnetic rack and resuspend the pellet by pipetting in 15 µL of Elution Buffer (EB).

**Note:** Pipette directly onto the pellet and check that entire pellet is resuspended. Quick spin down to collect the liquid to the bottom of the tube – it sometimes can be stuck on the side in small droplets.

- Incubate for 10 minutes at room temperature.
- Label a new 1.5 ml tube with "Clean Library" and Run details e.g. Date. Worksheet #
- Place the tube back on the magnet until there is a pellet and the eluate is clear. DO NOT DISCARD ELUATE – this contains your library.
- Keep the tube on the magnet while you pipette the eluate from the bottom of the tube. Transfer this eluate to the "Library tube". This is your barcoded DNA library.
- Quantify the DNA Library using the Qubit dsDNA BR Assay kit (Use 2 µL of sample).

Expected Library concentration: 20-50ng/µL for 8 x pooled positive samples.

- Take forward the 200-800 ng of the library and if needed make up the volume to 11 µL with Elution Buffer.
- In a new 1.5 ml tube labeled "diluted RA" add 1.5 µL of RA to 3.5 µL of Adapter Dilution Buffer.
- Add 1µL of the "diluted RA" to 11µL of the barcoded DNA. Tip mix gently.
- Incubate at room temperature for 5 minutes.
- This prepared library is ready for loading onto the MinION flow cell. Store the library on ice or in fridge until ready to load.

## Priming and Loading the SpotON Flow Cell

### Consumables and Equipment Required

- Flow Cell Flush (FCF)
- Flow Cell Tether (FCT)
- Library Beads (LB)
- Sequencing Buffer (SB)
- 1.5 mL tubes
- MinION Mk1B/Mk1D or GridION device
- R10.4.1 flow cell (FLO-MIN-114)

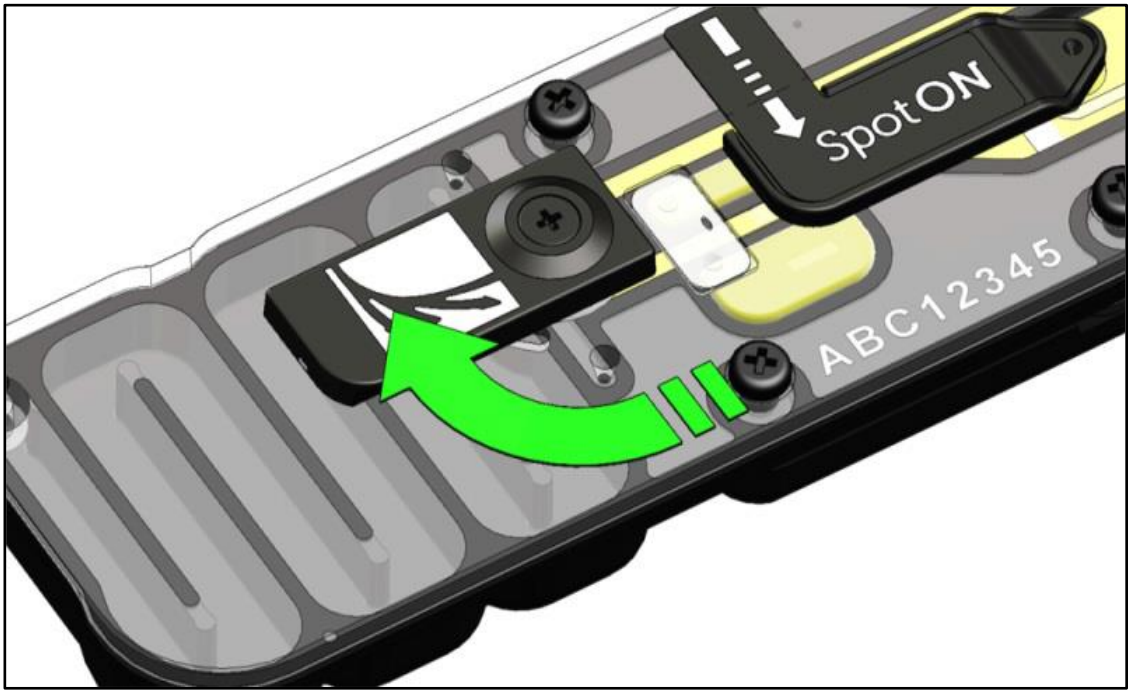
**Note:** If you are loading onto a flowcell that has run another library before – refer to ONT website and protocols for guidance.

### Procedure

- Thaw the Sequencing Buffer (SB), Library Beads (LB), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature.
- Vortex the Sequencing Buffer (SB), Flow Cell Flush (FCF), Flow Cell Tether (FCT) reagents. Spin down the SB and FLT tubes.
- To prepare the flow cell priming mix, add 30 µL of Flow Cell Tether (FCT) to 1.17 mL of Flow Cell Flush (FCF). Mix by vortexing at room temperature.

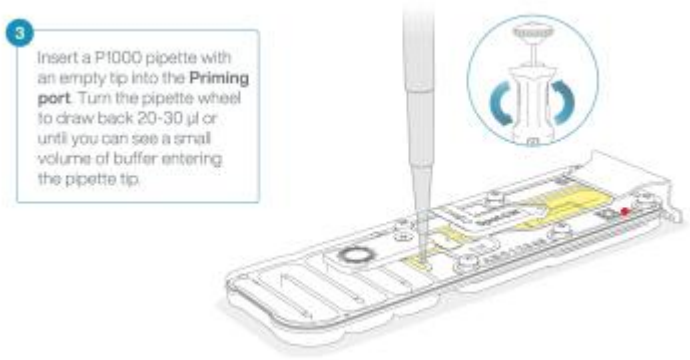
### Loading the flow cell

- Slide the priming port cover clockwise to open the priming port (see image below)



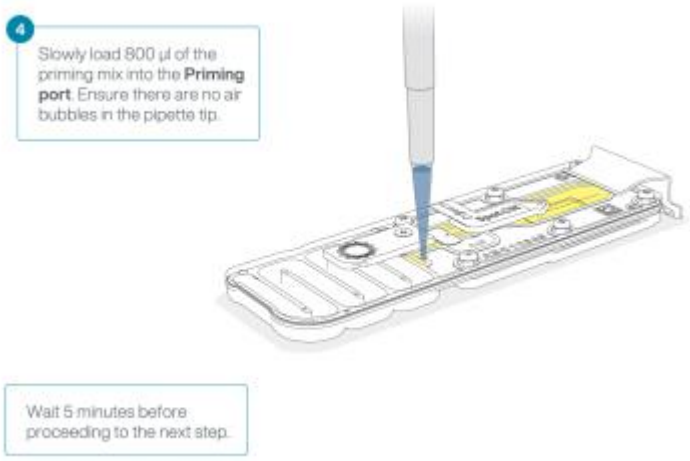

- After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µL).
    - Set a P1000 pipette to 200 µL
    - Insert the tip into the priming port
    - Turn the wheel slowly until the dial shows 220-230µL or until you can see a small volume of buffer entering the pipette tip – it is yellow in colour.
    - Visually check that there is continuous buffer from the priming port across the sensor array

    **Note:** 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.




- Load 800 µL of the flow cell priming mix into the flow cell via the priming port avoiding

the introduction of air bubbles. Close the priming port. Wait 5 mins. During this time, prepare the library for loading.

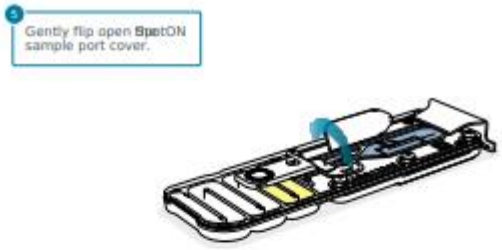


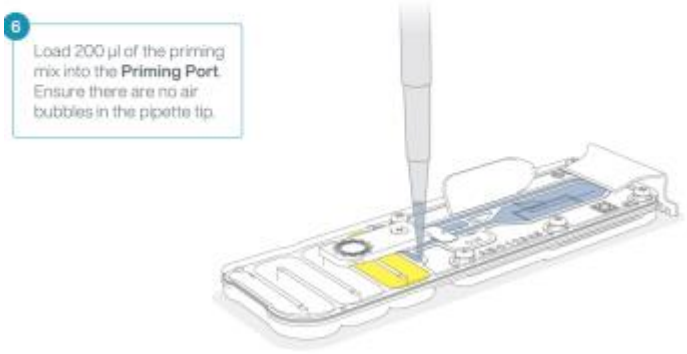

- Thoroughly mix the contents of the Library Beads (LB) by pipetting.
  **Note:** these beads are white and will settle very quickly and it is vital that they are mixed immediately before use.
- In a new tube, prepare the library for loading as follows:

| Reagent | Volume |
|---|---|
| Sequencing Buffer (SB) | 37.5 µL |
| Library Beads (LB) | 25.5 µL |
| Library ("Clean Library") | 12.0 µL |
| **Total** | **75.0 µL** |

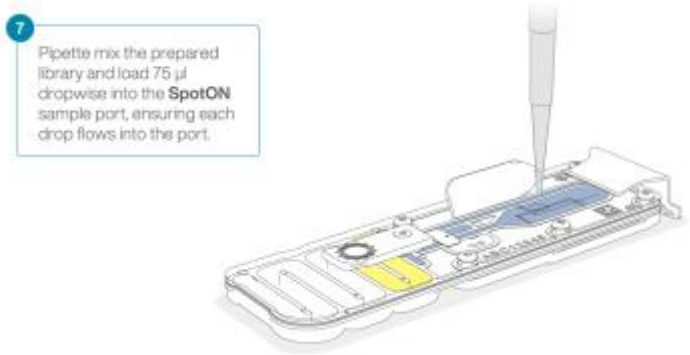
- Complete the flow cell priming:
  - Open the Priming port
    - Gently lift the SpotON sample port cover to make the SpotON sample port accessible
    - Load 200 µL of the priming mix via the priming port (**not** the SpotON sample port) avoiding the introduction of air bubbles

  **Note:** load the library as soon as possible after this step

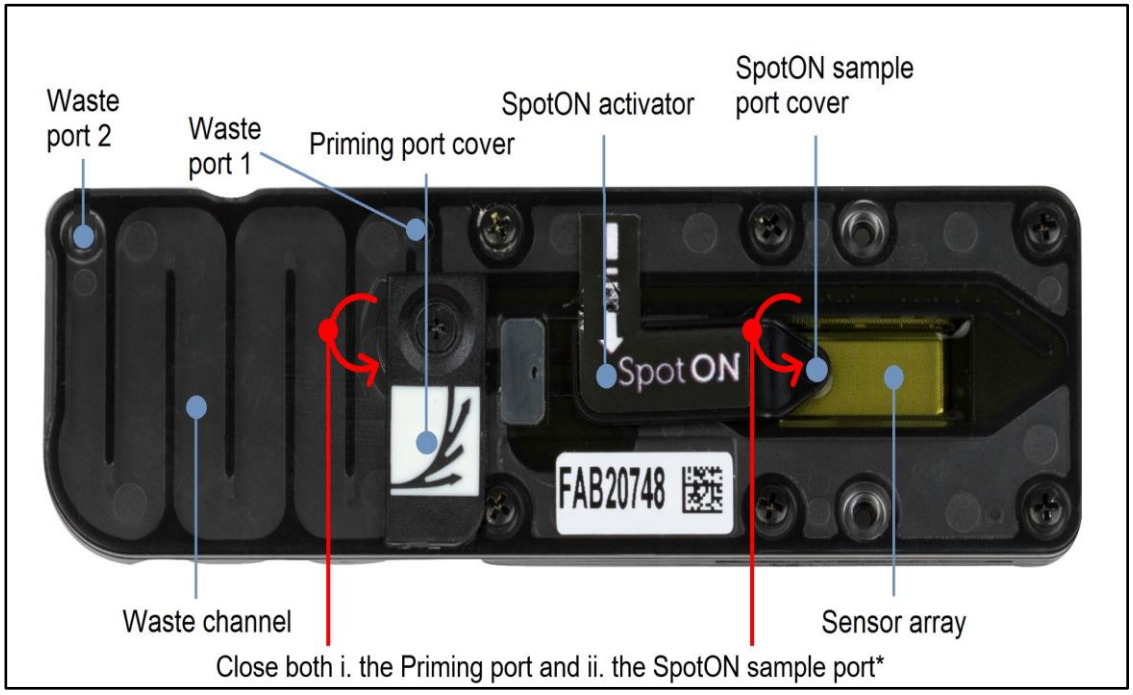



- Mix the prepared library gently by pipetting up and down just prior to loading.
- Add 75 µL of Library Mix to the flow cell via the SpotON sample port in a dropwise fashion. Do not allow the pipette tip to come into contact with the SpotON port. Ensure each drop flows into the port before adding the next.




- Gently replace the SpotON sample port cover making sure the bung enters the SpotON port and close the priming port. Add the light shield onto the flow cell.


Waste port 2
Waste port 1
Priming port cover
SpotON activator
SpotON sample port cover
SpotON
FAB20748
Waste channel
Sensor array
Close both i. the Priming port and ii. the SpotON sample port*