2023 UFIT Workshop Protocol

funded by USDA-NIFA-AFRI-Tactical Sciences for Agricultural Biosecurity-2021-68013-33758

Day 1 (March 7th) Library Prep and Target Enrichment Part 1

Day 2 (March 8th) Target Enrichment Part 2 and Nanopore Sequencing

Day 3 (March 9th) Data Analysis

Library Preparation EF 2.0 with Enzymatic Fragmentation and Twist Universal Adapter System

- For use with the Nanopore sequencing

This protocol is modified based on the Twist protocol (short reads library preparation). The target fragment size is 5 kb; therefore, the conditions are changed.

PROTOCOL COMPONENTS

Please read the product packaging and storage recommendations carefully for each kit and store components as recommended immediately upon arrival.

CATALOG #	NAME	DESCRIPTION	STORAGE
104206: 16 rxn 104207: 96 rxn	Twist Library Preparation EF Kit 2.0	Reagents for library construction	–
	Twist Library Preparation EF Kit 1, 2.0	• Frag/AT Enzymes • Frag/AT Buffer • Ligation Master Mix • Equinox Library Amp Mix (2x) • P5/P7 Primers (10x)	-20°C
	Twist Library Preparation Kit 2	DNA Purification Beads	2-8°C
101307: 16 rxn 101308, 101309, 101310, 101311: 96 rxn	Twist Universal Adapter System - TruSeq Compatible	Twist Universal Adapters and Twist UDI Primers	-20°C

MATERIALS SUPPLIED BY USER

The following materials or their equivalent are required to generate libraries using the Twist Library Preparation Kit 2.0 with Enzymatic Fragmentation and Twist Universal Adapter System.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	–
Molecular biology grade water	–
10 mM Tris-HCl pH 8 (optional)	–
Buffer EB (optional)	Qiagen
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
96-well compatible magnetic plate	Alpaqua, Permagen Labware
Qubit dsDNA Broad Range Quantitation Assay	Thermo Fisher Scientific
Agilent DNA 7500 Kit	Agilent Technologies
EQUIPMENT	
Pipettes and tips	–
Vortex mixer	–
Benchtop mini centrifuge for 0.2-ml tubes	–
Thermomixer for 1.5-ml tubes	Eppendorf
Thermal cycler (96 well) with heated lid	–
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer	Agilent Technologies

Protocol Overview

Procedure (**200 ng** starting DNA material)	Time
Perform DNA fragmentation, end repair, and dA-tailing	1 hour
Ligate Twist universal adapters and purify	1 hour
PCR amplifying using Twist UDI Primers, purify, and perform QC	2.5 hour

Step1. Perform DNA fragmentation, end repair, and dA-tailing

Reagents required:

1. Genomic DNA (gDNA): **200 ng** per sample (*determined by Qubit quantitation assay*)

2. Molecular biology grade water (Fisher)
3. Qubit dsDNA BR Quantitation Assay (Room temperature)
4. Twist Library Preparation EF Kit 1, 2.0
    a. Frag/AT Enzyme (FAE)
    b. Frag/AT Buffer (FAB)

Prepare the thermal cycler, samples, and reagents:

1.1 Program the thermal cycler with the following conditions. Set the temperature of the heated lid to 105°C. **Start the program to pre-chill the thermal cycler*.

Temperature	Time
4 °C	Hold *
20 °C	2 min
65 °C	30 min
4 °C	Hold

1.2 Mix gDNA by flicking the tube with finger. ***DO NOT VORTEX***.

1.3 Dilute gDNA sample to a final concentration of 5 ng/ul with water (H_2O) in a thin-walled PCR 0.2-ml tube. Mix well with gentle pipetting. *Note: Dilute the target to a volume of 40 ul*

1.4 Pulse-spin the tube and place on ice.

1.5 Vortex the Frag/AT Buffer (FAB) for 5 seconds. Pulse-spin the buffer and place on ice.

1.6 Invert Frag/AT Enzyme (FAE) more than 5 times to homogenize. ***DO NOT VORTEX***. Pulse-spin and place on ice.

1.7 Prepare an enzymatic fragmentation mix in a 1.5-ml microcentrifuge tube on ice. Mix thoroughly by gentle pipetting (avoid formation of bubbles). *Prepare master mix if needed.*

Reagent	Volume per reaction	4.5X
Frag/AT Buffer (FAB)	4 ul	18
Frag/AT Enzymes (FAE)	6 ul	27
Total	10 ul	45

1.8 Add 10 ul enzymatic fragmentation mix to each 40 ul gDNA sample tube and mix well by gentle pipetting. Seal or cap the sample tube and keep on ice.
Note: Complete mixing is critical to achieve desired fragment lengths.

1.9 Pulse-spin the sample tube(s) and immediately transfer to the pre-chilled thermal cycler.

1.10 Initiate steps 2 to 4 of the thermal cycler program (step 1.1)

Note: While the program is running, prepare the reagents for Step 2

1.11 When the program is complete and the sample block has returned to 4 °C, remove the samples from the block and place them on ice.

Step 2. Ligate Twist Universal Adapters and purify

Reagents required:

1. dA-tailed DNA fragments (50 ul from step 1.12)
2. Ethanol (Molecular Biology Grade, Fisher BioReagents; under the fume hood)
3. Molecular Biology Grade Water
4. From Twist Library Preparation EF Kit 1, 2.0
    a. Ligation Master Mix (LMM)
5. From Twist Universal Adapter System
    a. Twist Universal Adapters (TUA)
6. From the Twist Library Preparation Kit 2
    a. DNA purification beads (DPB)

Before you start:

1. Thaw or place on ice:
    a. Twist Universal Adapters
    b. Ligation Master Mix
2. Prepare 1 ml 80% ethanol for each sample (for step 2 and 3)
3. Keep DNA purification beads in the room temperature for a least 30 min (for both step 2 and 3)
4. Program the thermal cycler to incubate temperature at 20 °C (infinite time). Start the program while preparing samples, so the cycler has reached 20 °C. ***DO NOT CLOSE THE LID.*** *Note: Can also set the lid to the minimum temperature or turned off*

Ligate Twist universal adapters:

2.1 Add 5 ul Twist Universal Adapters (TUA) into each sample tube containing dA-tailed DNA fragments from step 1.12. Mix by gently pipetting and keep on ice.

2.2 Invert the Ligation Master Mix (LMM) more than 5 times until homogenized. ***DO NOT VORTEX!!!***

2.3 Add 20 ul of Ligation Master Mix (LMM) to each sample from step 2.1 and mix well by gentle pipetting. Seal/cap the tube and pulse-spin.

2.4 Incubate the ligation reaction at 20 °C for 15 min in the thermal cycler, and them move the sample to the bench top. ***DO NOT CLOSE THE LID.***

Note: While the program is running, prepare the reagents for step 3

Purification:

2.5 Vortex the pre-equilibrated room temperature DNA Purification Beads (DPB) until well mixed.

2.6 Add 37.5 ul of homogenized beads to each ligation sample (75 ul) from step 2.4. Mix well by vertexing.

2.7 Incubate for 5 min at room temperature.

Note: Keep the tube on magnetic rack from step 2.8 - 2.13 and 2.16-2.17

2.8 Place sample on a magnetic rack for 2 min.

2.9 Without removing tube from the magnetic rack, remove and discard the supernatant.

Note: If beads are disturbed by accident, put the supernatant back and let it form pellet.

2.10 While keeping samples on the magnetic rack, wash the beads by gently adding 200 ul freshly prepared 80% ethanol without disturbing the pellet. Incubate for 1 min, and then remove the ethanol.

2.11 Repeat step 2.10, a total of two washes.

2.12 Carefully remove all remaining ethanol with 10-ul pipette without disturbing the beads.

Note: Before pipetting, tubes can be briefly spun to collect ethanol at the bottom of the tube, and then returned to the magnetic rack.

2.13 Air-dry the bead pellet on the magnetic rack *no more than 5 min*. Do not overdry the bead pellet.

2.14 Remove the tube from the magnetic rack and add 17 ul water (H_2O) to each sample. Mix by pipetting until homogenized.

2.15 Incubate at room temperature for 2 min.

2.16 Place the tube back on the magnetic rack and let stand util the beads form a pellet.

2.17 Transfer 15 ul of the clear supernatant containing the ligated libraries to a new thin-walled PCR 0.2-ml tube, making sure not to disturb the bead pellet.

Step 3. PCR amplify using Twist UDI Primers, purify and performing QC

Reagents required:

1. Ligated libraries (from step 2.17)
2. Freshly prepared 80% ethanol (form step2)
3. Equilibrated DNA purification beads (from step 2 [DPB]) to room temperature
4. Molecular Biology Grade water (H_2O)
5. From the Twist library Preparation EF Kit 1, 2.0
    a. Equinox Library Amp Mix (2x; [ELAM])
6. From the Twist Universal Adapter System
    a. Twist UDI Primers (TUP)

Before you start:

1. Thaw or place on ice
    a. Equinox Library Amp Mix (2x; [ELAM])
    b. Twist UDI Primers (plate with single-use primers; [TUP]): 96-UDI Plate A (PN101308)

3.1 Program the thermal cycler. Set the temperature of the heated lid to 105 °C.

Step	Temperature	Time	Number of cycles
Initiation	98°C	2 min	1
Denaturation	98°C	15 sec	8
Annealing	60°C	30 sec	
Extension	72°C	8 min	
Final extension	72°C	1 min	1
Final hold	4°C	Hold	-

3.2 Add 10 ul of Twist UDI Primer (TUP) from the provided 96-well plate to each of the gDNA libraries from step 2.17 and mix well by gentle pipetting.

Note: Please document which index was used for EACH sample; It is critical for the following data analysis.

Sample name	Index
	TUP-___
	TUP-___
	TUP-___
	TUP-___

3.3 Invert Equinox Library Amp Mix (ELAM) 5 times before use. Add 25 ul of Equinox Library Amp Mix (ELAM) to the gDNA libraries from step 3.2 and mix well by gentle pipetting.
3.4 Pulse-spin sample tube and transfer to the thermal cycler. Start the program. (It takes about an hour and half)
3.5 Remove the samples from the block when the program is over.
Purification:
3.6 Vortex the pre-equilibrated DNA Purification Beads (DPB) until mixed.
3.7 Transfer the mixtures from 0.2 ml PCR tube to a clean 1.5 ml microcentrifuge tube.
3.8 Add 25 ul of homogenized DNA Purification Beads to each sample (50 ul) from step 3.5. Mix well by vertexing.
3.9 Incubate the samples for 5 min at room temperature.

Note: Keep the tube on magnetic rack from step 3.9 - 3.14 and 3.17-3.18

3.10 Place the samples on magnetic rack for 2 min.

3.11 Without removing the sample from magnetic rack, discard the supernatant.

3.12 Wash the bead pellet by gently adding 200 ul freshly prepared 80% ethanol. Do not disturb the pellet. Incubate for 1 min and then remove the ethanol.

3.13 Repeat step 3.11. A total of 2 washes.

3.14 Carefully remove all remaining ethanol with 10-ul pipet, making sure not to disturb the bead pellet.

Note: Before pipetting, tubes can be briefly spun to collect ethanol at the bottom of the tube, and then returned to the magnetic rack.

3.15 Air-dry the bead pellet on the magnetic rack for no more than 5 min. Do not overdry the bead pellet.

3.16 Remove the tube from the magnetic rack and add 22 ul water to each sample. Mix by pipetting until homogenized.

3.17 Incubate at room temperature for 2 min.

3.18 Place the samples on magnetic rack for 2 min.

3.19 Transfer 20 ul of clear supernatant containing the Amplified Indexed Libraries to a clean 0.2-ml PCR tube. Do not disturb the beads.

3.20 Quantify and validate the size range of each library using Qubit dsDNA BR Quantitation Assay and Tape Station (ICBR)

DNA sample	Qubit HS working solution
1 ul	199 ul

3.21 Vortex mixture for 2 seconds, spin down, and incubate the reaction for 2 minutes at RT.

Sample name: _____; concentration: _____ ng/ul

Note: If not proceeding immediately to a Twist Target Enrichment System, store the amplified indexed libraries at -20°C.

Twist Target Enrichment Protocol

- For use with the Nanopore sequencing

This protocol is modified based on the Twist protocol (short reads library preparation). The target fragment size is 5 kb; therefore, the conditions are changed.

CATALOG #	NAME	DESCRIPTION	STORAGE
TWIST HYBRIDIZATION AND WASH KIT WITH AMP MIX (For target enrichment with standard hybridization)			
101279: 2 rxn* 104178: 12 rxn 104179: 96 rxn	Twist Hybridization Reagents (Box 1)	• Hybridization Mix • Hybridization Enhancer • Amplification Primers	–20°C
	Twist Wash Buffers (Box 2)	• Binding Buffer • Wash Buffer 1 • Wash Buffer 2	2–8°C
	Equinox Library Amp Mix (Box 3)	• Equinox Library Amp Mix (2x)	–20°C
TWIST PROBE PANELS (Ordered separately)			
Choice of panel type and reaction size	Twist Fixed Panel	Fixed content enrichment panel for hybridization reactions (for example, Twist Human Core Exome Panel)	–20°C
	Twist Custom Panel	Custom enrichment panel for hybridization reactions	–20°C
	(Optional) Secondary Twist Probe Panel	Custom or fixed enrichment panel for adding content to a fixed or custom panel	–20°C
TWIST BLOCKERS & BEADS FOR TARGET ENRICHMENT			
100856: 2 rxn 100578: 12 rxn 100767: 96 rxn	Twist Universal Blockers	For the prevention of nonspecific capture: • Universal Blockers • Blocker Solution	–20°C
101262: 2 rxn 100983: 12 rxn 100984: 96 rxn	Twist Binding and Purification Beads	For target enrichment and purification: • Streptavidin Binding Beads • DNA Purification Beads	2–8°C

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
10 mM Tris-HCl pH 8	—
Buffer EB	Qiagen
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates (optional)	VWR
1.5-ml compatible magnetic stand	Beckman Coulter
96-well compatible magnetic plate	Alpaqua
Qubit dsDNA High Sensitivity Quantitation Assay	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit	Agilent Technologies
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
Benchtop mini centrifuge for 0.2-ml tubes	—
Thermomixer for 1.5-ml tubes	Eppendorf
Thermal cycler (96-well) with heated lid	—
Lab shaker, rocker, rotator	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer	Agilent Technologies
Vacuum concentrator (if unavailable, contact technical support)	—

Protocol Overview

Workflow	Time
Prepare library for hybridization	1 hour
Hybridize capture probes with pools	16 hours
Bind hybridized targets to streptavidin beads	1.5 hour
Post-capture PCR amplify, purify, and perform QC	4 hours
Sequence on an Nanopore platform	-

Step1. Prepare libraries for hybridization

Reagents Required:

1. Fragmented and indexed library from previous step in "Library Preparation EF 2.0 with Enzymatic Fragmentation and Twist Universal Adapter System"

Before you start:

This protocol supports a single or multiplex (up to 8-plex) hybridization capture. The amount of indexed library to use depends on the number of indexed samples per pool. For this workshop, 4-plex will be used. Four ug total DNA will be used.

Note: use more than 4 ug total DNA might lead to reduced performance of the enrichment.

1.1 Transfer 1 ug of each indexed library to an indexed library pool reaction tube (1 tube per team). Clean, thin-walled PCR 0.2-ml tube is recommended to avoid unnecessary transfers in downstream steps.

Sample name	Concentration of indexed DNA sample (ng/ul)	Calculated volume of DNA sample (ul)

1.2 Pulse-spin the indexed library pool tube(s) to minimize the amount of bubbles present.

1.3 Dry the indexed library pool(s) using a vacuum concentrator for 30 minutes or until it is dried.

STOP POINT: If not proceeding immediately to Step 2, store the dried indexed library pool at –20°C for up to 24 hours.

Step 2. Hybridize capture probes with pools

Reagents Required:

1. Dried library pool(s) from Step 1.3
2. Twist custom panel (probe set; [TCP])
3. From Twist Hybridization Reagents:
   a. Hybridization Mix (HM)
   b. Hybridization Enhancer (HE)
4. From Twist Universal Blockers:
   a. Universal Blockers (UB)
   b. Blocker Solution (BS)

Before You Begin:

1. Thaw all required reagents on ice, then pulse-vortex for 2 seconds to mix and then pulse-spin.
2. Set a heat block to 65°C.
3. Program a 96-well thermal cycler to 95°C and set the heated lid to 105°C.

2.1 Heat the Hybridization Mix (HM) at 65°C in the heat block for 10 minutes, or until **all precipitate is dissolved**, then cool to room temperature on the benchtop for 5 minutes.
2.2 Prepare a probe solution in a clean thin-walled PCR 0.2-ml tube as indicated in the table below. Mix by flicking the tube(s).

Read the note below before preparing mixture:

1. Hybridization Mix is very viscous. Pipette slowly to ensure accurate pipetting.
2. Small white particles may be present in the Twist Fixed or Custom Panel tube(s). This will not affect the final capture product.

Reagents	Volume
Hybridization Mix (HM)	20 ul
Twist Custom Panel (TCP)	4 ul
Water (H_2O)	4 ul
Total	28 ul

2.3 Resuspend the dried indexed library pool (from Step 1.3) by adding the reagents described below. Mix by flicking the tube.
Note: Vortex the Universal Blockers first, and then pulse-spin.

Reagent	Volume
Dried library pool	-
Blocker solution (BS)	5 ul
Universal Blockers (UB)	7 ul
Total	12 ul

2.4 Heat the probe solution (Step2.2) to 95°C for 2 minutes in a thermal cycler with the lid at 105°C, then immediately cool on ice for 5 minutes.
2.5 While probe solution is cooling on ice, heat the tube containing the resuspended indexed library pool (Step2.3) at 95°C for 5 minutes in a thermal cycler with the lid at 105°C, then equilibrate both the probe solution and resuspended indexed library pool to room temperature on the benchtop for 5 minutes.
2.6 Vortex and spin down the probe solution, then transfer the entire volume to the resuspended indexed library pool. Mix well by vortexing.
2.7 Pulse-spin the tube(s) to ensure all solution is at the bottom of the tube(s).
2.8 Add 30 µl Hybridization Enhancer (HE) to the top of the entire capture reaction.
2.9 Pulse-spin the tube(s) to ensure there are no bubbles present.
Note: Seal the tubes well to prevent excess evaporation over 16-h incubation.
2.10 Incubate the hybridization reaction at 70°C for 16 hours in a thermal cycler with ***the lid at 85°C***.
NOTE: Halting hybridization between 15–17 hours will not affect downstream capture quality.

Step 3. Bind hybridized targets to streptavidin beads

Reagents Required:

1. Hybridization reactions (from Step 2.10)
2. From the Twist Hybridization Reagents:
    a. Amplification Primers (AP)
    b. Equinox Library Amp Mix (2x; [ELAM])
3. From the Twist Wash Buffers:
    a. Binding Buffer (BB)
    b. Wash Buffer 1 (WB1)
    c. Wash Buffer 2 (WB2)
4. From Twist Binding and Purification Beads:
    a. Streptavidin Binding Beads (SBB)
    b. DNA Purification Beads (DPB*).
    Note: Double check these two vials.

Before You Begin:

1. Preheat the following tubes at 48°C until any precipitate is dissolved:
    a. Binding Buffer (BB)
    b. Wash Buffer 1 (WB1)
    c. Wash Buffer 2 (WB2)
2. For each hybridization reaction:
    a. Equilibrate 800 µl Binding Buffer to room temperature
    b. Equilibrate 200 µl Wash Buffer 1 to room temperature
    c. Leave 700 µl Wash Buffer 2 at 48°C
3. **Equilibrate the Streptavidin Binding Beads to room temperature for at least 30 minutes**.
4. In preparation for Step 4 (Post-Capture PCR Amplify, Purify, and Perform QC):
    Thaw on ice:
    a. Equinox Library Amp Mix (2x; [ELAM])
    b. Amplification Primers (AP)
5. **Equilibrate DNA Purification Beads (DPB) from the Twist Binding and Purification Beads to room temperature for at least 30 minutes**.

<u>Prepare the beads:</u>

3.1 Vortex the pre-equilibrated Streptavidin Binding Beads (SBB) until mixed. ***Vortex it hard!!***

3.2 Add 100 µl Streptavidin Binding Beads (SBB) to a 1.5-ml microcentrifuge tube. Prepare one tube for each hybridization reaction.

3.3 Add 200 µl Binding Buffer (BB) to the tube(s) and mix by pipetting.

3.4 Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.

3.5 Repeat step 3.3 and 3.4 two more times for a total of ***three washes***.

3.6 After removing the clear supernatant from the third wash, add a final 200 µl Binding Buffer (BB) and resuspend the beads by vortexing until homogenized.

Important: At step 3.7, rapid transfer directly from the thermal cycler is a critical step for minimizing off-target binding. Do not let the hybridization reaction cool to less than 70 °C before transferring it to the washed Streptavidin Binding Beads.

3.7 After the hybridization (Step2.10) is complete, open the thermal cycler lid and ***DO NOT remove the tube(s) of hybridization reaction from the thermal cycler.*** Directly transfer the volume (70 ul) of each hybridization reaction into a corresponding tube of washed Streptavidin Binding Beads from Step 3.6. Mix by pipetting and flicking.

3.8 Mix the tube(s) of the hybridization reaction with the Streptavidin Binding Beads for 30 minutes at room temperature on hula mixer at 20 rpm. **DO NOT VORTEX!!**

3.9 Remove the tube(s) containing the hybridization reaction with Streptavidin Binding Beads from the mixer and pulse-spin to ensure all solution is at the bottom of the tube(s).

3.10 Place the tube(s) on a magnetic stand until the supernatant is clear.

3.11 Remove and discard the clear supernatant including the Hybridization Enhancer. Do not disturb the bead pellet.

3.12 Remove the tube(s) from the magnetic stand and add 200 µl Wash Buffer 1 (WB1). Mix by pipetting.

3.13 Pulse-spin to ensure all solution is at the bottom of the tube(s).

3.14 Transfer the entire volume from Step 3.13 (~200 µl) into a new 1.5-ml microcentrifuge tube, one per hybridization reaction. Place the tube(s) on a magnetic stand for 1 minute.

IMPORTANT: This step reduces background from non-specific binding to the surface of the tube.

3.15 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

From step 3.16 to 3.20, work next to the block heater.

3.16 Remove the tube(s) from the magnetic stand and add 200 µl of 48°C Wash Buffer 2 (WB2). Mix by pipetting, then pulse-spin to ensure all solution is at the bottom of the tube(s).

3.17 Incubate the tube(s) for 5 minutes at 48°C.

3.18 Place the tube(s) on a magnetic stand for 1 minute.

3.19 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

3.20 Repeat the wash (Steps 3.16–3.19) two more times, for ***a total of three washes***.

3.21 Before removing supernatant, the bead pellet may be briefly spun to collect supernatant at the bottom of the tube and returned to the magnetic rack. Use a 10 µl pipette to remove all traces of supernatant. ***Proceed immediately to the next step. Do not allow the beads to dry.***

3.22 Remove the tube(s) from the magnetic stand and add 22.5 µl water (H2O). Mix by pipetting until homogenized, then incubate this solution, hereafter referred to as the Streptavidin Binding Bead Slurry, on ice.

Step 4. Post-capture PCR amplify, purify, and perform QC

Reagents Required:

1. Streptavidin Binding Bead Slurry (from Step 3.22)
2. Ethanol
3. Molecular biology grade water
4. Reagents thawed and equilibrated in Step 3:

a. DNA Purification Beads (DPB*)
b. Equinox Library Amp Mix (2x; [ELAM])
c. Amplification Primers (AP)

5. Agilent Tape Station (ICBR)
6. Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantitation Assay (4 °C).

Before You Begin:

1. Prepare 500 µl 80% ethanol for each Streptavidin Binding Bead slurry to be processed.

Prepare the beads, thermal cycler, and PCR mix:

4.1 Program a thermal cycler with the following conditions. Set the heated lid to 105°C.

Step	Temperature	Time	# of cycles
Initiation	98 °C	2 min	1
Denaturation	98 °C	15 sec	15
Annealing	60 °C	30 sec	
Extension	72 °C	8 min	
Final extension	72 °C	1 min	1
Hold	4 °C	Hold	-

*Number of amplification cycles vary depending on hybridization reaction size.

Panel size	# of cycles singleplex	# of cycle multiplex
> 100 Mb	6	5
50-100 Mb	8	7
10-50 Mb	9	8
1-10 Mb	10	9
500 – 1000 kb	12	11
100 – 500 kb	14	13
50 – 100 kb	15	14
< 50 kb	16	15

4.2 Vortex the Streptavidin Binding Bead slurry (SBBS; from step 3.22).

4.3 Transfer 22.5 ul SBBS to a 0.2-ml thin-walled PCR tube(s). Keep on ice until ready to use in the next step.

4.4 Prepare a PCR mixture by adding the following reagents to the tube(s) containing the SBBS. Mix by pipetting.

Reagent	Volume per reaction
SBBS	22.5 ul
Amplification Primer (AP)	2.5 ul

Equinox Library Amp Mix (2x; [ELAM])	25 ul
Total	50 ul

4.5 Pulse-spin the tubes, transfer them to the thermal cycler and start the cycling program. (*It will take about 3 hours*)

4.6 When the thermal cycler program is complete, remove the tube(s) from the block and immediately proceed to the Purify step.

Purification:

4.7 Vortex the pre-equilibrated DNA Purification Beads (DPB*) until well mixed.

4.8 Add 25 µl homogenized DNA Purification Beads (DPB*) to the tube(s) from Step 4.6. Mix well by vortexing.

4.9 Incubate for 5 minutes at room temperature.

4.10 Place the tube(s) on a magnetic rack until the supernatant is clear.

4.11 The DNA Purification Beads (DPB) form a pellet, leaving a clear supernatant. Without removing the tube(s) from the magnetic rack, remove and discard the clear supernatant.

4.12 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

4.13 Repeat this wash once, for a total of two washes, while keeping the tube on the magnetic rack.

4.14 Briefly spun the bead pellet to collect ethanol at the bottom of the tube and returned to the magnetic rack. Carefully remove all remaining ethanol using a 10 µl pipette, making sure to not disturb the bead pellet.

4.15 Air-dry the bead pellet on the magnetic rack no more than 5 minutes. Do not overdry the bead pellet.

4.16 Remove the tube(s) from the magnetic rack and add 32 µl water (H_2O) to each capture reaction. Mix by pipetting until homogenized.Transfer 30 µl of the clear supernatant containing the enriched library to a clean thin-walled PCR 0.2-ml tube, making sure to not disturb the bead pellet.

4.17 Validate and quantify each enriched library using the Thermo Fisher Scientific Qubit 1x dsDNA HS assay.

DNA sample	Qubit HS working solution
1 ul	199 ul

4.18 Vortex mixture for 2 seconds and incubate the reaction for 2 minutes at RT.

Sample name: _____; concentration: _____ ng/ul

STOP POINT: If not proceeding immediately, store the enriched library sample at –20 °C for up to 24 hours.

Ligation sequencing DNA for use with Twist Target Target Enrichemnt Protocol

Protocol adated from Community - Protocol - Ligation sequencing gDNA (nanoporetech.com) (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/genomic-dna-by-ligation-sqk-lsk110/v/gde_9108_v110_revw_10nov2020).

Last updated: 03/03/2023

Before Start checklist

Materials

☐1 ug of enriched library

☐Ligation Sequencing Kit (SQK-LSK110)

Consumables

☐Agencourt AMPure XP beads (BeckmanCoulter™, A63881, [AXB])

☐NEBNext FFPE Repair Mix (M6630, [FFPE buffer & FFPE mix])

☐ NEBNext Ultra II End repair/dA-tailing Module(E7546, [EPB & EPM])

☐ NEBNext Quick Ligation Module (E6056, [T4])

☐ 1.5 ml Eppendorf DNA LoBind tubes

☐ 0.2 ml thin-walled PCR tubes

☐ Nuclease-free water (H_2O)

☐ Freshly prepared 70% ethanol in nuclease-free water (70%)

☐ Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit

Equipment

☐ Hula mixer (gentle rotator mixer)

☐ Magnetic rack, suitable for 1.5 ml microcentrifuge tubes

☐ Microfuge

☐ Vortex mixer

☐ Thermoal cycler

☐ Pipettes P10, P20, P200, P1000

Flow Cell Number: _____

Enriched library name: _____

Concentration of enriched library : _____ ng/ul

INSTRUCTIONS	NOTE
1. DNA repair and end-repair (~45 min)	
Before you start: ☐ Thaw DNA Control Sample (DCS) at RT, spin down, mix by pipetting, and place on ice. Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End Repair / dA-tailing Module reagents inaccordance with manufacturer's instructions, and place on ice. ☐ Thaw all reagents on ice. ☐ Flick and/or invert the reagent tubes to ensure they are well mixed. *Note: Do not vortex the FFPE DNA Repair Mix (FFPE mix) or Ultra II End Prep Enzyme Mix (EPM).* ☐ Always spin down tubes before opening for the first time each day. ☐ The Ultra II End Prep Buffer (EPB) and FFPE DNA Repair Buffer (FFPE bfr) may have a little precipitate. Allow the mixture tocome to RT and pipette the buffer up and down several times to break up the precipitate, followed byvortexing the tube for 30 seconds to solubilise any precipitate.Note: It is important the buffers are mixed well by vortexing. ☐ The FFPE DNA Repair Buffer (FFPE bfr) may have a yellow tinge and is fine to use if yellow. **Prepare the DNA in Nuclease-free water:** ☐ **1.1** For R9.4.1 flow cells, transfer 1 ug enriched library into a 0.2 ml PCR tube. ☐ **1.2** Adjust the volume to 47 ul with Nuclease-free water ☐ **1.3** Mix thoroughly by flicking the tube, then spin dow briefy in a microfuge	

Enriched library (1ug)	
Nuclease-free water (H_2O)	
Total volume	47 ul

INSTRUCTIONS	Note
1. DNA repair and end-repair (continued)	
☐ **1.4** In the 0.2 ml thin wall PCR tube, mix the following: ☐ 47 ul DNA from the step 1.3 ☐ 1 ul DNA CS (DCS) ☐ 3.5 ul NEBNext FFPE DNA Repair Buffer (FFPE bfr) ☐ 2 ul NEBNext FFPE DNA Repair Mix (FFPE mix) ☐ 3.5 ul Ultra II End-prep Reaction Buffer (EPB) ☐ 3 ul Ultra II End-prep Enzyme Mix (EPM) ☐ **1.5** Ensure the components are thoroughly mixed by pipetting, and spin down. ☐ **1.6** Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes. **AMPure XP bead clean-up:** ☐ **1.7** Resuspend the AMPure XP beads (AXB) by vortexing. ☐ **1.8** Transfer the DNA sample from step to a clean 1.5 ml Eppendorf DNA LoBind tube. ☐ **1.9** Add 60 µl of resuspended AMPure XP beads (AXB) to the end-prep reaction and mix by flicking the tube.Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT. ☐ **1.10** Prepare 500 µl of fresh 70% ethanol in Nuclease-free water. ☐ **1.11** Spin down the sample and pellet on a magnet until supernatant is clear and colourless. ☐ **1.12** Keep the tube on the magnet, and pipette off the supernatant. ☐ **1.13** Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol withoutdisturbing the pellet. Remove the ethanol using a pipette and discard. ☐ **1.14** Repeat the previous step. ☐ **1.15** Spin down and place the tube back on the magnet. ☐ **1.16** Pipette off any residual ethanol. Allow to dry for ~30seconds, but do not dry the pellet to the point of cracking. ☐ **1.17** Remove the tube from the magnetic rack and resuspend the pellet in 63 µl Nuclease-free water. ☐ **1.18** Incubatefor 2 minutes at RT.Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute. ☐ **1.19** Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.	

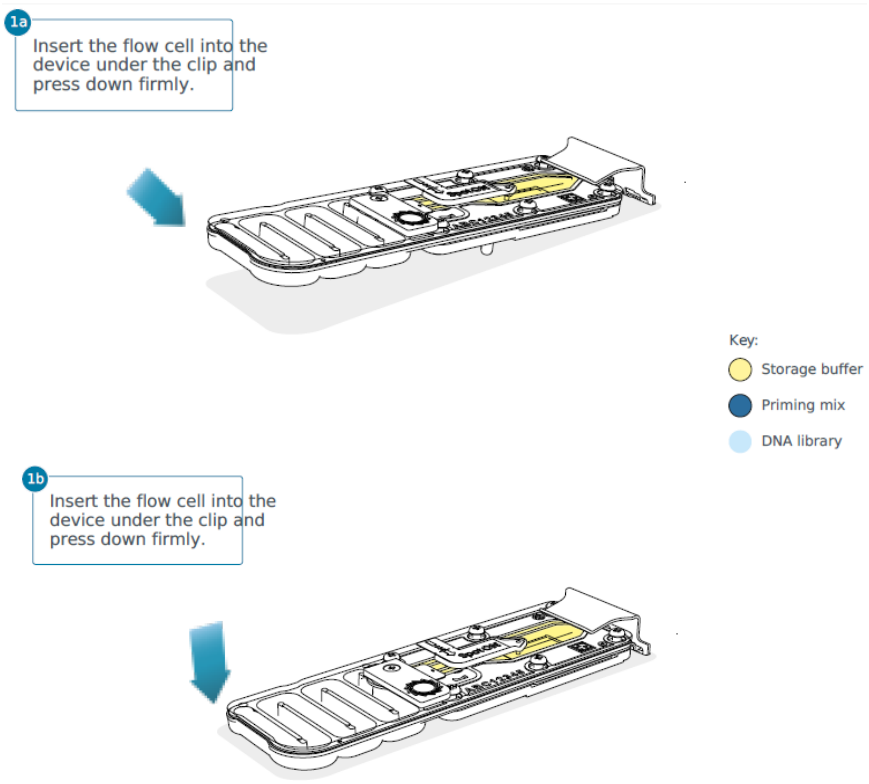
<table>
<tr><td>INSTRUCTIONS</td><td>Note</td></tr>
<tr><td>1. DNA repair and end-repair (continued)</td><td></td></tr>
<tr><td>Quantify 1 ul of eluted sample using a Qubit fluorometer:
☐ 1.20 Add 1 ul of eluted sample into Qubit 1x dsDNA working solution (total volume of 200 ul)
☐ 1.21 Vortex the mixture for 2 seconds then incubate the tube at room temperature for 2 minutes.
☐ 1.22 Quantify eluted sample on the Qubit fluorometer

<table><tr><td>Eluted sample Name</td><td>Concentration (ng/ul)</td></tr><tr><td></td><td></td></tr></table>Please report the concentration.</td><td></td></tr>
<tr><td>2. Adapter ligation and clean-up (~35 min)</td><td></td></tr>
<tr><td>☐ 2.1 Spin down the Adapter Mix F (AMF) and Quick T4 Ligase (T4), and place on ice.
☐ 2.2 Thaw Ligation Buffer (LNB) at RT, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.
☐ 2.3 Thaw the Elution Buffer (EB) at RT, mix by vortexing, spin down and place on ice.
NOTE: Depending on the wash buffer (LFB or SFB) used, the clean-up step after adapter ligation is designed to either enrich for DNA fragments of >3 kb, or purify all fragments equally.
☐ 2.4 To retain DNA fragments of all sizes, thaw one tube of <u>Short Fragment Buffer (SFB)</u> at RT, mix by vortexing, spin down and place on ice.
☐ 2.5 In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:
☐ 60 µl DNA sample from step 1.19
☐ 25 µl Ligation Buffer (LNB)
☐ 10 µl NEBNext Quick T4 DNA Ligase (T4)
☐ 5 µl Adapter Mix F (AMF)
☐ 2.6 Ensure the components are thoroughly mixed by pipetting, and spin down.
☐ 2.7 Incubate the reaction for 10 minutes at RT.
☐ 2.8 Resuspend the AMPure XP beads (AXB) by vortexing.
☐ 2.9 Add 40 µl of resuspended AMPure XP beads (AXB) to the reaction and mix by flicking the tube.
☐ 2.10 Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.</td><td></td></tr>
</table>

<table>
<tr><td>INSTRUCTIONS</td><td>NOTE</td></tr>
<tr><td>2. Adapter ligation and clean-up (continued)</td><td></td></tr>
<tr><td>☐ 2.11 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
☐ 2.12 Wash the beads by adding 250 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.
☐ 2.13 Repeat the previous step.
☐ 2.14 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.
☐ 2.15 Remove the tube from the magnetic rack and resuspend the pellet in 17 µl Elution Buffer (EB). Spin down and incubate for 10 minutes at 37°C.
☐ 2.16 Pellet the beads on a magnet rack until the eluate is clear and colorless, for at least 1 minute.
☐ 2.17 Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.
Quantify 1 ul of eluted sample using a Qubit fluorometer:
☐ 2.18 Add 1 ul of eluted sample into Qubit 1x dsDNA working solution (total volume of 200 ul)
☐ 2.19 Vortex the mixture for 2 seconds then incubate the tube at room temperature for 2 minutes.
☐ 2.20 Quantify elued sample on the Qubit fluorometer
Store the library on ice until ready to load

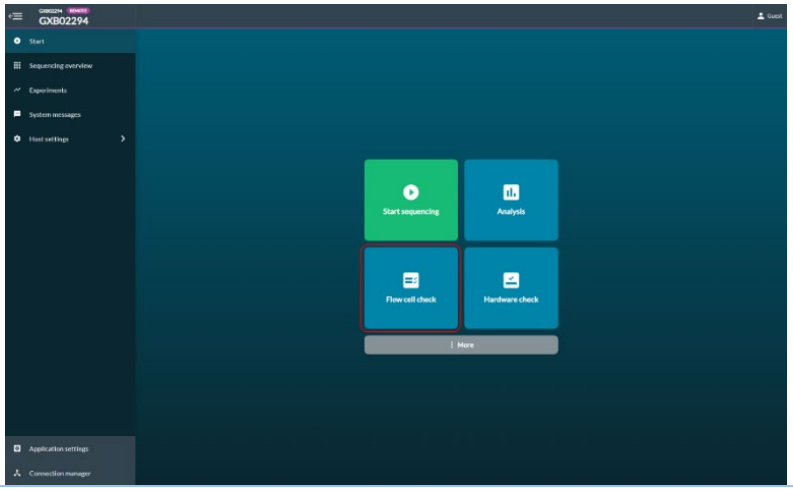
<table><tr><td>Eluted sample Name</td><td>Concentration (ng/ul)</td></tr><tr><td></td><td></td></tr></table>Note: Recovery aims > 250 ng</td><td></td></tr>
<tr><td>3. Priming and loading the SpotON flow cell</td><td></td></tr>
<tr><td>New user? Watch the Priming and loading your flow cell https://youtu.be/Pt-iaemrM88 video before your first run.
☐ 3.1 Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) or Loading Solution (LS), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before mixing the reagents by vortexing and spin down at RT.
☐ 3.2 To prepare the flow cell priming mix, add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at RT.</td><td></td></tr>
</table>

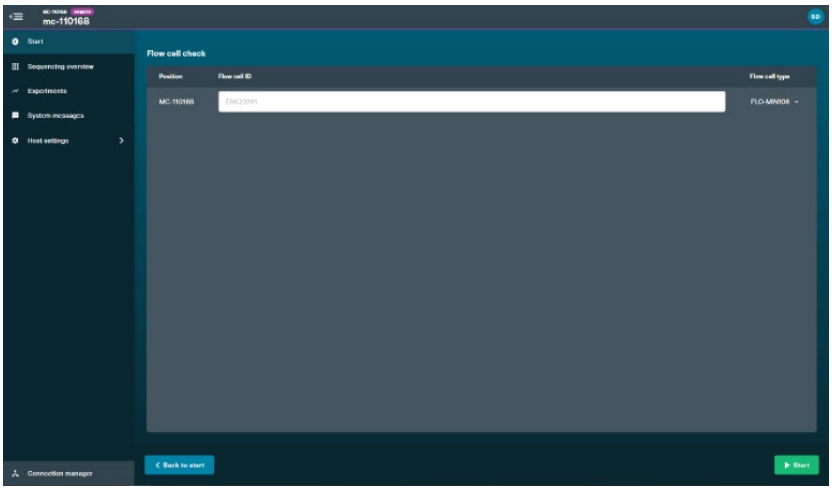
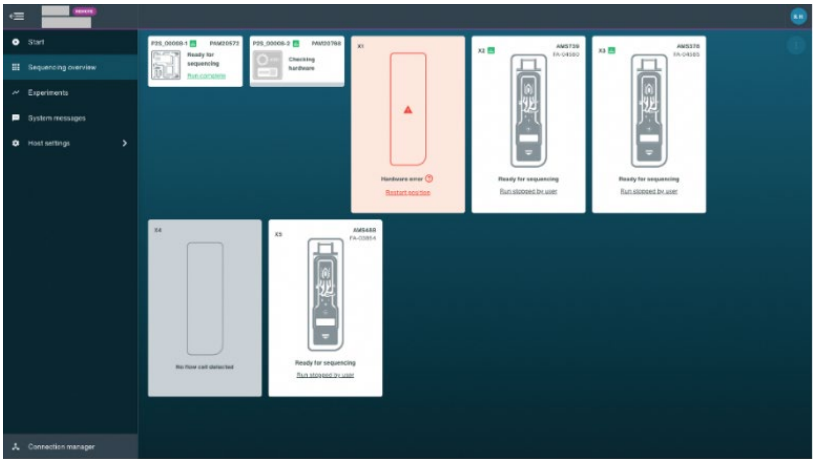
INSTRUCTIONS	NOTE
3. Priming and loading the SpotON flow cell (continued)	
☐ **3.3** Open the MinION device lid and slide the flow cell under the clip.	



Complete a flow cell check to assess the number of pores available before loading the library:

☐ **3.4** Navigate to the start homepage and select 'Flow Cell Check'.

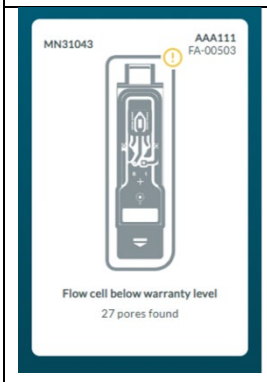
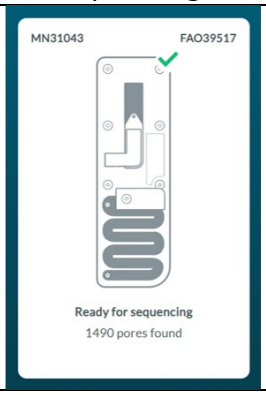
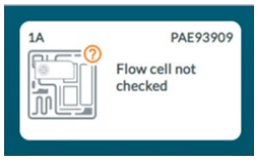


INSTRUCTIONS	NOTE
3. Priming and loading the SpotON flow cell (continued)	
☐ **3.5** When you see the flow cell type and flow cell IDs recognised, click 'Start' to begin.  ☐ **3.6** You will be automatically navigated to the Sequencing Overview page. 	

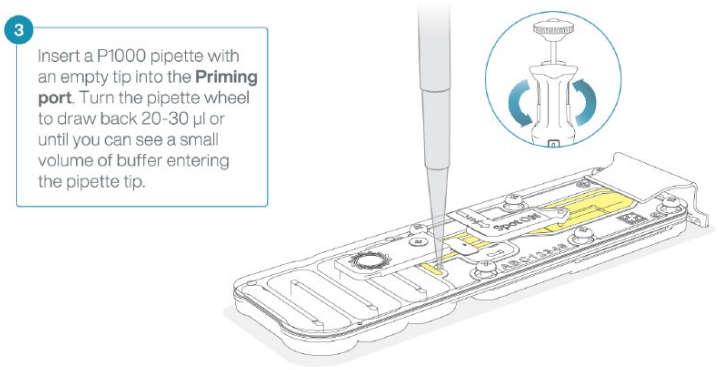
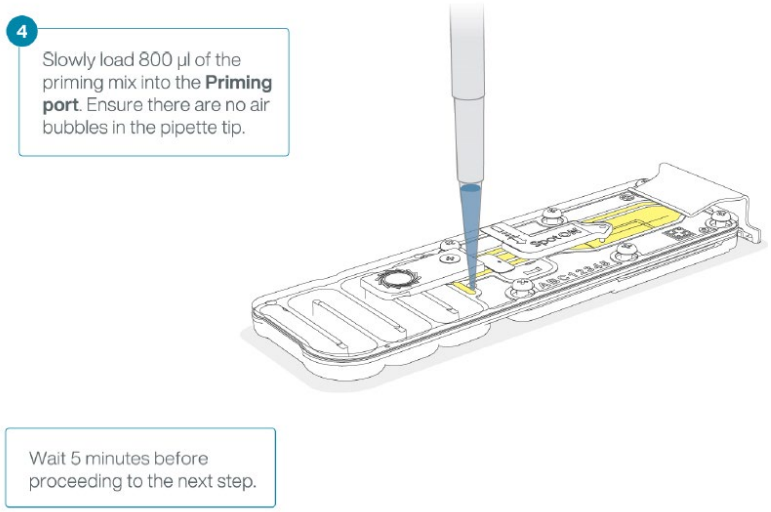
Flow cell	Minimum number of active pores covered by warranty
Flongle Flow Cell (FLO-FLG001)	50
MinION/GridION Flow Cell	800
PromethION Flow Cell	5000

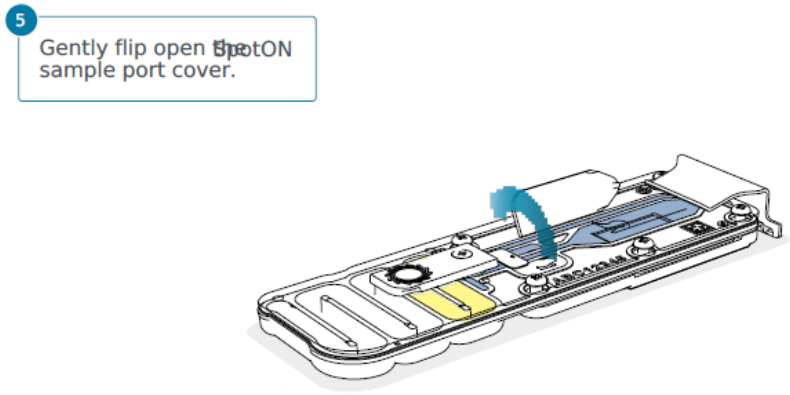
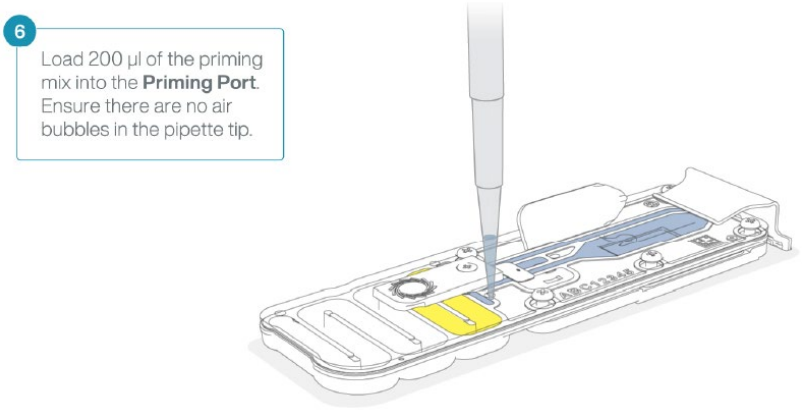
INSTRUCTIONS	NOTE
3. Priming and loading the SpotON flow cell (continued)	

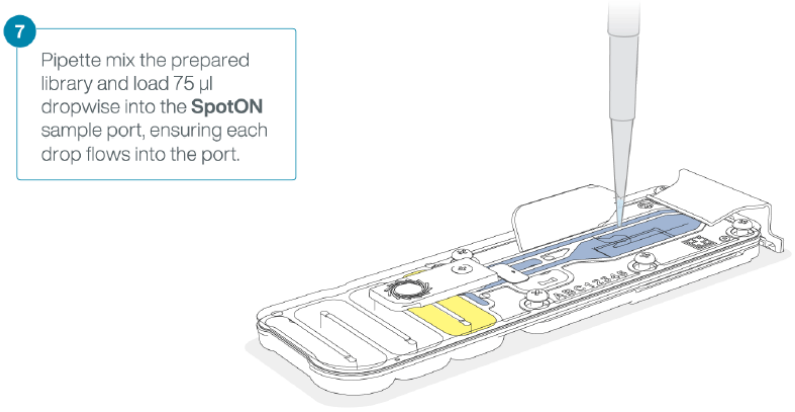
Flow cell health indicators:

Yellow exclamation mark	Green tick	Question mark
The number of sequencing pores is below warranty.	The number of sequencing pores is above warranty and ready for sequencing.	A flow cell check has not been run on the flow cell during this MinKNOW session
		

☐ **3.7** Slide the priming port cover clockwise to open the priming port.

INSTRUCTIONS	NOTE
3. Priming and loading the SpotON flow cell (continued)	
☐ **3.8** 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 until the dial shows 220-230 ul, to draw back 20-30 ul. NOTE: 1. ***DO NOT remove more than 30 ul***; 2. Visually check that there is continuous buffer from the priming port across the sensor array.  ☐ **3.9** Load 800 µl of the priming mix (from step 3.2) into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes. During this time, prepare the library for loading by following the steps below.  	

INSTRUCTIONS	NOTE
3. Priming and loading the SpotON flow cell (continued)	
☐ **3.10** Thoroughly mix the contents of the Loading Beads II (LBII) by pipetting. **NOTE: The Loading Beads II (LBII) tube contains a suspension of beads. These beads settle very quickly. It is vital** that they are mixed immediately before use. ☐ **3.11** In a new 1.5-ml tube, prepare the library for loading as follows: ☐ 37.5 µl Sequencing Buffer II (SBII) ☐ 25.5 µl Loading Beads II (LBII) mixed immediately before use ☐ 12 µl DNA library from step 2.17 ☐ **3.12** Complete the flow cell priming: ☐ Gently lift the SpotON sample port cover to make the SpotON sample port accessible.  ☐ Load 200 µl of the priming mix into the flow cell via the **PRIMING PORT** (not the SpotON sample port), avoiding the introduction of air bubbles. 	

INSTRUCTIONS	NOTE
3. Priming and loading the SpotON flow cell (continued)	
☐ **3.13** Mix the prepared library gently by pipetting up and down just prior to loading. ☐ **3.14** Add 75 µl of sample to the flow cell via the **SpotON sample port** in a dropwise fashion. Ensure each drop flows into the port before adding the next.  ☐ **3.15** Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.