Schedule DAY 2

TUESDAY	ACTIVITY	PRESENTER
9.00 – 9.10	Review of Day 1	Jean
9.10 – 10.00	**Lab: AMPLICON QC**	**Louise**
10.00 – 10.30	Morning tea	
10.30 – 12.30	**Lab:** Library Preparation & Loading	Jean
12.30 – 13.30	Lunch	
13.30 – 14.15	**Lab:** Flow cell loading practice and Run Reviewing	Louise
14.15 – 14.30	**Lecture**: Review of laboratory workflow	Jean
14.30 – 15.30	Panel: Ask us anything!	
15.30 – 16.00	Afternoon Tea	
16.00 – 17:00	**Lecture:** MPXV Illumina Sequencing Options	Jean

WET LAB DAY 2:
ONT Library Prep and Sequencing

Dr. Louise Judd
Centre for Pathogen Genomics- Innovation Hub
Lead Laboratory Scientist and Genomics Trainer

CENTRE FOR PATHOGEN GENOMICS

World Health Organization
Western Pacific Region

IPSN International Pathogen Surveillance Network
WHO hosted network

QUANTIFY AMPLICONS

Quantify DNA (Qubit)

15 mins

Qubit dsDNA BR Assay Kit

Thermo Fisher Scientific

- *Contains dye that is highly specific for **dsDNA** (will not bind to ssDNA or RNA)*
- 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

- Before library prep measure concentration of at least the following samples
  - NTC, one high Ct sample and one low Ct sample for both the Pool 1 and Pool 2 reactions
  - Expect the concentration of both low and high Ct samples to be greater than NTC
  - Expect similar concentrations for Pool 1 and Pool 2 reactions, if more than 40% difference in concentration between pools repeat PCR reaction

Prepare samples for Quantitation

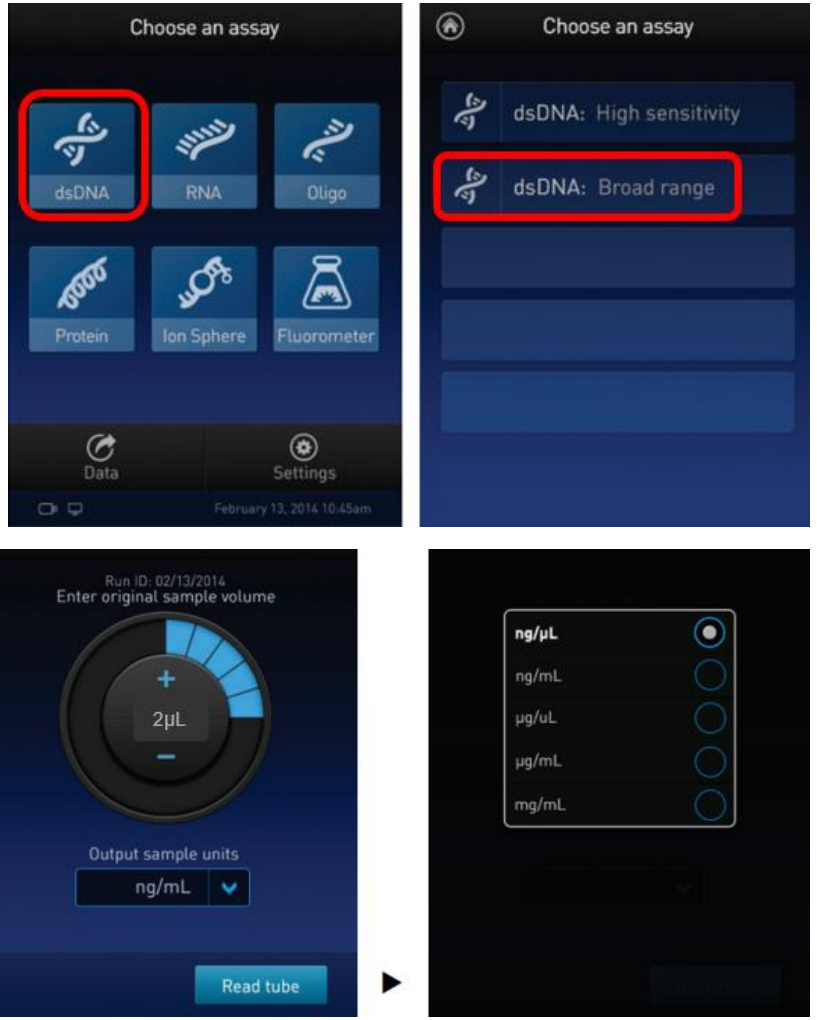
15 mins

1. Label the top of the two standard tubes S1 and S2
2. Label your sample tubes P1 1-8 and P2 1-8
3. Add 190 µl of the Qubit working solution to standard tubes
4. Add 198 µl of the Qubit working solution to sample tubes
5. Add 10 µl of standard 1 to S1 tube
6. Add 10 µl of standard 1 to S1 tube
7. Add 2 µl of each of the samples to the appropriate sample tubes
8. Vortex all tubes for 2-3 secs
9. Incubate tubes at room temperature for 2 minutes

Read concentrations in Qubit

1. Ensure there are no large bubbles in any of your tubes
2. On the Qubit select “dsDNA”
3. Next select “dsDNA: Broad range”
4. Follow instructions to read standard 1 and standard 2
5. Record the result of the standards in your worksheet
6. Next select ”Run samples”
7. On the assay screen select the sample volume used (2 µl) and the units for the output of concentration (ng/µl)
8. Read samples and record concentrations in worksheet

Expected results
NTC- 5-10 ng/µl
High Ct samples- 10-30 ng/µl
Low Ct samples- 40-90 ng/µl



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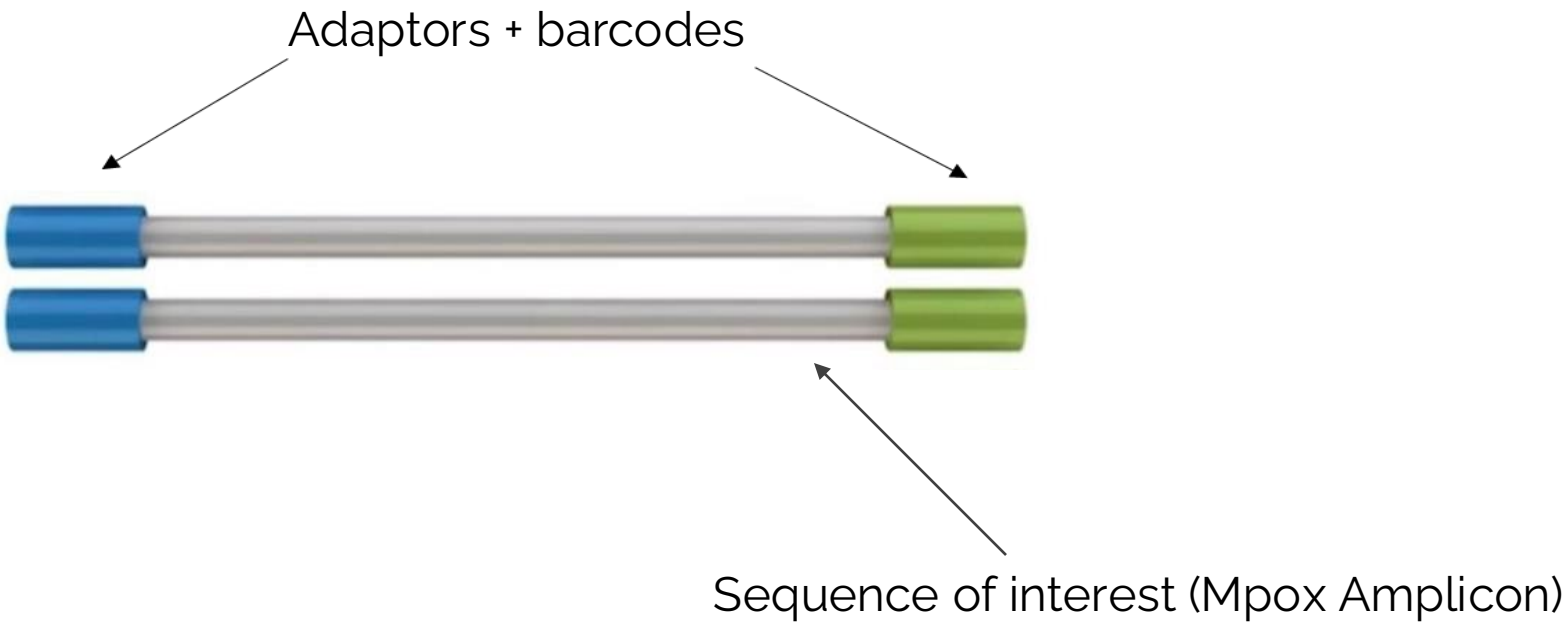
PREPARE ONT SEQUENCING LIBRARY

Mpox Library Preparation

Adapters are added to the sample (amplicon) to interact with the ONT platform flow-cell surface and pores

The goal of library prep is to add sequencing adaptors to molecules (genetic material) to be sequenced on a NGS platform.



Overview of ONT Rapid Library Prep

Simple and rapid library preparation

Transposase (enzyme) simultaneously:

cleaves/cuts template molecules (amplicon)
&
attaches barcoded tags to the cleaved ends

Amplicons need to be >200bp

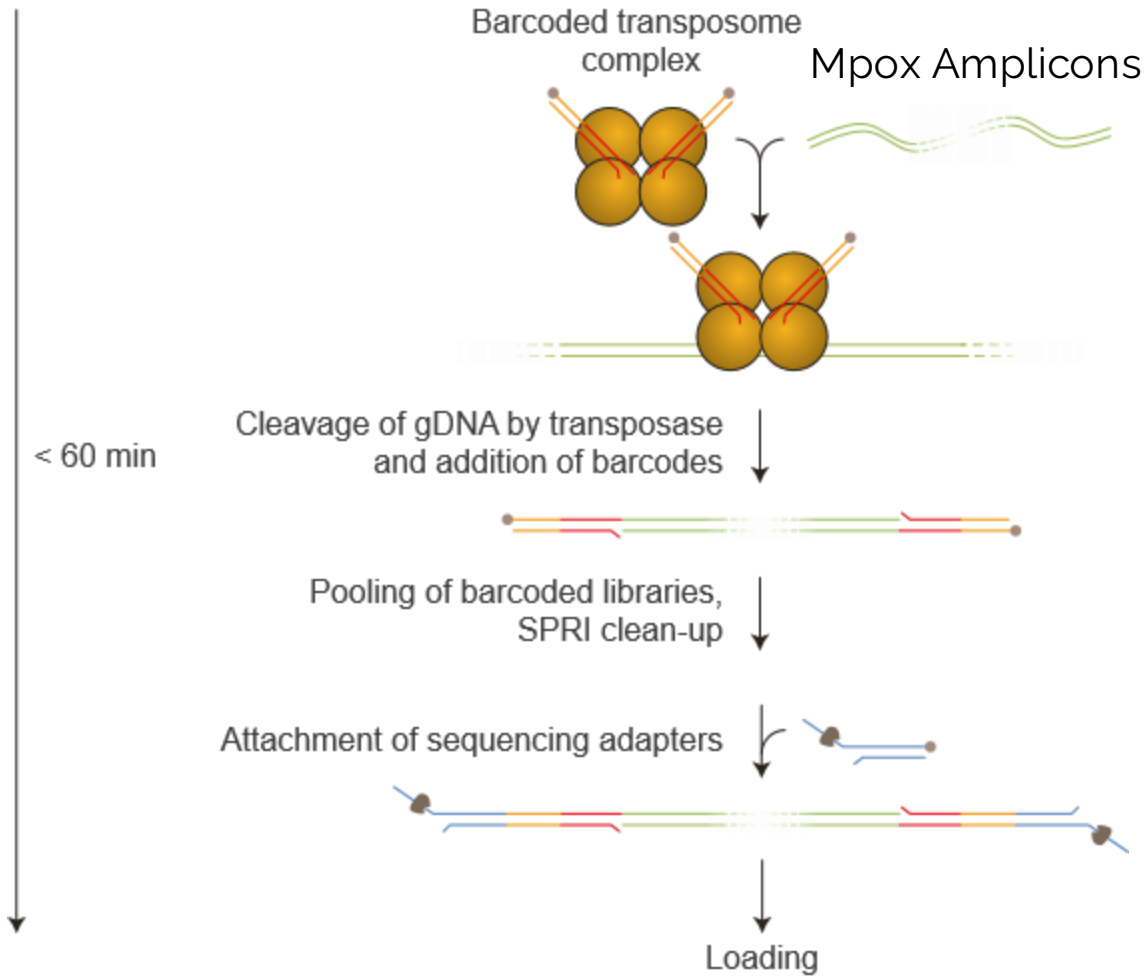
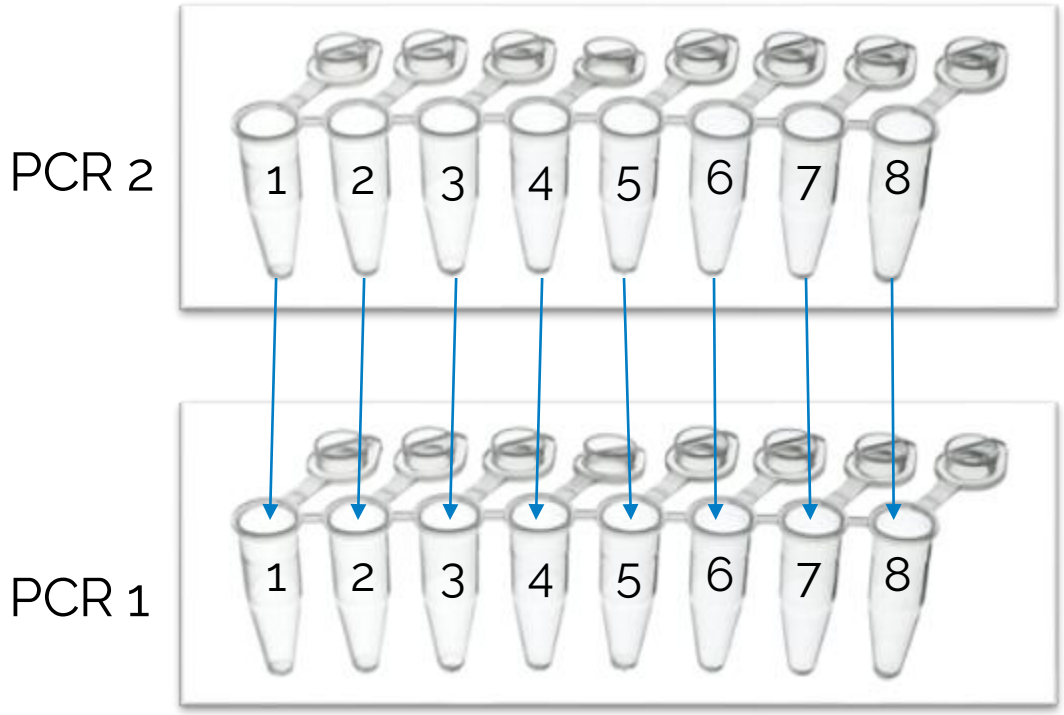


Image Source: https://store.nanoporetech.com/rapid-barcoding-kit-1.html

Combine Pool 1 and 2 PCR reactions

1. Transfer the **entire** contents (~22 µl) of each well of Pool 2 PCR strip tube to the corresponding well of the Pool 1 PCR strip tubes
2. Change pipette tips between samples
3. Gently mix the two PCR reactions together



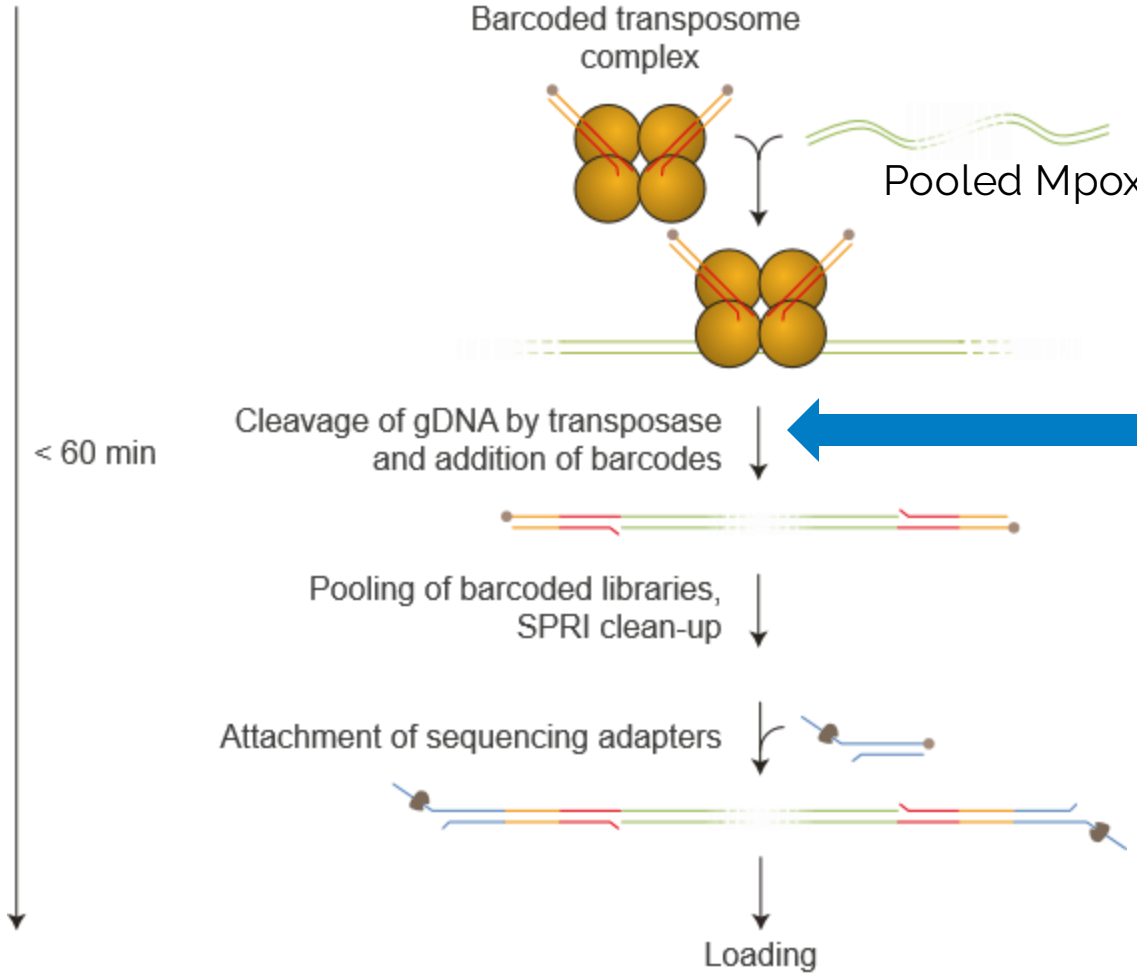
Barcoding tagmentation reaction

1. Put your initials and numbers 1-8 on a new strip tube, this is the **barcoding strip tube**
2. Add 2.5 µl Nuclease Free Water (NFW) to each well of strip tube
3. From the Pool 1 PCR strip tubes Transfer 5 µl of the combined pool 1 and 2 PCR products to the barcoding strip tube
4. Add 2.5 µl of your assigned barcodes (see sequencing workbook) to each well of the barcoding strip tube

N.B. Each person in your group **MUST** use a different set of barcodes

1. Tip mix the contents
2. Quick spin the tubes
3. Place strip tubes in the thermocycler and run the RBK program

ONT Rapid Library Prep Thermocycler



RBK Program

Heated Lid: Yes

Reaction Volume: 10 µL

30˚C for 2 minutes

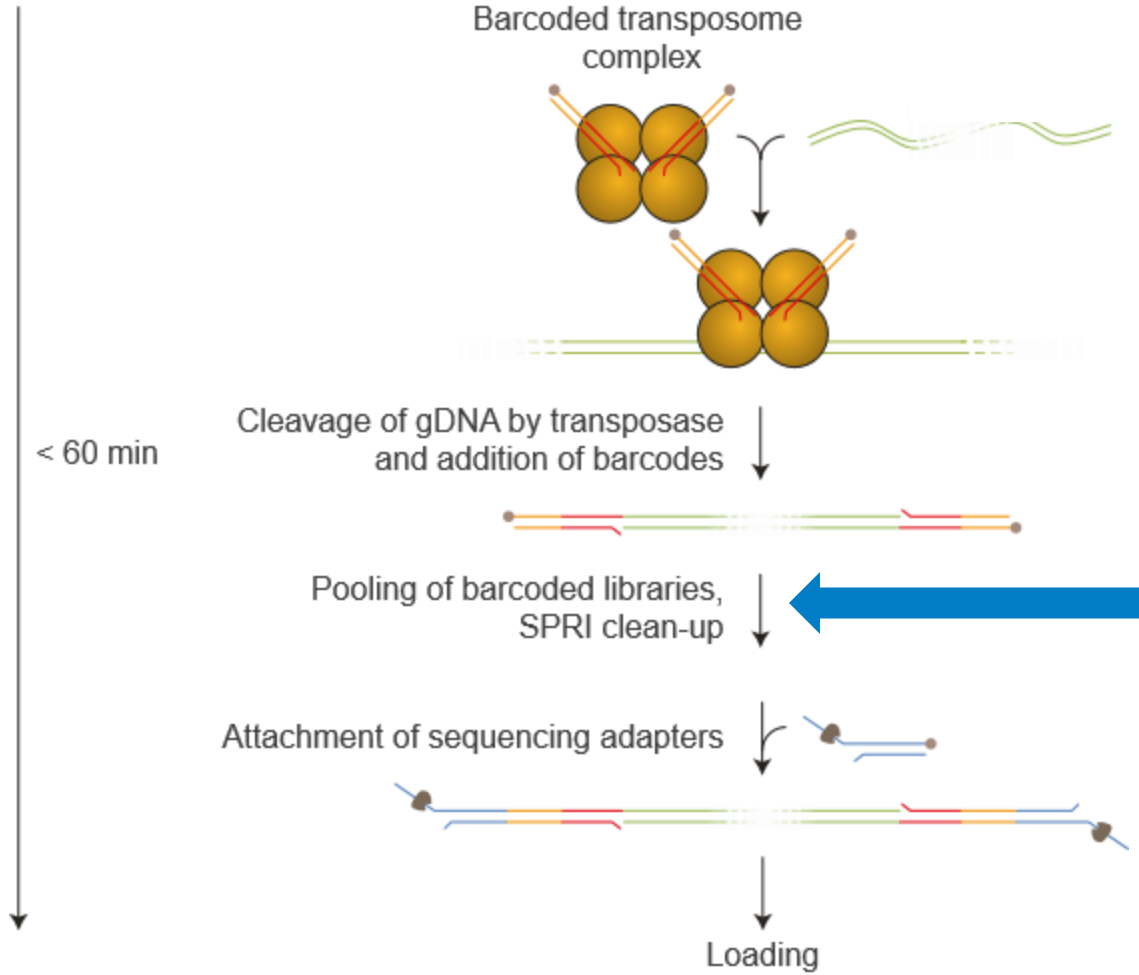
80˚C for 2 minutes

4˚C for 2 minutes

Image Source: https://store.nanoporetech.com/rapid-barcoding-kit-1.html

SAMPLE POOLING

ONT Rapid Library Prep Thermocycler



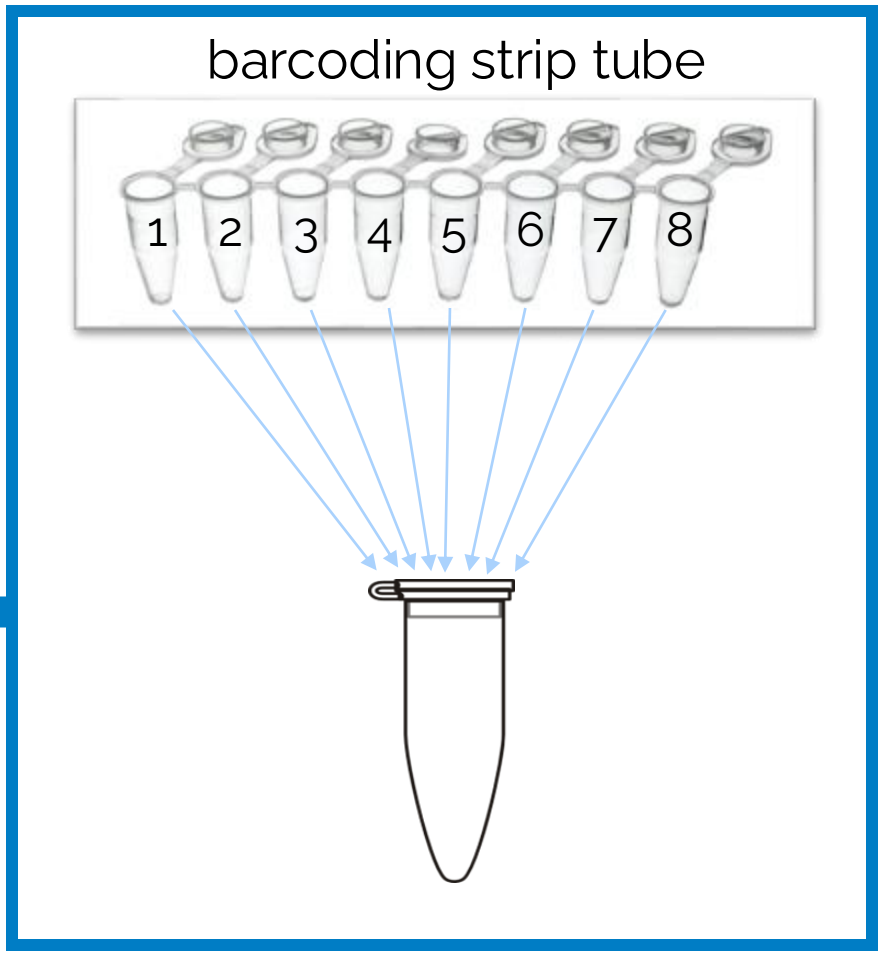


Image Source: https://store.nanoporetech.com/rapid-barcoding-kit-1.html

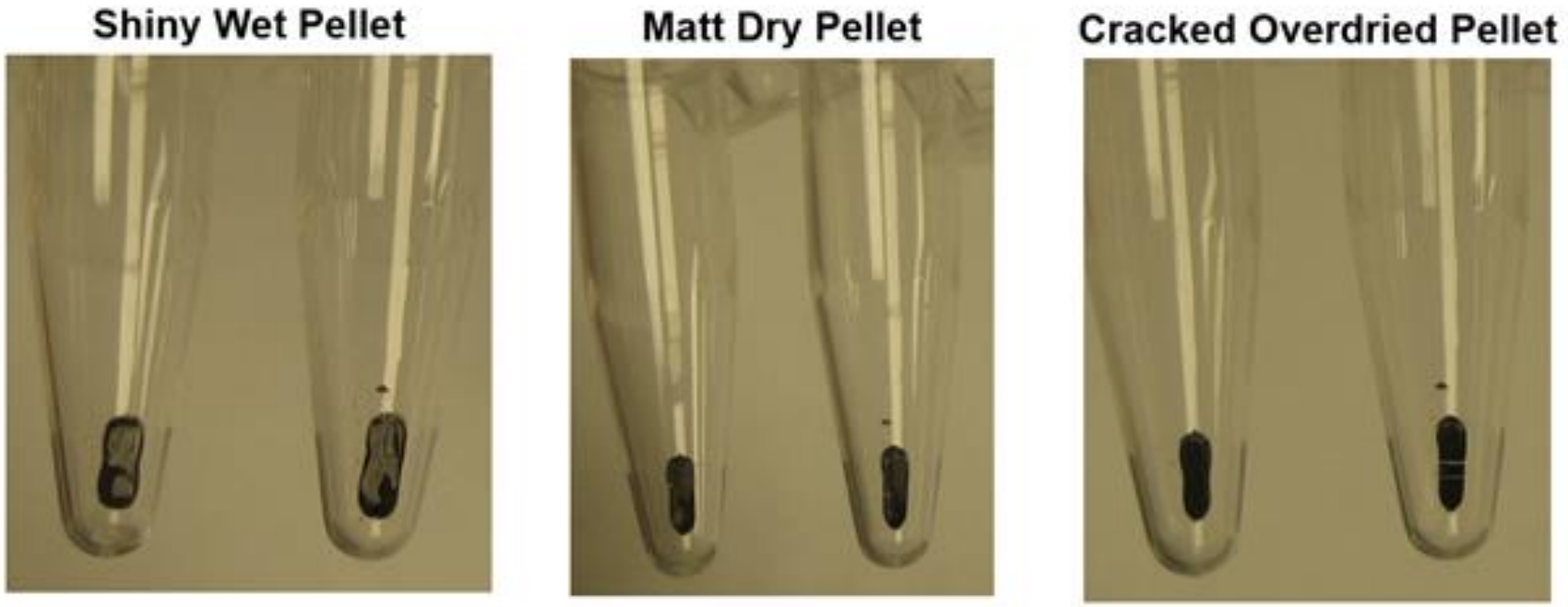
Sample Pooling

1. Each individual will pool their own samples
2. Label an Eppendorf tube with your Group Name and Initials (e.g. 1A-HW)
3. Combine 10 µL of each of the barcoded samples into your Eppendorf tube
4. Make note of the final pooled volume, should be 80 µl

CLEAN UP LIBRARIES

Ampure XP Beads

- Always make sure beads are vortexed thoroughly immediately before use
- Don't spin beads down --> you can tap gently on the bench to remove any liquid on the lid
- Be careful not to leave pellets too wet or over-dry pellets



Add Ampure XP Beads

1. Add an equal Ampure XP Beads (AXP) to your pooled sample (1:1 ratio) (80 µl)
2. Gentle flick mix
3. Incubate at room temperature for 5 minutes

Ethanol in Clean-Ups

- Always needs to be made up fresh
- Make it up during your incubations for clean-up

Ethanol evaporates rapidly. If your ethanol solution is mostly water you will end up eluting the DNA prematurely during your washing steps.

Prepare 80% Ethanol

1. Label a fresh tube "80% EtOH"
2. Add 1.2 mL of 100% Ethanol (EtOH) to a fresh tube
3. Add 300 µl of NFW to the same tube
4. Invert well to mix

Clean-Up

1. Spin down samples
2. Place samples on magnetic stand for ~3 minutes or until liquid is clear
3. Discard **supernatant**
4. Wash twice
    a. Keep samples on magnet
    b. Add 750 ul of 80% ethanol without disturbing the pellet
    c. Wait 10 seconds
    d. Remove supernatant
5. Remove residual ethanol
    a. Quick spin
    b. Place on magnet
    c. Pipette any liquid off

Elution

1. Remove tube from magnet
2. Add 10 µL of Elution Buffer (EB)
3. Incubate at room temperature for 10 minutes
4. Place sample on magnet and wait until pellet forms
5. Quantify 2 µL of the sample --> note this value down

    Expected concentration 40-80 ng/µl

Dilution and combining of libraries

1. Calculate the volume required for **200 ng of your library**

Each member needs to add in 50ng total – for a group of 4.

Eg. Your library is 20 ng/µl -> Volume required = 50ng/20ng/µl = 2.5 µl

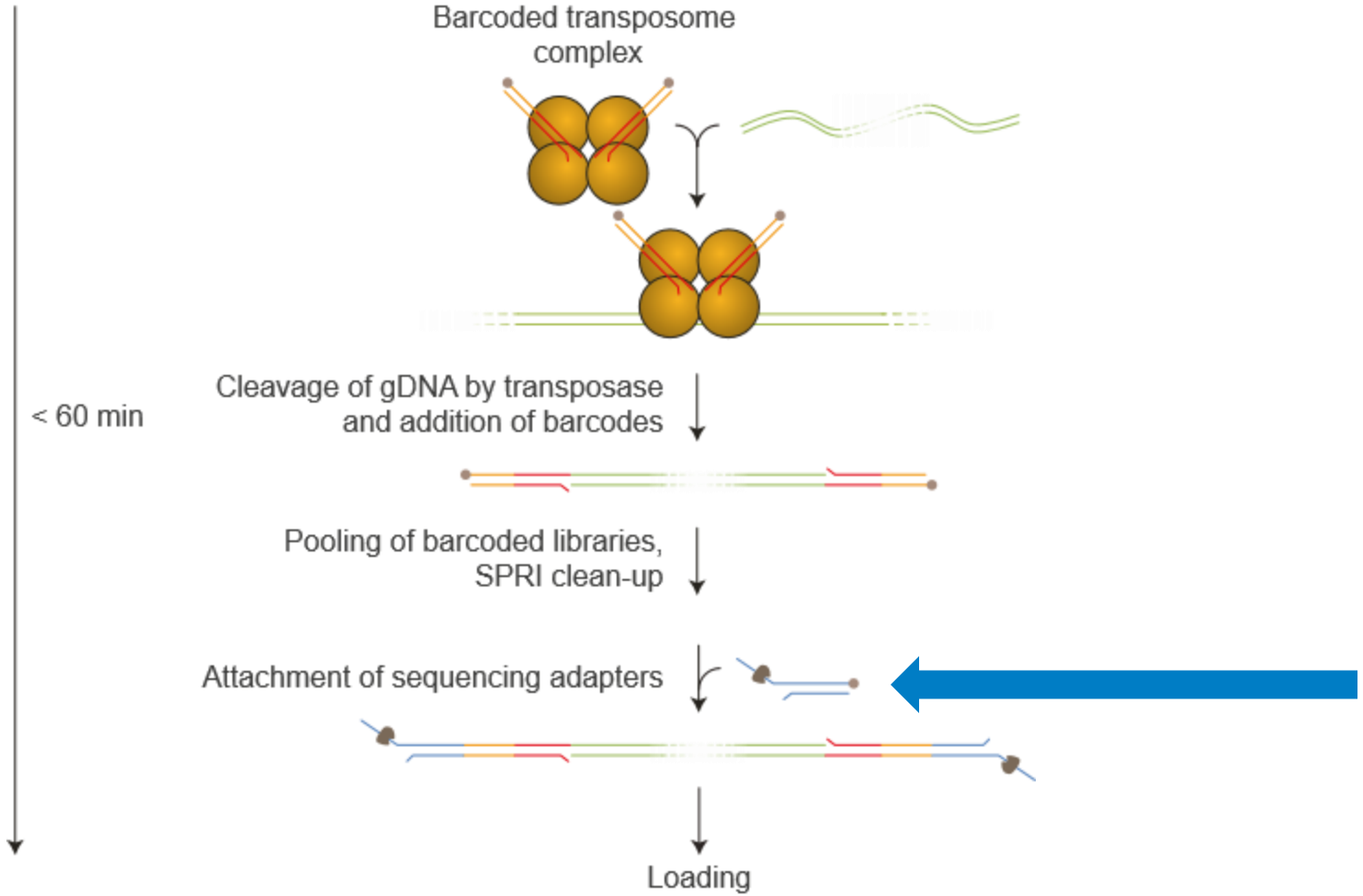
2. All members of the group should pipette calculated volume of their library into a new single 1.5 ml tube

3. Tip mix groups combined (200ng) libraries

4. Transfer 11 µl of the combined libraries to a new 1.5 ml tube labelled "Load"

(Optional: if final volume of combined libraries is less than 11 µl make up to 11 µl with EB)

ONT Rapid Library Prep Thermocycler



combined group libraries "LOAD" tube

+

Rapid Adapter (RA)

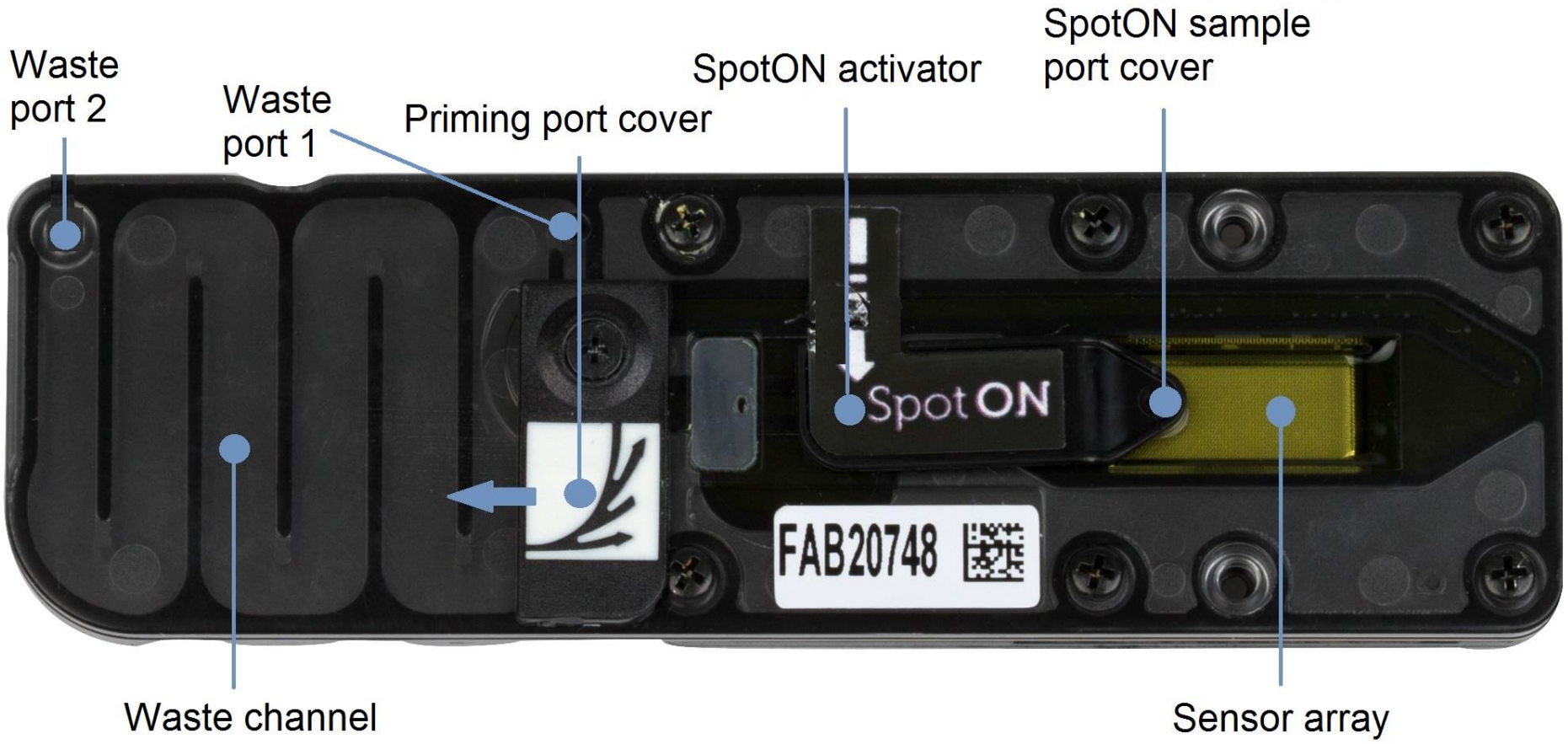
Image Source: https://store.nanoporetech.com/rapid-barcoding-kit-1.html

Adapter Addition

1. Your Rapid Adapter (RA) tube contains exactly 1.5 µL - enough for the ENTIRE GROUP
2. Have **ONE PERSON** from your group add 3.5 µL of Adapter Dilution Buffer (ADB) to the RA tube
3. Flick mix and spin down
4. Have one person add 1 µL of this RA mix to the "Load" tube
5. Mix gently by flicking
6. Incubate the reaction for 5 minutes at room temperature
7. Proceed immediately to loading flow cell or store prepared library on ice temporarily

LOADING ONT LIBRARY

MinION Flow Cell



Begin Sequencing Run
Priming MinION flow cell

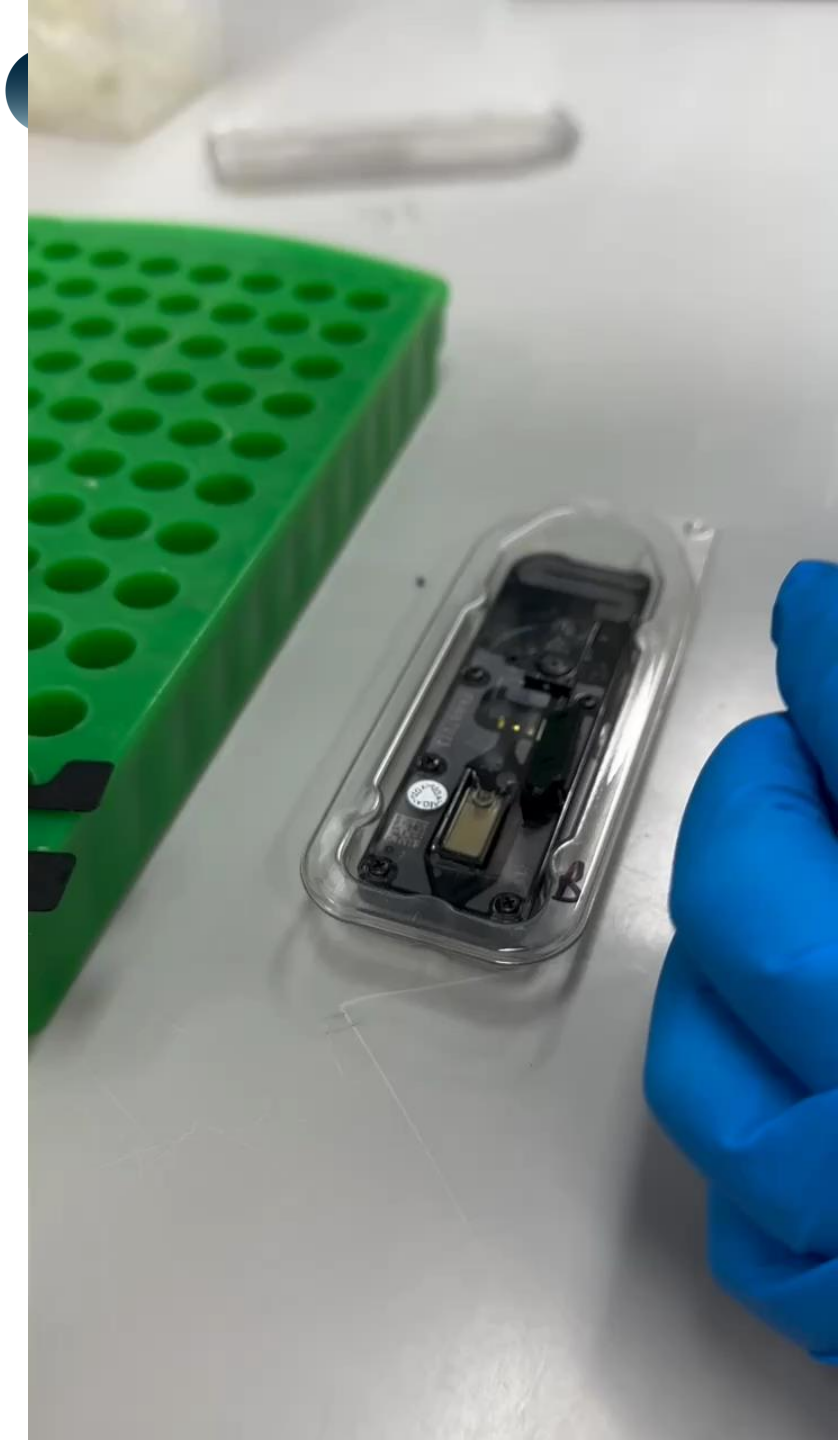
1. Open priming port fully 90° clockwise
2. Aspirate 20-30 µl of storage buffer out of Priming port
3. Dispense 800 µl priming solution into Priming port
4. Close Priming port
5. Incubate 5 minutes

Begin Sequencing Run
Priming MinION Spot-ON port

1. Open Priming port fully 90° clockwise
2. Open SpotON port and fold back to hold open
3. Slowly dispense 200 µl priming solution into priming port – watch solution bubble up through SpotON port and be drawn back in
4. Proceed immediately to loading library

Begin Sequencing Run
Loading ONT library

1. Flick mix to resuspend library solution (library PLUS Sequencing buffer PLUS Library beads)
2. Aspirate library
3. Dispense library drowpwise over SpotON port, do not touch SpotON port
4. Close SpotON port
5. Close Priming port
6. Cover with light shield
7. Proceed to sequencing



Flow Cell Prime: Make Priming Buffer

1. Label a 1.5 mL tube "Flow Cell Prime"
2. Add 1170 µL Flow Cell Flush (FCF)
3. Add 30 µL Flow Cell Tether (FCT)
4. Vortex

Flow Cell Prime: Prime Flow Cell

1. Open priming port by rotating cover 90°C clockwise
2. Remove air from priming port
   - Set a p1000 pipette to 200 µl,
   - Insert tip into priming port and turn wheel on pipette until the dial reads 230 µl, you should see the yellow storage buffer move into the tip
3. Add Priming Buffer
   - Without introducing air, pipette 800 µl of the prepared FCF/FLT "Flow Cell Prime" mix into **priming port**
   - Do **not** go down to the second stop of your pipette as this will introduce air
   - Close priming port
   - 5 minute incubation

Prepare Sample

1. To your "Load" tube, add the following:
   - 37.5 µl Sequencing Buffer (SB)
   - 25.5 µl Library Beads (LIB)

Your "Load" tube should now have 75 µl of volume in it

Flow Cell Prime: Load Sample

Establish vacuum

1. Open both the priming port and the SpotON port
2. Slowly add 200 µl of the prepared FCF/FLT "Flush" mix into **priming port**
3. Do not go down to the second stop of your pipette as this will introduce air
4. You should see gentle bubbling from the spot on port
5. Proceed immediately to next step

Flow Cell Prime: Load Sample

Add sample

1. Gently pipette mix your "Load" tube ~5 times
2. Aspirate 75 µl into pipette
3. Hold pipette tip above SpotON port, close but not touching
4. Add 75 µL in a drop wise fashion to the SpotON port
5. Sample should get sucked onto the SpotON port

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THANK YOU

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CENTRE FOR PATHOGEN GENOMICS