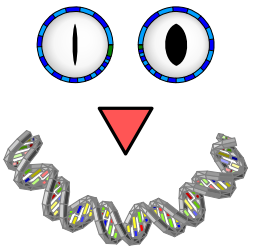
Flow Cell Loading - Flongle

GrinGene Bioinformatics



Version: 2.2.0 Written by: David Eccles
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Whakapapa / Tohutoro (References)

- Based on the 'Library preparation - Priming and loading the SpotON Flow Cell' step of ONT Community Protocols for Rapid Barcoding Sequencing (SQK-RBK004) [RBK_9054_v2_revM_14Aug2019, MinION / GridION / Flongle (https://community.nanoporetech.com/protocols/rapid-barcoding-sequencing-sqk-rbk004/v/RBK_9054_v2_revM_14Aug2019)]
- Gentle Flongle loading protocol from Graham Wiley (Oklahoma Medical Research Foundation); https://community.nanoporetech.com/posts/a-very-gentle-relatively (https://community.nanoporetech.com/posts/a-very-gentle-relatively)]
- Additional explanations by David Eccles (knowledge from ONT talks, workshops, other ONT community members, and practical experience)

Quick Summary

Loading - Flongle Flow Cells

This uses the gentle negative-pressure loading method from the community, with 5µl of library loaded in 30µl mix:

https://community.nanoporetech.com/posts/a-very-gentle-relatively (https://community.nanoporetech.com/posts/a-very-gentle-relatively)

1. Prepare Flow Cell Flush solution:
    - 117 µl Flow Cell Flush (FCF)
    - 3 µl Flow Cell Tether (FCT)
    - mix ***by pipetting***
2. Unseal the flow cell
3. Add tape to the rectangular waste port (see the sticker)
4. Drop 30 µl of flush solution onto the loading port to form a dome
5. If liquid is not draining from the loading port, there is a liquid flow blockage. Place a pipette through the drop and into the loading port, then dial up about 5 µl to check for bubbles (dial up to remove bubbles if they exist).
6. If liquid is still not draining from the loading port, place a pipette into the exposed waste port (with a circular hole) and dial up 30 µl or until liquid starts dropping from the loading port.

7. If liquid is still not draining from the loading port, set the pipette to 30 µl, press down while in mid air to expel air, then place into the exposed waste port and slowly release the plunger. If liquid starts dropping from the loading port, stop releasing the plunger and lift up the pipette.
8. If liquid is still not draining from the loading port, Repeat the last step with a faster release speed until liquid starts dropping from the loading port.
9. Wait 5 minutes (while preparing library for loading)
    - 15 µl Sequencing Buffer (SB)
    - 10 µl Library Beads (LB), mixed ***by pipetting*** immediately before use
    - 5 µl DNA library
    - mix ***by pipetting***
10. Remove the tape from the waste port
11. Drop another 30 µl of flush solution onto the loading port, wait for it to drain through
12. Drop 30 µl of sequencing library onto the loading port, wait for it to drain through
13. Re-seal the flow cell by rolling a finger across the plastic adhesive cover, trying to avoid putting pressure on the flow cell matrix

Scope and Purpose

For optimal sequencing, adapter-bound template molecules to be sequenced need to reach the nanopore with enough energy to allow sequencing to proceed. The sequencing process involves the ratcheting of a nucleic acid through a helicase motor protein, an ATP-dependant process. There is an optimal concentration of adapter-bound template required for sequencing. Too little template means that the pores will not be fully-loaded, which leads to an increased flow of carrier ions through the pores and faster depletion of the flow cell. Too much adapter-bound template will deplete the ATP reserves (ATP is depleted by unbound adapter proteins), leading to a reduction in the sequencing capacity of the flow cell. Good input quality is essential for good sequencing: pores and adapters can be easily damaged by some commonly-used chemicals (e.g. phenol, ethanol), and the ionic concentration of the solution should be maintained at an optimal level for sequencing.

Potential Hazards

Nanopore flow cells contain small volumes (<5ml) of a solution containing <10% Potassium Ferrocyanide (CAS#: 14459-95-1; EC#: 237-722-2). Due to its small quantity, it is unlikely to be considered a significant hazard, as long as standard laboratory practises are observed. For more details about this chemical, and information about management / disposal, please refer to the Flow Cell Wash Kit Safety Data Sheet (docs/MSDS-EXP-WSH003_Wash_Kit.pdf).

Safety / Procedural Precautions

- The flow cell matrix (containing an array of pores) must be covered by buffer at all times. It is extremely important to avoid the introduction of bubbles into the flow cell, and especially important to avoid the *movement* of bubbles within the flow cell matrix area. Removing more than 20-30µl from a flow cell with an empty waste channel risks a loss of sequencing yield.
- Avoid fast pipetting, and stop pipetting if flow stops, or moves in an unexpected direction.
- Where there is a high risk of introducing bubbles, use the dial technique to give more control over pipetted volume.
- The method of mixing reagents depends on their composition. Take extra care to mix only via the prescribed method (by pipetting, vortexing, or flicking).
- Never let the waste channel of the flow cells overflow. Prior to loading, use a pipette to aspirate waste liquid from the waste channel, and dispose of in the appropriate container.
- After a sequencing run has finished, tape up the waste channels to prevent fluid leakage during movement.

Procedure

This proceedure assumes a starting input of a prepared DNA sequencing library, i.e. the end-product from one of the Main Sequencing Protocols (http://10.0.0.15/HGCC-SOP/_site/#main-sequencing-protocols). This should be an adapted DNA or RNA library of approximately 100 fmol (for MinION flow cells), or 50 fmol (for Flongle flow cells). This procedure also assumes that the Flow Cell performance has been evaluated in MinKNOW *before* loading the library (see the SOP for Flow Cell QC with MinKNOW (flow-cell-QC.html)).

Equipment & Consumables (ONT)

- MinION
- Flongle device - flow cell and adapter
- Flow Cell Flush (FCF), thawed at RT, then stored on ice [117 µl]
- Flow Cell Tether (FCT), thawed at RT*, then stored on ice [3 µl]
- Sequencing Buffer (SB), thawed at RT, then stored on ice [15 µl]
- Library Beads (LB), thawed at RT, then stored on ice [10 µl]

* Room Temperature (RT) - 18-23°C

Equipment & Consumables (Other Suppliers)

- P200 pipette and tips
- 2 x 1.5 ml tube
- 5 µl DNA library (from previous sequencing protocols)

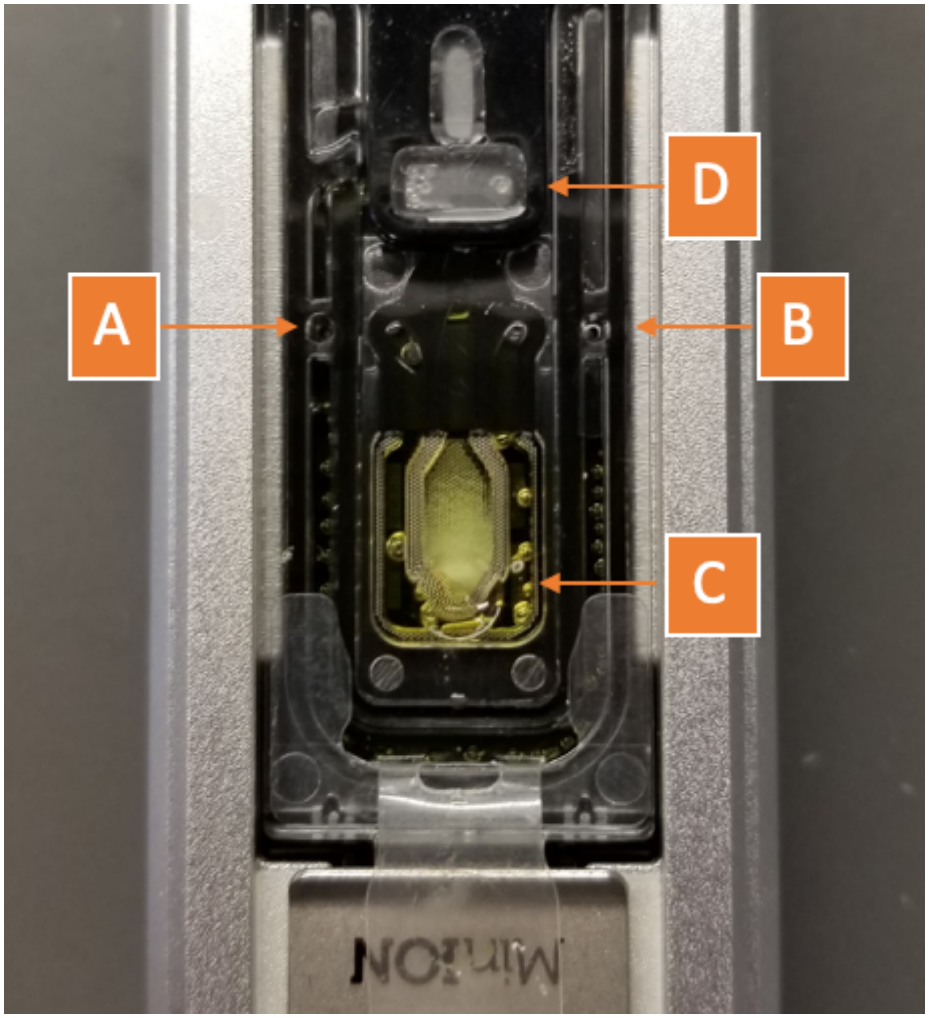
Flow Cell QC

1. Place the Flongle adapter into the MinION Mk1B.
2. Place the flow cell into the Flongle adapter, clip side first, and press the button side of the flow cell down until you hear a click.
3. Run a flow cell QC check (prior to any seal removal or loading).

Buffer preparation

1. Mix the Flow Cell Flush (FCF) and Flow Cell Tether (FCT) tubes **by vortexing**, then spin down.
2. In a new 1.5 ml tube, mix 117 µl of Flow Cell Flush (FCF) with 3 µl of Flow Cell Tether (FCT) and mix by pipetting. This is the flow cell priming mix.

Priming The Flow Cell



Flongle loading diagram - from Graham Wiley's ONT community post

1. Carefully remove the Flongle port sealing tape by lifting at the arrows, then pulling firmly and slowly towards the hinge on the MinION device. Pull until the tape releases from the end of the fluid-filled area - there is a small semicircular indentation in the tape at this point. Stick the dots of the sealing label onto the lid of the MinION device (e.g. the silver sticker).

2. Cover the rectangular waste port (B; indicated on the sticker with a rectangle) with a small piece of lab tape, pressing down to seal.

3. Drop 30 µl of flow cell priming mix onto the loading port. The priming mix should form a large dome of fluid over the port. Do not add so much that priming solution flows freely over the top of the flowcell. All that is needed is a small "reservoir" of solution to pull from.

4. If the liquid is draining from the loading port, then there is sufficient liquid flow; you can continue on to the next section ("Preparing Library for Loading"). Otherwise, there is a liquid flow blockage. Continue on with the subsequent steps as needed, until liquid starts to drain from the loading port.

5. Using a P200 set at 30 µl, check for bubbles through the priming mix dome in the loading port by dialing up (see bubble checking steps below).

6. If the liquid is *not* draining from the loading port, insert the P200 tip into the left waste port (A). Use a tip with proportions large enough to seal around the waste port. The pipette should be previously set to 30µl to allow for gentle, wheel-turning aspiration.
7. Turn the pipette volume wheel up to 60µl to start aspiration. After a short period you should begin to see area (D) darken as fluid moves into the waste channel. You should also begin to see the dome of fluid over loading port (C) decrease in size.
8. As soon as you notice liquid moving from the loading port (i.e. the dome gets smaller), stop aspirating with the pipette and let it drain through. Gently remove the pipette by lifting upwards. If there is no fluid movement after aspirating ~30µl, see troubleshooting steps below.

Preparing Library for Loading

1. Mix the Sequencing Buffer (SB) tube **by vortexing**, then spin down.
2. In a new 1.5 ml DNA LoBind tube, prepare the library in the following order, mixing slowly **by pipetting** after each addition:
    a. 15 µl Sequencing Buffer (SB)
    b. 10 µl Library Beads (LB), mixed **by pipetting** immediately before use
    c. 5 µl DNA library

Loading The Prepared Library Into The Flow Cell

1. Remove the tape from (B).
2. Add 30 µl remaining priming buffer dropwise onto the loading port (C), allowing time for the drop to drain into the flowcell before adding another, similar in method to library Spot-On loading for the MinION flow cell.
3. Add 30 µl prepared library in a similar dropwise fashion.
4. Re-seal the flow cell by rolling a finger across the plastic adhesive cover, trying to avoid putting pressure on the flow cell matrix.

The flow cell is now ready for sequencing. Proceed with the next protocol, Sequencing with MinKNOW (sequencing-with-minknow.html).

Flongle Troubleshooting

Checking for bubbles in the loading port

Note: be careful pressing down on the loading port area. The nanopores are very sensitive to pressure and fast fluid flow near the loading port, and can be easily destroyed.

1. Gently place the pipette tip into into the loading port *through* the dome of liquid over (C) so that it's resting in the middle of the loading port. The tip should "set" into the port, but don't press down (as this could damage the pores).
2. Gently dial the wheel of the pipette up (e.g. from 30µl to 35 µl) to confirm that there is no bubble in the loading port. If there *is* a bubble:
    a. slowly dial up until the entire bubble is sucked up
    b. gently lift the pipette upwards from the loading port
    c. dispense the air from the pipette into a waste container by dialing down
    d. place the pipette back into the loading port, and dial up about 10µl to ensure there is a continuous liquid flow
3. Gently remove the pipette from the loading port by lifting upwards.

Gentle Unblocking From The Waste Port

If the (D) area doesn't darken and/or the dome of fluid over (C) doesn't decrease after ~30ul of pipette aspiration at (A) it is likely that fluid flow has been blocked (e.g. by a sticky valve, or a bubble in the inlet port).

Initially, try the following steps to unblock the fluid flow:

1. Gently remove the pipette by lifting upwards.
2. Lift up and replace the tape at port (B), pressing down to form a firm seal
3. Reset the P200 to 30µl and empty the pipette tip of liquid
4. While the pipette is still in the air, press down on the plunger to expel 30µl air.
5. While still holding the plunger at the resistance point, place the pipette tip into the circular waste port (A).
6. Slowly release the plunger. If liquid starts dropping from the loading port (i.e. the dome gets smaller), stop releasing the plunger and lift up the pipette.
7. If liquid is still not draining from the loading port, repeat these steps (i.e. 30µl of suction) with a faster plunger release speed until liquid starts draining from the loading port.
8. If liquid still doesn't drain from the loading port, try again with 50µl of suction by setting the pipette to 50µl.

9. If liquid still doesn't drain from the loading port, follow the forced unblocking method below.

Forced Unblocking From The Loading Port

If the above steps still don't unblock the fluid flow, then use the pipette dialing method to pipette liquid through a dome of priming liquid. There should be a dome of priming solution over the port that is not getting smaller; this indicates that liquid is not draining through the flow cell.

Because the pipetting is happening very close to the flow cell matrix, these steps are likely to destroy pores, and should only be used as a last resort to save an otherwise useless flow cell. Only proceed with these steps if the dome is still there, and stop as soon as liquid starts to flow:

1. Using a P200 set at 30µl, check for bubbles through the priming mix dome in the loading port by dialing up (see bubble checking steps above).
2. Gently remove the pipette by lifting upwards.
3. Set the pipette to 30µl.
4. Place the plunger into the priming mix tube, and draw up 20µl by dialing the pipette up to 50µl.
5. Dial the pipette down until a small drop of solution forms on the end of the tip.
6. Gently place the pipette tip into into the loading port *through* the dome of liquid over (C) so that it's resting in the middle of the loading port. The tip should "set" into the port, but don't press down (as this could damage the pores).
7. Slowly dial the pipette up to ensure that there is a continuous liquid flow through the pipette (e.g. from 50µl to 60µl).
8. Slowly dial the pipette down to 30µl (or until the pipette tip is almost empty) to force liquid through the flow cell. Stop as soon as the liquid from the dome starts to flow through.
9. Gently remove the pipette by lifting upwards.