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Flongle DirectRNA Library preparation

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The Nordic Nanopore-S...

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Protocol status: Working
This protocol was used on four different occasions and regularly produced >80,000 reads.

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Abstract

Oxford Nanopore Technologies allows sequencing of native RNA for the first time. Additionally they released tiny devices that democratize sequencing among scientists. However, the smallest sequencing device - Flongle - is so far not officially supported for RNA sequencing experiments.

This protocol provides users with a personally tested framework protocol to use Flongle flow-cells for native RNA sequencing. It is based on the protocol for **ONT RNA library preparation** and changed to fit the volume and input requirements for Flongle.
The here advised workflow will routinely result in 100,000 native RNA reads within 24 hours of sequencing.

Guidelines

One of the main considerations to take for any Nanopore sequencing experiment is that read length affects output quality and quantity. Therefore EVERY experimental step should be reviewed for forces that could generate molecule degradation. Thus we advise against any vortexing and forceful pipetting during the following procedures. Instead, we advise to handle samples with care and mix by tube inversion wherever possible. Keeping samples on ice is not recommended, as it could reduce ligation efficiencies, but could be considered for any short pausing steps.

The actual Library preparation protocol has **NO safe stopping point.** Thus please make sure you have sufficient time for the final steps of the library.

Oxford Nanopore library preparation is based on the ligation of a bridge adapter specific to the poly(A) tail, and the subsequent addition of a Motor Protein adapter based on sequence complementarity to the first adapter. The efficiency of library preparation thus solely depends on the efficiency of DNA-RNA ligation procedures. Any contaminant that reduces ligation efficiency will impact the final library performance.
Additionally, any RNA species without poly(A) tails that could interfere with the ligation (unspecific binding) have an effect on ligation efficiency. It is thus important to follow the recommendations given in the Nanopore protocols (nanoporetech.com) for RNA quality and quantity measures.
Finally, it is crucial to proceed quickly from the final ligation to actual sequencing and avoid harsh chemicals and temperatures with that library, as an active protein is added whose function is essential for sequencing.

Materials

MATERIALS

- NEBNext Quick Ligation Module - 20 rxns **New England Biolabs** **Catalog #**E6056S
- Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32851
- Agencourt RNAClean XP Beads **Beckman Coulter** **Catalog #**A63987
- PCR Machine
- 95% EtOH
- Nuclease-free water **Thermo Fisher Scientific** **Catalog #**R0581
- Centrifuge 5424 R refrigerated with Rotor FA-45-24-11 rotary knobs 120 V/50–60 Hz (US) **Eppendorf** **Catalog #**5404000537
- Qubit Fluorometer **Life Technologies** **Catalog #**Q33216
- DNA LoBind Tubes, 1.5 mL **Eppendorf** **Catalog #**0030108051
- Qubit RNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32852
- Qubit assay tubes **Thermo Fisher Scientific** **Catalog #**Q32856
- DynaMag™-2 Magnet **Thermo Fisher** **Catalog #**12321D
- HulaMixer™ Sample Mixer **Thermo Fisher** **Catalog #**15920D
- SuperScript™ III Reverse Transcriptase **Thermo Fisher** **Catalog #**18080044
- Thin-walled, frosted lid, RNase-free PCR tubes (0.2 mL) **Thermo Fisher** **Catalog #**AM12225
- dNTP Mix (10 mM each) **Thermo Fisher** **Catalog #**R0191
- Direct RNA Sequencing kit (SQK-RNA002) **Oxford Nanopore Technologies** **Catalog #**SQK-RNA002
- Flow Cell Priming Kit (EXP-FLP002) **Oxford Nanopore Technologies** **Catalog #**EXP-FLP002
- MinION sequencer **Oxford Nanopore Technologies**
- ONT Flongle Flow-Cell **Oxford Nanopore Technologies** **Catalog #**FLO-FLG001
- ONT Flongle adapter **Oxford Nanopore Technologies** **Catalog #**FLGIntSP

The specific enzymes recommended for use in the library preparation are under constant review by Oxford Nanopore Technologies. Please visit the company's website and protocols for possible updates on performance-enhancing chemistry.

The Flow Cell Priming Kit (EXP-FLP002 in this instance) is usually a component of the Library preparation kit and does not have to be ordered extra.

Troubleshooting

Before start

This protocol is based on the "Direct RNA sequencing (SQK-RNA002)" protocol from Oxford Nanopore Technologies. The protocol is available for Community members **here**.
Please check for updates on these protocols, and check your RNA kit availability, as the kit chemistry develops fast. A new version of this kit (SQK-RNA003) is already inbuilt in the recent MinKNOW update. However, the comments and recommendations for basic incubation steps in this protocol will be valid for upcoming versions as well.

RNA should be extracted as fresh as possible, or alternatively stored at -80°C in RNA storage medium (TRI reagent or RNALater). The sample size should be chosen big enough to yield the required amount of poly(A)-selected RNA - currently 200ng. As mRNA is routinely only 1-2% of total RNA, it should be aimed for extracting 10ug of total RNA from the sample. A full workflow including RNA extraction and poly(A) enrichment can be found **here**.

General idea

- Volumes for RTA and RMX were reduced to fit the Flongle input requirements (1/2 and 1/3 respectively.
- The volumes for the initial RTA ligation and Reverse transcription were not changed, to avoid pipetting of too small volumes.
- The volume of the RMX adapter ligation was reduced to 1/2 to account for smaller amount of RNA and save reagent costs.
- The volumes of the final library mix was adjusted to fit the volumes recommended in other Flongle sequencing protocols.

Flongle native RNA library preparation (SQK-RNA002)

1 Take 200 ng poly(A)-selected RNA into a 0.2ml thin-walled DNA-free PCR tube and bring volume to 9.5 µL with RNase-free water

Note

The following description of Nanopore Library preparation is based on the protocols and consumable recommendations available at the date of publication (product version SQK-RNA002). However, experience has shown that Oxford Nanopore regularly updates protocols and the associated reagents to increase performance. Please check the current version of protocols at nanoporetech.com

Note

If your RNA concentration is too low and upconcentration is necessary, use RNAClean XP bead procedures to increase the concentration of your RNA.

2 Add the following reagents and carefully mix by pipetting:

- 0.5 µL Nanopore RT adapter (RTA)
- 3 µL of NEBNext Quick Ligation buffer
- 1.5 µL T4 DNA Ligase ([M] 2000 U/ul same as Quick T4 Ligase)

3 (optionally) add 0.5 µL RNA CS from the Nanopore kit to monitor sequencing quality

4 Incubate for 00:15:00 at Room temperature 15m

Note

Longer time can increase ligation efficiency, yet increase the chance of further RNA degradation

5 Meanwhile, mix the following ingredients for a reverse-transcription Master Mix from SuperScript III kit:

- 9 µL RNase-free water
- 2 µL [M] 10 Molarity (M) dNTPs
- 8 µL First-Strand RT Buffer
- 4 µL [M] 0.1 Molarity (M) DTT

Note

The following reverse transcription reaction is optional to remove secondary structures from RNA and increase RNA stability (in an RNA-DNA hybrid). Yet it may not be necessary for sequencing performance and can be omitted if wished. If these steps are omitted, the volume of RNAclean XP beads in step 60 have to be adjusted to 27ul

6 After RNA incubation, add the Master Mix to the RNA sample and mix by careful pipetting

7 Add 2 µL SuperScript III RT enzyme and mix by careful pipetting

8 In a thermocycler, incubate at 50 °C for 00:50:00 , 70 °C for 00:10:00 and finally bring to 4 °C 1h

Note

The incubation times can be reduced upon experience, as reverse transcription is optional and these incubation times are for most complete reverse transcription

9 Transfer whole volume into a fresh Eppendorff 1.5ml Lo-Bind safe-lock tube

Note

It is **extremely important** to work with the recommended DNA LoBind 1.5ml Eppendorff tubes. A series of experiments has shown that unknown plastic components from other tube do not only reduce the efficiency of DNA recovery, but also severely disturb the final sequencing chemistry, resulting in poor sequencing performance!

10 Add 72 µL RNAClean XP beads and resuspend by careful pipetting

11 Incubate at Room temperature under constant agitation for 00:10:00 10m

Note

Every incubation step for purification in *this* protocol is slightly longer as recommended in Nanopore protocols. This is to increase efficiency of the reaction while at the same time minimizing RNA degradation. Shorter times might give more contiguous RNA reads at the expense of RNA quantity and thus library performance efficiency.

12 Pellet beads on a magnetic stand and aspirate supernatant

13 Wash the beads on the magnet with 200 µL fresh [M] 70 % volume EtOH without resuspending the beads. Instead, turn the tube quickly by 180°C to let the magnets float through the EtOH

14 Aspirate EtOH, spin down briefly on tabletop centrifuge and remove residual EtOH

15 Resuspend beads with 10 µL RNase-free water by tapping the tube

16 Incubate 00:10:00 at Room temperature 10m

17 Pellet beads on the magnetic stand and transfer to a new 1.5ml Lo-bind Safe-lock tube

18 Add the following reagents for Sequencing adapter ligation:
- 4 µL NEBNext Quick Ligation buffer
- 2 µL Nanopore RNA Adapter Mix (RMX)
- 2.5 µL RNase-free water
- 1.5 µL T4 DNA Ligase ([M] 2000 U/ul same as Quick T4 Ligase)

19 Carefully mix by pipetting and incubate 00:15:00 at Room temperature 15m

20 Add 20 µL RNAClean XP beads and resuspend by careful pipetting

21 Incubate at Room temperature under constant agitation for 00:10:00 10m

22 Pellet beads on a magnetic stand and aspirate supernatant

23 Wash the beads on the magnet with 100 µL Nanopore Wash Buffer (WSB) by resuspending the beads by tube-flicking

24 Aspirate Wash Buffer and repeat washing go to step #23

25 Aspirate Wash Buffer, spin down briefly on tabletop centrifuge and remove residual liquid

26 Resuspend beads with 9 µL Elution buffer water by tapping the tube

27 Incubate 00:10:00 at Room temperature 10m

28 Use the time to quality-control the Flongle Flow Cell (Insert Flongle adapter and Flow Cell in the MinION sequencer, and run the "Test Flow Cell" program in MinKNOW).

29 Pellet beads on the magnetic stand and transfer to a new 1.5ml Lo-bind Safe-lock tube

30 Use 1 µL to quantify final library on Qubit DNA HS Kit

Note

Use the RNA HS kit if you omitted the cDNA synthesis, as the DNA kit is sensitive to double-stranded nucleotide sequences only. Recovery aim is around 80ng

31 To the recovered 8 µL RNA library, add 7 µL RNase-free water and 15 µL Nanopore Sequencing Buffer (RRB), mix by careful pipetting

32 Mix 117 µL Flush Buffer (FLB) and 3 µL Flush Tether (FLT) in a new 1.5ml Lo-bind Safe-lock tube

33 Prime the Flongle Flow cell with the 120 µL Flush Buffer mix (a detailed description including video documentation can be found **here**)

Note

Most important during Priming and loading is to not use any force when applying reagents, and to avoid introduction of air bubbles. Both physical force and air bubble introduction can rupture sequencing arrays and clog essential microfluidic valves, which make later use of flow cells impossible.

Note

If unsure, dispense volume by turning the adjustment wheel of your pipet instead of dispensing as usual

34 Immediately after priming, add the 30ul Library mix carefully in the Flongle Flow Cell

Note

If unsure, dispense volume by turning the adjustment wheel of your pipet instead of dispensing as usual

35 Start sequencing on MinKNOW (at least version 19.12.5) by choosing Flongle as Flow-cell and RNA-002 as kit. (Other options depending on preference for data output; external Link: **Start Sequencing**)