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ONT DirectRNA Library preparation for poly(A) estimation

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We use this protocol in our group and it is working.

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Abstract

This protocol provides a detailed explanation of of the steps necessary for successful Direct RNA Library preparation for Oxford Nanopore Sequencing. The protocol explains the steps needed for RNA sample preparation based on TRIzol extraction and Poly(A)Purist Mag kit enrichment prior to Direct RNA library preparation protocol. The library preparation protocol is based on the Library preparation protocols for RNA-002 kits, yet offers additional advice on what we think is important for a successful library with minimal RNA degradation.

The protocol is used to assess poly(A) tail length using the *tailfindr* package. The poly(A) tail is a homopolymeric stretch of adenosine at the 3`-end of mature RNA transcripts and its length plays an important role in nuclear export, stability, and translational regulation of mRNA. With the introduction of native RNA sequencing by Oxford Nanopore Technologies (ONT), it is now possible to sequence full-length native RNA. A single long read contains both the transcript and the associated poly(A) tail, thereby making genome-wide transcript-specific poly(A) tail length assessment in native RNA feasible. For more information on *tailfindr* visit the **publication** or **the GitHub repository**

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
- 2-Propanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**190764
- 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
- Chloroform **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**372978
- TRIzol™ LS Reagent **Thermo Fisher** **Catalog #**10296028
- 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
- Poly(A)Purist™ MAG Kit **Thermo Fisher** **Catalog #**AM1922
- GlycoBlue™ Coprecipitant (15 mg/mL) **Thermo Fisher** **Catalog #**AM9515
- Sodium Acetate (3 M), pH 5.5, RNase-free **Thermo Fisher** **Catalog #**AM9740
- 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 MinION Flow Cell R9.4.1 **Oxford Nanopore Technologies** **Catalog #**FLO-MIN106D

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.

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. 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 500ng. As mRNA is routinely only 1% of total RNA, it should be aimed for extracting 25ug of total RNA from the sample.

Extraction should be chosen to avoid any contaminants, as these could be detrimental to the sequencing chemistry. In our experience, silica-column based purification strategies not only cause RNA degradation by physical force, but also are prone to retain Guanidine-hydrochloride contamination. We thus advise on the use of phenol-chloroform extraction methods, such as the use of TRI reagent. These are more time-consuming, but in our hands yield higher quality RNA with minimal contaminant carry-over.

Poly(A) enrichment (or any small RNA depletion strategy) is necessary to ensure efficient sequencing analysis, as the essential Motor Protein is added to the RNA via poly(A)-guided ligation. Non-poly(A)-containing RNA thus acts as an inert contaminant that affects proper sequencing. We routinely use the Poly(A)Purist MAG Kit, but any other strategies that do not involve vortexing, vigorous pipetting or column-based purification would work as well.

Described below is the full workflow from total RNA to sequencing using TRI reagent and the Poly(A)Purist MAG kit.

After poly(A) RNA enrichment, the Library preparation protocol has **NO safe stopping point.** Thus please make sure you plan with sufficient time for this part of the experiment

RNA extraction and quality control

1 Resuspend and homogenize necessary amount of fresh sample in TRIZol reagent (1ml of TRIZol per 50mg tissue or 3×10^7 cells) in an Eppendorf Safe-Lock 1.5ml tube

> Note
>
> Homogenization should be kept as gentle as possible to avoid RNA molecule degradation. Reduce number of pestle strokes, pipetting, or replace by vigorous shaking.

2 Incubate 00:05:00 at Room temperature, with regular tube inversion 5m

3 Add 200 µL chloroform per 1 mL TRIZOL and shake by tube inversion

4 Incubate 00:05:00 at Room temperature, with regular tube inversion 5m

5 Centrifuge 00:10:00 at 12-15,000g at 4 °C to separate phases 10m

6 Carefully transfer the aqueous phase to a new Eppendorf Safe-lock 1.5ml tube by angling the tube for most efficient transfer

> Note
>
> Care should be taken to avoid any transfer of TRI reagent. A small drop of aqueous phase can be left behind to make sure that the sample is as clean as possible.

7 Add 500 µL chloroform per 1 mL TRIZOL and shake by tube inversion

8 Centrifuge 00:10:00 at 12-15,000g at 4 °C to separate phases

9 Carefully transfer the aqueous phase to a new Eppendorf Safe-lock 1.5ml tube by angling the tube for most efficient transfer

10 Add 1 µL GlycoBlue reagent, 0.1 Vol [M] 3 Molarity (M) NaOAc and 1 Vol Isopropanol and mix by inversion of the tube

By experience, samples with an initial volume of 1ml TRI reagent will need 50 µL [M] 3 Molarity (M) *NaOAc and* 500 µL *Isopropanol*

11 Incubate 01:00:00 at -20 °C for most efficient yields 1h

12 Centrifuge for 00:10:00 at 15-20,000g at 4 °C 10m

Note

Total RNA should form a strong white pellet. Care should be taken to not aspirate the pellet during the following washing steps

13 Aspirate the supernatant without disturbing the RNA pellet

14 Wash the RNA pellet with 1 mL freshly-prepared [M] 75 % volume EtOH

15 Centrifuge for 00:10:00 at 15-20,000g at 4 °C 10m

16 Aspirate supernatant and repeat ethanol wash go to step #13 once

17 Aspirate the supernatant and air-dry the pellet for 00:05:00 can be reduced to 2 min 5m

Note

If necessary, briefly spin down on a tabletop centrifuge to collect remaining EtOH, and pipet off with a 200ul pipet

18 Add 50 µL of RNase-free water and resuspend by tapping the tube or shaking in a thermoshaker at Room temperature

19 Record quantity and quality by Nanodrop measurement and Qubit RNA Broad Range kit. Test RNA integrity by BioAnalyzer RNA chip

Note

All measurements are necessary for Nanopore Experiments.
Nanodrop 260/280 and 260/230 measurements are important to assess possible remnant contaminants that are detrimental to Nanopore's sequencing chemistry.
Qubit measurements offer the most sensitive RNA quantification, and are regularly used during the library preparation protocols.
BioAnalyzer traces yield an RNA integrity measurement that allows to assess the biological quality of the sample

poly(A) enrichment

20 Bring RNA concentration to [M] 600 ng/ul , but minimal 50 µL (30 µg RNA)

Note

Only consider total RNA samples with BioAnalyzer RIN (RNA Integrity number) higher than 9 for further procedures as RNA quality directly affects sequencing quality and quantity

21 Add an equal volume of the Poly(A)Purist Mag Kit 2x Binding Solution (minimal 50 µL) and mix by tube inversion

22 Store RNA On ice until further processing

23 Vortex the Poly(A)Purist Magnetic Bead solution and pipet the necessary amount of beads to a 1.9ml tube provided with the kit. For each 100 µg of total RNA from

above, use 10 µL Magnetic Beads solution and in subsequent washing steps 50 µL Wash Buffer

Note

Never use smaller volumes than 10 ul beads and 50 ul Wash Buffer, as it will reduce efficiency of washes and RNA elution during the protocol

24 Precipitate the beads on a magnetic stand and aspirate the buffer

Note

Beads might take several minutes to fully precipitate. Observe the buffer to check for clarity. Occasional slow rotation of the tubes on the magnetic stand may increase the collection efficiency

25 Take the tube out of the magnetic stand and resuspend the beads in Kit Wash Solution 1 with volume depending on the amount of magnetic beads used. For example,
go to step #23

26 Repeat once from go to step #24

27 Add the total RNA sample + Binding Solution from go to step #22 to the beads, mix by tube inversion

28 Heat the bead-RNA mixture to 65 °C for 00:05:00 5m

Note

Longer time and higher temperatures are not advised, to avoid additional RNA degradation

29 Incubate 00:30:00 at Room temperature under constant agitation 30m

Note

Longer incubation time to up to 1h is possible, but increases the chance of RNA degradation

30 Meanwhile preheat the Kit THE elution buffer to 70 °C

31 Precipitate the magnetic beads with the RNA attached on the magnetic stand and aspirate supernatant

32 Take the tube out of the magnetic stand and resuspend the beads in Kit **Wash Solution 1** with volume depending on go to step #23

33 Repeat once from go to step #31

34 Take the tube out of the magnetic stand and resuspend the beads in Kit **Wash Solution 2** with volume depending on go to step #23

35 Precipitate beads on the magnetic stand and aspirate the supernatant

36 Repeat o go to step #34

37 Briefly spin down on a tabletop centrifuge to remove residual Wash Solution

38 Remove the tube from the magnetic stand and resuspend in 100 µL hot THE buffer from go to step #30

39 Incubate 00:01:00 can be extended to 2 min at 70 °C 2m

Note

This additional incubation is to make sure that elution efficiency is as high as possible. However, heat treatment of RNA should be kept short to avoid RNA degradation.

40 Capture the magnetic beads on the magnetic stand, and transfer the supernatant into a clean Eppendorf 1.5ml Safe-lock tube and store on ice

41 Repeat from go to step #38 and pool the supernatants into one tube

42 Put the fresh sample tube on a magnet again to collect residual beads for 00:03:00 3m

On ice

43 Transfer the cleaned supernatant into a fresh Eppendorf 1.5ml tube

44 Add 1 µL GlycoBlue reagent, 20 µL [M] 3 Molarity (M) NaAc and 250 µL Isopropanol, mix by inversion

45 Store 01:00:00 at -20 °C 1h

46 Centrifuge for 00:10:00 at 15-20,000g at 4 °C 10m

Note

poly(A)-selected RNA should form a small white pellet, with blue coloring from the coprecipitant. Care should be taken to not aspirate the pellet during the following washing steps. If a small brown coloring is observed, it is residual magnetic beads that should not affect downstream processes.

47 Aspirate the supernatant without disturbing the RNA pellet

48 Wash the RNA pellet with 1 mL freshly-prepared [M] 75 % volume EtOH

49 Centrifuge for 00:10:00 at 15-20,000g at 4 °C 10m

50 Repeat once from go to step #48

51 Aspirate the supernatant and air-dry the pellet for 00:02:00 2m

Note

If necessary, briefly spin down on a tabletop centrifuge to collect remaining EtOH, and pipet off with a 200ul pipet

52 Add 15 µL of RNase-free water and resuspend by tapping the tube or shaking in a thermoshaker at 25 °C .

53 Record quantity and quality by Nanodrop measurement and Qubit RNA Broad Range kit. Test rRNA removal by BioAnalyzer RNA chip.

Note

All measurements are necessary for Nanopore Experiments.
Nanodrop 260/280 and 260/230 measurements are important to assess possible remnant contaminants that are detrimental to Nanopores sequencing chemistry.
Qubit measurements offer the most sensitive RNA quantification, and are regularly used during the library preparation protocols.
BioAnalyzer traces at this step will provide an estimate for effective rRNA removal. RIN numbers should be low, as RIN is calculated based on rRNA peaks. The length distribution should give an estimate of which average read length can be expected from Nanopore sequencing.

Nanopore Direct RNA library preparation (SQK-RNA002)

54 Take 500 ng poly(A)-selected RNA into a 0.2ml thin-walled DNA-free PCR tube and bring volume to 9 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.

55 Add the following reagents and carefully mix by pipetting:

- 1 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)

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

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

Note

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

58 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

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

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

61 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

62 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!

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

64 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.

65 Pellet beads on a magnetic stand and aspirate supernatant

66 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

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

68 Resuspend beads with 20 µL RNase-free water by tapping the tube

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

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

71 Add the following reagents for Sequencing adapter ligation:

- 8 µL NEBNext Quick Ligation buffer
- 6 µL Nanopore RNA Adapter Mix (RMX)
- 3 µL RNase-free water
- 3 µL T4 DNA Ligase ([M] 2000 U/ul same as Quick T4 Ligase)

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

73 Add 40 µL RNAClean XP beads and resuspend by careful pipetting

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

75 Pellet beads on a magnetic stand and aspirate supernatant

76 Wash the beads on the magnet with 150 µL Nanopore Wash Buffer (WSB) by resuspending the beads by tube-flicking

77 Aspirate Wash Buffer and repeat washing go to step #76

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

79 Resuspend beads with 21 µL Elution buffer water by tapping the tube

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

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

82 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

83 Add 17.5 µL RNase-free water and 37.5 µL Nanopore Sequencing Buffer (RRB) to the library

84 Prime a MinION flow cell as specified in Nanopore protocols, and finally load the library drop-wise through the **Sample port** (a detailed description including video documentation can be found here: **Flow Cell Priming**)

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

Library loading by drop-wise application should neither be too slow nor too fast. Too slow loading yields to poor sequencing array coverage, while too fast loading might flush out RNA from the array into the waste sink.

85 Sequence under the settings recommended for your flow cell (depending on prior use, storage, and kit components; external Link: **Start Sequencing**)