Nov 17, 2019

ONT DirectRNA Library preparation for poly(A) estimation

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1 Works for me dx.doi.org/10.17504/protocols.io.9cjh2un

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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

EXTERNAL LINK

http://doi:10.1261/rna.071332.119

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Krause M, Niazi AM, Labun K, Torres Cleuren YN, Müller FS, Valen E.tailfindr: alignment-free poly(A) length measurement for Oxford Nanopore RNA and DNA sequencing.RNA. 2019;25(10):1229–1241. doi:10.1261/rna.071332.119

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

NAME	CATALOG #	VENDOR
NEBNext Quick Ligation Module - 20 rxns	E6056S	New England Biolabs
Qubit dsDNA HS Assay Kit	Q32851	Thermo Fisher Scientific
Agencourt RNAClean XP Beads	A63987	Beckman Coulter
PCR Machine	View	
95% EtOH		
Nuclease-free water	R0581	Thermo Fisher Scientific
Centrifuge 5424 R refrigerated with Rotor FA-45-24-11 rotary knobs 120 V/50 – 60 Hz (US)	5404000537	Eppendorf Centrifuge
Qubit Fluorometer	Q33216	Life Technologies
2-Propanol	190764	Sigma Aldrich
DNA LoBind Tubes, 1.5 mL	0030108051	Eppendorf
Qubit RNA HS Assay Kit	Q32852	Thermo Fisher Scientific
Qubit assay tubes	Q32856	Thermo Fisher Scientific
Chloroform	372978	Sigma Aldrich
TRIzol™ LS Reagent	10296028	Thermo Fisher
DynaMag™-2 Magnet	12321D	Thermo Fisher
HulaMixer™ Sample Mixer	15920D	Thermo Fisher
SuperScript™ III Reverse Transcriptase	18080044	Thermo Fisher
Thin-walled, frosted lid, RNase-free PCR tubes (0.2 mL)	AM12225	Thermo Fisher
Poly(A)Purist™ MAG Kit	AM1922	Thermo Fisher
GlycoBlue™ Coprecipitant (15 mg/mL)	AM9515	Thermo Fisher
Sodium Acetate (3 M), pH 5.5, RNase-free	AM9740	Thermo Fisher
dNTP Mix (10 mM each)	R0191	Thermo Fisher
Direct RNA Sequencing kit (SQK-RNA002)	SQK-RNA002	Oxford Nanopore Technologies
Flow Cell Priming Kit (EXP-FLP002)	EXP-FLP002	Oxford Nanopore Technologies
MinION sequencer	View	Oxford Nanopore Technologies
ONT MinION Flow Cell R9.4.1	FLO-MIN106D	Oxford Nanopore Technologies

MATERIALS TEXT

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 STARTING

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 3x10^7 cells) in an Eppendorf Safe-Lock 1.5ml tube

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

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** **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** **3 Molarity (M)** *NaOAc and* **500 µl** *Isopropanol*

11 1h

Incubate **01:00:00** at **-20 °C** for most efficient yields

12 10m

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

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 **75 % volume** EtOH

15 10m

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

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

17 5m

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

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

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)

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

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

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

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

29 ⚠ 30m

Incubate **00:30:00** at **Room temperature** under constant agitation

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 2m

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

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 3m

Put the fresh sample tube on a magnet again to collect residual beads for 00:03:00 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 1h

Store 01:00:00 at -20 °C

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

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 **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

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.

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

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

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

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** **10 Molarity (m)** dNTPs
- **8 µl** First-Strand RT Buffer
- **4 µl** **0.1 Molarity (M)** DTT

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 1h

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

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

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 ⚠ 10m

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

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

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)

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.

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)