Feb 09, 2021

RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing

Eric RA Pederson[1]

[1]Uppsala University

1 Works for me dx.doi.org/10.17504/protocols.io.bj7tkrnn

methods

Eric Pederson
Uppsala University

SUBMIT TO PLOS ONE

ABSTRACT

RNA extraction of extraction using the Qiagen Allprep RNA extraction kit followed by nanopore sequencing on the MinION.

DOI

dx.doi.org/10.17504/protocols.io.bj7tkrnn

PROTOCOL CITATION

Eric RA Pederson 2021. RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . **protocols.io** https://dx.doi.org/10.17504/protocols.io.bj7tkrnn

CREATED

Aug 23, 2020

LAST MODIFIED

Feb 09, 2021

PROTOCOL INTEGER ID

40915

MATERIALS TEXT

MATERIALS

b-mercaptoethanol **Sigma-aldrich Catalog #M3148-25ML** Step 5

Zirconium Oxide Beads 2.0 mm RNase Free 4 mL **Contributed by users Catalog #ZROB20-RNA** Step 3

- Forceps

RNaseZap® **Thermo**

- **Scientific Catalog #AM9780** In 2 steps

Qiagen AllPrep DNA/RNA Mini
- Kit **Qiagen Catalog #80204** In 2 steps

Flow Cell Priming Kit **Oxford Nanopore**
- **Technologies Catalog #EXP-FLP002** Step 54

DNase I (RNase-free) - 1,000 units **New England**
- **Biolabs Catalog #M0303S** Step 9
- Nuclease-free water

cDNA-PCR Sequencing (SQK-PCS109) **Oxford Nanopore**
- **Technologies Catalog #SQK-PCS109** Step 36

Prime STAR GXL DNA
- polymerase **Takara Catalog #R050B** Step 46.1

NEBNext Ultra II DNA Library Prep Kit for
- Illumina **NEB Catalog #E7645S** Step 48.1

T4 RNA Ligase 2 - 750 units **New England**
- **Biolabs Catalog #M0239L** Step 50.1

PCR barcoding expansion 96 **Oxford Nanopore**
- **Technologies Catalog #EXP-PBC096** Step 46.1

Flow Cell (R9) **Oxford Nanopore**
- **Technologies Catalog #FLO-MIN106D** Step 54

HALT Protease and Phosphatase Inhibitor
- Cocktail **Abcam Catalog #78440** Step 11.1

Qubit 2.0 Fluorometer instrument Q33226
with Qubit RNA HS Assays

4200 TapeStation System
Electrophoresis tool for DNA and RNA sample quality control.
TapeStation Instruments G2991AA

SAFETY WARNINGS

b-mercaptoethanol is dangerous to work with
Always use gloves and a flow hood

BEFORE STARTING

See pre-experiment steps

Citation: Eric RA Pederson (02/09/2021). RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . https://dx.doi.org/10.17504/protocols.io.bj7tkrnn

Pre-experiment

1 Book RNA extraction room

2 RNaseZap® **Thermo Scientific Catalog #AM9780**

Spray with ETOH and then in the fume hood

3 Prepare labelled 2 ml low bind tubes with 4 Zirconium Oxide Beads 2.0 mm RNase Free 4 mL beads in each

Zirconium Oxide Beads 2.0 mm RNase Free 4 mL **Contributed by users Catalog #ZROB20-RNA**

4 Make 3 1.5 ml eppendorf tubes for each sample; 1 with sterile water, 1 with 99% ETOH and 1 with RNaseZap® **Thermo Scientific Catalog #AM9780**

5 Qiagen AllPrep DNA/RNA Mini

From the Kit **Qiagen Catalog #80204**

Add 1 ml buffer RLT to 10 ul b-mercaptoethanol

Each sample requires 600 ul of buffer RLT + b-mercaptoethanol

So for example for 6 samples one would need 3.6 ml of the buffer RLT + b-mercaptoethanol mix. Thus, one would need to prepare 4 ml buffer RLT + 40 ul b-mercaptoethanol.

b-mercaptoethanol **Sigma-aldrich Catalog #M3148-25ML**

6 Buffer AW1 is supplied as a concentrate. Ensure that ethanol is added to Buffer AW1 before use

Buffer AW2 is supplied as a concentrate. Ensure that ethanol is added to Buffer AW2 before use

7 Label all the spin columns and the collection tubes properly

8 Turn on centrfuge to 4°.

9 DNase I (RNase-free) - 1,000 units **New England Biolabs Catalog #M0303S**

From the

Make DNase solution.

Citation: Eric RA Pederson (02/09/2021). RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . https://dx.doi.org/10.17504/protocols.io.bj7tkrnn

9.1 Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex.

9.2 For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20oC for up to 9 months. Thawed aliquots can be stored at 2–8oC for up to 6 weeks. Do not refreeze the aliquots after thawing.

Tissue destruction

10 Add to the 2.0 ml eppidendorf tube that already has the beads (Zirconium Oxide Beads 2.0 mm RNase Free 4 mL) with 600 ul RLT buffer and b-mercaptoethanol mixture.

11 Get tissue from the -80 freezer and place on dry ice

11.1 Materials to take to the basement -80° freezer;

- gloves
- white warm gloves
- dry ice
- blades
- wipes
- forceps
- ethanol spray
- petri-dishes (tissue type written on the lid)
- marker
- paper tape
- normal ice
- tubes with Beads, PBS and ⊠HALT Protease and Phosphatase Inhibitor Cocktail **Abcam Catalog #78440** on ice

12 In the most sterile way possible, cut a small piece of tissue, half a pea size or so, and put it into one of the labelled 2ml tubes with Beads and the RLT mixture on ice

Make sure to use the ethanol to clean everything and not to cut yourself.

Use the forceps to grab the tissue that flies away.

13 Use the bullet blender to destroy the tissue (Tape on lids)

- 3 minutes
- speed setting 8

14 Centrifuge the lysate for 3 min at maximum speed.

Citation: Eric RA Pederson (02/09/2021). RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . https://dx.doi.org/10.17504/protocols.io.bj7tkrnn

15 Qiagen AllPrep DNA/RNA Mini

From the Kit **Qiagen Catalog #80204**

Carefully remove the supernatant by pipetting, and transfer it to the AllPrep DNA spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 30 s at ≥ 8000 x g (≥10,000 rpm).

16 Place the AllPrep DNA spin column in a new 2 ml collection tube (supplied), and store at room temperature (15–25oC) or at 4oC for later DNA purification in steps 14–17. Use the flow-through for RNA purification in the following steps

RNA extraction

17 Add 1 volume (usually 600 µl) of 70% ethanol to the flow- through from step 5, and mix well by pipetting. Do not centrifuge.

18 Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥ 8000 x g (≥ 10,000 rpm). Discard the flowthrough.

19 Add 350 µl Buffer RW1 to the RNeasy spin column, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.*
Reuse the collection tube in step E4.

20 Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.

Buffer RDD is supplied with the RNase-Free DNase Set.

Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

21 Add the DNase I incubation mix (80 µl) directly to the RNeasy spin column membrane, and incubate at room temperature (20–30oC) for 15 min.

Note: Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

22 Add 350 µl Buffer RW1 to the RNeasy spin column, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.

Continue with step 9 (22) of the protocol on page 26 (i.e., the first wash with Buffer RPE).

Reuse the collection tube in step 9 (22).

Citation: Eric RA Pederson (02/09/2021). RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . https://dx.doi.org/10.17504/protocols.io.bj7tkrnn

23 Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥ 8000 x g (≥ 10,000 rpm) to wash the spin column membrane. Discard the flow-through.

24 Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥ 8000 x g (≥ 10,000 rpm) to wash the spin column membrane

25 Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Centrifuge at full speed for 1 min.

26 Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥ 8000 x g (≥10,000 rpm) to elute the RNA.

27 If the expected RNA yield is > 30 µg, repeat the previous step using another 30 µl of RNase-free water, or using the eluate from step 12 (if high RNA concentration is required). Reuse the collection tube from the previous step.

28 Run the tapestation and the nanodrop on the RNA.

29 Store the RNA in the -80 freezer for long term storage, or leave on ice until after the DNA extractions to preform the cDNA synthesis

DNA extraction

30 Add 500 µl Buffer AW1 to the AllPrep DNA spin column from before the RNA extraction. Close the lid gently, and centrifuge for 15 s at ≥ 8000 x g (10,000 rpm). Discard the flow-through.

Citation: Eric RA Pederson (02/09/2021). RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . https://dx.doi.org/10.17504/protocols.io.bj7tkrnn

31 Add 500 µl Buffer AW2 to the AllPrep DNA spin column. Close the lid gently, and centrifuge for 2 min at full speed to wash the spin column membrane.

32 Place the AllPrep DNA spin column in a new 1.5 ml collection tube (supplied). Add 100 µl Buffer EB directly to the spin column membrane and close the lid. Incubate at room temperature (15–25oC) for 1 min, and then centrifuge for 1 min at ≥ 8000 x g (10,000 rpm) to elute the DNA.

33 Repeat previous step to elute further DNA.

34 Run the tapestation (genomic) and the Qubit on the DNA.

35 Store the DNA in the -20° or -80° freezer for long term storage.

cDNA synthesis

36 cDNA-PCR Sequencing (SQK-PCS109) **Oxford Nanopore**

From the **Technologies Catalog #SQK-PCS109**

Prepare the following reaction in a 0.2 ml PCR tube

36.1 (X ul) 1 ng PolyA+ RNA (or ~50 ng total RNA)
(1 ul) VN primers (VNP), at 2 uM
(1 ul) 10 mM dNTPs
(9-X ul) RNase-free water
Total volume of 11 ul

37 Mix gently by flicking the tube and spin down

38 Incubate at 65° for 5 minutes and then snap cool on a pre-chilled freezer block

39 In a separate 0.2 ml PCR tube, mix the following reaction

Citation: Eric RA Pederson (02/09/2021). RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . https://dx.doi.org/10.17504/protocols.io.bj7tkrnn

39.1 (4 ul) 5x RT buffer
(1 ul) RNase Out
(1 ul) Nuclease-free water
(2 ul) Strand-Switching Primer (SSP, at 10 uM)

Total vollume of 8 ul

40 Mix gently by flicking the tube and spin down

41 Add the strand-switching buffer to the snap-cooled, annealed mRNA and mix by flicking the tube and spin down

42 Incubate at 42° for 2 minutes

43 Add 1 ul of Maxima H minus Reverse Transciptase.

The total volume should now be 20 ul

44 Mix by flicking the tube and spin down

45 Incubate using the following protocol

45.1

Cycle step	Temp	Time	# of cycles
Reverse Transcription and strand-switching	42°	90 minutes	1
Heat inactivation	85°	5 minutes	1
Hold	4°	∞	-

Adapator extension

46 Add adaptors to the cDNA using PCR

46.1 ☒Prime STAR GXL DNA
From the polymerase **Takara Catalog #R050B**
And
☒PCR barcoding expansion 96 **Oxford Nanopore Technologies Catalog #EXP-PBC096**

Citation: Eric RA Pederson (02/09/2021). RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . https://dx.doi.org/10.17504/protocols.io.bj7tkrnn

Reaction mix;

(10 ul) 5x Prime STAR GXL buffer
(1 ul) 10 mM KAPA dNTP mix
(1 ul) Prime STAR GXL DNA polymerase
(5 ul) cDNA
(1 ul) primer (Barcode mix from nanopore

46.2 PCR program

1 cycle
98° - 30 seconds

10 cycles
98° - 10 seconds
64° - 15 seconds
68° - 10 seconds

1 cycle
68° - 5 minutes

4° - ∞

47 Serapure/Ampure purification

47.1 Bead extraction:

- Add 1.8X beads to samples
- flick to mix
- incubate for 10 minutes at room temperature
- use the magnet to collect the DNA-beads
- discard supernatant
- wash with 70% ethanol
- dry at 37° for 3 minutes
- elute with 20 ul TE buffer
- incubate for 5 minutes at room temperature
- use the magnet to collect the DNA-beads
- collect supernatant into a new tube

Ligation reaction

48 First step

48.1 NEBNext Ultra II DNA Library Prep Kit for

From the Illumina **NEB Catalog #E7645S**

(7 ul) Ultra II End prep reaction buffer (NEB, E7647AA)
(3 ul) Ultra I End prep Enzyme mix (NEB, E7646AA)
(50 ul) cDNA with adapters

Total volume of 60 ul

Citation: Eric RA Pederson (02/09/2021). RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . https://dx.doi.org/10.17504/protocols.io.bj7tkrnn

48.2 PCR program

20° for 5 minutes
65° for 5 minutes

49 Serapure/Ampure purification using a 1:1 mix ration (60:60)

49.1 Bead extraction:

- Add 1.8X beads to samples
- flick to mix
- incubate for 10 minutes at room temperature
- use the magnet to collect the DNA-beads
- discard supernatant
- wash with 70% ethanol
- dry at 37° for 3 minutes
- elute with 20 ul TE buffer
- incubate for 5 minutes at room temperature
- use the magnet to collect the DNA-beads
- collect supernatant into a new tube

50 Ligation reaction

50.1 T4 RNA Ligase 2 - 750 units **New England**

From the **Biolabs Catalog #M0239L**

(60 ul) purified DNA
(25 ul) Ligation buffer
(10 ul) Ligase
(5 ul) adaptor mix

51 Incubate for 10 minutes at room temperature

52 ⚠

Modified Serapure/Ampure bead purification

52.1
- Add 1.8X beads to samples
- flick to mix
- incubate for 10 minutes at room temperature
- use the magnet to collect the DNA-beads
- discard supernatant
- **wash with SFB (250 ul)**
- dry at 37° for 3 minutes
- **elute with 15 ul TE buffer**
- incubate for 5 minutes at room temperature
- use the magnet to collect the DNA-beads

Citation: Eric RA Pederson (02/09/2021). RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . https://dx.doi.org/10.17504/protocols.io.bj7tkrnn

- collect supernatant into a new tube

53 Run Qubit

Loading Nanopore flow cell

54 Flow Cell Priming Kit **Oxford Nanopore**
From the **Technologies Catalog #EXP-FLP002**

Flow Cell (R9) **Oxford Nanopore**
And using a **Technologies Catalog #FLO-MIN106D**

DNA library - 12 ul (of the 14 ul)
Sequencing buffer (SQB) - 37.5 ul
Loading Beads - 25.5 ul

Total volume of 75 ul

55 Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT before placing the tubes on ice as soon as thawing is complete

56 Mix the Sequencing Buffer (SQB) and Flush Buffer (FB) tubes by vortexing, spin down and return to ice.

57 Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice.

58 Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover clockwise so that the priming port is visible.

59 After opening the priming port, check for small bubbles under the cover. Draw back a small volume to remove any bubble (a few µl) as follows:

59.1 Set a P1000 pipette to 200 µl

59.2 Insert the tip into the priming port

59.3 Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip
Care must be taken when drawing back buffer from the flow cell. The array of pores must be covered by buffer at all times. Removing more than 20-30 µl risks a loss of sequencing channels.

59.4 Care must be taken when drawing back buffer from the flow cell. The array of pores must be covered

Citation: Eric RA Pederson (02/09/2021). RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . https://dx.doi.org/10.17504/protocols.io.bj7tkrnn

by buffer at all times. Removing more than 20-30 µl risks a loss of sequencing channels.

60 Prepare the flow cell priming mix as follows:

60.1 Add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by pipetting up and down.

61 Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.

62 Thoroughly mix the contents of the Loading Beads (LB) by pipetting. Note that The Loading Beads (LB) tube contains a suspension of beads.

These beads settle very quickly. It is vital that they are mixed immediately before use.

63 In a new tube, prepare the library for loading as follows:

63.1
- 37.5 µl Sequencing Buffer (SQB)
- 25.5 µl Loading Beads (LB), mixed immediately before use
- 12 µl cDNA library prepared as described in SOP ID:0005

75 ul Total

64 Complete the flow cell priming:

64.1
- Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
- Load 200 µl of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

65 Mix the prepared library gently by pipetting up and down just prior to loading.

66 Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

67 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.

Citation: Eric RA Pederson (02/09/2021). RNA and DNA extraction (Qiagen) of frozen tissue to nanopore sequencing . https://dx.doi.org/10.17504/protocols.io.bj7tkrnn