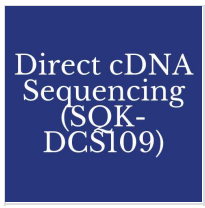

Sep 13, 2022

Direct cDNA Sequencing (SQK-DCS109)

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dx.doi.org/10.17504/protocols.io.yxmvmkpxng3p/v1

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ABSTRACT

This protocol describes how to prepare direct cDNA Sequencing libraries for nanopore sequencing without using PCR.

ATTACHMENTS

_Direct_cDNA_Sequencing_(SQK-DCS109)-promethion.pdf

DOI

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PROTOCOL CITATION

e.gustavsson 2022. Direct cDNA Sequencing (SQK-DCS109). **protocols.io** https://protocols.io/view/direct-cdna-sequencing-sqk-dcs109-bseunbew

KEYWORDS

direct cDNA sequencing, reverse transcription, strand-switching, RNA degradation, second strand synthesis, adapter ligation, AMPure XP bead binding, ASAPCRN

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OWNERSHIP HISTORY

Feb 15, 2021	Emily Hasser	University of Washington
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47284

MATERIALS TEXT

Materials

- 100 ng PolyA+ RNA, or 70-200 ng alreadyprepared cDNA
- Direct cDNA Sequencing Kit (SQK-DCS109)
- Flow Cell Priming Kit (EXP-FLP002)

Consumables

- Agencourt AMPure XP beads
- NEBNext End repair / dA-tailing Module (E7546)
- NEB Blunt/TA Ligase Master Mix (M0367)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Freshly prepared 70% ethanol in nucleasefree water
- 10 mM dNTP solution (e.g. NEB N0447)
- LongAmp Taq 2X Master Mix (e.g. NEB M0287)
- Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0751)
- RNaseOUT™, 40 U/µl (Life Technologies, 10777019)
- RiboShredder (Epicentre, RS12500), or RNase Cocktail Enzyme Mix (ThermoFisher, AM2286)

Equipment

- Hula mixer (gentle rotator mixer)
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- Microfuge
- Vortex mixer
- Thermal cycler
- Ice bucket with ice
- Timer
- Pre-chilled freezer block at -20° C for 200 µl tubes (e.g. Eppendorf 022510509)
- Multichannel pipette capable of 20-200 µl
- Pipettes and pipette tips P2, P10, P20, P100, P200, P1000

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

Reverse transcription and strand-switching

1

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If you have already prepared your cDNA, use 70-200 ng cDNA and start from the End-prep step.

2 Prepare the RNA in Nuclease-free water.

2.1 Transfer **100 ng PolyA+ RNA** into a 1.5 ml Eppendorf DNA LoBind tube.

2.2 Adjust the volume to up to **7.5 µL** with Nuclease-free water.

2.3 

Mix by flicking the tube to avoid unwanted shearing.

2.4 

Spin down briefly in a microfuge.

3 Prepare the following reaction in a 0.2 ml PCR tube:

- x µl poly A+ RNA, 100 ng
- **2.5 µL VNP**
- **1 µL 10 mM dNTPs**
- 7.5-x µl RNase-free water

4 

Mix gently by flicking the tube, and spin down.

5 

5m

Incubate at **65 °C** for **00:05:00** and then snap cool on a pre-chilled freezer block.

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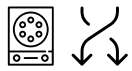
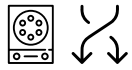
6 In a separate tube, mix together the following:

- **4 µL 5x RT Buffer**
- **1 µL RNaseOUT**
- **1 µL Nuclease-free water**
- **2 µL Strand-Switching Primer (SSP)**

7 

Mix gently by flicking the tube, and spin down.

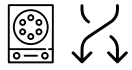
8 

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

9  2m

Incubate at **42 °C** for **00:02:00** .

10 Add **1 µL Maxima H Minus Reverse Transcriptase** . The total volume is now **20 µL** .

11 

Mix gently by flicking the tube, and spin down.

12  1h 35m

Incubate using the following protocol:

- Reverse transcription and strand-switching **01:30:00** @ **42 °C** (1 cycle)
- Heat inactivation **00:05:00** @ **85 °C** (1 cycle)
- Hold @ **4 °C**

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RNA degradation and second strand synthesis

13 Add **1 µL RiboShredder or RNase Cocktail Enzyme Mix (ThermoFisher, AM2286)** to the reverse transcription reaction.

14 10m

Incubate the reaction for **00:10:00** at **37 °C** .

15 Resuspend the AMPure XP beads by vortexing.

16 Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

17

Add **17 µL resuspended AMPure XP beads** to the reaction and mix by flicking the tube.

18 5m

Incubate on a Hula mixer (rotator mixer) for **00:05:00** at **Room temperature** .

19 Prepare **500 µL** fresh **70 %** ethanol in Nuclease-free water.

20

Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

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21

Keep the tube on the magnet and wash the beads with
200 µL freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

22 Repeat the previous step.

23 30s

Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~ **00:00:30** , but do not dry the pellet to the point of cracking.

24 Remove the tube from the magnetic rack and resuspend pellet in
20 µL Nuclease-free water .

25 10m

Incubate on a Hula mixer (rotator mixer) for **00:10:00** at **Room temperature** .

26 Pellet beads on magnet until the eluate is clear and colourless.

27 Remove and retain **20 µL eluate** into a clean 1.5 ml Eppendorf DNA LoBind tube.

28 Prepare the following reaction in a 0.2 ml thin-walled PCR tube:

- **25 µL 2x LongAmp Taq Master Mix**
- **2 µL PR2 Primer (PR2)**
- **20 µL Reverse-transcribed sample from above**
- **3 µL Nuclease-free water**

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29 17m

Incubate using the following protocol:

- **94 °C** **00:01:00** 1
- **50 °C** **00:01:00** 1
- **65 °C** **00:15:00** 1
- **4 °C** ∞

30 Resuspend the AMPure XP beads by vortexing.

31 Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

32

Add **40 µL resuspended AMPure XP beads** to the reaction and mix by flicking the tube.

33 5m

Incubate on a Hula mixer (rotator mixer) for **00:05:00** at **Room temperature** .

34 Prepare **500 µL fresh 70% ethanol in Nuclease-free water** .

35

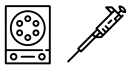
Citation: e.gustavsson Direct cDNA Sequencing (SQK-DCS109) https://dx.doi.org/10.17504/protocols.io.yxmvmkpxng3p/v1

Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

36 

Keep the tube on the magnet and wash the beads with **200 µL freshly prepared 70% ethanol** without disturbing the pellet. Remove the ethanol using a pipette and discard.

37 Repeat the previous step.

38  30s

Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~ **00:00:30** , but do not dry the pellet to the point of cracking.

39 Remove the tube from the magnetic rack and resuspend pellet in **21 µL Nuclease-free water** .

40  10m

Incubate on a Hula mixer (rotator mixer) for **00:10:00** at **Room temperature** .

41 Pellet beads on magnet until the eluate is clear and colourless.

42 Remove and retain **21 µL eluate** into a clean 1.5 ml Eppendorf DNA LoBind tube.

43 

Analyse **1 µL strand-switched DNA** for size, quantity and quality.

Citation: e.gustavsson Direct cDNA Sequencing (SQK-DCS109) https://dx.doi.org/10.17504/protocols.io.yxmvmkpxng3p/v1

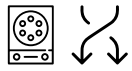
End-prep

44

If you have prepared your own cDNA instead of performing reverse transcription using the Direct cDNA Sequencing Kit, please start this step with 70-200 ng cDNA in 20 µl Nuclease-free water

45 Perform end repair and dA-tailing of fragmented DNA as follows:

- **20 µL cDNA sample**
- **30 µL Nuclease-free water**
- **7 µL Ultra II End-prep reaction buffer**
- **3 µL Ultra II End-prep enzyme mix**

46 

Mix gently by pipetting and spin down.

47 

10m

Using a thermal cycler, incubate at **20 °C** for **00:05:00** and **65 °C** for **00:05:00** .

48 Resuspend the AMPure XP beads by vortexing.

49 Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube.

50 

Add **60 µL resuspended AMPure XP beads** to the end-prep reaction and mix by pipetting.

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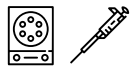
51 5m

Incubate on a Hula mixer (rotator mixer) for **00:05:00** at **Room temperature** .

52 Prepare **500 µL fresh 70% ethanol in Nuclease-free water** .

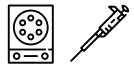
53 

Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

54 

Keep the tube on the magnet and wash the beads with **200 µL freshly prepared 70% ethanol** without disturbing the pellet. Remove the ethanol using a pipette and discard.

55 Repeat the previous step.

56  30s

Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~ **00:00:30** , but do not dry the pellet to the point of cracking.

57 2m

Remove the tube from the magnetic rack and resuspend pellet in **30 µL Nuclease-free water** . Incubate for **00:02:00** at **Room temperature** .

58 Pellet the beads on a magnet until the eluate is clear and colourless.

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59 Remove and retain **30 µL eluate** into a clean 1.5 ml Eppendorf DNA LoBind tube.

60 Take forward **30 µL end-prepped cDNA** into adapter ligation.

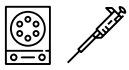
Adapter ligation

61 Check the contents of each tube are clear of any precipitate and are thoroughly mixed before setting up the reaction.

61.1 

Mix the contents of each tube by flicking.

61.2 Check that there is no precipitate present (DTT in the Blunt/TA Master Mix can sometimes form a precipitate).

61.3 

Spin down briefly before accurately pipetting the contents in the reaction.

62 

Taking the end-prepped DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition.

- **30 µL End-prepped DNA**
- **5 µL Adapter Mix**
- **50 µL Blunt/TA Ligation Master Mix**
- **15 µL Nuclease-free water**

63 

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Mix gently by flicking the tube, and spin down.

64  10m

Incubate the reaction for **00:10:00** at **Room temperature** .

AMPure XP bead binding

65 Resuspend the AMPure XP beads by vortexing.

66 

Add **40 µL resuspended AMPure XP beads** to the adapter ligation reaction from the previous step and mix by pipetting.

67  5m

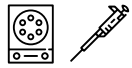
Incubate on a Hula mixer (rotator mixer) for **00:05:00** at **Room temperature** .

68 Place on magnetic rack, allow beads to pellet and pipette off supernatant.

69 

Add **200 µL Wash Buffer (WSB)** to the beads. Resuspend the beads by pipetting up and down. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant.

70 Repeat the previous step.

71 

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Spin down and place the tube back on the magnet. Pipette off any residual supernatant.

72 Remove the tube from the magnetic rack and resuspend pellet in **25 µL Elution Buffer (EB)** .

73 

10m

Incubate on a Hula mixer (rotator mixer) for **00:10:00** at **Room temperature** .

74 Pellet the beads on a magnet until the eluate is clear and colourless.

75 Remove and retain **25 µL eluate** into a clean 1.5 ml Eppendorf DNA LoBind tube.

76 

Quantify **1 µL eluted cDNA** using a Qubit fluorometer - recovery aim ~60 fmol.

Priming and loading the flow cell

77

Please be aware that the flow cell's pore occupancy could be compromised when loading lower amounts of cDNA. Please use the table below as a guide:

- Please check the Mass to Molarity table in the protocol.

The prepared library is used for loading onto the flow cell. Store the library on ice until ready to load.

78 Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at **Room temperature** .

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79

Mix the Sequencing Buffer (SQB), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing and spin down at **Room temperature** .

80

To prepare the flow cell priming mix, add **30 µL thawed and mixed Flush Tether (FLT)** directly to 1 tube of thawed and mixed Flush Buffer (FB), and mix by vortexing.

81 Load the flow cell(s) into the docking ports within the PromethION.

82 Prime the flow cell using the following steps, taking care to avoid the introduction of air bubbles.

82.1 Turn the valve to expose the inlet port (Port 1).

82.2

A small tract of air will be visible beyond the inlet port. Draw back a small volume to remove any air bubbles (a few µls):

a. Set a P1000 pipette to 200 µl
b. Insert the tip into the inlet port
c. Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip.

82.3

Using a P1000 pipette, flush **500 µL Priming Mix** into the inlet port of the flow cell, avoiding the introduction of air bubbles.

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82.4 Wait **00:05:00** . During this time you can prepare your library for loading, as described in the next steps. 5m

82.5 Repeat the priming step with another **500 µL flush** .

83 

Thoroughly mix the contents of the Loading Beads (LB) tubes by vortexing.

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

- **75 µL SQB**
- **51 µL LB**
- **24 µL DNA library**

85 Load your sample.

85.1 Load **150 µL sample** through the inlet port.

85.2 Close the valve to seal the inlet port and close the PromethION lid when ready.

85.3 Wait a minimum of **00:10:00** after loading the flow cells onto the PromethION before initiating any experiments. This will help to increase the sequencing output. 10m

Ending the experiment

86 After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at **2 °C** - **8 °C** , OR

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87 Follow the returns procedure by washing out the flow cell ready to send back to Oxford Nanopore.

88

If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Guide that can be found in the online version of this protocol.

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