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Synthesis of double-strand cDNA (ds-cDNA) from viral dsRNA by using Random primers

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Nanovirseq

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We use this protocol and it's working

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Abstract

Double-stranded cDNA synthesis from viral dsRNAs:
For dsRNA sequencing by nanopore sequencing, this protocol was used. Before treating samples with RNase T1, you should measure the total concentration of RNAs in the samples by using a nanodrop or Qubit device, as RNase T1 has the ability to partially digest double-stranded RNAs in the absence of single-stranded RNA.

Materials

RNase T1 and DNase I digestion

- DNase I (RNase-free)
- DNase I Reaction Buffer with MgCl2 (10X)
- RNase T1

Synthesize the first strand of cDNA (Reverse Transcription)

- Random primers (60 µM)
- dNTP (10 mM)
- H_2O
- First Strand cDNA synthesis Buffer
- RNase out or RNasin® Ribonuclease Inhibitor (40 u/µl)
- Superscript III or Maxima H minus

Removal of the residual RNA by RNase H

- RNase H

Synthesize the second strand of cDNA

- Klenow 10X buffer+NAD,
- dNTP (10 mM),
- Klenow DNA Polymerase I,
- E. coli DNA Ligase I

cDNA purification by AMPure XP beads

- Agencourt AMPure XP
- Magnet plate
- Ethanol 80%
- Nuclease free water
- 1.5 ml eppendorf tube

Troubleshooting

RNase T1 and DNase I digestion

1h 20m

1 20m

- Add 10X DNase Buffer with MgCl2 (final concentration should be 1X).
- Add 50 units RNase T1 per 1µg of total RNA and 1 unit DNase I per 2ug of total RNA.
- Incubate at 37 degrees C for 20 min.

1.1 1h

Stop reaction
Do a phenol-chloroform extraction and Ethanol precipitation of dsRNA.

Synthesis of the first strand of cDNA

2h

2 5m

Mix well below components by pipetting and centrifuge or spin briefly.

	A	B
	Treated dsRNA	5 µl
	Random primers (60 µM)	2µl
	dNTP (10 mM)	1 µl
	H2O	6 µl
	Total	14 µl

- Incubate at 99C for 5 mins and put tubes **immediately on water ice** (it is better to do this step with a thermocycler).

2.1

Add below components and mix well by pipetting and centrifuge or spin briefly.

	A	B
	First Strand Buffer	4 µl
	RNase out or RNasin® Ribonuclease Inhibitor (40 u/µl)	1 µl
	Maxima H minus (Point: just use 200 unit)	1 µl
	Total	20 µl

2.2 1h 55m

Incubation step.

	A	B
	25 C	20 mins
	55 C	90 mins
	85 C (Inactivation step)	5 mins

Removal of residual RNA by RNase H

20m

3 Mix well below components by pipetting and centrifuge or spin briefly.

20m

	A	B
	cDNA	20 µl
	RNase H	1 µl
	Total	21 µl

Incubate at 37 C for 20 mins

Synthesis of the second strand of cDNA

2h 40m

4 Mix well below components by pipetting and centrifuge or spin briefly.

	A	B
	cDNA	21 µl
	Klenow 10X buffer	2.7 µl
	dNTP (10 mM)	1 µl
	Klenow DNA Polymerase I	0.7 µl
	E. coli DNA Ligase I	1 µl
	H2O	0.6 µl
	Total	27 µl

4.1 Incubation step. 2h 40m

	A	B
	16 C	150 mins
	75 C; (Inactivation step) Point: check this part based on enzyme brand.	10 mins

cDNA purification by AMPure XP 17m

5 Mix well below components by pipetting and incubate at room temperature for 5 mins. 7m
Point: Put bead stock in room temperature for 20 min (before using). Vortex the bead stock very well (before using)

	A	B
	double stranded-cDNA	27 µL
	AMPure XP	49 µL

Place the reaction tube onto a magnet plate for 2-5 mins
IMPORTANT: Wait for the solution to clear before proceeding to the next step.

5.1 This step must be performed while the reaction tube is situated on the magnet plate:
- Aspirate the cleared solution from the reaction tube and discard. Leave 5 µL of supernatant behind, otherwise, beads are drawn out with the supernatant.

IMPORTANT: Do not disturb the ring of separated magnetic beads.

5.2 This step must be performed while the reaction tube is situated on the magnet plate: 1m
Dispense 200 µL of 80% ethanol to the reaction tube and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard.
Repeat previous step.

5.3 After removing and discarding all supernatant, **A dry time is optional (2 to 5 mins)** to ensure all traces of Ethanol are removed. 2m
NOTE: **For fragments 10 kb and larger, do not over-dry the bead ring** (bead ring appears cracked if over dried) as this will significantly decrease elution efficiency.

5.4 Remove the reaction tube from the magnet plate, and then add 40 µL of nuclease-free water to the reaction tube and pipette mix 10 times. Incubate for 2 minutes. 2m
NOTE The liquid level will be high enough to contact the magnetic beads at a 40 µL nuclease-free water. A greater volume of nuclease-free water can be used, but using less than 40 µL will require extra mixing (to ensure the liquid comes into contact with the beads), and may not be sufficient to elute all cDNAs.

5.5 Place the reaction tube onto the magnet plate for 5 minute to separate beads from the solution. 5m
IMPORTANT Wait for the solution to clear before proceeding to the next step.
Transfer the eluate to a new plate.

Use 1 µL of eluted ds-cDNA for concentration measurement by Qubit.