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Preparation of dsRNA viruses for MinION - CLM

Forked from Preparation of dsRNA viruses for next-generation sequencing

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In Development Share This protocol is published without a DOI.

Clmattson

ABSTRACT

Updated protocol by CLM

EXTERNAL LINK

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PROTOCOL CITATION

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FORK NOTE

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MATERIALS TEXT

MATERIALS

NEBNext Second Strand Synthesis (dNTP-free) Reaction Buffer - 0.4 ml **New England Biolabs Catalog #B6117S**

E.coli DNA Ligase - 1,000 units **New England Biolabs Catalog #M0205L**

DNA Polymerase I (E.coli) - 2,500 units **New England Biolabs Catalog #M0209L**

DMSO **Sigma Aldrich Catalog #D1435**

Nuclease-free water **Ambion Catalog #AM9932**

Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32851**

DNase I (e.g., NEB #M0303) and DNase I Cleanup Reagants or Kit for Removal of DNA Prior to Depletion **New England Biolabs**

Millex-GP Syringe Filter Unit, 0.22 µm **Emd Millipore Catalog #SLGP033RS**

Qubit RNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32852**

RNase H **New England Biolabs Catalog #M0297S**

RNeasy® Mini Kit **Qiagen Catalog #74104**

SuperScript™ III First-Strand Synthesis System **Thermo Fisher Scientific Catalog #18080051**

RNAse AT/1 Mix **Thermo Scientific Catalog #EN0551**

RNeasy MinElute Cleanup Kit **Qiagen Catalog #74204**

Nucleospin Gel and PCR cleanup **Macharey Nagal Catalog #740609.250**

1 Viral lysates should be passed through a 0.22µm filter to remove host debris, then treated with nucleases to degrade extracapsular nucleic acids.

To 1ml filtrate:
25µl DNAse I,
50µl RNAse A/T1
107.5 DNAse I Buffer

Scale down for concentrated lysates, which should be in the range of 50-500uL.

Incubate for 1 hour 30 minutes at 37ºC.

Inactivate nucleases at 65ºC for 10 minutes and proceed to RNA extraction.

2 RNA should be extracted using a commercially available kit. We used an RNeasy Mini Kit (Qiagen). Kits that require carrier RNA are not recommended because this will interfere with sequencing.

Follow manufacturer instructions with the following modifications:
1. Pass all lysate through column at the first step (i.e. add buffer + lysate mix multiple times until lysate is used up)
2. Elute with 45uL of Elution Buffer. Let column sit at 25ºC for 5 minutes before spinning.
3. Split into 20uL aliquots. RNA may be frozen at -80ºC for future processing or may move on to next step.

3 Quantify the RNA using Qubit RNA High Sensitivity Kit (HS). Note that low viral titres and/or small viral genomes may result in the amount of RNA being below the Qubit's limit of detection.

4 Add 50% DMSO (v/v) to RNA sample, and incubate for 1 hour 30 minutes at 65ºC.

5 Purify RNA using a column cleanup kit (we recommend Qiagen's RNeasy MinElute Cleanup Kit) or your preferred other method (e.g. ethanol precipitation, TRIzol purification). This step removes DMSO from sample.

If using RNeasy MinElute Cleanup Kit:
1. Adjust sample volume to 100 uL using RNade free water (ex: if sample volume = 20 uL RNA + 10 uL DMSO, add 70 uL RNase free water)
2. Centrifuge 'full speed' == 21000 rcf or highest speed on centrifuge
3. Store at -80 ºC or place on ice and proceed to first strand synthesis

6 First strand synthesis - Superscript IV (in BSC):

Calculate reagent volumes for MM:

A	B
Reagent	Vol for 1 rxn
dNTPs	1 uL
Random hexamers	1 uL
RNase free water	4 uL

Step 1

A	B
Reagent	Vol for 1 rxn
5x SSIV buffer	4 uL
100 mM DTT	1 uL
RNase out *	1 uL
SSIV RT	1 uL

Step 2: (* = RNase out instead of RNase inhibitor)

1. Mix appropriate volume (# samples+ 1) of 1st step mastermix and add 6 uL of MM to each tube
   Keep MM tube on ice
   Start with RNase free water

2. Add 7 uL of each RNA eluate to a tube strip and label + record scheme in notebook
   keep on cooling tray, do not allow to freeze
   Total vol of each tube is 13 uL
   Minifuge

3. Place on thermal cycle, start incubation at 65ºC for 5 minutes. At end of 5 min, place on ice for at least 1 min

4. During 5 min incubation, mix appropriate volume (# samples+ 1) of 2nd step mastermix.
   Get SSIV RT on cold block from freezer
   Keep MM tube on ice
   Mix SSIV buffer
   Add SSIV RT last (pipette slowly)

5. Add 7 uL (vol for 1 rxn) of MM to each sample on the tube strip
    Keep tube strip on cooling tray
    Total vol of each tube is now 20 uL
    Minifuge

6. Place back on thermal cycler and proceed thru the following incubation steps:
    10 min at 23 ºC
    10 min at 52.5 ºC
    10 min at 80 ºC

7. Store at 4 ºC or place reactions on ice and proceed to second strand synthesis.

7 Second strand synthesis.

Calculate reagent volumes for MM:

A	B
Reagent	Vol for 1 rxn
dNTPs	1 uL
E. Coli DNA ligase	0.5 uL
DNA polymerase I	2 uL
RNase H	0.5 uL
5x second strand synthesis buffer (OR 10x second strand synthesis buffer)	8uL(4uL)
Nuclease free water	8 uL (use 12 uL if used 10x buffer)

1. Mix appropriate volume (# samples+ 1) of second strand mastermix.
    Keep MM tube on ice
    Keep enzymes on cold block
    Start with nuclease free water
    Add enzymes last

2. Add 20 uL (vol for 1 rxn) of MM to each sample on the tube strip
    Keep tube strip on cooling tray
    Total vol of each tube is now 20 uL
    Minifuge
3. Place on thermal cycler and begin 5 hour incubation at 16 ºC

4. Store at 4 ºC or proceed to cleanup

8 Purify DNA using your preferred method (we used a Macharey-Nagal Nucleospin Gel and PCR Clean-up kit, per manufacturer instructions). Quantify DNA using Qubit.

9 Prepare DNA libraries for sequencing using your chosen platform and library preparation kit (we used Nextera XT, we will modify this protocol for Oxford Nanopore Native Barcode sequencing).