Library construction for viral analysis

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The aim of the protocol is to construct a viral enriched library for mass sequencing, using reclaimed water as a source of water, to detect viral pathogens.

Equipment:

Centrifuge	☐
Thermocycler	☐
Micropipettes	☐
Timer	☐
0.5 mL Eppendorf	☐
0.2 mL Eppendorf	☐
1.5 mL Eppendorf	☐
PCR cooler or Ice	☐
Termoblock	☐
Qubit® fluorometer	☐
Vortex	☐

Reagents:

TurboDNAse (AM1907-with inactivation reagent, AM2238 - no inactivation reagent) - Ambion.	☐
QIAmp Viral RNA Mini Kit (22906) - QIAGEN.	☐
Ethanol (96-100%).	☐
SUPERSCRIPT III reverse transcriptase (18080093) - life technologies	☐
RNAseOUT (40U/µl) (10777-019) - Thermofisher	☐
Random primer A (5'-GTTTCCCAGTCACGATCNNNNNNNNN-3') - 50 pmols/µl	☐
Primer B (5'-GTTTCCCAGTCACGATC-3')	☐
UltraPure™ DEPC-Treated Water (750023) - Thermofisher	☐
dNTPs mix (10 mM each)	☐
RNAse H (2U/ µl) (18021-071) - Thermofisher	☐
Sequenase 2.0 (70775Y 200 UN) - Thermofisher	☐
Amplitaq Gold (4311806) - life technologies	☐
Qubit® dsDNA HS Assay Kit, (Q32854) - life technologies	☐
Zymo kit clean and concentrator (D4013) - Zymo research	☐
HV Filter Unit 0,45 µm (SLHV033RS) - Millex	☐

Removal of free DNA from sample:

Library will be constructed by using 300 µl of reclaimed water viral concentrate previously filtrated by using HV Filter Unit 0,45 µm. To avoid the loss of the viral concentrate filters should be pre-conditioned with 200 µL of PBS. We recommend to filtrat 400 µl in order to ensure a filtrated reclaimed water volume of 300 µl.

1- Add 50 µl of filtrated viral concentrate to six different 0,5 mL eppendorf tube.

2- For each tube add:

- 10 µl de TurboDNAse (2 U/ µL)
- 10 µl 10X TurboDNase reaction Buffer

3- Incubate 1 h- 37ºC. After the first 30 minutes of incubation 3 µl turbo DNAse enzyme are added per tube. Incubate for half an hour. Regular agitation during incubation is recommended. This step is performed in the TERMOBLOCK.

4- Put in a single 1.5 mL Eppendorf tube the DNAse treated viral concentrate.

5- Inactivate DNAse by adding the inactivation reagent provided (AM1907-Ambion) using 44 µl per sample tested (10% of the total reaction volume: 300 µl sample + 140 µl reagents). Once the reagent is added the tube should be inverted a couple of times

6- Incubate it 5 min at room temperature (RT = 22-26ºC).

7- Centrifuge the tubes at 10000g during 1, 5 minutes.

8- Keep the supernatant without disturbing the pellet and discard the pellet.

Nucleic acid extraction:

A nucleic acid extraction is performed using **QIAmp Viral RNA Mini Kit** (QIAGEN 22906) following the manufacturer's instructions. NA extraction is performed by charging the same column in two consecutive steps with 140 µl of DNAse treated viral concentrate. The total volume of viral concentrate used is **280 µl**.

1- Pipet 560 µl of prepared Buffer AVL containing carrier RNA* into a 1.5 ml microcentrifuge tube. *If bigger volumes of sample concentrates are going to be extracted AVL buffer has to be increased in proportion. I.e. 280* µl of viral concentrate with 1120 50 µl of AVL.

2- Add 140 µl of viral concentrate to the 560 µl of Buffer AVL-Carrier. Mix the tube 15 seconds. *In our case use 60 µl of viral concentrate plus 80 µl of water DNA/RNA free to achieve a sample of 140 µl. Mix the 140 µl of viral concentrate with another 560 µl of AVL.*

3- Incubate at room temperature (15-25ºC) for 10 min.

4- Spin the tubes to remove drops from the inside of the lid.

5- Add 560 µl of ethanol (96%-100%) to the sample. Mix by vortexing during 15 seconds. Spin the tubes to remove drops from the inside of the lid. *If the volume is bigger than 140 µl, increase the amount of ethanol proportionally. In our case we will require 1120 µl of ethanol per sample.*

6- Carefully apply 630µl of the solution from the step 5 to a QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim. Centrifuge at 6000xg (8000rpm) for 1 min. Place the QIAamp spin column into a clean 2mL collection tube and discard the tube containing the filtrate.

7- Repeat the step 6 until all sample has passed through the spin column.

8- Add 500 µl of buffer AW1. Centrifuge at 6000xg (8000rpm) for 1 min. Keep the spin column, and discard the collection tube and the filtrate.

9- Add 500 µl of buffer AW2. Centrifuge at 20.000xg (14.000 rpm) for 3 min. Keep the spin column and discard the collection tube and the filtrate.

10- Repeat step number 9 but centrifuge for 1 min.

11- Place the QIAamp Mini Spin Column into a clean 1.5mL centrifuge tube (collection tube). Open the column and add 60 µl of AVE. Incubate the Spin Column for 2 min and centrifuge at 6000xg (8000rpm) for 1 min.

12- Store the eluted NA at -80ºC.

**The use of carrier is not recommended.*

cDNA synthesis:

Prepare a mastermix of components common to all reactions to minimize pipetting error, then dispense appropriate volumes into each reaction tube prior to add primers and RNA template.

1- Prepare the **mastermix** 1 for each sample in a 0.2 µl eppendorff:

- 2 µl 5X RT buffer Superscript III
- 4 µl 25mM $MgCl_2$
- 2 µl 0'1M DTT
- 1 µl RNAse Out
- 1 µl superscript III

2- Prepare as well a sample mix for each sample in a 0.2 µl eppendorff:

- 1µl dNTPs Mix (10 mM each)
- 1µl random primers - 50 pmols/µl (5'-GTTTCCCAGTCACGATCNNNNNNNNN-3')

- 2 µl H20 DNA/RNA free. *Adjust the water volume to a maximum final volume of 13 µl.*

3- Distribute sample mix in each sample PCR tube and add 6 µl of the **nucleic acid extraction** (1-500 ng total RNA). *This amount can be scaled up to 11 µl*

4- Denature the RNA and primer incubating 5 min – 65ºC.

5- Chill in ice for 5 minutes.

6- Add 10 µl of **mastermix** 1 to each of the samples.

7- Run the RT program as follows:

- 25ºC – 10 min.
- 50ºC- 50 min.
- 85ºC -5 min (to inactivate the reaction).

8- Chill on ice

9- To denature ds RNA-DNA hybrids add 1 µl of RNAse H.

10- Incubate 20 minutes at 37ºC.

Construction of second strand DNA:

Using the same tube we used to construct de cDNA:

1- Incubate tubes at 95ºC - 5 min and

2- Chill on ice - 5 minutes

2- Add to each tube:

- 2 µl of sequenase buffer
- 0.3 µl of sequenase enzyme (13U/µl)
- 7.7 µl H20 RNAsa/DNAsa free

3- Perform an incubation ramp (pass from 10ºC to - 37 ºC in 8 minutes. In our PCR devices we do it by increasing temperature by 1ºC every 18 seconds). After achieving the 37ºC:

- 37ºC – 8 min
- 94ºC – 2 min
- 10ºC – 5 min

4- A second step of sequenase enzyme is performed. This time the enzyme is diluted using the enzyme dilution reagent provided (1:4). To each tube add 1.2 µl of diluted sequenase (1:4).

5- Repeat the temperature ramp from 10ºC to 37ºC in 8 minutes (every 18s increase 1ºC the Temperature).

6- Maintain the temperature at 37ºC during 8 minutes.

7- 94ºC – 8 min

8- Keep the samples at 4ºC.

DNA amplification:

Environmental samples contain few DNA/RNA to construct the library directly. Therefore and amplification step is usually needed to have enough DNA to construct the library.

Nextera needs at least 1ng of dsDNA to prepare the library. Prior to PCR amplification is recommended to check the amount of dsDNA available using a fluorometric method such as Qubit. If enough **DNA is available (1ng/ µl)** no amplification is needed. However this concentration is rarely found in environmental samples. Therefore a PCR using primer B (constant region of random primer A) is performed.

The number of PCR amplification cycles affects the diversity of sequences found in the library. Hence a minimal number of PCR cycles amplification should be performed. 25 PCR cycle amplification should be enough to obtain the desired dsDNA concentration.

In order to obtain it is recommended to perform two PCR-B amplifications using 20 µl of sequenase template.

PCR mastermix preparation:

25mM MgCl	28 µl
10X PCR Buffer	10 µl
100 mM dNTPs	1 µl
Taq polymerase	1 µl
Primer B (100pmol/µl)	1 µl
H2O	69 µl
Sequenase template	10 µl*

** The sequenase double stranded DNA tubes from previous steps contain enough volume to perform 2 PCR-B amplification reactions.*

PCR Cycle program:

95ºC – 10 min

94ºC – 30 sec
40ºC – 30 sec
50ºC – 30 sec
72ºC – 1 min
} 25 cycles

72 º C -10 min

4ºC – hold

Cleaning and concentration

We can concentrate the two PCR-B products using the Zymo kit clean and concentrator. Follow the manufacturer's instructions (amplicon purification protocol) briefly described here:

1- Put the PCR-B products in a 1,5mL Eppendorf tube.
2- Add the DNA binding buffer in 5:1 proportion with the sample (5 times more buffer than sample).
3- Transfer the mix in a zymo column.
4- Centrifuge at 14000g during 30 seconds. Discard the eluate.
5- Add 200 µl of DNA wash buffer to the column.
6- Centrifuge at 14000g during 30 seconds. Discard the eluate.
7- Repeat step number 5 and 6. Discard the eluate.
8- Pre-warm at 70ºC one Eppendorf containing DNase/RNAse free water.
9- Add 15 µl pre-warmed water DNase/RNAse free to the column and place a new Eppendorf under the column.
10- Incubate 5 minutes (this step enhances the elution of fragments bigger than 6kbs)
11- Centrifuge at 14000g during 1 minute.
12- Keep the eluate.

Final library quantification:

Use the Qubit fluorometer to ensure that a final concentration of 1 ng/ µl is obtained.