cDNA library preparation using ARTIC v3 primers and NEB UDI UMI adaptors for NGS V.1

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Marion Walker: Developed the protocol
Nick Gilbert: Developed the protocol and supervised the study
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bio_rdm

This is a cDNA library preparation protocol with the aim to quantify SARS-CoV-2 in wastewater and potentially detect its different variants using Next Generation Sequencing (Illumina).

In this protocol, we amplified cDNA from viral RNA extracted from wastewater.

The main steps involve:

- Amplification of cDNA with ARTIC v3 primers
- Ligation of amplified cDNA to NEB UDI UMI adaptors
- PCR enrichment of the libraries
- Pooling libraries together for sequencing

The further analysis pipeline used to perform assembly and intra-host/low-frequency variant calling is the nf-core/viralrecon: https://nf-co.re/viralrecon

This protocol is still under revision

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CREW (Scotland's Centre of Expertise for Waters)
Grant ID: CD2019_06 Tracking SARS-CoV-2 via municipal wastewater

SARS-CoV-2, Covid-19, wastewater, coronavirus, viral RNA, sewage

protocol ,

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- NEBNext® ARTIC SARS-CoV-2 Library Prep Kit (Illumina®)
- 80% Ethanol (freshly prepared with nuclease free water)
- Nuclease-free Water
- DNA LoBind Tubes (Eppendorf #022431021)
- Magnetic rack/stand (NEB #S1515, Alpaqua®, cat. #A001322 or equivalent)
- Thermal cycler 2
- Vortex Mixer
- Microcentrifuge
- Agilent® Bioanalyzer® or similar fragment analyzer and associated consumables •
- DNase RNase free PCR strip tubes (ex. USA Scientific 1402-1708)
- External positive control
- Template RNA

cDNA synthesis

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A	B
NTC	No-template control
EPC	External positive control
Sample	wastewater sample

It is recommended to add as templates a positive and a negative control.

For cDNA synthesis, refer to

NEBNext® ARTIC SARS-CoV-2 Library Prep Kit (Illumina®) NEB #E7650S/L **NEB ARTIC E7650.pdf**

Chapter 2 Standard Protocol with cDNA Amplicon and Ligation Bead Cleanups (Three clean-up steps)

- Follow steps 2.1 to 2.3

2 After cDNA clean up, you can run 1 µL of the cDNA, diluted 1:10 on **Agilent TapeStation with HS D1000 Tapes** to look at the size distribution.

If you are confident the sizes would be correct, just use **Qubit HS dsDNA protocol** to quantify the input DNA amount.

This cDNA quantification step is crucial for this protocol because it affects the experiment set up in subsequent procedures

3 Store the amplified cDNA at -20 °C.

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Ligate amplified cDNA to NEB UDI UMI adaptors

4 For choosing adaptors, follow the following manual:

NEBNext® Multiplex Oligos for Illumina® - Unique Dual Index UMI Adaptors DNA Set 1
NEB #E7395S
NEB E7395.pdf

"Designed for use in library prep for DNA, ChIP DNA and RNA (but not Small RNA), the NEBNext Unique Dual Index UMI (Unique Molecular Identifier) Adaptors enable high-efficiency adaptor ligation and high library yields.
These adaptors contain all necessary sequences for sequencing on the Illumina platform, and are compatible with PCR-free applications and sample pooling prior to PCR amplification".

5 Refer to tables 1-4 for information on index sequences

6 Check Index Pooling guidelines in the manual mentioned above (NEB #E7395S)

7 **End Prep**

Follow the **step 2.4** of **NEBnext End Prep** protocol in the NEB kit for SARS-CoV-2 detection manual (**E7650)**

8 **Adaptor dilution and ligation**

In this step we use parts of both protocols (E7395 and E7650)
- Adaptors with UMI are used
- A smaller reaction volume is used following that of the ARTIC kit

8.1 **Adaptor dilution (optional depending on amount of input DNA)**
In this step the cDNA quantification information is detrimental. Depending on the amount of DNA input, adaptor dilution is a necessary step.

"If DNA input is ≤ 100 ng, dilute NEBNext Adaptor"

Please refer to NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®
NEB #E7645S/L, #E7103S/L
NEBNext Ultra II DNA library prep for Illumina E7103-E7645.pdf

Step 2. "Adaptor Ligation - Determine whether adaptor dilution is necessary"

8.2 **Adaptor ligation**

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Use NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® NEB #E7645S/L, #E7103S/L as a guide

The E7650 protocol uses NEBNext adaptors for Illumina which are truncated and must be PCRed to add the full P5 and P7 sequences.

Those NEBNext adaptors also need the USER enzyme because the adaptors need to be cleaved prior to the PCR step.

The E7395 protocol does not require this.

Mixing together these features the ligation recipe was rewritten as follows:

COMPONENT	VOLUME (µL)	10.4
End Prep Reaction Mixture	30	-
Diluted or undiluted NEBNext UMI Adaptors for Illumina	1.25	-
(red) NEBNext Ultra II Ligation Master Mix	15	156
(red) NEBNext Ligation Enhancer	0.5	5.2
Total Volume	46.75	

modified from step 2.2 of NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® NEB #E7645S/L, #E7103S/L

8.3 Follow to steps 2.3 and 2.4 of the aforementioned manual (NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® NEB #E7645S/L, #E7103S/L)

Samples can be stored at - 20 overnight

Cleanup of Adaptor-ligated DNA

9 As we know most amplicons are 400 bp in size, it is not necessary to use the size selection protocol.

Rather, we move to **section 3B Cleanup of Adaptor-ligated DNA without size selection** for all samples

Protocol: NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® NEB #E7645S/L, #E7103S/L

Note:

The final volume from the last step is 46.75 µL.

It will be necessary to top it up to 93.5 µL to continue following the NEB's protocol.

***Add 46.75 µL of 0.1X TE for the top up.**

***Prepare fresh 80% EtOH with nuclease-free water**

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Continue with cleanup according to the protocol (section 3B in Protocol for E7645)

PCR enrichment

10 Follow on to **Step 4. PCR Enrichment of Adaptor-ligated DNA**
Protocol: NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® NEB #E7645S/L, #E7103S/L

Determine the number of cycles in your PCR reaction:

*cDNA quantification information is important for determining the number of cycles in the PCR reaction- refer to **Table 4.2.**

11 Proceed to **Step 4.1. PCR Amplification**

From NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® NEB #E7645S/L, #E7103S/L

12 **PCR enriched library clean up**

Follow on to **Step 5** of the same manual

Checking library size distribution and library quantification

13 After clean up, run 0.5 µL of the cDNA (diluted 1:10 4.5 µL of water) on a Agilent TapeStation with HS D1000 Tapes to look at the size distribution.

We recommend running a few randomly selected libraries to confirm size distirbution.
If this the first time doing it and we see consistent aberrant patterns showing up, then it will be necessary to run all libraries to judge how widespread the issue is.

Below is an example of expected size distribution

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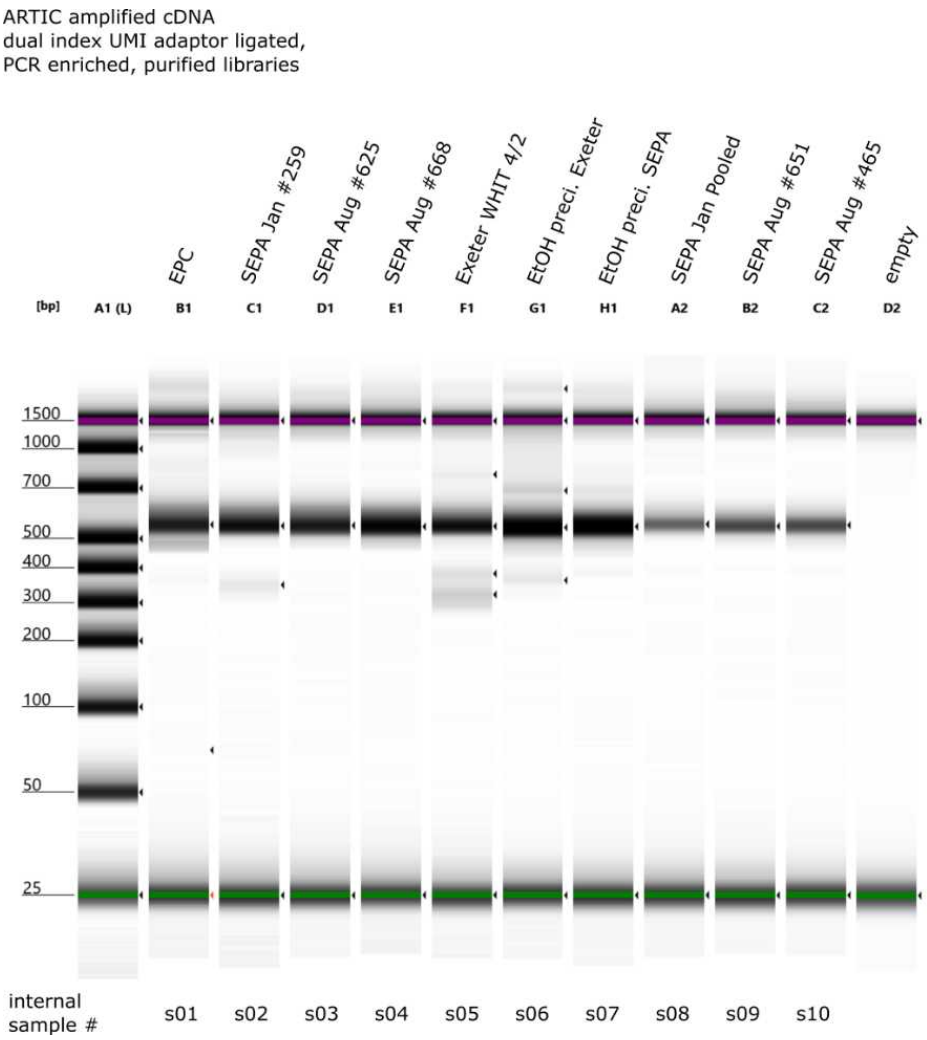

14 Use a quantification method such as Qubit HS dsDNA protocol to quantify the input DNA amount. DNA quantification here is very important

Pooling the libraries for sequencing

15 The samples that are going to be sequenced have to be pooled together in equimolar concentrations.

Check the requirements of your sequencing facility

In this example we will use the requirements of the Edinburgh Genomics facility (information from 2021)

A	B	C	D	E
Sequencer type	Diluent	Recommended Concentration	Minimum Concentration	Minimum Volume (uL)
MiSeq and NovaSeq	EB-Tween	>5nM*	>2.5nM	50 (1 lane)

*Use the following equation for nM = (ng per uL of DNA x 1,000,000) / (Number of base pairs x 660)

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Example of how samples were prepared

A	B	C	D	E	F	G	H	I	J
Sample ID	Total library DNA amount (µg)	Remaining volume (µL)	Concentration (ng/µL)	Volume ratio to reach lowest concentration	Scaled volume to mix (µL)*	Rounded volume (µL)	DNA amount in mixed volume (ng)	Mixed DNA concentration (ng/µL)	Mixed DNA concentration (nM)
s001	1.9942	29.5	67.6	0.4452662722	6.2337278107	6.23	421.148		
s002	2.22725	29.5	75.5	0.3986754967	5.5814569536	5.58	421.29		
s003	2.03845	29.5	69.1	0.4356005789	6.0984081042	6.1	421.51		
s004	2.65795	29.5	90.1	0.3340732519	4.6770255272	4.68	421.668		
s005	2.1594	29.5	73.2	0.4112021858	5.7568306011	5.76	421.632		
s006	3.363	29.5	114	0.2640350877	3.6964912281	3.7	421.8		
s007	3.5105	29.5	119	0.2529411765	3.5411764706	3.54	421.26		
s008	0.88795	29.5	30.1	1	14	14	421.4		
s009	1.1859	29.5	40.2	0.7487562189	10.4825870647	10.48	421.296		
s010	1.1918	29.5	40.4	0.745049505	10.4306930693	10.43	421.372		
					Total volume	70.5	4214.376	59.7783829787	164.6787409882

Scaled volume to mix was determined by looking at the sample with lowest concentration (s008), to which we should add 14 µL first (out of 29.5 µL)

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