

PROTOCOL

Multiplexed library preparation for viral whole genome sequencing of SARS-CoV-2 using ARTIC v3 RT-PCR and the plexWell™ 384 Library Prep Kit

Overview

This protocol describes how to generate NGS libraries for whole genome sequencing of SARS-CoV-2 isolates, combining methods for RT-PCR from the ARTIC Network for generating tiled amplicons from coronavirus RNA [1] along with downstream library prep with standard plexWell multiplexed NGS library prep reagents.

Background

The ARTIC Network is an international consortium of laboratories established to share protocols and sequencing results for monitoring pathogen outbreaks and genetic epidemiology in real-time. Viral genome data generated prospectively during outbreaks help provide information about relatedness to other viruses, mode and tempo of evolution, geographical spread and adaptation to human hosts.

The sample preparation methods initially developed by the ARTIC network for sequencing other pathogens have been rapidly adopted by scientists worldwide to address the urgent need to understand the short-term genomic epidemiology and evolution of the SARS-CoV-2 human coronavirus. The method generates amplicons which cover the full length of the SARS-CoV-2 viral genome.

Sequencing data can assist in COVID-19 epidemiological investigations, particularly when combined with other types of data (e.g., case counts). Although NextGen Sequencing (NGS) instruments are the most highly multiplexed assay platforms and broadly deployed worldwide, one obstacle to scaling up sequencing for COVID-19 testing has remained--how to easily prepare NGS libraries from 100's or 1,000's of SARS-CoV-2 samples per day. The plexWell workflow was designed to specifically address this challenge through scalable and highly multiplexed library preparation, enabling sequencing of up to 1152 samples in a single sequencing run.

[1] <https://artic.network/ncov-2019>

Required Materials

Part 1 of this protocol was adapted from the ARTIC nCoV-19 sequencing protocol v3 (dx.doi.org/10.17504/protocols.io.bdp7i5rn). Required reagents:

- SuperScript® IV Reverse Transcriptase (200 reactions), Thermo (P/N: 18090200)
- dNTP mix (10 mM each), Thermo (P/N: R0192)
- Random Hexamers (50 µM), Thermo (P/N: N8080127)
- RNaseOUT™ Recombinant Ribonuclease Inhibitor (125 reactions), Thermo (P/N: 10777019)
- Q5® Hot Start High-Fidelity DNA Polymerase, NEBiolabs (P/N: M0493S)
- 10 µM ARTIC v3 primer pools 1 and 2, Integrated DNA Technology (contact IDT)
- Quant-iT™ dsDNA assay kit, high sensitivity, Thermo (P/N: Q33120)

Additional equipment needed:

- Mastermix hood or cabinet
- Extraction and sample addition hood or cabinet*

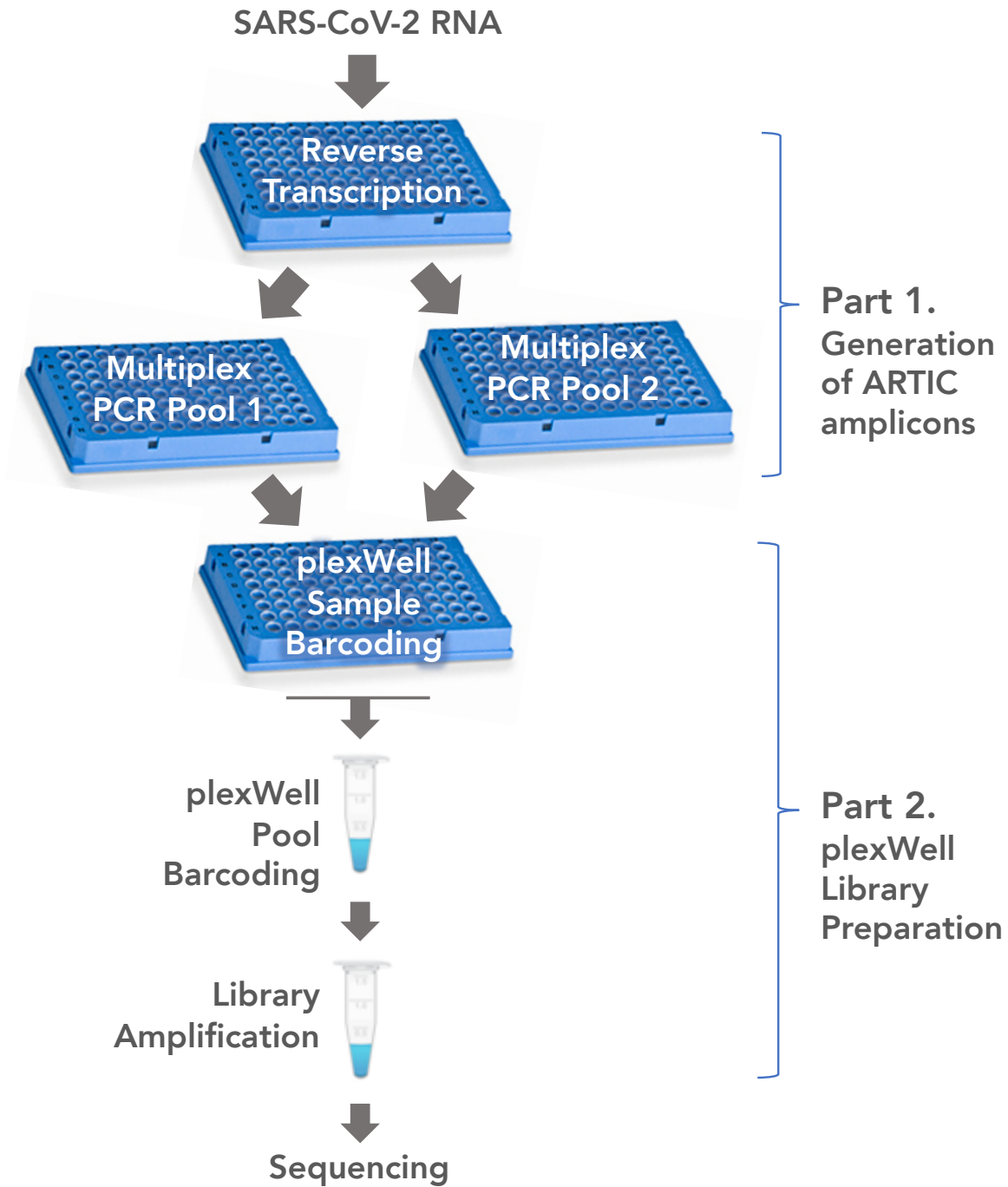
*Should be cleaned with decontamination wipes and UV sterilized before and after use.

Part 2 of this protocol is the implementation of plexWell for high-throughput library preparation.

- plexWell™ 384 Library Preparation Kit, seqWell (P/N: PW384)
- KAPA HiFi HotStart ReadyMix, Roche (P/N: KK2602 or KK2601)
- 80% Ethanol (freshly prepared)
- 10 mM Tris-HCl, pH 8.0

Please follow the instructions in the online version of the plexWell 384 Library Preparation Kit User Guide on the seqWell website, with protocol modifications as outlined in Part 2.

plexWell ARTIC Protocol Workflow Diagram



plexWell ARTIC Protocol

Part 1: Generation of ARTIC amplicons

Set up RT reactions:

Note: It is recommended to run at least one no template control (NTC) that receives purified human total nucleic acid as input on each 96 well plate of reverse transcription (RT) reactions.

1. Prepare the **Hexamer/dNTP mastermix** in a mastermix cabinet with the following components. Aliquot into PCR 8-strip tubes which should be wiped down when entering and exiting the **mastermix cabinet**.

Reagent	Per Sample	Per 96 reactions (~10% overage)
50 μ M random hexamer	1 μ l	106 μ l
10 mM dNTPs	1 μ l	106 μ l

2. Array 11 μ l of each purified sample into a PCR plate in the extraction and sample addition cabinet. *Note: The recommended input for RT reactions is purified nucleic acid from positive COVID-19 clinical samples with Ct values between 18 - 33. This Ct working range assumes that a concentration factor of 3X or greater was applied by eluting in a smaller volume than the initial sample volume used for purification. If the Ct is between 12 - 15, then dilute the sample 100-fold in water, if between 15 - 18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition.*
3. Add 2 μ l **Hexamer/dNTP mastermix** to each well and tip mix.
4. Gently mix by pipetting 10 μ L up and down five times, seal the PCR plate, and pulse spin the plate to collect liquid at the bottom of the wells.
5. Anneal the hexamers to the template RNA in a thermal cycler, as follows:
65 °C for 5 minutes; Hold at 4 °C.
Immediately remove from thermal cycler after incubating at 65 °C, and place on ice.

6. Prepare the **RT mastermix** on ice in a mastermix cabinet with the following components. Aliquot into PCR 8-strip tubes which should be wiped down when entering and exiting the mastermix cabinet.

Reagent	Per Sample	Per 96 Reactions (~10% overage)
SuperScript IV Buffer	4 μ l	424 μ l
100 mM DTT	1 μ l	106 μ l
RNaseOUT	1 μ l	106 μ l
SuperScript IV	1 μ l	106 μ l

7. the PCR plate on ice, add 7 μ l of **RT mastermix** to each annealed RNA sample inside an extraction and sample addition cabinet.
8. Gently mix by pipetting, reseal and pulse spin the PCR plate to collect the RT reactions at the bottom of the wells.
9. Incubate the RT reaction in a thermal cycler, as follows:
 42 °C for 50 min
 70 °C for 10 min
 Hold at 5 °C

Set up Multiplex PCR

10. In the mastermix hood, set up the **Pool 1 Multiplex PCR mastermix** with Primer Pool 1 as follows. Dispense 22.5 μ l of mastermix into each well of a PCR plate.

Reagent	Per Sample	Per 96 Reaction (~10% overage)
5X Q5 Reaction Buffer	5 μ l	528 μ l
10 mM dNTPs	0.5 μ l	52.8 μ l
Nuclease free water	13.15 μ l	1389 μ l
10 μ M Primer Pool 1	3.6 μ l	380 μ l
Q5 Hot Start DNA Polymerase	0.25 μ l	26.4 μ l

11. Repeat Step 10 with 10 μ M Primer Pool 2 to set up the **Pool 2 Multiplex PCR mastermix**.

Note: *Pool 1 and Pool 2 reactions **must be amplified separately and kept separate** until after PCR.*

12. PCR plates should be wiped down when entering and exiting the mastermix cabinet.
13. In the extraction and sample addition cabinet add 2.5 µl of cDNA (from step 9) to the corresponding wells of the **Pool 1 and Pool 2 Multiplex PCR mastermix** plates and tip mix.
14. Seal the plates and pulse centrifuge the PCR plates to collect the contents at the bottom of the wells.
15. Set-up and run the following PCR program on the thermal cycler:

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 seconds	1 cycle
Denaturation	98 °C	15 seconds	35 cycles
Annealing/Extension	65 °C	5 minutes	
Hold	4 °C	Hold	NA

16. After PCR, combine 3 µl of "Pool 1" and 3 µl of "Pool 2" amplicons from each plate into to the corresponding wells of a new PCR plate and tip mix.
17. Assay 1 µl of each pooled amplicon by PicoGreen™ and subtract the residual primer background using the measured NTC concentration. Note: NTC wells should not amplify during multiplex PCR.
18. Calculate the average amplicon concentration across the plate.
19. From the average concentration, calculate a global dilution factor that will generate an average diluted concentration of 2.5 ng/µl:

$$\text{Global Dilution Factor} = \text{Average Concentration}/2.5$$

20. Dilute all wells by the global dilution factor using 10 mM Tris-HCl. Do not use EDTA-containing solutions (e.g., TE buffer) as EDTA can inhibit enzymatic activity during downstream processing.

- a. Determine volume of 10 mM Tris to dilute the remaining 5 uL of pooled amplicon:

$$\text{Tris Volume} = (5 * \text{Global Dilution Factor}) - 5$$

- b. Add the calculated Tris Volume to each well of the pooled amplicon plate and tip mix.

Note: After gaining a better sense of the range of ARTIC amplicon concentrations that multiplex PCR generates, the number of wells assayed per plate using PicoGreen can be safely reduced (e.g., 16 - 24 wells per 96 well plate).

Part 2: plexWell library preparation

Follow the instructions in the plexWell 384 Library Preparation Kit User Guide (linked below):

https://seqwell.com/wp-content/uploads/2020/02/plexWell_384_and_96_Library_Preparation_Kit_User_Guide.pdf

Note the following important modification to the standard plexWell protocol:

1. Purify the final amplified plexWell library using **1 volume equivalent** of MAGwise (1:1) to ensure good recovery of the library fragments produced from tagging the relatively short ARTIC amplicons.

Please also note that the plexWell library amplification step requires KAPA HiFi HotStart ReadyMix, and not the NEB Q5 polymerase which is used for ARTIC amplification in Part 1 of the protocol.

Additional Questions?

Please contact support@seqwell.com for any further support or assistance.