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

v20210604 Updates (prior v20200527)

- Incorporates protocols for reverse transcription using LunaScript in addition to SuperScript IV
- Instructions for use with ARTIC v3 or MIDNIGHT multiplex primer sets
- Reduction of multiplex PCR annealing temperature from 65°C to 63°C
- Eliminates quantification of multiplex PCR and normalization prior to library preparation
- Includes example library QC results and recommended sequencing configuration and multiplexing capabilities

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 2,304 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 (https://www.protocols.io/view/covid-19-artic-v3-illumina-library-construction-an-bibtkann). ARTIC nCoV-19 sequencing protocols have been developed using both SuperScript™ IV (ThermoFisher) and LunaScript (NEB) reagents for the cDNA synthesis. This document includes protocols for use of either reagent during cDNA synthesis.

Additional protocols for alternate primer sets have been evaluated by the ARTIC network. Within this document, we also include multiplex PCR conditions for the 1200 bp primer set (dx.doi.org/10.17504/protocols.io.btsrnnd6).

Part 2 of this protocol is the implementation of plexWell for high-throughput library preparation following the plexWell 384 library preparation user guide (available on the seqWell website) with the modifications outline in Part 2 of this protocol.

Required reagents

- SuperScript™ IV First-Strand Synthesis System (ThermoFisher P/N: 18091200) or LunaScript® RT SuperMix Kit (NEB P/N: E3010L)
- Q5® Hot Start High-Fidelity 2X Master Mix (NEB P/N: M0494L)
- ARTIC nCoV-2019 V3 Panel, 500rxn (IDT P/N: 10006788) or SARS-Cov2-Midnight-1200, 500rxn (IDT P/N: 10007184) Integrated DNA Technology (contact IDT)
- 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

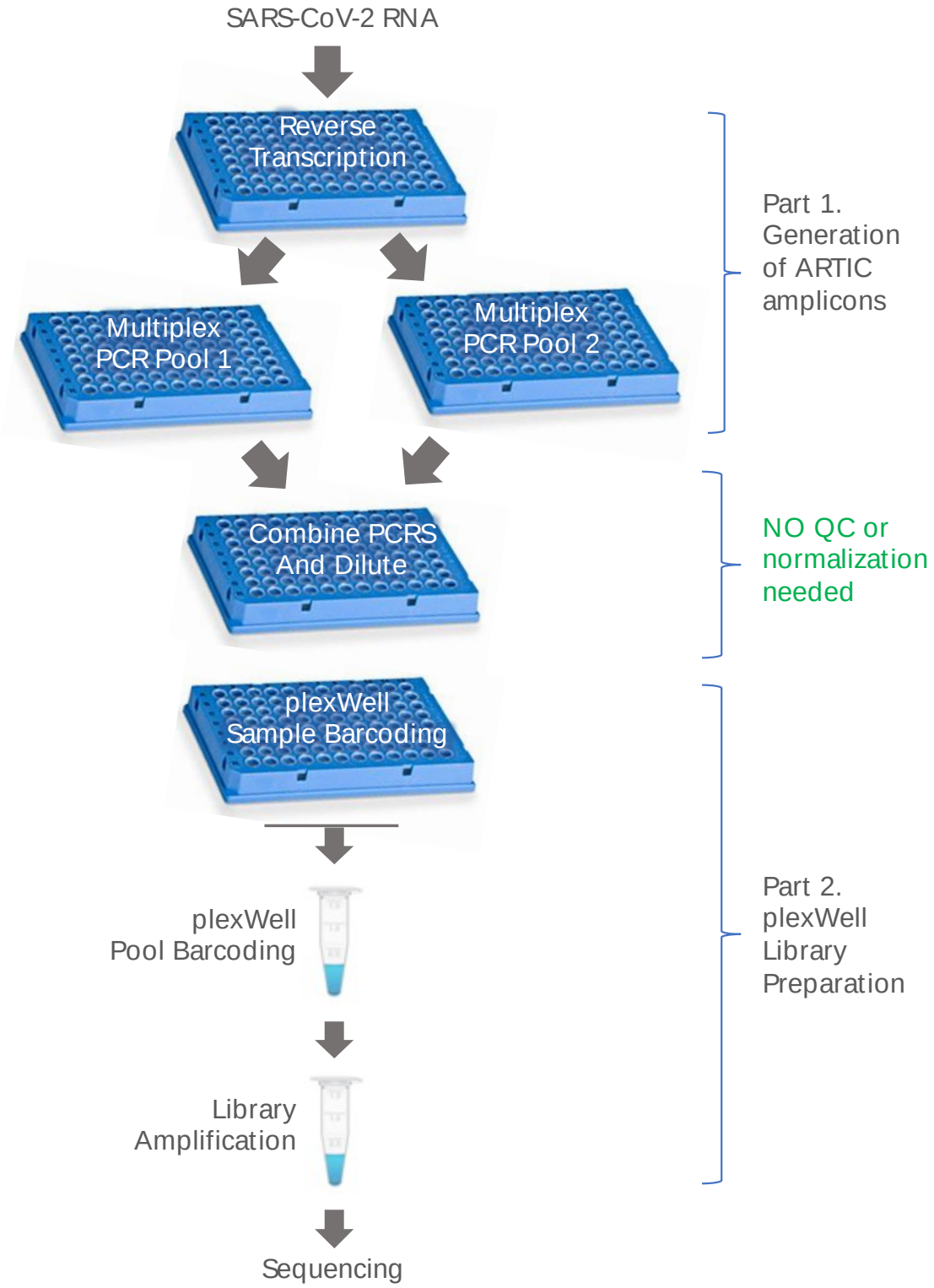
Required Equipment & Consumables

- Single-channel & multi-channel pipettors & appropriate tips
- Eppendorf Tubes® (1.5 ml & 2.0 ml, DNA LoBind Tubes)
- PCR plates & evaporation resistant plate seals
- Thermal cycler (compatible with 96 well low profile fully skirted PCR plates, BioRad HSP 9611)
- Magnetic stand for 1.5 ml and 2 ml tubes
- 0.2 ml PCR 8-tube strips and caps/seals
- Benchtop centrifuge to pulse-spin 1.5/2 mL tubes and PCR 8-tube strips
- Plate centrifuge
- Vortex mixer

Additional equipment recommended:

- Mastermix hood or cabinet
- Extraction/sample addition hood or cabinet (decontaminate/sterilize between uses)

plexWell ARTIC Protocol Workflow Diagram



plexWell ARTIC Protocol

Part 1: Reverse Transcription and enrichment for SARS-CoV2 amplicons

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

A. Reverse Transcriptase with SuperScirpt™ IV

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.**

Table 1. Hexamer/dNTP mastermix volumes

Reagent	Per Sample	Per 96 reactions (~10% overage)
50 µM random hexamer	1 µl	106 µl
10 mM dNTPs	1 µl	106 µl

2. Array 11 µl of each purified sample into a PCR plate in the extraction and sample addition cabinet.
3. Add 2 µl **Hexamer/dNTP mastermix** to each well and tip mix.
4. Gently mix by pipetting 10 µL 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.
6. Immediately remove from thermal cycler, and place on ice.
7. 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.

Table 2. SuperScript IV RT mastermix volumes

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

8. With the PCR plate on ice, add 7 µl of **RT mastermix** to each annealed RNA sample inside an extraction and sample addition cabinet.
9. Gently mix by pipetting, reseal and pulse spin the PCR plate to collect the RT reactions at the bottom of the wells.
10. Incubate the RT reaction in a thermal cycler, as follows:

    42 °C for 50 min
    70 °C for 10 min
    Hold at 5 °C

B. Reverse Transcriptase with LunaScript® SuperMix

1. Array 8 µl of each purified sample into a PCR plate in the extraction and sample addition cabinet.
2. Add 2 µl **LunaScript® Supermix** to each well.
3. Gently mix by pipetting up and down five times at 8 µl. Seal the PCR plate, then pulse spin the plate to collect liquid at the bottom of the wells.
4. Incubate the RT reaction in a thermal cycler, as follows:

   25 °C for 2 min
   55 °C for 20 min
   95 °C for 1 min
   Hold at 4 °C

C. Set up Multiplex PCR

Note: *Pool 1 and Pool 2 reactions **must be amplified separately***

1. In the mastermix hood, set up the Multiplex PCR pool 1 mastermix. You will have two mastermixes, one for pool 1, one for pool 2. The volume of primer pool used differs for ARTIC primers MIDNIGHT primers

Table 3. Multiplex PCR Pool 1 Reagent Volumes

Reagent	Per Sample (ARTIC)	96 reactions (ARTIC)	Per Sample (MIDNIGHT)	96 reactions (MIDNIGHT)
Q5® Hot Start High-Fidelity 2X Master Mix	12.5 µl	1320 µl	12.5 µl	1320 µl
10 µM Primer Pool 1	3.6 µl	380 µl	1.1 µl	116.2 µl
Nuclease free water	6.4 µl	676 µl	8.9 µl	940 µl

2. Repeat Step 1 with 10 µM Primer Pool 2 to set up the **Pool 2 Multiplex PCR mastermix.**

Table 4. Multiplex PCR Pool 2 Reagent Volumes

Reagent	Per Sample (ARTIC)	96 reactions (ARTIC)	Per Sample (MIDNIGHT)	96 reactions (MIDNIGHT)
Q5® Hot Start High-Fidelity 2X Master Mix	12.5 µl	1320 µl	12.5 µl	1320 µl
10 µM Primer Pool 2	3.6 µl	380 µl	1.1 µl	116.2 µl
Nuclease free water	6.4 µl	676 µl	8.9 µl	940 µl

3. Label one PCR plate for multiplex PCR pool 1. Distribute 22.5 µl of Pool1 multiplex PCR mastermix into the labelled PCR plate
4. Label a 2nd PCR plate for multiplex PCR pool 2. Distribute 22.5 µl of Pool 2 multiplex PCR mastermix into the labelled PCR plate.
5. Transfer multiplex PCR plates to the extraction and sample addition cabinet. Wipe down outside of plates before entering extraction and sample addition cabinet.
6. In the extraction and sample addition cabinet add 2.5 µl of cDNA (from part A or B) to the corresponding wells of the **Pool 1 and Pool 2 Multiplex PCR mastermix** plates. Pipette 5x at 20 µl to mix.

7. Seal the plates and pulse centrifuge the PCR plates to collect the contents at the bottom of the wells.
8. Set-up and run the following PCR program on the thermal cycler, with lead heating on

Table 5. Multiplex PCR Thermal Cycling Parameters

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	30 seconds	1 cycle
Denaturation	98 °C	15 seconds	35 cycles
Annealing/Extension	63 °C	5 minutes	
Hold	4 °C	Hold	NA

Note: The thermal cycling conditions are the same for both the ARTIC and MIDNIGHT primer sets.

9. 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 labelled "Combined amplicons".

10. Dilute combined multiplex PCRs by adding 10 mM Tris to the "Combined amplicons" PCR plate and tip mixing 10x. See chart below for volumes and recommended dilutions. For the ARTIC primer set, first time users should use a 1:8 dilution. For the MIDNIGHT primer set, first time users should use a 1:10 dilution.

Table 6. Recommended Dilution of combined multiplex PCRs prior to library preparation.

Amplicon Set	Range	Volume Tris	Comments	Typical Median Insert**
ARTIC	1:5 to 1:10	24 µl (1:5)	Final library fragment analysis may show residual amplicon peaks.	180 nt
		42 µl (1:8)		165 nt
		54 µl (1:10)		150 nt
MIDNIGHT	1:8 to 1:15	42 µl (1:8)	Final library fragment analysis may show residual amplicon peaks.	400 nt
		54 µl (1:10)		350 nt
		66 µl (1:12)		320 nt
		84 µl (1:15)		275 nt

*Typically residual amplicon peaks do not contribute to sequencing and are not a problem if library concentration is determined by qPCR. Optimization may be required if these peaks are significant and library concentration is derived by picogreen.
**Assumes the final library purification conditions used are 1 volume equivalent for ARTIC and 0.8 volume equivalents for MIDNIGHT.

Part 2: plexWell library preparation

Follow the instructions in the plexWell 384 Library Preparation Kit User Guide

Notes on PW384 User Guide and protocol modifications

- 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.
- Final library purification conditions should be altered to 1 volume equivalent (100 µl of MAGWise) for the ARTIC primer set only.
- Final library purification conditions for MIDNIGHT should use 0.75 to 0.8 volume equivalents of MAGwise (75 to 80 µl of MAGwise)

Expected Performance, recommended QC and sequencing

- Best practice for QC is to run a fragment analysis of the final library (Bioanalyzer, Tapestation, Fragment Analyzer etc.) followed by qPCR for concentration determination.
- ARTIC libraries typically have an average fragment size of 400 bp which can be used for size correction for SYBR based qPCR analysis. Residual peaks of the multiplex PCR amplicons may be visible in the fragment analysis and can influence the average size when using a smear or region analysis, thus it is recommended to use a size of 400 bp.
- MIDNIGHT libraries typically have a longer size distribution with an average fragment of 650-750 bp. Follow the instructions in the user guide for additional information on determining fragment size for the MIDNIGHT libraries.
- Typical library concentrations are >50 nM for a pool of 96 samples
- Recommended read configurations for an ARTIC based library are 2x76 while those for a MIDNIGHT library are 2x151
- Load the Illumina sequencer according to Illumina's recommendations.
- For surveillance sequencing purposes, we recommend >50K read pairs per sample. This equates to 96-192 samples per MiSeq (v2 300 or v3 150) flow cell or 384-768 samples per NextSeq 550 mid output flow cell.

Example Final Library traces on D500 tapescreen analysis

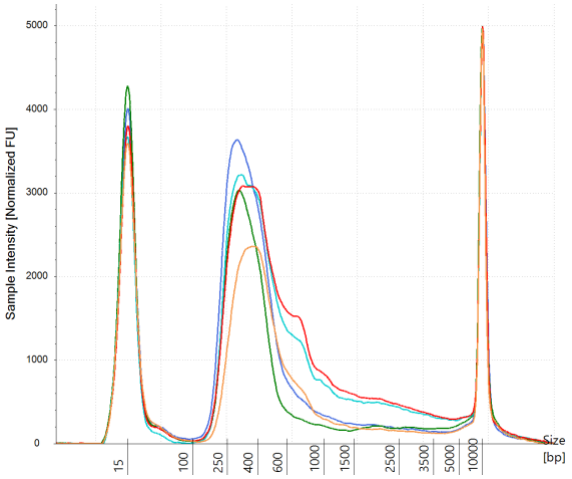


Figure 1. ARTIC library fragment distribution varies depending on how the multiplex ARTIC PCRs are diluted prior to library preparation. All traces generated using D5000 tapescreen analysis (Tapestation). All dilution points generate usable library with slight variations in insert size. 1:12 dilution (green) of ARTIC multiplex amplicons resulted in a median insert of 140-150 nt; 1:10 dilution (turquoise, blue) of amplicons resulted in a median insert of 150-160 nt; 1:8 dilution (red) and 1:5 dilution (orange) results in a median insert of 170-175 nt.

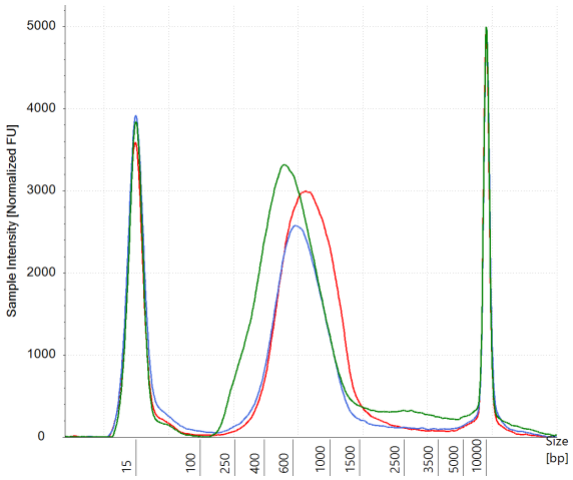


Figure 2. Midnight library fragment distribution varies depending on how the multiplex MIDNIGHT amplicons are diluted prior to library preparation. All traces generated using D5000 tapescreen analysis (Tapestation). All dilution points generate usable library with slight variations in insert size. 1:15 dilution (green) of Midnight multiplex amplicons resulted in a median insert of 285 nt; 1:10 dilution (blue) resulted in a median insert of 340-380 nt; 1:5 dilution (red) resulted in a median insert of 430 nt.

Additional Questions?

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