

Oct 18, 2021

SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1

DOI

<https://dx.doi.org/10.17504/protocols.io.bwv5pe86>



Emmanuel Kagning Tsinda¹, Masahiro Sakamoto¹, Michiko Okamoto¹, Clyde Dapat¹, Mariko Saito¹, Mayuko Saito¹, Hitoshi Oshitani¹

¹Department of Virology, Tohoku University Graduate School of Medicine

NGS_Virology_tohoku



Emmanuel KT

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.bwv5pe86>

Protocol Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani 2021. SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1.

protocols.io <https://dx.doi.org/10.17504/protocols.io.bwv5pe86>

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

Our collaborators at the Research Institute for Tropical Medicine (RITM, Manilla, Philippines) and our members at the Virology Department of Tohoku University Graduate School of Medicine, use this protocol and it works well !

Created: July 26, 2021

Last Modified: October 18, 2021

Protocol Integer ID: 51869

Keywords: High-throughput sequencing, Library prep, Illumina, DNA, Next generation Sequencing, illumina dna prep library kit, low input dna samples as the starting material, original illumina dna prep reference guide, dna libraries from pcr amplicon, using low input dna sample, dna library, ml pcr tube, hundreds of dna library, dna sample, current library preparation protocol, magnetic stand for pcr plate, pcr plate, pcr reaction, well pcr plate, dna, pcr amplicon, pcr, running few specimen, laboratory equipment, reduced library preparation cost, few samples for library preparation, well pcr, original illumina protocol, ml tube, cheaper than the original illumina protocol, library preparation cost, tiled virus amplicon, microseal adhesive seal, laboratory equipment such as the eppendorf

Funders Acknowledgements:

Japanese Agency for Medical Research and Development (AMED)

Grant ID: JP21wm0125001

Disclaimer

Funder had no role in the conception and design of this protocol.

Abstract

This protocol describes the steps to prepare DNA libraries from PCR amplicons using the Illumina DNA prep library kit. The current library preparation protocol was adapted from the original Illumina DNA Prep Reference Guide (document #1000000025416 v09) using low input DNA samples as the starting material.

We omitted laboratory equipment such as **the Eppendorf 96-well PCR plate, microseal adhesive seals**. In addition, we replaced **96-well plate magnetic stand with a magnetic stand suitable for 1.5 ml tubes**.

The added value of this protocol is that PCR reactions happen in 0.2 ml PCR tubes, and it can be implemented without a separate purchase of 96-well PCR plates or a magnetic stand for PCR plates. Using individual 0.2 ml tubes increases the user flexibility when running few samples for library preparation. The other advantage of using our adapted protocol is the reduced library preparation cost when running few specimens. For instance, implementing the current protocol might be cheaper than the original Illumina protocol when using the Illumina® DNA Prep, (M) Tagmentation (24 Samples) catalog number 20018704.

The protocol has proven effective for processing hundreds of DNA libraries from tiled virus amplicons such as Sapovirus and SARS-CoV-2 submitted to public repositories such as GISAID and GenBank.



Guidelines

To minimize the risk of contamination, steps in this protocol must be performed in a DNase-free environment, preferably in a disinfected biosafety cabinet.

This protocol was found suitable to prepare a sequencing library for SARS-CoV-2 amplicons generated using the ARTIC method and implemented without using PCR plates during library prep. However, this protocol may be effective for other kinds of amplicon sequencing. Please follow the appropriate reagent storage and handling precautions defined by the manufacturer.



Materials

	A	B	C
	Catalog number	Reagent / Item name	Manufacturer
	20018707	Nextera DNA CD Indexes (24 Indexes, 24 Samples)	Illumina
	20018704	Nextera DNA Flex Library Prep (24 Samples)	Illumina
	MS-102-2003	MiSeq Reagent Kit v2 (500 Cycles)	Illumina
	Q32854	Qubit ds DNA HS Assay Kit, 500 assays	ThermoFisher Scientific
	20015892	HT1 Hybridization Buffer	Illumina
	FC-110-3001	PhiX Control v3	Illumina
	E3010L	LunaScript RT SuperMix Kit-100 rxns	NEB
	M0494X	Q5 Hot Start High-Fidelity 2X Master Mix-500 rxns (1 x 12.5 ml)	NEB
	A63880	Agencourt AMPure XP 5ml	Beckman Coulter
	12321D	DynaMag™-2 magnetic stand	ThermoFisher Scientific
	E7023	Ethanol	Sigma-Aldrich



	A	B	C
	AM9937	Nuclease free water	ThermoFisher Scientific
	BM4006	Low binding tubes (0.6 ml)	BMBio
	509-GRD-Q	1.5 ml graduated microcentrifuge tubes	BMBio
	430-V-Q	0.2 ml thin walls PCR tubes with cap	BMBio
	Not applicable	Milli-Q water or ultrapure water	
	Not applicable	Micropipettors	
	Not applicable	Heatblocks and Thermocyclers	

Table of required reagents

Protocol materials

☒ Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #Q32851**

☒ Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

☒ HT1 Hybridization Buffer **Illumina, Inc. Catalog #20015892**

☒ phiX V3 control **Illumina, Inc. Catalog #FC-110-3001**

Safety warnings

❗ Comply with principles of Good Laboratory Practice (GLP) and/or the ISO15189 safety rules. In addition, please follow safety regulations disclosed by reagents' manufacturers



Before start

- Please follow steps 1 to 25 of the protocol below ARTIC protocol below.

Protocol



NAME

COVID-19 ARTIC v3 Illumina library construction and sequencing protocol

CREATED BY

Diana Rajan

[Preview](#)

- After the multiplex PCR using the ARTIC protocol (<https://www.protocols.io/view/covid-19-artic-v3-illumina-library-construction-an-bgxjjxkn>), please pool P1 and P2 amplicons into a single 1.5 ml tube; then clean-up the amplicons using magnetic beads following the manufacturer's protocol.
- Quantify the cleaned DNA product Qubit DNA High-sensitivity kit (important).
- Dilute the genomic DNA of each sample to 1ng/ul in 30 µl of RNase/DNase free water.

Overview of the library prep workflow

1

Figure 1 Nextera DNA Flex Library Prep Workflow

Source: https://sapac.support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/illumina_prep/illumina-dna-prep-reference-guide-1000000025416-09.pdf

Please make sure you review and understand this library preparation workflow.

DNA tagmentation

2 This step uses the Nextera Transposome to tagment genomic DNA. Tagmentation is a process that fragments DNA and then tags the fragmented DNA with adapter sequences in a single reaction.

2.1 Thaw EPM and indexes on ice.

- 2.2 Bring the following reagents at room temperature: BLT (beads linked transposomes), TB1(Tagmentation buffer), TSB (Tagment Stop buffer), and TWB (Tagment Wash Buffer).
- 2.3 Vortex BLT and TB1 to mix before use.
- 2.4 Pre-heat the lid of the Thermocycler (by Running the "**TAG**" program, and press "Pause")
The TAG program is set as follows:
 - Set the reaction volume to  50 μL
 -  55 °C for  00:15:00 15 min
 - Hold at  10 °C
- 3 Vortex BLT vigorously for  00:00:10 seconds to resuspend. Do not centrifuge BLT.
- 4 Prepare Tagmentation master mix by adding the following to a separate tube for the master mix.
 - BLT:  11 μL
 - TB1:  11 μLMultiply the volume of BLT and TB1 by the number of samples.
- 5 Vortex tagmentation master mix thoroughly to resuspend BLT in tagmentation buffer.
- 6 Distribute  20 μL into each 0.2 tubes containing  30 μL of DNA samples ( 1 ng/ μL) and gently pipette 10 times to resuspend. Gentle pipetting prevents liquid drops splits on the walls of tubes. Do not spin down.
- 7 Close caps and place tubes into the pre-heated thermocycler and resume the "**TAG**" program.
- 8 Immediately proceed to the next step → Post-Tagmentation cleanup.

Post tagmentation cleanup

- 9 This step washes the adapter-tagged DNA on the BLT before PCR amplification.



- 9.1 Prepare the following reagents:
- Tagment Stop Buffer (TSB): if precipitates are observed, heat at 37°C for 10 min (using a heat-block) and vortex to dissolve the precipitate.
 - Tagment Wash Buffer (TWB)
 - DynaMag magnetic stand
 - 0.2 ml PCR tubes
- 9.2 Run the **PTC** program below on the thermocycler and press "pause".
- Choose the preheat lid option and set it to 100 °C
 - Set the reaction volume to 60 µl
 - 37 °C for 00:15:00 min
 - Hold at 10 °C
- 10 Add 10 µL of TSB to the tagmentation reaction and slowly pipette each well 10 times to resuspend the beads.
- 11 Place tubes in the pre-programmed thermal cycler, and run the "PTC" program.
- 12 Transfer content of each PCR tube (60 µL) to a 1.5 ml low adhesion microcentrifuge tube.

Equipment

1.5 ml graduated microcentrifuge tube Flat Top Cap		NAME
Tube		TYPE
Quality Scientific Plastics		BRAND
509-GRD-Q		SKU
certified RNase and DNase free		SPECIFICATIONS

- 13 Place tubes on the magnetic stand and wait until liquid is clear (~3 minutes).
- 14 Using a P100 pipette, carefully remove and discard supernatant.
- 15 **Washing steps:**
 - 15.1 Remove from the magnetic stand and use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads. The deliberately slow pipetting technique minimizes the potential of TWB foaming to avoid incorrect volume aspiration and incomplete mixing.
 - 15.2 Mix at 1400 rpm for 2 min at room temperature (or pipette mix slowly 10 times until beads are re-suspended).
 - 15.3 Place the tubes on the magnetic stand and wait until the liquid is clear (~3 minutes).
 - 15.4 Using a P200 pipette, remove and discard the supernatant.
 - 15.5 Repeat the washing steps 15.1 to 15.3 above.
 - 15.6 Keep tubes on the magnetic stand until step 22 of the section **Amplify Tagmented DNA section** below. The TWB remains in the tube to prevent overdrying of the beads.

Amplify tagmented DNA

- 16 This step amplifies the tagmented DNA through a limited-cycle PCR program. The PCR step adds Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing cluster generation.

Note

- Items to prepare: powder-free gloves (number of gloves = number of adapter indexes to use)
- Thawed indexes on ice: vortex to mix, then briefly centrifuge
- Thaw EPM: invert to mix, then briefly centrifuge
-  Nuclease-Free Water (not DEPC-Treated) **Thermo Fisher Scientific Catalog #AM9937**
- 0.2 ml thin Wall PCR tube

Equipment

0.2 ml Thin Wall PCR Tube with Cap

Tube

QSP

430-V-Q

https://www.bmbio.com/img/freepage/download/2018_QSP_catalog.pdf^{LINK}

Certified RNAase and DNAse free

NAME

TYPE

BRAND

SKU

SPECIFICATIONS

17 Fill the table below including index information.

	A	B	C	D	E	F	G	H	I
		H503	H505	H506	H517				
	H705	sample 1	sample 2	sample 3					
	H706								
	H707								
	H710								
	H711								



	A	B	C	D	E	F	G	H	I
	H714								

Note: Index names may vary depending on the purchased Illumina index kit.

- 18 Pre-heat the thermocycler by running the "**BLT**" program below and press "Pause".

Choose the preheat lid option and set it to 100°C. set thermocycler:

- 68°C for 3 minutes
- 98°C for 3 minutes
- (6) cycles of: 98°C for 45 seconds, 62°C for 30 seconds, 68°C for 2 minutes
- 68°C for 1 minute
- Hold at 10°C

- 19 Label 0.2 ml PCR tubes with the sample number.

- 20 Combine the following volumes to prepare the PCR master mix. Multiply each volume by the number of samples being processed. Reagent overage is included in the volume to ensure accurate pipetting

- EPM :  22 µL
- Nuclease-free water  22 µL

- 21 Briefly vortex then centrifuge for 10 seconds.

- 22 With the 1.5 ml tubes on the magnetic stand, use a P300 pipette to carefully remove and discard the supernatant.

Foam that remains on the well-walls does not adversely affect the library.

- 23 Remove tubes from the magnetic stand.

- 24 **Immediately** add  40 μL of PCR master mix directly onto the beads in each sample tube, then gently pipette to mix until the beads are fully re-suspended and transfer all the contents into labeled PCR tubes. Gentle pipetting avoids bubbles and liquid drop splits on the walls of the tube.
- 25 Add the 5 μL of I5 index adapters and 5 μL of I7 index adapters to each sample, using 1 pair of gloves per index adapters to minimize contamination risk.
- 26 After adding each index, mix by gently pipetting up and down 5 times. Careful pipetting ensures the absence of bubbles and liquid splits on the tube walls.
- 27 Place on the thermal cycler and run the "BLT" PCR program.

Expected result

At the end of this PCR reaction, you should expect beads pellet at the bottom of PCR tubes.

Note

SAFE STOPPING POINT

If you are stopping here, store at 2°C to 8°C for up to 3 days.

Cleaning the library

- 28 This step uses a double-sided bead purification procedure to purify the amplified libraries.
Items to prepare :
- Sample Purification Beads (SPB): bring at room temperature 30 min before use, vortex to resuspend the beads, and invert to mix.
 - Freshly prepared 80% ethanol (EtOH). use milli-Q water to prepare the 80% ethanol.
 - RSB (Resuspension Buffer): thaw and bring to room temperature; vortex to mix.
 - 1.5 ml low binding microcentrifuge tubes; label two tubes per sample.
 - Nuclease-free water.
- 29 Transfer the contents to a 1.5 ml tube.

- 30 Spin down for 10 seconds to collect contents at the bottom of tube.
- 31 Place the tubes on the magnetic stand and wait until the liquid is clear (~5 minutes).
- 32 Transfer 45 μ l supernatant from each tube to new 1.5 ml tubes.
- 33 Vortex and invert SPB multiple times to resuspend.
- 34 The starting DNA was a small PCR amplicon input. Perform the following steps:
 - 34.1 Add 81 μ l SPB to each 1.5 ml tube containing supernatant.
 - 34.2 Mix using a plate shaker at  1600 rpm , at room temperature for 1 minute.
- 35 Incubate capped tubes at room temperature for 5 minutes.
- 36 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
- 37 Without disturbing the beads, remove and discard supernatant.
- 38 Wash two times as follows:
 - 38.1 With the plate on the magnetic stand, add 200 μ l fresh 80% EtOH without mixing.
 - 38.2 Incubate for 30 seconds.



- 38.3 Without disturbing the beads, remove and discard supernatant.
- 39 Use a 20 µl pipettor to remove and discard residual EtOH.
- 40 Air-dry on the magnetic stand for 5 minutes, then remove from the magnetic stand.
- 41 Add 32 µl RSB to the beads and gently pipette 5 times to resuspend.
- 42 Incubate at room temperature for 2 minutes.
- 43 Place the tube on the magnetic stand and wait until the liquid is clear (~2 minutes).
- 44 Transfer 30 µl supernatant to a new low binding 0.5 ml tube. This supernatant contains double stranded DNA library.

Note

SAFE STOPPING POINT

If you are stopping, close cap and store at -25°C to -15°C for up to 30 day

Pool and dilute library

45 **Library quality control**

Check the concentration of library using

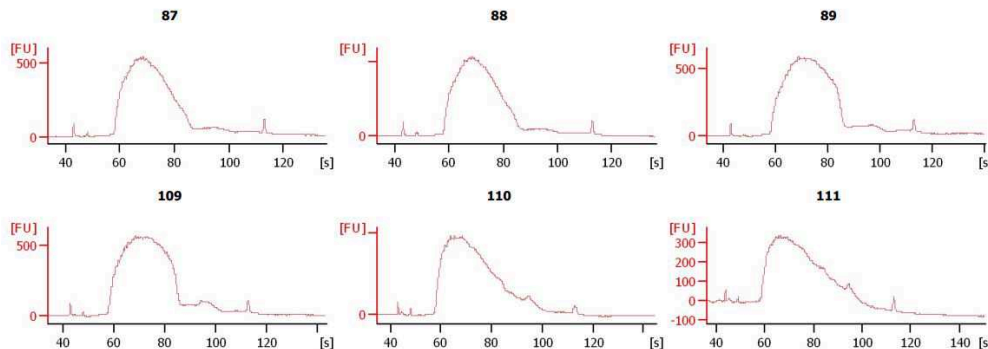
 Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #Q32851**

46 Run 1 µl of undiluted library on an Agilent Technology 2100 Bioanalyzer using the

 Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626** .

Expected result

The following figure shows example traces of libraries we successfully sequenced on a MiSeq system.



Note: Make sure the library concentration values from Qubit and Bioanalyzer are within the expected range.

47 Dilute Libraries to the starting concentration

- 1 Calculate the molarity value of the library or pooled libraries using the following formula.
 - For libraries qualified on a Bioanalyzer, use the average size obtained for the library.
 - For all other qualification methods, use 600 bp as the average library size.

$$\frac{\text{ng} / \mu\text{L} \times 10^6}{660 \frac{\text{g}}{\text{mol}} \times \text{average library size (bp)}} = \text{Molarity (nM)}$$

E.g: In case of sample P1743, the library size is on average 400 bp, the library concentration is 1.65 ng/ul.
The molarity using this formula is 6.25 nM.

Chemistry	Compatible Denature and Dilute Steps
MiSeq Reagent Kit v3	4 nM library —Results in a 6–20 pM loading concentration.
MiSeq Reagent Kit v2	4 nM library —Results in a 6–20 pM loading concentration. 2 nM library —Results in a 6–10 pM loading concentration.

This table shows the possible loading concentrations for MiSeq v2 and v3 reagent kits.
Source : https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-denature-dilute-libraries-guide-15039740-10.pdf

- 48 Dilute the library using RSB to **[M] 2 nanomolar (nM)** (**10 µL**) into a low binding 0.6 ml tubes.

Equipment

Platinum 0.6 ml tube	NAME
Tube	TYPE
BMBio	BRAND
BM4006	SKU

	A	B	C	D	E	F
	sample	Library concentration [Qubit(ng/ul)]	Molarity(nM)	DNA2nM(ul)	RSB (ul)	total
	P1743	1.65	$1.65 \times 10^6 / 60 / 400 = 6.25$	$20 / 6.25 = 3.2$	$10 - 3.2 = 6.8$	10
	P1866	0.968	$0.95 \times 10^6 / 60 / 400 = 3.5985$	5.57	4.43	10

sample P1743: add 3.2 DNA + 6.8 ul RSB to make 2nM (10ul) DNA library

49 Library pooling

Label a clean 1.5 ml low binding microcentrifuge tube.

49.1

Add 5ul of each 2nM diluted libraries into the 1.5 ml tube to make a pooled 2nM DNA library.

49.2 Mix for 2 min 30 seconds at  1400 rpm , at room temperature .



50 Library denaturation before the sequencing using Illumina MiSeq v2

Source: https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-denature-dilute-libraries-guide-15039740-10.pdf

Prepare :  HT1 Hybridization Buffer Illumina, Inc. Catalog #20015892 , NaOH (10 M), milli-Q water (ultrapure water), 1.5 ml low binding tubes.

- thaw HT1 ((Hybridization Buffer), RSB,

 phiX V3 control Illumina, Inc. Catalog #FC-110-3001 and place on ice.

- prepare 1N NaOH = 10M NaOH (50ul) + MilliQ H2O (450ul)
- prepare fresh [0.2N NaOH] from 1N NaOH stock solution :

○ Mix  800 μ L milli-Q water +  200 μ L of Stock 1N NaOH

○ Invert tube several times to mix

NB: 0.2N NaOH should be used within 12 hours.

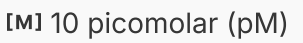
50.1 Label a new 1.5 ml low binding microcentrifuge tube and combine the following volumes in the tube:

- 5 μ L of pooled library (2nM)
- 5 μ L of 0.2N NaOH

50.2 Briefly vortex the mixture.

50.3 briefly centrifuge for 2- 3 seconds.

50.4 incubate for 5 minutes at room temperature.

50.5 Add  990 μ L of pre-chilled HT1 into the tube to obtain  10 picomolar (pM) of denatured library ( 1 mL) $\rightarrow$ then flick tube to mix.

50.6 Dilute denatured library (if needed)

Concentration	6 pM	8 pM	10 pM
10 pM library	360 μ L	480 μ L	600 μ L
Prechilled HT1	240 μ L	120 μ L	0 μ L



- 50.7 Please make a [M] 10 picomolar (pM) of final solution ( 600 μL). Therefore,
- transfer  600 μL of denatured library into new low binding 1.5 ml tube.
 - Invert to mix and spin down

51 Preparation of PhiX control

PhiX Control v3 is a reliable, adapter-ligated library used as a control for Illumina sequencing runs. The library is derived from the small, well-characterized PhiX genome, offering several benefits for sequencing and alignment.

This is a small, ready-to-use Illumina library with a balanced nucleotide representation. Adding a 2% PhiX spike-in to your library provides additional metrics. For low-diversity libraries, use a 10% spike-in to increase base diversity.

- 51.1 In a 0.2 ml PCR tube, prepare [M] 4 nanomolar (nM) Phix from the [M] 10 nanomolar (nM) stock.

Mix:

-  2 μL (of 10 nM stock)
-  3 μL of RSB

- 51.2 In a clean 1.5 ml tube, Mix :
- 4 nM PhiX library:  5 μL
 - 0.2 N NaOH:  5 μL

- 51.3 Vortex the modified PhiX solution vigorously.

- 51.4 Briefly centrifuge.

- 51.5 Incubate at room temperature for 5 minutes.

- 51.6 Dilute denatured PhiX to [M] 20 picomolar (pM)

- 51.7 Mix :
- Modified PhiX solution:  10 μL
 - Ice-cooled HT1:  990 μL



The newly prepared 1 mL of PhiX solution has a final concentration of 20 pM. It can be kept frozen for up to 3 weeks.

51.8 To prepare 10 pM of PhiX control, mix :

-  240 μL of PhiX solution (20 pM),
-  240 μL of ice cooling HT1.

51.9 In a new e-tube, prepare the PhiX 10% solution by mixing :

- PhiX (10 pM):  60 μL
- DNA library (10 pM):  540 μL

51.10 Store the prepared PhiX control and library pool at  -30 °C until sequencing using MiSeq flowcell reagents.