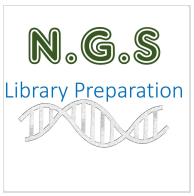

Oct 18, 2021

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

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NGS_Virology_tohoku

Emmanuel KT

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

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.

DOI

dx.doi.org/10.17504/protocols.io.bwv5pe86

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Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1 https://dx.doi.org/10.17504/protocols.io.bwv5pe86

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High-throughput sequencing, Library prep, Illumina, DNA, Next generation Sequencing

protocol ,

Jul 26, 2021

Oct 18, 2021

51869

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.

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

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

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
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 Thermocylcers	

Table of required reagents

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

:

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

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

COVID-19 ARTIC v3 Illumina library construction and sequencing protocol
by Diana Rajan

PREVIEW RUN

- 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

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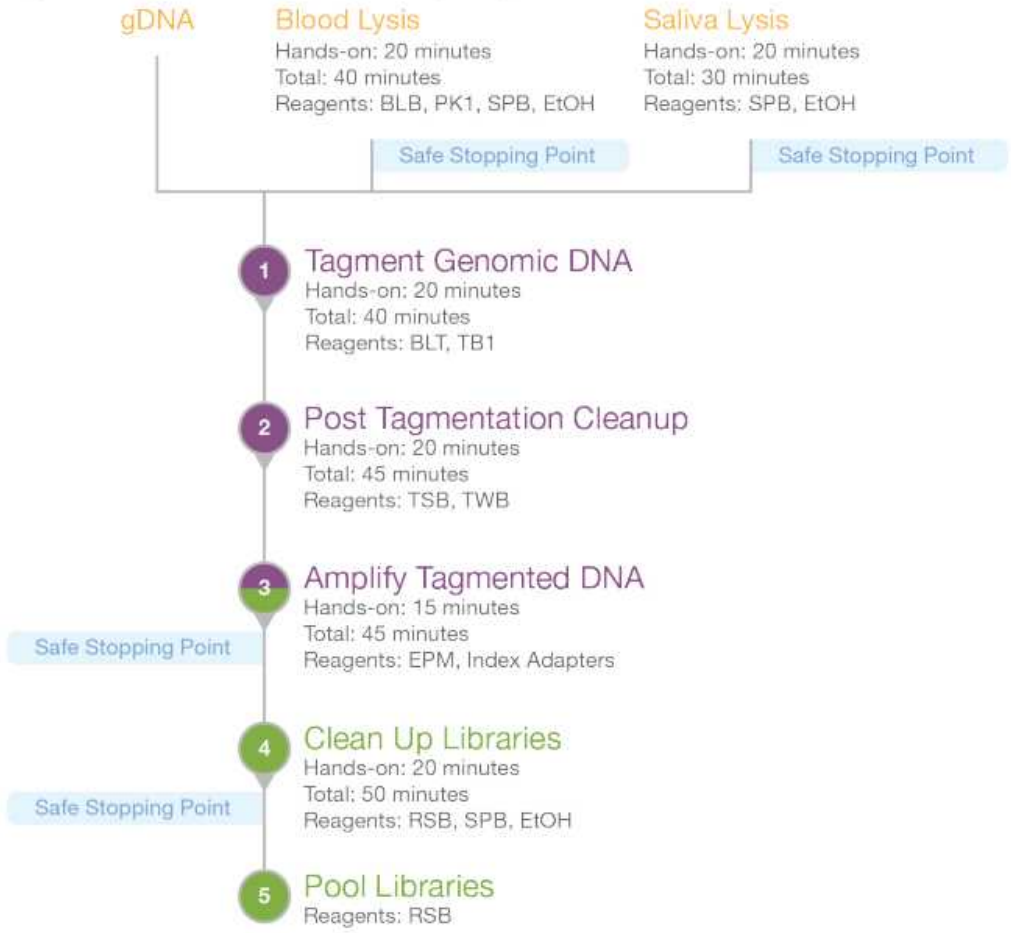
single 1.5 ml tube; then clean-up the amplicons using magnetic beads following the manufacturer's protocol.

- 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.

Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1 https://dx.doi.org/10.17504/protocols.io.bwv5pe86

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 µL**

Multiply 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.

Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1
https://dx.doi.org/10.17504/protocols.io.bwv5pe86

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.

1.5 ml graduated microcentrifuge tube Flat Top Cap
Tube
Quality Scientific Plastics **509-GRD-Q**
certified RNase and DNase free

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.

Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1
https://dx.doi.org/10.17504/protocols.io.bwv5pe86

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.

- 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

Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1 https://dx.doi.org/10.17504/protocols.io.bwv5pe86

0.2 ml Thin Wall PCR Tube with Cap
Tube

QSP 430-V-Q

Certified RNAase and DNAse free

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								
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

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

- 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 5µl of I5 index adapters and 5µl 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.

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

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 :

Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1
https://dx.doi.org/10.17504/protocols.io.bwv5pe86

- 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 µl 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 µl 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 caped tubes at room temperature for 5 minutes.

Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1 https://dx.doi.org/10.17504/protocols.io.bwv5pe86

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 µl 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).

Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1
https://dx.doi.org/10.17504/protocols.io.bwv5pe86

44 Transfer 30 µl supernatant to a new low binding 0.5 ml tube. This supernatant contains double stranded DNA library.

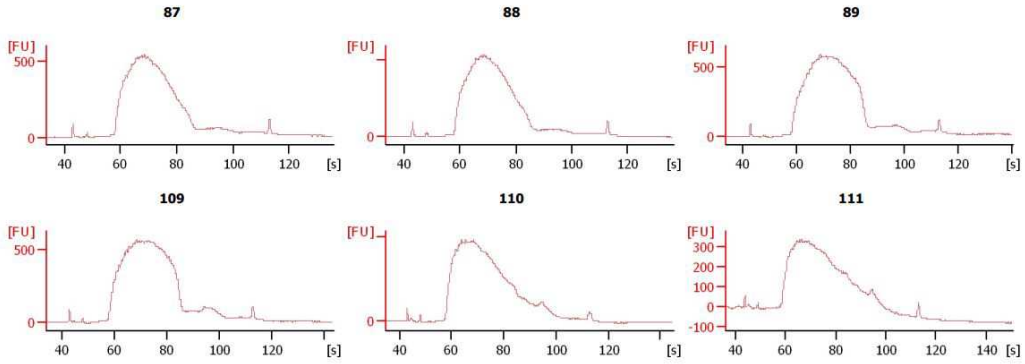
SAFE STOPPING POINT
If you are stopping, close cap and store at -25°C to -15°C for up to 30 day

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** .

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

Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1
https://dx.doi.org/10.17504/protocols.io.bwv5pe86

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.

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

E.g: In case of *s*ample 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.

Platinum 0.6 ml tube
Tube
BMBio **BM4006**

A	B	C	D	E	F
sample	Library concentration [Qubit(ng/ul)]	Molarity(nM)	DNA2nM(ul)	RSB (ul)	total
P1743	1.65	1.65*1E6/660/400=6.25	20/6.25=3.2	10-3.2=6.8	10
P1866	0.968	0.95*1E6/660/400=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.

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

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

HT1 Hybridization

Prepare : Buffer **illumina 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 µL** milli-Q water + **200 µL** 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 µl of pooled library (2nM)
- 5 µl 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 µL** of pre-chilled HT1 into the tube to obtain **10 Picomolar (pM)** of denatured library (**1 mL**) --> then flick tube to mix.

50.6 Dilute denatured library (if needed)

Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1 https://dx.doi.org/10.17504/protocols.io.bwv5pe86

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

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 clean1.5 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)**

Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1
https://dx.doi.org/10.17504/protocols.io.bwv5pe86

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.

Citation: Emmanuel Kagning Tsinda, Masahiro Sakamoto, Michiko Okamoto, Clyde Dapat, Mariko Saito, Mayuko Saito, Hitoshi Oshitani SARS-CoV-2 DNA library preparation using an adapted version of Illumina DNA prep protocol v.1.1 https://dx.doi.org/10.17504/protocols.io.bwv5pe86