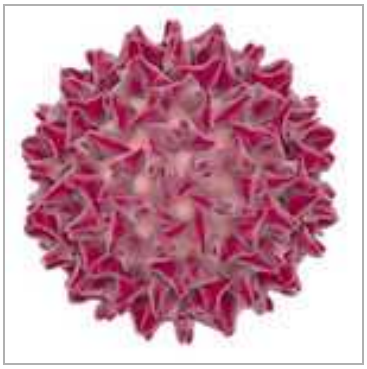

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Protocol status: Working
We use this protocol and it's working

Created: Jan 14, 2021

SARS-CoV-2 Whole Genome Sequencing on Illumina V.2

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DISCLAIMER

In development
We are still developing and optimizing this protocol. Comments and feedback appreciated.

ABSTRACT

This SOP describes the procedure for generating cDNA from SARS-CoV-2 viral nucleic acid extracts and subsequently obtaining, through the amplicons tiling, the whole viral genome using V3 nCov-2019 primers (ARTIC). This is followed by library construction and pooling of samples and quantitation, prior to sequencing on the Illumina MiSeq.
The SOP is adapted from the nCoV-2019 sequencing protocol: https://www.protocols.io/view/ncov-2019-sequencing-protocol-bbmuik6w, and it was used in this study:
Lucey M, Macori G, Mullane N, Sutton-Fitzpatrick U, Gonzalez G, Coughlan S, Purcell A, Fenelon L, Fanning S, Schaffer K. Whole-genome Sequencing to Track Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Transmission in Nosocomial Outbreaks. Clinical Infectious Diseases. 2020. https://academic.oup.com/cid/advance-article/doi/10.1093/cid/ciaa1433/5909421

Last Modified: Jul 20, 2023

PROTOCOL integer ID: 46297

Keywords: Tiling PCR, WGS, SARS-CoV-2, nCoV-2019, nCoV19, WvGS

MATERIALS

MATERIALS

- NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) - 96 rxns **New England Biolabs Catalog #E7600S**
- Q5 Hot Start High-Fidelity 2X Master Mix - 100 rxns **New England Biolabs Catalog #M0494S**
- Deoxynucleotide Solution Mix - 40 umol of each **New England Biolabs Catalog #N0447L**
- Agencourt AMPure XP SPRI beads **Beckman Coulter Catalog #A63881**
- NEBNext Ultra II FS DNA Library Prep Kit for Illumina - 96 rxns **New England Biolabs Catalog #E7805L**
- Random primer mix **New England Biolabs Catalog #S1330S**
- SuperScript™ IV Reverse Transcriptase **Thermo Fisher Scientific Catalog #18090050**
- RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Catalog #10777019**
- MiSeq Reagent Nano Kit v2 (500 cycles) **Illumina, Inc. Catalog #MS-103-1003**

- Deoxynucleotide Solution Mix - 40 umol of each **New England Biolabs Catalog #N0447L**
- DTT, 100mM (Dithiothreitol) **Promega Catalog #P1171**
- ARTIC v3 Primer Pools **IDT Technologies**
- Nuclease-free Water - 100 ml **New England Biolabs Catalog #B1500L**
- TE Buffer (1X) **New England Biolabs Catalog #E7808**
- NEBNext End Repair Module - 100 rxns **New England Biolabs Catalog #E6050L**
- Sodium Hydroxide NaOH 1M **Gibco, ThermoFisher Catalog #A4782601**
- PhiX Control v3 **Illumina, Inc. Catalog #FC-110-3001**
- MiSeq Reagent Kit V2 (300-cycles) **Illumina, Inc. Catalog #MS-102-2002**

1 cDNA/Reverse Transcription Section Date/Initials:_____

In this section, the nucleic acid is extracted and used for the qPCR diagnostic test as starting material for sequencing.

1.1 [ ] In a PCR hood, mix the following reagents in a 0.2 mL PCR tube or PCR plate:

	A	B	C
	Reagent	Volume (µL)	MM for N+2 samples
	60 µM random hexamers	1.0	
	10 mM dNTPs mix (10 mM each)	1.0	
	Template RNA	11.0	
	Total	13.0	

Master mix calculations

Note

Mastermix should be made up in the mastermix cabinet and aliquoted into PCR tubes.
Tubes should be wiped down when entering and leaving the mastermix cabinet.
Each reaction should have 13 µL when mixed.

If using master mix, it is recommended to add the 2 µL of the master mix to the PCR tube/plate first, then add the 11 µl of RNA to help prevent contamination.

Random Primer Mix-6 nmol **New England Biolabs Catalog #S1330S**

Lot# _____ Exp. Date _____

Deoxynucleotide Solution Mix - 8 umol of each **New England Biolabs Catalog #N0447S**

Lot# _____ Exp. Date _____

MicroAmp™ Reaction Tube with Cap, 0.2 mL **Thermo Fisher Catalog #N8011540**

1.2 [ ] Mix gently and briefly centrifuge to spin down the components, and return On ice.

1.3 [ ] Preheat Thermocycler to 65 °C, with heated lid at 105 °C

1.4 [ ] Incubate the reaction at 65 °C for 00:05:00, followed by an immediate snap-cool On ice for at least 00:01:00.

1.5 [ ] In a clean 1.5 mL LoBind tube (96 well plates can also be used), mix together the following reagents:

	Reagent	Volume (uL)	MM for N+2 samples
	SuperScript IV RT 5X Buffer	4.0	
	100mM DTT	1.0	
	RNaseOUT RNase Inhibitor	1.0	
	Superscript IV Reverse Transcriptase	1.0	
	Total	7.0	

Master mix for RT reaction.

Note

The mastermix should be made up in the mastermix cabinet and added to the denatured RNA in the extraction and sample addition cabinet. Tubes should be wiped down when entering and leaving the mastermix cabinet.

RNaseOUT Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #10777019**

Lot# _____ Exp. Date _____

SuperScript™ IV Reverse Transcriptase **Thermo Fisher Catalog #18090050**

Lot# _____ Exp. Date _____

twin.tec PCR Plate 96 LoBind semi-shirted clear 25 pcs. **Eppendorf Catalog #30129504**

1.6 [ ] Add the above mastermix (7 µL) to the annealed DNA (13 µL) giving a total volume 20 µL

1.7 [ ] Cap the tube (or seal the plate), mix and then briefly centrifuge the contents.

1.8 [ ] Preheat thermocycler to 42 °C , with heated lid at 105 °C

1.9 [ ] Incubate sample using the following reverse transcription program:

	Step	Temperature (°C)	Time	Cycle
	Reverse Transcription	42	50:00	1
	RT Inactivation	70	10:00	1
	Cool	4	Hold	Hold

PAUSE POINT cDNA can be stored at** 4 °C **(same day) or** -20 °C **(up to a week).

SARS-CoV-2 WvGS protocol - ARTIC protocol - Tiled PCR

1h

2 Tiled PCR Section Date/Initials:_____

This section outlines the process for the tiled PCR approach from the ARTIC protocol.

Note

Primer pool sequences (v3) can be found here: https://github.com/joshquick/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V3/nCoV-2019.tsv

If required, resuspend lyophilised primers at a concentration of 100 µM each.
Prepare the primer working solution diluting to [M] 10 micromolar (µM) using [M] 0.1 % volume TE buffer.

2.1 [ ] Set up two individual reactions using primer pool 1 (set 1) and primer pool 2 (set 2) in 0.2 mL PCR tubes according to the following table:

	A	B	C	D	E
	Reagent	Pool 1 (uL)	MM for N+2 samples	Pool 2 (uL)	MM for N+2 samples
	Q5 Hot Start HiFi 2x MM	12.5		12.5	
	Primer pool at 10uM (1 or 2)	3.7		3.7	
	Nuclease-free water	6.3		6.3	
	Total	22.5		22.5	

Master Mix for Tiled PCR

Q5 Hot Start High-Fidelity 2X Master Mix - 100 rxns**New England Biolabs Catalog #M0494S**

Lot# _____ Exp. Date _____

2.2 [ ] Aliquot 22.5 µL from the mastermix into 2 96-well PCR plates or 2 sets of PCR tubes.

2.3 [ ] Add 2.5 µL of sample cDNA (from step 1.9) to each pool giving a total volume 25 µL and mix by pipetting. Spin briefly.

2.4 [ ] Heat seal and place the plates onto a thermocycler and run the following program. 3h 30m
Important! Heat seal to minimise evaporation.

Note: Amplification should ideally be performed in a different lab to minimise the risk of contaminating other samples.

	A	B	C	D
	Step	Temperature	Time	Cycles
	Initial Denaturation	98°C	0:30	1
	Denaturation	98°C	0:15	35
	Anneal and Extension	63°C	5:00	35
	Cool	4°C	Hold	Hold

SARS-CoV-2 Tiled PCR Program

Note

Cycle number should be 25 for Ct 18-21 up to a maximum of 35 cycles for Ct 35

Note

Pause point, Amplified cDNA can be stored at 4 °C **(overnight) or** -20 °C **up to a week.**

SARS-CoV-2 WvGS protocol - ARTIC protocol - PCR Clean-Up...

3 Section for Clean-Up and Size Selection Date/Initials:_____

Reagent preparation:

- Allow AMPure XP beads to equilibrate to room temperature (~30 minutes). Ensure solution is homogenous prior to use, mixing gently by inversion.

Note

AMPure XP beads are needed in later steps. As the beads will need to equilibrate to room temperature before use, please consult steps 5.6 and 7.1 to ensure enough beads to cover all steps and save time.

IMPORTANT: At all stages, ensure to homogenise beads before use.

Ampure XP beads **Beckman Coulter Catalog #A63881**

- Prepare the [M] 80 % volume ethanol (EtOH) using the following calculation:

0.360 mL x (# Sample + 1: _____) = _____ mL total volume (EtOH 100%)

mL total volume x 0.8 = _____ mL EtOH

Total volume _____mL - _____mL EtOH = _____mL H2O

3.1 [ ] Combine the entire volumes of pool 1 and pool 2 PCR reactions (50 µL in total) into one clean PCR plate (or PCR tubes set).

3.2 [ ] Add 0.8X volume of SPRI beads per sample (40 µL SPRI : 50 µL amplified cDNA), mix well by pipetting.
Incubate 00:10:00 at Room temperature .

3.3 [ ] Transfer the plate on the magnet and incubate for 00:05:00 at Room temperature .

3.4 [ ] Keep the plate on the magnet and remove the superanatant by pipetting from the bottom.

Note

Keep the supernatant in case you have to go back for quality assessment. You may recycle one of the PCR plates used during the pool 1/pool 2 PCR stage to retain supernatant.

Ensure to label plate correctly with step no. 3.4 and any unique identifiers for ease of finding later on.

3.5 [ ] Wash the beads in the magnet with 180 µL of freshly prepared 80 % volume EtOH without disturbing the pellet and incubate for 00:00:30 and remove the EtOH.

3.6 [ ] Repeat previous step (total 2 washes).

3.7 [ ] Spin down and place the tubes back on the magnet. Pipette off any residual ethanol with a P10 pipette and allow to dry for approximately 00:10:00 .

Note

Do not over-dry the beads. This may result in a lower recovery of DNA. Beads should appear dark brown and glossy. If they have become light brown or start to crack, this may be a sign they have become too dry.
Dry beads may result in a lower recovery of DNA

3.8 [ ] Remove the plate from the magnet and add 30 µL of nuclease-free water, resuspend the beads pipetting up and down at least 10 times or vortex at 1800 rpm for 00:01:00

3.9 [ ] Incubate at room temperature for 00:02:00

3.10 [ ] Transfer the plate on the magnet and incubate for 00:05:00 at Room temperature

3.11 [ ] Carefully transfer the supernatant (28µl) into a new plate, **taking care not to disturb the bead pellet.**

Note

PAUSE POINT
Purified amplified cDNA can be stored at -20°C for several weeks prior to library preparation.

3.12 [ ] Quantify the sample on Qubit fluorometer or similar instrument and store completed PCR amplified cDNA prep at -20 °C

Note

Purified amplified cDNA is quantified with the use of the dsDNA HS Assay kit.
30 uL of samples should contain 50 ng to 1 ug of DNA (optimal 100-500 ng of DNA). If the DNA concentration at this step is less than ~3ng/uL, the sample did not amplify well and it could be under-represented in the final sequencing reaction.
To streamline the workflow, the samples are not normalised but used as input for library preparation, the entire volume is used for the library preparation.

To normalise, add enough DNA to reach a total of at least 100 ng** and add molecular grade water to bring the total volume to 30 µl.

**NOTE: Preferred amount is 100 ng to 500 ng. Less than that can lead to under-representation of the sample in the final pool.

Qubit dsDNA HS Assay Kit **Invitrogen Catalog #Q32851**

NEBNext library preparation protocol - Fragmentation/End p.

4

Note

At this point in the protocol, there are two options, enzymatic fragmentation and end repair. The method used is dependant upon preference and equipment/consumable/budgetary constraints in the lab.

The enzymatic fragmentation (using NEBNext FS Library Prep Kit E7658) generates library inserts in the 150bp range compatible with 2 x 75 sequencing on illumina instruments. Follow steps 4.1 to 4.3 for this method.

The end repaire method (using NEBNext Library prep kit E7650) repairs the ends of the ~400bp amplicons generated in the tiling PCR. These libraries will be ~400bp, compatible with 2 x 250 sequencing. Follow steps 4.4 to

This section is an adaptation protocol for FS DNA Library Prep Kit (E7805, E6177) with Inputs ≥ 100 ng

Note

For inputs ≤ 100 ng, size selection is not recommended. For 100 ng inputs, either the no size selection protocol or a size selection protocol can be followed.

4.1 [ ] Prepare enzyme Master Mix using the following table:

	A	B	C
	Reagent	Volume (uL)	* (#samples +2)
	NEBNext Ultra II FS Reaction Buffer	3.5 µl	
	NEBNext Ultra II FS Enzyme Mix	1 µl	
	Total Volume	4.5 µl	

Note

Ensure that the Ultra II FS Reaction Buffer is completely thawed. If a precipitate is seen in the buffer, pipette up and down several times to break it up, and quickly vortex to mix. Place on ice until use.

Vortex the Ultra II FS Enzyme Mix 5-8 seconds prior to use and place on ice.

4.2 [ ] Add 4.5 µL of prepared mastermix (above) to each well. Add 13 µL of purified DNA to the PCR tube or to the wells of the PCR plate.Vortex the reaction for 5 seconds and briefly spin in a microcentrifuge.

4.3 [ ] In a Thermocycler, with the heated lid set to 75°C, run the following program:

	A	B	C
	Step	Temp	Time

	A	B	C
	1	37°C	30 min
	2	65°C	30 min
	Hold	4°C	Hold

Note

OPTIMIZATION
Fragmentation occurs during the 37°C incubation step.
Use the chart below to determine the incubation time required to generate the desired fragment sizes. Incubation time may need to be optimized for individual samples. Run the fragmented suspension on Bioanalyzer to visualize the size distribution.

	A	B	C
	Fragmentation size	Incubation at 37°C	Optimization
	100 bp-250 bp	30 min	30-40 min
	150 bp-350 bp	20 min	20-30 min
	200 bp-450 bp	15 min	15-20 min
	300 bp-700 bp	10 min	5-15 min
	500 bp-1 kb	5 min	5-10 min

	A	B
	NEBNext End Prep	Vol/PCR RXN (µl)
	NEBNext Ultra II End Prep Enzyme Mix	1.2
	NEBNext Ultra II End Prep Reaction Buffer	2.8
	Total	4

	A	B
	Temperature	Time

	A	B
	20°C	30 minutes
	65°C	30 minutes
	4°C	∞

https://www.protocols.io/view/covid-19-artic-v3-illumina-library-construction-an-bgttjwnn?step=26

Note

If necessary, samples can be stored at – -20 °C , however, a slight loss in yield (~20%) may be observed. It is recommend continuing with adaptor ligation before stopping.

Continue with this protocol from step 5.

4.4

Steps 4.1 to 4.3 detailed enzymatic fragmentation. The following steps (4.4 to 4.6) detail the end repair option.

If you have carried out steps 4.1 to 4.3, this protocol continues from step 5

[ ] Prepare the following mastermix in a sterile nuclease-free tube:

	A	B
	Component	Volume
	NEBNext Ultra II End Prep Enzyme Mix	1.5 µl
	NEBNext Ultra II End Prep Reaction Buffer	3.5 µl
	Total Volume	5 µl

4.5

[ ] Add 5 µL of mastermix (above) to each well. Add 25 µL of purified DNA to the PCR tube or to the wells of the PCR plate.Vortex the reaction for 5 seconds and briefly spin in a microcentrifuge.

4.6

[ ] In a thermocycler, with the heated lid set to 75 °C, run the following program:

	A	B
	Temperature	Time
	20 °C	30 min
	65 °C	30 min
	4 °C	∞

Note

If necessary, samples can be stored at – -20 °C, however, a slight loss in yield (~20%) may be observed. It is recommend continuing with adaptor ligation before stopping.

NEBNext library preparation protocol - Adapter ligation

5 [ ] Add the following components directly to the FS Reaction Mixture:

	A	B
	Component	Volume
	FS Reaction Mixture (Step 4.3) or End Prep Reaction Mixture (step 4.6)	17.5 µl/ 30 µl
	NEBNext Ultra II Ligation Master Mix	15 µl
	NEBNext Adaptor for Illumina	1.25µl
	Total Volume	33.75 µl/ 46.25 µl

Note

It is not recommended to add adaptor to a premix in the Adaptor Ligation Step.

For this reason, add Ligation Master mix to each well/PCR tube, then add 1.25µl adapter at the end. Seal plate, vortex for 00:00:10 & spin briefly

5.1 [ ] Incubate at 20 °C for 00:15:00 in a thermocycler with the **heated lid off**.

5.2 [ ] Add 1.5 µL µl of USER Enzyme to the ligation mixture from Step 5.1. Vortex for 00:00:10 & spin briefly. 10s

5.3 [ ] Mix well and incubate in thermocycler at 37 °C for 00:15:00 with the heated lid set to ≥ 47 °C

Note

Samples can be stored overnight at -20 °C

Cleanup of Adaptor-ligated DNA

5.4

Note

The volumes of Ampure XP Breads will vary depend on fragmentation method used in section 4.

5.5 [ ] Vortex SPRIselect or NEBNext Sample Purification Beads to resuspend.

5.6 [ ] Add 28 µL (FS fragmentation) or 43 µL (end repair) of the Ampure XP Beads to the ligation reaction mixture and mix well by pipetting up and down, or vortex. Spin briefly.

5.7 [ ] Incubate at room temperature for 00:05:00

5.8 [ ] Place the plate on magnetic block for 00:05:00

5.9 [ ] Carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets

5.10 [ ] Wash the beads adding 200 µL of freshly prepared 80% ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 00:00:30, and then carefully remove and discard the supernatant.
Be careful not to disturb the beads that contain DNA targets.

5.11 [ ] Repeat Step 5.10 once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

5.12 [ ] Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Note

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

5.13 [ ] Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 10 µL [M] 0.1 % volume TE (dilute 1X TE Buffer 1:10 in water).

5.14 [ ] Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 00:02:00 at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

5.15 [ ] Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 7.5 µL to a new PCR tube.

Note

Samples can be stored at -20 °C

NEBNext library preparation protocol - PCR Enrichment of A..

6 [ ] Add the following reagents to each well from step 5.15

	A	B
	Component	Volume
	Adaptor Ligated DNA Fragments (Step 5.15)	7.5 µl
	NEBNext Ultra II Q5 Master Mix	12.5 µl
	Index Primer/i7 Primer	2.5 µl
	Universal PCR Primer/i5 Primer	2.5 µl

	A	B
	Total Volume	25 µl

Note

Ensure to take note of what index set (1 or 2) is used and their sequence numbers,

Index set no. _____

Index Range (A) _____ Index Range (B) _____

6.1 [ ] Set a 100 µl or 200 µl pipette to 40 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

6.2 [ ] Place the tube/plate on a thermocycler with the heated lid set to 105 °C and perform PCR amplification using the following PCR cycling conditions:

	CYCLE STEP	TEMP	TIME	CYCLES
	Initial Denaturation	98°C	30 seconds	1
	Denaturation	98°C	10 seconds	5*
	Annealing/Extension	65°C	75 seconds	
	Final Extension	65°C	5 minutes	1
	Hold	4°C	∞	

Note

*Cycle number was determined by size of input DNA ~100ng is 4-5 cycles.

NEBNext library preparation protocol - Clean up of PCR reac..

7 **Allow the Ampure XP beads to warm to room temperature for at least 30 minutes before use.**

[ ] Vortex SPRIselect to resuspend.

7.1 [ ] Add 22.5 µL (0.9X) resuspended beads to the PCR reaction. Mix well by pipetting up and down at least 10 times. Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

7.2 [ ] Incubate samples on bench top for at least 00:05:00 at Room temperature

7.3 [ ] Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

7.4 [ ] After 00:05:00 (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads).**

7.5 [ ] Add 200 µL of [M] 80 % volume freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 00:00:30 , and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

7.6 [ ] Repeat Step 7.5. once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

7.7 [ ] Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Note

Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

7.8 [ ] Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 17 µL of [M] 0.1 % (v/v) TE (dilute 1X TE Buffer 1:10 in water).

7.9 [ ] Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 00:02:00 at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

7.10 [ ] Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 15 µL to a new PCR tube and store at -20 °C.

Assess Library quality

8 Set up dilutions and standards as laid out in the kit protocol for dsDNA high sensitivity kit. Record Qubit readings before normalization.

Note

In this protocol 2 µL of library (198 µL buffer)

8.1 [ ] Run Samples on Agilent Bioanalyser or Agilent Tapestation to check that the library shows a narrow distribution with an expected peak size based on fragmentation time and size selection. Record the the average peak bp size.

Note

Tape station D1000 HS 2 µL of library in 2 µL buffer (ladder 2 µL in 2 µL buffer for each cartridge)

8.2 [ ] Calculate the dilutions required to normalise each sample to a 4nM concentration using the following formula:
((LibraryConc*(1000)*(1/expected.length)*(1/Average.fragment.length))*1000)

Note

Note: If a peak ~80 bp (primers) or 128 bp (adaptor-dimer) is visible in the Bioanalyzer trace, bring up the sample volume (from Step 2.5.11.) to 50 µl with 0.1X TE Buffer and repeat the Cleanup of PCR Reaction in Section 2.5.

9 [ ] Run Samples on a bioanalyser or tapestation and check that the library shows a narrow distribution with an expected peak size based on fragmentation time and size selection. Record the the average peak bp size

Note

Calculate the molar concentration of each library to be diluted using average size from the TapeStation and mass from Qubit, using the following equation:
(????????????? (??/??)) / (660 ?/??? × ??? ??????? ???????? ????) × 10^6 ??/? = ?????????????? (??)
Make a 4nM dilution of each library

MiSeq Sequencing

6m

10 Pooling and Library Denaturation Date/Initials:_____

This section demonstrates how to generate a pooled library for V2 reagents on the MiSeq.

Note

Thaw the MiSeq reagents overnight or in a Room temperature waterbath.
Remove HT1 from freezer and thaw at Room temperature.
Store at 2 °C to 8 °C until you are ready to dilute denatured libraries.

Note

Label 3 eppendorfs for:
(1) the pooled library
(2) denaturation of library
(3) 0.2N NaOH

Make a fresh dilution of 0.2N of NaOH by combining the following volumes in a microcentrifuge tube: 800 µL laboratory-grade water and 200 µL stock [M] 1.0 nanomolar (nM) NaOH

10.1 [ ] Pool 5 µL of each normalised sample into an eppendorf tube. This will be (1) pooled library.

10.2 [ ] Combine the following volumes in a microcentrifuge tube (2):
5 µL 4nM pooled library and 5 µL of 0.2 N NaOH.

[ ] Vortex briefly and then centrifuge at 280 x g for 1 minute.
[ ] Incubate at room temperature for 00:05:00

10.3 [ ] Add 990 µL of pre-chilled HT1 to the tube containing the denatured library (2). The result is 1 mL of a 20 pM denatured library.

10.4 [ ] Dilute the 20 pM library to the desired concentration, see table below:

	Concentration	6 pM	8 pM	10 pM	12 pM	15 pM	20 pM
	20 pM library	180 uL	240 uL	300 uL	360 uL	450 uL	600 uL
	Pre-chilled HT1	420 uL	360 uL	300 uL	240 uL	150 uL	0 uL

Note

We recommend diluting the library to 10pM for optimal cluster density during Miseq runs with V2 reagents.

[ ] Invert to mix and then pulse centrifuge

Note

The following steps 10.5 to 10.7 can be carried out ahead of time and PhiX library can be stored at -20 °C for a number of weeks

10.5 [ ] Dilute stock PhiX to 4nM by combining:
- 2 µL of [M] 10 nanomolar (nM) PhiX library
- 3 µL of [M] 10 millimolar (mM) Tris-Cl, pH 8.5 with 0.1% Tween 20

10.6 Denature the PhiX control by adding the following volumes in a microcentrifuge tube:
- 5 µL of [M] 4 nanomolar (nM) PhiX library
- 5 µL of [M] 0.2 nanomolar (nM) NaOH

Note

Remaining [M] 4 nanomolar (nM) PhiX can be frozen and reused

6m

10.7 [ ] Vortex briefly to mix and centrifuge at 280 x g for 00:01:00.

[ ] Incubate at Room temperature for 00:05:00

10.8 [ ] Dilute denatured PhiX library to 20 pM by adding 990 uL pre-chilled HT1 to the PhiX tube. Invert to mix.

Note

If using a MiSeq reagent kit v2, dilute 20 pM PhiX library to 12.5 pM by adding the following volumes in a microcentrifuge tube:
- 375 µL 20 pM denatured PhiX library
- 225 µL pre-chilled HT1

10.9 [ ] Combine library and PhiX control according to the table below:

	A	B
	Denatured and diluted PhiX (12.5pM)	30 µl
	Denatured and diluted library (10 pM)	570 µl

10.10 [ ] Set aside on ice until you are ready to load it onto the reagent cartridge.

10.11 [ ] Mix reagents of the MiSeq cartridge thoroughly by inverting several times.

[ ] Using a fresh 1000 µL pipette tip, transfer the denatured and library (with PhiX spiked) into position 17.

10.12 [ ] Load the sample sheet and reagents according to onscreen instructions in the MiSeq Control software.