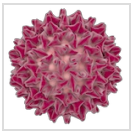

Dec 16, 2020

SARS-CoV-2 Whole Genome Sequencing on Illumina - Tiling PCR

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In Development This protocol is published without a DOI.

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

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MATERIALS TEXT

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** Step 2.1

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**

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.
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https://academic.oup.com/cid/advance-article/doi/10.1093/cid/ciaa1433/5909421

SARS-CoV-2 WvGS protocol - cDNA Preparation Reverse Transcription

1 cDNA/Reverse Transcription Section Date/Initials:_____

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

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

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

A mastermix should be made up in the mastermix cabinet and aliquoted into PCR strip 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 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** 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.

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

SARS-CoV-2 Reverse Transcription Program

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

2 Tiled PCR Section Date/Initials:_____

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

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 **10 Micromolar (µM)** using **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:

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 [ ] Aliquotate **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.

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.

Step	Temperature	Time	Cycles
Initial Denaturation	98°C	0:30	1
Denaturation	98°C	0:15	35
Anneal and Extension	65°C	5:00	35
Cool	4°C	Hold	Hold

SARS-CoV-2 Tiled PCR Program

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

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 and Size Selection

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.

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

- Prepare the **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 primer 1 and primer 2 PCR reactions (**50 µl** in total) into one clean PCR plate (or PCR tubes set). Add 0.8X volume of SPRI beads per sample (**40 µl** SPRI : **50 µl** amplified cDNA), mix well by pipetting.

3.2 [ ] 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.

Keep the supernatant in case you have to go back for quality assessment.

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).

[ ] Spin down and place the tubes back on the magnet. Pipette off any residual ethanol and allow to

3.7 dry for approximately **00:10:00** .

Do not over-dry the beads. This 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 into a new plate, taking care not to disturb the bead pellet.

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 **-20 °C**

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 prep

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

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.

Starting Material
100–500 ng purified DNA. If the input DNA is less than 26 µl, add molecular grade water or 1X TE ([M]**10 Milimolar (mM)** Tris pH**8.0**, [M]**1 Milimolar (mM)** EDTA to a final volume of **26 µl** .

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

Reagent	Volume (uL)	* (#samples+2)
NEBNext Ultra II FS Reaction Buffer	7 µl	
NEBNext Ultra II FS Enzyme Mix	2 µl	
Total Volume	**9 µl**	

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 **26 µl** of purified DNA to the mix. 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:

Step	Temp	Time
1	37°C	10 min
2	65°C	30 min
Hold	4°C	Hold

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.

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

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

Temperature	Time
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

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:

Component	Volume
FS Reaction Mixture (Step 4.3)	35 µl
NEBNext Ultra II Ligation Master Mix	30 µl
NEBNext Ligation Enhancer	1 µl
NEBNext Adaptor for Illumina	2.5 µl
Total Volume	**68.5 µl**

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. It is not recommended adding adaptor to a premix in the Adaptor Ligation Step.

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

5.2 [ ] Add **3 µl** µl of USER Enzyme to the ligation mixture from Step 5.1.

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

Samples can be stored overnight at **-20 °C**

5.4 Size Selection 275-475bp of Adaptor-ligated DNA for DNA Input ≥100 ng.
Volume of SPRIselect for 1st bead selection **25 µl**
Volume of SPRIselect for 2nd bead selection **10 µl**

[ ] Bring the volume of the reaction up to **100 µl** by adding **28.5 µl** of 0.1% TE Buffer.

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

5.6 [ ] Add **25 µl** of the Ampure XP Beads to the **100 µl** sample and mix well by pipetting up and down.

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 transfer the supernatant ~ **125 µl** into a new wells. **(Caution: do not discard the supernatant)**. Discard the beads that contain the unwanted large fragments.

5.10 [ ] Add **10 µl** of the Ampure XP Beads to the supernatant from step 38. Mix well by pipetting up and down.

5.11 [ ] Incubate at room temperature for **00:05:00**

5.12 [ ] Place plate on magnetic block for **00:05:00**

5.13 [ ] Carefully remove and discard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA **(Caution: do not discard beads).**

5.14 [ ] Wash the beads adding **200 µl** of 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.

5.15 [ ] Repeat Step 5.14 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.

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

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.

5.17 [ ] Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding **17 µl** **0.1 % volume** TE (dilute 1X TE Buffer 1:10 in water).

5.18 [ ] 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.

5.19 [ ] 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.

Samples can be stored at **-20 °C**

NEBNext library preparation protocol - PCR Enrichment of Adapter-ligated DNA

6 [ ] Add the following reagents to each well from step 5.19

Component	Volume
Adaptor Ligated DNA Fragments (Step 5.19)	15 µl
NEBNext Ultra II Q5 Master Mix	25 µl
Index Primer/i7 Primer	5 µl
Universal PCR Primer/i5 Primer	5 µl
Total Volume	**50 µl**

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 on a thermocycler 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	∞	

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

NEBNext library preparation protocol - Clean up of PCR reaction

7 **The volumes of AMPure XP beads are for use with the sample contained in the exact buffer at this step. Allow the beads to warm to room temperature for at least 30 minutes before use.**

[ ] Vortex SPRIselect to resuspend.

7.1 [ ] Add **45 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 **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 7.6. 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.

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 **33 µl** of **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 **30 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.

In this protocol **2 µl** of library (**198 µl** buffer)

8.1 [ ] Run Samples on a bioanalyser 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.

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: 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 and check that the library sthows a narrow distribution with an expected peak size based on fragmentation time and size selection. Record the the average peak bp size

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

10 Pooling and Library Denaturation Date/Initials:_____

This section demonstrates how to generate a pooled library for V3 reagents on the MiSeq.

Thaw the MiSeq reagents overnight or in a RT 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.

Make a fresh dilution of 0.2N of NaOH and Tris by combining the following volumes in a microcentrifuge tube: 800 uL laboratory-grade water 200 stock 1.0N NaOH
microcentrifuge tube: 800 uL laboratory-grade water 200 stock 1.0M Tris

10.1 [ ] Pool **5 µl** of each normalised sample into one eppendorf tube.

10.2 [ ] Combine the following volumes in a microcentrifuge tube:
5 µl 4nM 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 **5 µl** of 0.2N TrisHCL and **985 µl** of pre-chilled HT1 to the tube containing the denatured library. 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

[ ] Invert to mix and then pulse centrifuge

10.5 [ ] Dilute stock PhiX to 4nM by combining: 2 uL 10 nM PhiX library 3 uL 10 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 uL 4nM PhiX library
5 uL 0.2N NaOH

Remaining 4nM PhiX can be frozen and reused

10.7 [ ] Vortex briefly to mix and centrifuge at 280 x g for 1 minute.
[ ] Incubate at room temperature for 5 minutes

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

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 uL 20 pM denatured PhiX library
225 uL pre-chilled HT1

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

Denatured and diluted PhiX	30 uL
Denatured and diluted library	570 uL

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 uL 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.