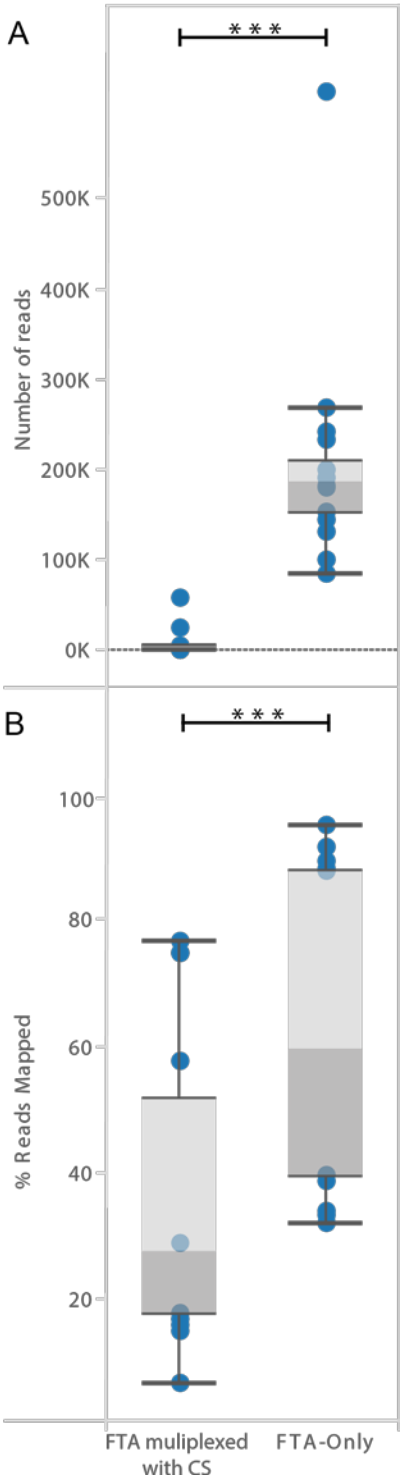
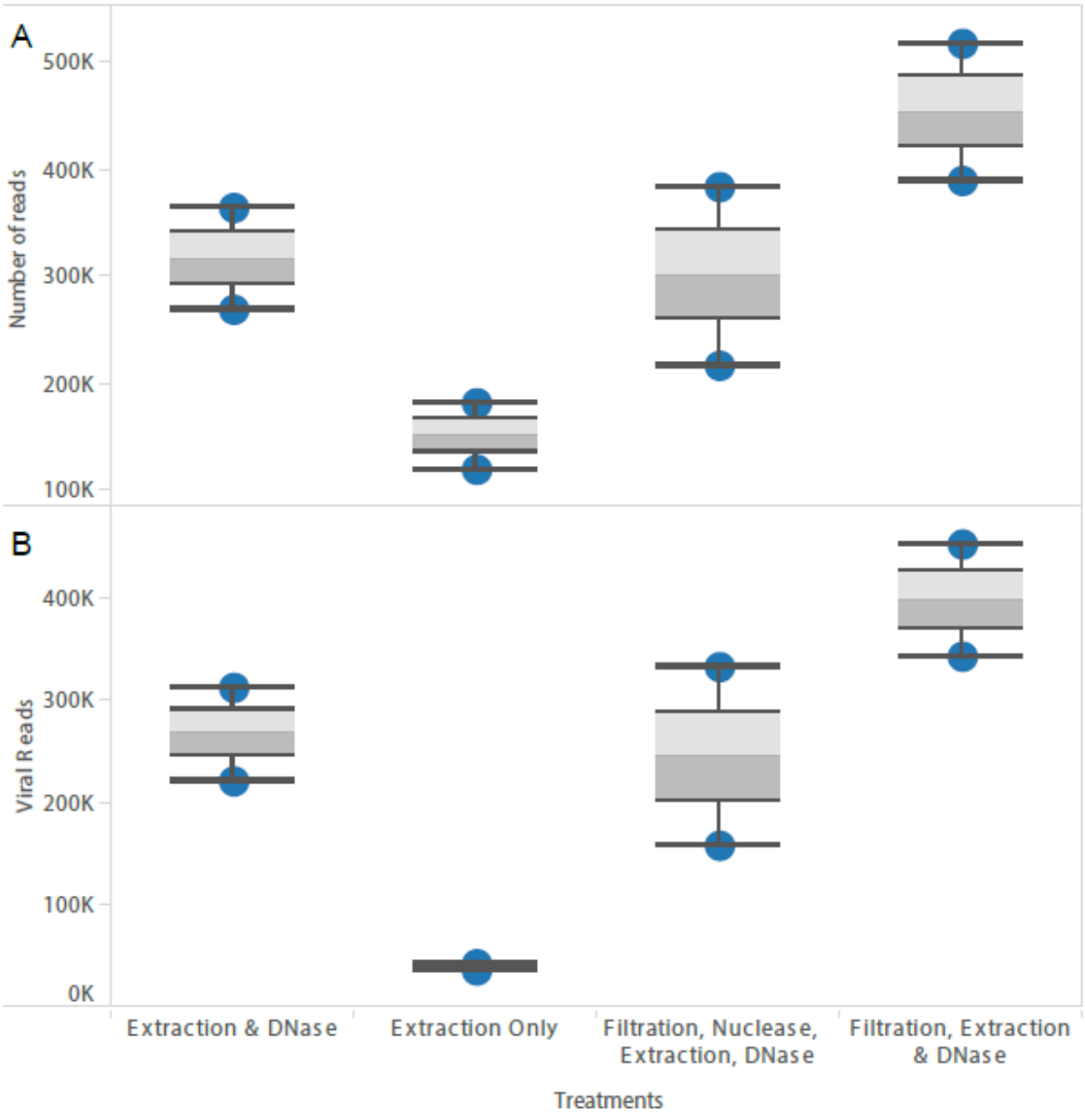
Supplementary Figure 1. Comparison of the number of trimmed and de-duplicated sequencing reads and percentage of those reads mapping to targeted poliovirus genomes for an FTA-only library versus an FTA and CS pooled library. * P<0.05; **P<0.01; *** P<0.0001

Suppl. Fig. 1



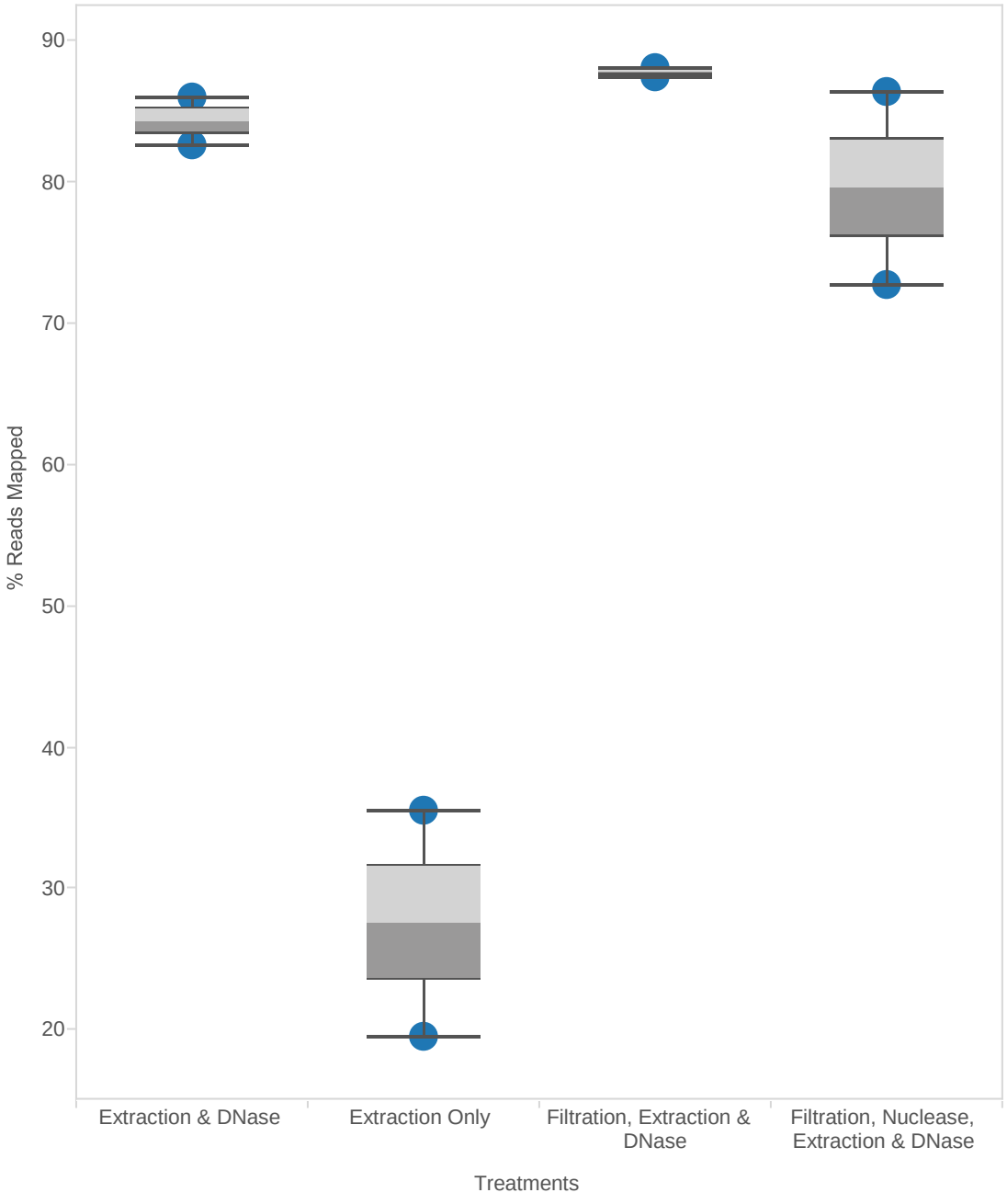
Supplementary Figure 2. Comparison of four specimen pre-treatments showing both total number of trimmed, de-duplicated sequencing reads and viral-specific reads.

Suppl. Fig. 2



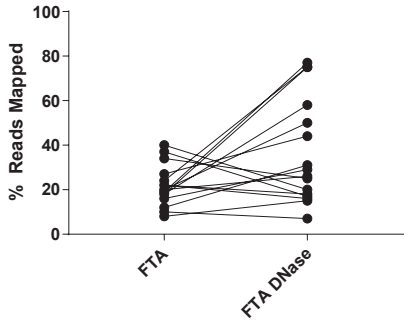
Supplementary Figure 3. Comparison of the percentage of poliovirus genome sequenced in four specimen pre-treatment groups.

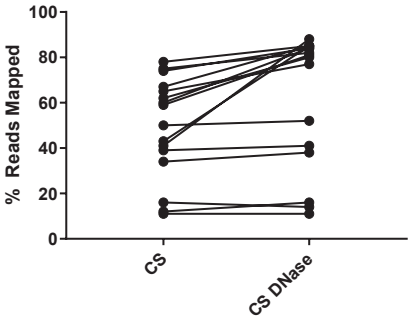
Suppl. Fig. 3



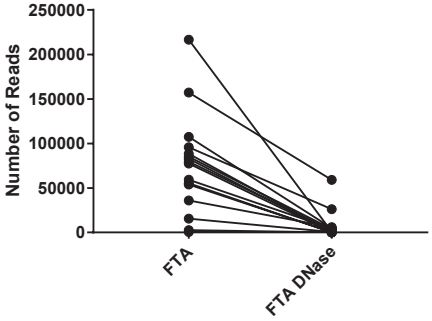
Supplementary Figure 4. Analysis of sample pairs in the experiments.

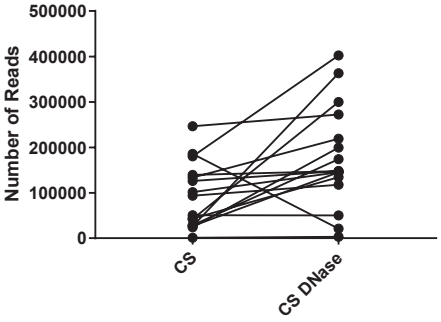
Corresponding to Fig. 1A



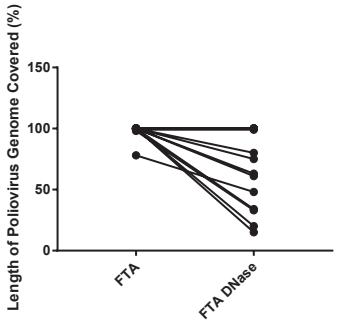


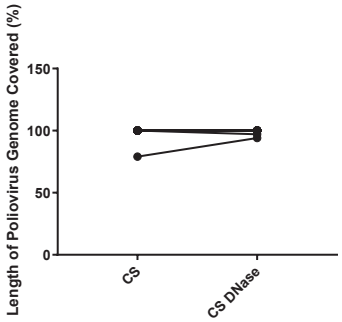
Corresponding to Fig. 1B



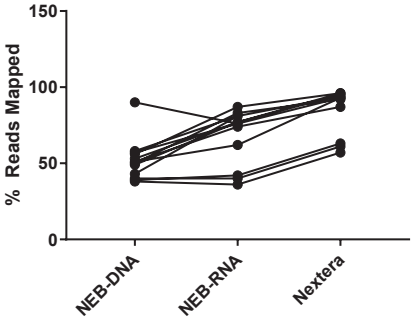


Corresponding to Fig. 1C

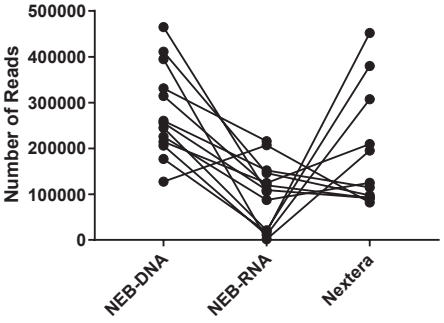




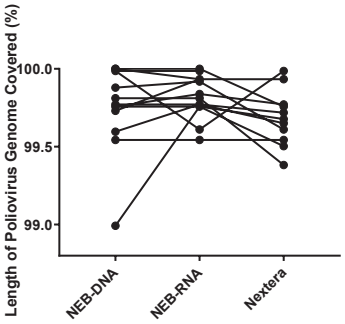
Corresponding to Fig. 2A



Corresponding to Fig. 2B



Corresponding to Fig. 3



Suppl. Method: NGS Protocol for Poliovirus Isolate Sequencing. Ver.TN16.11

Relevant references:

- **Ng TFF, Marine R, Wang C, Simmonds P, Kapusinszky B, Bodhidatta L, Oderinde BS, Wommack KE, Delwart E.** 2012. High variety of known and new RNA and DNA viruses of diverse origins in untreated sewage. Journal of Virology **86:**12161-12175.
- **Delwart EL.** 2007. Viral metagenomics. Reviews in Medical Virology **17:**115-131.
- **Ng TFF, Kondov NO, Deng X, Van Eenennaam A, Neibergs HL, Delwart E.** 2015. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. Journal of Virology **89:**5340-5349.

1. Setting up

1.1 If a commercial decontamination solution is not used, prepare 10% bleach solution in PCR prep room or RNA work area by adding 10 ml of bleach to 90 ml of water.

1.2 10% bleach solution has a shelf life of 10 days and should be prepared fresh weekly.

1.3 In the clean PCR prep room, clean all surfaces with 10% bleach solution: ice bucket, cooling chamber and pipettes. Rinse with 70% ethanol followed by ≥ 10 minutes of UV light exposure.

1.4 In the RNA work area, clean surfaces with 10% bleach: ice bucket, cooling chamber and pipettes. Rinse with 70% ethanol followed by ≥ 10 minutes of UV light exposure.

1.5 In a biosafety cabinet, clean surfaces with 10% bleach solution, followed by 70% ethanol and ≥ 10 minutes of UV light exposure.

2. Sample extraction

2.1 Polioviruses were isolated and propagated in culture according to the WHO Polio Laboratory Manual (10).

2.2 Prior to nucleic acid extraction, culture supernatant (CS) was first frozen and thawed three times and clarified at 15,294 x g for 10 minutes at 4°C.

2.3 For FTA cards, a detailed, previously described card processing procedure was used to extract PV RNA from the card (10) (12).

3. Viral nucleic acid extraction

3.1 Follow the QIAamp Viral RNA Mini Kit (Qiagen Cat No. 52904) instructions, with the following modification

3.2 Carrier RNA should **NOT** be added to the lysis buffer.

3.3 After the first wash of buffer AW1, perform an “on-column” DNase treatment. First, add 10 µl DNase I stock solution (Qiagen, Catalog no. 79254) to Buffer RDD. Mix by gently inverting the tube. Then, Add the DNase I incubation mix (80 µl) directly to the spin column. Place on the bench top at room temperature for 15 min.

3.4 Perform another wash of buffer AW1, and follow the rest of the protocol beginning with wash of buffer AW2.

3.5 At the elution step (step 11 in the manual), before pipetting any elution buffer onto the spin column, let the column air dry for 10 min. Clean any residual buffer AW2 the inside rim with pipette tips. This removes any residual ethanol that will compromise downstream enzymatic reactions. Then add ~80 µl AVE buffer onto the column. Let elution buffer incubate in the spin column for 10 min at room temperature before eluting.

3.6 For better stability of RNA, add 0.5 µl RNase Inhibitor (RiboLock EO0381) into the eluted DNA/RNA. The eluted nucleic acids is purified viral DNA/RNA.

4. Order the following primers, as per Ng et al. 2015

N1_8N ; CCTTGAAGGCGGACTGTGAGNNNNNNNN (100 µM; HPLC purified)

N1 ; CCTTGAAGGCGGACTGTGAG (100 µM; HPLC purified)

5. Random reverse transcription and amplification for viral DNA/RNA (SISPA)

RT

5.2 Prepare the sample mix and master mix as below

5.3 Combine the following to obtain sample mix

5 µl	Viral DNA/RNA
0.5 µl	dNTP (10 mM)
0.5 µl	"N1-8N primer" (100 µM)

6 µl, incubate at 72°C, 2 min

Incubate on ice, 2 min

5.4 Combine the following to obtain master mix

Master mix for 1X (Superscript IV)

2 µl	5X Buffer
0.5 µl	DTT
0.5 µl	RNAse inhibitor (Ribolock)
0.5 µl	Reverse Transcriptase (Superscript IV)

3.5 µl

5.5 Put 3.5µl of master mix into each of the sample mix (6 µl)

5.6 Incubate with the following temperate using a thermocycler designated for cDNA

23°C for 10 min

52°C for 10 min,

80°C for 10 min,

Hold at 4°C.

5.7 This gives you "RT product"

Second- strand cDNA synthesis

5.8 Incubate RT product at 95°C, 2 min,

Incubate on ice, 2 min

5.9 Combine the following

9.5 µl	RT product
1 µl	Klenow 3'→5' exo- (NEB M0212S)

10.5 µl,

5.10 Incubate with the following temperature using a thermocycler designated for cDNA

37°C for 60 min

75°C for 20 min to inactivate Klenow.

5.11 This gives you cDNA. Keep on ice or -20°C until PCR.

PCR

5.12 Using fresh, DNase free PCR tubes. Combine the following into a master mix

# of 25 µl reactions	**1X**	**2X**	**3X**	**4X**	**5X**	**6X**	**7X**	**8X**	**9X**	**10X**
10× Taq buffer	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25
$MgCl_2$ (25 mM)	4	8	12	16	20	24	28	32	36	40
dNTP (10 mM)	0.63	1.26	1.89	2.52	3.15	3.78	4.41	5.04	5.67	6.3
N1 Primer (100 µM)**	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5
AmpliTaq Gold	0.37	0.74	1.11	1.48	1.85	2.22	2.59	2.96	3.33	3.7
H2O	14.5	29	43.5	58	72.5	87	102	116	131	145
Total	22.5	45	67.5	90	113	135	158	180	203	225

5.13 Pipette 22.5 µl of the master mix into PCR tubes/strips. Add 2.5 µl of the cDNA individually into each tubes.

5.14 Perform the following PCR cycles using a thermocycler designated for PCR, ideally in a different room.

95°C	5 min	
95°C	1 min	|
59°C	1 min	| 5 cycles
72°C	1.5 min	|
95°C	30sec	|
59°C	30sec	| 15 cycles - 25 cycles*
72°C	1.5min	|
72°C	10 min	
4°C	Hold	

*Depending on sample types and titer. To optimize, perform 15 cycles and 25 cycles in parallel in separate tubes and compare results.

5.15 This gives you random PCR product. Keep on ice or -20°C until PCR.

5.16 Evaluate the PCR product using Tapestation, Bioanalyzer, Agarose gel eletrophoresis, Lonza FlashGel, or equivalent methods. The PCR product should contain random amplicons (appear as a smear) with molecular weight between 150-500 bp.

6 Purify the PCR product using 1.8X Agencourt AMPure XP Beads

6.1 Bring AMPure XP beads to room temperature. Vortex AMPure XP beads to resuspend.

6.2 Add 1.8 volume (~36 µl) of resuspended AMPure XP beads to an aliquot of the random PCR product (~20 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.

6.3 Incubate for 5 minutes at room temperature.

6.4 Quickly spin the tube in a micro centrifuge to collect any sample on the sides of the tube. Place the tube on an appropriate magnetic rack to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

6.5 Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

6.6 Repeat Step 5 once for a total of 2 wash steps.

6.7 Air dry the beads for 10 minutes while the tube is on the magnetic rack with lid open.

6.8 Elute the DNA target from the beads into 25 µl nuclease-free water. Mix well by pipetting up and down. Quickly spin the tube and then place it in the magnetic rack until the solution is clear.

6.9 Remove 20 µl of the supernatant and transfer to a clean nuclease-free PCR tube.

6.10 This gives you cleaned random PCR product. Keep on ice or -20C until PCR.

6.11 (Optional) Perform another QC on the purified product using Tapestation, Bioanalyzer, Agarose gel eletrophoresis, Lonza FlashGel, or equivalent methods. Recommended to do this step if adaptors were visualized before the AMPure cleanup in Section 5.

7 Qubit dsDNA HS Assay Kits

7.1 Required Materials:
Qubit dsDNA HS Assay kit Cat. No. Q32851 for 100 assays and Q32854 for 500 assays (Invitrogen)
0.5 ml PCR tubes: 500 tubes, Cat. No. Q32856 (Invitrogen)
Sample concentrations from 10 pg/µl to 100 ng/µl.

7.2 Set up the number of 0.5 ml tubes you will need for standards and samples. The Qubit dsDNA HS assay requires 2 standards.

7.3 Label the tube lids.

7.4 Make the Qubit working solution by diluting the Qubit dsDNA HS reagent 1:200 in Qubit dsDNA HS buffer. Use a clean plastic tube each time you make Qubit working solution. Do not mix the working solution in a glass container.

7.5 Note: the final volume in each tube must be 200 µl. Each standard tube will require 190 µl of Qubit working solution, and each sample tube will require anywhere from 180 µl to 199 µl.

7.6 6 ml for 30 samples: 30 µl of Qubit reagent + 5,970 µl of Qubit buffer.

7.7 Load 190 µl of Qubit working solution into each of the tubes used for standards.

7.8 Add 10 µl of each Qubit standard to the appropriate tube and mix by vortexing 2-3 seconds, being careful not to create bubbles.

7.9 Load Qubit working solution into individual assay tubes so that the final volume in each tube after adding sample is 200 µl.

7.10 Use 2 µl DNA + 198 µl of working solution.

7.11 Add each of your samples to assay tubes containing the correct volume of Qubit working solution and mix by vortexing 2-3 seconds. The final volume in each tube should be 200 µl.

7.12 Allow all tubes to incubate at room temperature for 2 minutes.

7.13 On the Home Screen of the Qubit Fluorometer, press DNA, and then select dsDNA High Sensitivity as the assay tube. The Standards Screen is automatically displayed.

7.14 On the Standards Screen, press **YES** to run a new calibration or press No to use the last calibration.

7.15 Run a New Calibration

7.16 Insert the tube containing Standard #1 in the Qubit Fluorometer, close the lid, and press **GO**. The reading will take approximately 3 seconds.

7.17 Remove Standard #1.

7.18 Insert the tube containing Standard #2 in the Qubit Fluorometer, close the lid, and press **GO**.

7.19 Remove Standard #2.

7.20 To read the sample, remove the standard 2 from the Qubit Fluorometer, insert the sample, and press **GO**.

7.21 Repeat sample readings until all samples have been read.

7.22 Using the Qubit value, each sample should be normalized to 0.2 ng/µl. 5 µl is need for Nextera XT.

8 Proceed to Nextera XT. (See manufacturer's manual). This should be followed by QC of the library

9 Library normalization and pooling (See manufacturer's manual)

10 Create MiSeq Sample Sheet (See manufacturer's manual)

11 Preparing DNA Libraries for Sequencing on the MiSeq (see below and refers to MiSeq Manual)

Denature DNA

NOTE
If your application requires higher than a 20 pM final concentration of your library, make sure that your concentration of NaOH is equal to 0.2 N in the denaturation solution and not more than 0.0025 N (2.5 mM) in the final solution after diluting with HT1. Higher concentrations of NaOH in the library will inhibit library hybridization to the flow cell and decrease cluster density.

Use the following instructions to denature the DNA to a concentration of 20 pM.

1 Combine the following volumes of sample DNA and 0.2 N NaOH in a microcentrifuge tube:
- 2 nM sample DNA (10 µl)
- 0.2 N NaOH (10 µl)

NOTE
0.2 N NaOH is required to denature and dilute PhiX. If you plan to prepare a PhiX control within the next **12 hours**, set aside remaining 0.2 N NaOH. For more information, see *Denature and Dilute PhiX Control* on page 81.
Otherwise, discard the remaining dilution of 0.2 N NaOH.

2 Vortex briefly to mix the sample solution, and then centrifuge the sample solution to 280 xg for one minute.

3 Incubate for five minutes at room temperature to denature the DNA into single strands.

4 Add the following volume of pre-chilled HT1 to the tube containing denatured DNA to result in a 20 pM denatured library:
- Denatured DNA (20 µl)
- Pre-chilled HT1 (980 µl)

5 Place the denatured DNA on ice until you are ready to proceed to final dilution.

Dilute Denatured DNA for 4 nM Library

Use the following instructions to dilute the 20 pM DNA further to give 600 µl of the desired input concentration.

1 Dilute the denatured DNA to the desired concentration using the following example:

Final Concentration	6 pM	8 pM	10 pM	12 pM	15 pM	20 pM
20 pM denatured DNA	180 µl	240 µl	300 µl	360 µl	450 µl	600 µl
Pre-chilled HT1	420 µl	360 µl	300 µl	240 µl	150 µl	0 µl

2 Invert several times to mix and then pulse centrifuge the DNA solution.

3 Place the denatured and diluted DNA on ice until you are ready to load your samples onto the MiSeq reagent cartridge.

12 Sequencing sample libraries using the Illumina MiSeq sequencer

12.1 Load 600 µl of the diluted ~12 pM libraries into the sample well of the Miseq Cartridge.

12.2 Proceed directly to the run setup steps using the MiSeq Control Software (MCS) interface.

12.3 From the Welcome screen, select Manage Instrument.

12.4 From the Manage Instrument screen, select Reboot to reboot the system software.

12.5 From the Welcome screen, select Sequence to begin the run setup steps. A series of run setup screens open in the following order: BaseSpace (optional) Option, Load Flow Cell, Load Reagents, Review, and Pre-Run check.

12.6 Since the BaseSpace is not available, select **NEXT**. The Load Flow Cell screen opens.

12.7 Clean the flow cell (see the Miseq manual for procedure cleaning the flow cell). It is required before loading to MiSeq sequencer.

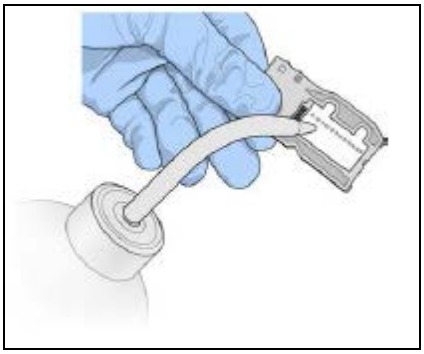
Cleaning the Flow Cell:

12.7.1 Please ensure that the cap color of the flow cell container is clear (the standard flow cell).

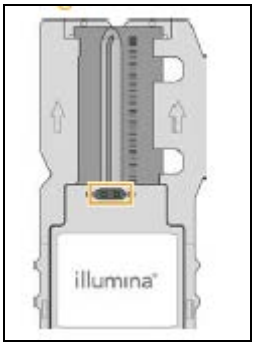
12.7.2 Wear a new pair of powder-free gloves.

12.7.3 Using plastic forceps, grip the flow cell by the base of the plastic cartridge and remove it from the flow cell container.

12.7.4 Rinse the flow cell with nuclease-free water, making sure that both the glass and plastic cartridge are thoroughly rinsed of excess salts.

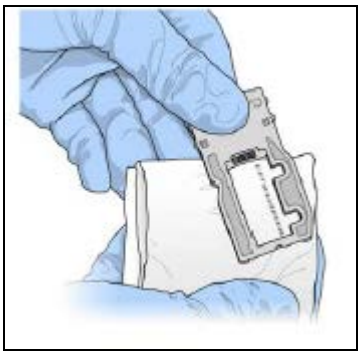


12.7.5 Thoroughly dry the flow cell and cartridge using a lint-free lens cleaning tissue. Gently pat dry in the area of the gasket and adjacent glass.



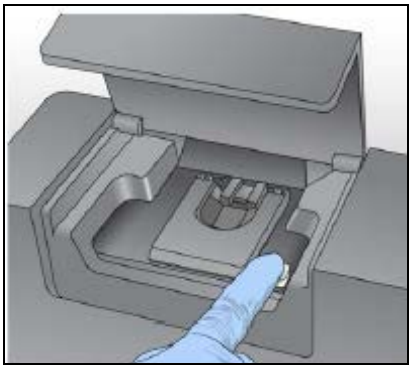
12.7.6 Using an alcohol wipe, clean the flow cell glass. Make sure that the glass is free of streaks, fingerprints, and lint or tissue fibers. Avoid using the alcohol wipe on the flow cell port gasket.

12.7.7 Dry any excess alcohol with a lint-free lens cleaning tissue. Visually inspect to make sure that the flow cell ports are free of obstructions and that the gasket is well-seated around the flow cell ports.

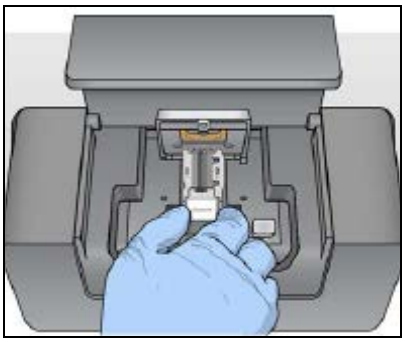


12.8 Inspect the flow cell stage to make sure that it is free of lint. Clean the flow cell stage using an alcohol wipe or a lint-free tissue moistened with ethanol or isopropanol if lint or other debris is present.

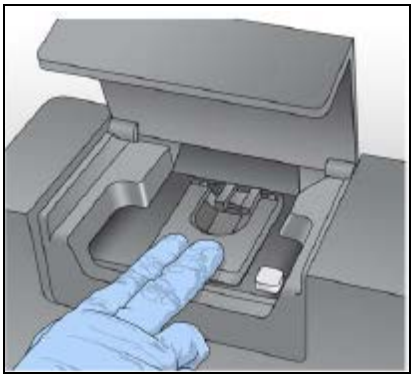
12.9 Open the Flow Cell Latch.



12.10 Place the flow cell on the flow cell stage.



12.11 Gently press down on the flow cell latch to close it over the flow cell.



12.12 Check the lower-left corner of the screen to confirm that the flow cell RFID was successfully read. Note: If the RFID cannot be read, see Resolve RFID Read Failure in the MiSeq System User Guide.

12.13 Close the flow cell compartment door.

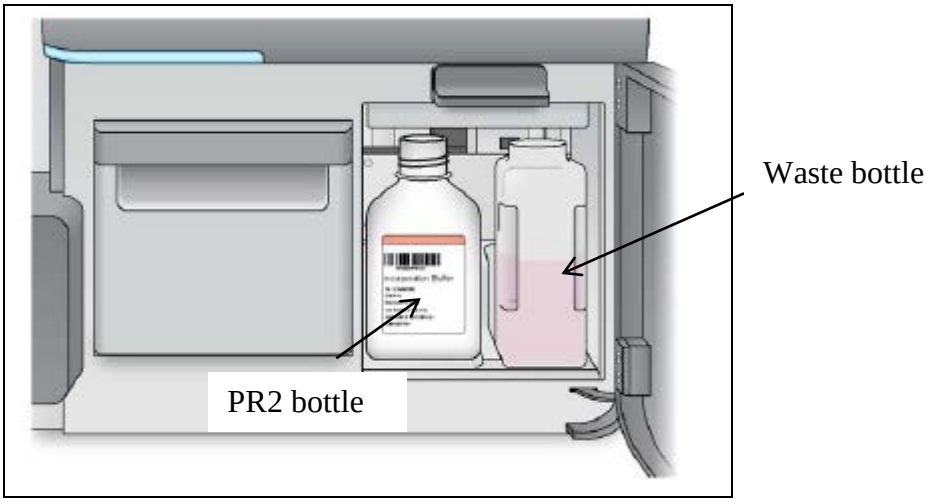
12.14 Select **NEXT** on the Load Flow Cell screen. The Load Reagents screen opens.

12.15 Remove the PR2 bottle from 4^0C storage. Gently invert the bottle to mix the PR2 bottle, and then remove the lid.

12.16 Open the reagent compartment door.

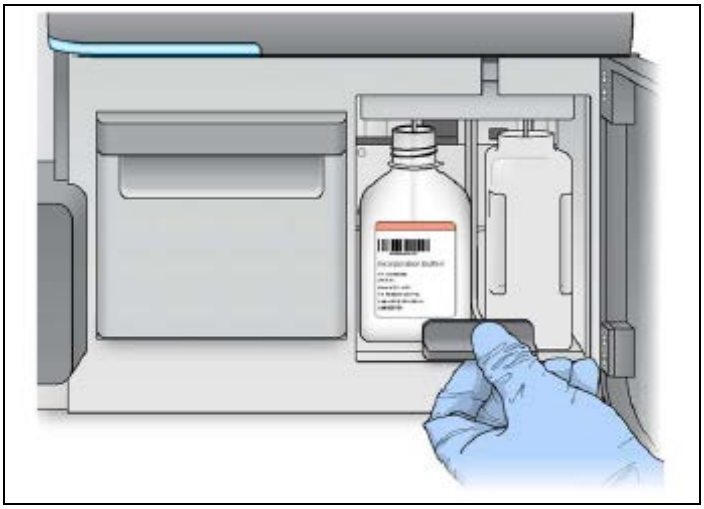
12.17 Raise the sipper handle until it locks into place.

12.18 Place the PR2 bottle in the indentation to the right of the reagent chiller.



12.19 Make sure that the waste bottle is empty. If it is not empty, empty the contents into the appropriate waste container.

12.20 Slowly lower the sipper handle. Make sure that the sippers lower into the PR2 and waste bottles.

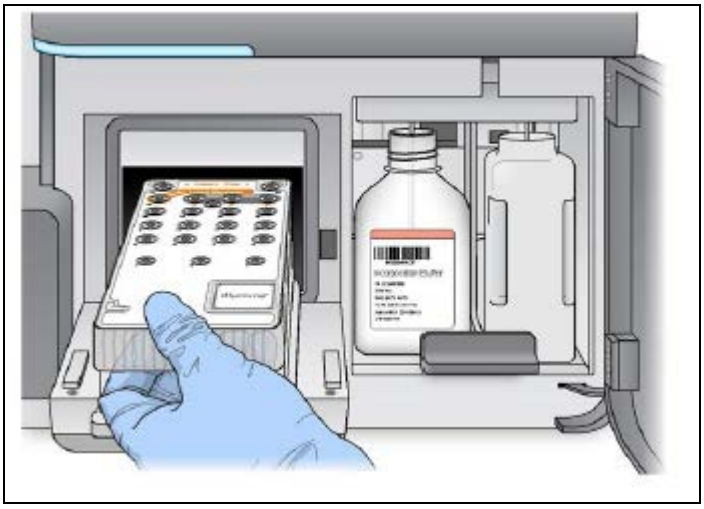


12.21 Check the lower-left corner of the screen to confirm that the RFID of the PR2 bottle was read successfully. Note: If the RFID cannot be read, see Resolve RFID Read Failure on page 79, ref. 12.2 MiSeq System User Guide.

12.22 Select **NEXT** on the Load Reagents screen.

12.23 Open the reagent chiller door.

12.24 Hold the reagent cartridge on the end with the Illumina label, and slide the reagent cartridge into the reagent chiller until the cartridge stops.



12.25 Close the reagent chiller door.

12.26 Check the lower-left corner of the screen to confirm that the RFID of the reagent cartridge was read successfully. Note: If the RFID cannot be read, see Resolve RFID Read Failure on page 79, ref. 12.2 MiSeq System User Guide.

12.27 Close the reagent compartment door.

12.28 Select **NEXT** on the Load Reagents screen. The Review screen opens.

12.29 Review the run parameters, experiment name, analysis workflow, and read length. These parameters are specified in the sample sheet.

12.30 By default, the software looks for a sample sheet file with a name matching the barcode number of the reagent cartridge loaded on the instrument. If a sample sheet is not found, a message appears that prompts you to browse to the location of the correct sample sheet for your run.

12.31 Review the folder locations in the lower-left corner.

12.32 Select **NEXT**. The Pre-Run Check screen opens.

12.33 The system performs a check of all run components, disk space, and network connections before starting the run. If any items do not pass the pre-run check, a message appears on the screen with the instructions on how to correct the error.

12.34 When all items successfully pass the pre-run check, select Start Run.

12.35 It will take approximately 44 hours to complete the run on Miseq instrument. Check that there is no power outage while the instrument is running. When the run is complete, the Next button appears, Review the results on the Sequencing screen before proceeding.

13 Post Run Wash

13.1 Perform Post-run wash on the same day when the run on Miseq is completed.

13.2 Select **NEXT** to exit the Sequencing screen and proceed to a post-run wash.

13.3 To perform a post-run wash and maintenance wash, please see chapter 3 of MiSeq System User Guide, Ref. 11.2. A diluted bleach line-wash (Post-run wash) is recommended to reduce run-to-run contamination.

14 Data Analysis

14.1 The default output folder (Fastq) is D:\MiseqOutput, under Run_Name\Data\Intensities\BaseCalls