DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service
Centers for Disease Control and Prevention (CDC)
Atlanta, GA 30333

Preparation of the DNA Libraries for Loading onto the MiSeq

Influenza Division, Virology, Surveillance and Diagnosis Branch, Genomics and Diagnostic Team

1.0 Purpose

1.1 This process describes how the Influenza DNA library is prepared before loading on to the MiSeq instrument.

2.0 Responsibility

2.1 The laboratory supervisor is responsible for ensuring that all staff performing this SOP is adequately trained.

2.2 Testing personnel are responsible for adhering to all safety procedures according to governmental and safety office guidelines.

3.0 Definitions

3.1 BMBL – Biosafety in Microbiological and Biomedical Laboratories

4.0 Critical Equipment

4.1 Freezer, -20°C

4.2 Refrigerator, +4°C

4.3 Microcentrifuge

4.4 Vortex

4.5 Fisher Scientific™ Isotemp™ Digital Dry Baths/Block Heaters (Catalog # 88-860-021 or equivalent)

4.6 Fisherbrand™ Certified Dry Block Thermometers: 50° to 110°C (Catalog # 13-201-911 or equivalent)

5.0 Materials

5.1 PhiX Control V3 (Illumina Catalog # 15017666)

5.2 MiSeq Reagent Kits V2 300-cycles 2 boxes (#MS-102-2002, MS-103-1001, or MS-103,1002) or MiSeq V3 Reagent kits 600 cycles 2 boxes (#MS-102-3003)

5.3 Ultra-Pure Pure nuclease-free water (Molecular Biology Grade) (ThermoFisher AM9938 or equivalent)

5.4 1.0 N NaOH, molecular biology-grade (Sigma-Aldrich #S2770 or equivalent)

5.5 Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20 (Teknova Catalog # T7724 or equivalent) or Illumina Resuspension Buffer (RSB) available with library kits

5.6 Sterile, nuclease free 1.5 ml micro-centrifuge tubes

5.7 Assorted Pipettes (2 µl, 10 µl, 20 µl, 200 µl, and 1000 µl)

6.0 Safety Precautions

6.1 Adhere to the safety guidelines provided in the Biosafety in Microbiological and Biomedical Laboratories and follow all established site-specific safety procedures, including wearing proper personal protective equipment (PPE).

7.0 Preparation of NaOH and PhiX (ϕX) Control

7.1 Turn on the Heat Block to 96°C.Verify thermometer temperature is @ 96°C.

7.1.1 Prepare 0.2 N NaOH

NOTE: Make 1 ml 1N NaOH aliquots and freeze at -20°C to avoid changes in pH. (*Using freshly diluted NaOH is essential in denaturing libraries for cluster generation on the MiSeq*.)

7.2 At room temperature, prepare 20 µl of **0.2 N NaOH** by combining the following volumes in a1.5 ml or smaller microcentrifuge tube:

Reagent	Volume (µl)
Sterile, nuclease free water	16.0
1.0 N NaOH	4.0
Total	**20 µl (0.2 N NaOH)**

7.2.1 Briefly vortex tube to mix.

7.3 Preparation of 20 pmol PhiX (ϕX) Control Stock

7.3.1 At room temperature, combine the following volumes of reagents to dilute the PhiX library to **4 nM**:

Reagent	Volume (µl)
10 nM PhiX library	4.0
10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20	6.0
Total	**10 µl (4 nM PhiX library)**

7.3.2 Combine the following volumes of **4 nM** PhiX library and freshly diluted **0.2 N NaOH** in a 1.5 µl microcentrifuge tube:

Reagent	Volume (µl)
4 nM PhiX library	5.0
0.2 N NaOH	5.0
Total	**10 µl (2 nM PhiX library)**

7.3.3 Vortex briefly to mix the **2 nM PhiX** library solution.

7.3.4 Briefly spin the template solution.

7.3.5 Incubate for **5 minutes** at room temperature to denature the PhiX library.

7.3.6 Add the following volume of pre-chilled HT1 to the tube containing denatured **2nM PhiX** for a final concentration of **20 pM PhiX** library.

Reagent	Volume (µl)
Denatured 2 nM PhiX library	10.0
Pre-chilled HT1 (must be cold)	990.0
Total	**1000 µl (20 pM PhiX library)**

NOTE: Make 40 µl aliquots of the denatured **20 pM PhiX** library and store up to 4 weeks at -15° to -25°C.

7.4 Dilute Denatured 20 pM PhiX Control

7.4.1 Make fresh before loading the MiSeq cartridge

7.4.2 Dilute an aliquot of the denatured **20 pM PhiX** library to a final concentration of **12.5 pM** as follows:

Reagent	Volume (µl)
20 pM Denatured PhiX	37.5 µl
Pre-chilled HT1	22.5 µl
Total	**60 µl (12.5 pM PhiX library)**

7.4.3 Vortex briefly to mix the solution and place on ice.

8.0 Normalization and Denaturing the DNA Library

8.1 Remove the reagent cartridge from -25° to -15°C storage.

8.2 Place the reagent cartridge in a water bath containing enough room temperature deionized water to submerge the base of the reagent cartridge up to the water line printed on the cartridge. Do not allow the water to exceed the maximum water line.

8.3 Allow the reagent cartridge to thaw in the room temperature water bath for approximately 20 minutes, then place in a 4°C refrigerator until ready to load the samples. The cartridge can also be placed in a water/ice bath.

8.4 Normalize the pooled DNA library to 2nM using the concentration from the Qubit assay and genome size determination from a fragment analyzer.

NOTE: (If needed, pooled libraries can be diluted to ~1.2 ng/µl or loading concentrations can be adjusted for optimal cluster density)

8.5 Combine the following volumes of pooled DNA library and freshly diluted 0.2 N NaOH in a microcentrifuge tube:

Reagent	Volume (µl)
Pooled 2 nM DNA library	5 µl
0.2 N NaOH	5 µl
Total	**10 µl**

8.6 Discard the remaining dilution of 0.2 N NaOH.

8.7 Vortex briefly to mix the sample solution, and then briefly centrifuge the sample solution.

8.8 Incubate for **5 minutes** at ***room temperature*** to denature the DNA into single strands.

8.9 After 5 minutes, add the following volume of pre-chilled HT1 to the tube containing denatured DNA:

Reagent	Volume (µl)
Pooled denatured DNA libraries	10 µl
Pre-chilled HT1	990 µl
Total	**1000 µl (10 pM Diluted Amplicon Library (DAL) in 1 mM NaOH)**

8.10 Place the Diluted Amplicon Library (DAL) on ice until you are ready to proceed.

8.11 **NOTE**: *Perform this heat denaturation step immediately before loading the Diluted Amplicon Library into the MiSeq reagent cartridge to ensure efficient template loading on the flowcell.*

8.12 Mix Diluted Amplicon Library (DAL) by vortexing briefly.

8.13 Using a heat block set at **96°C**, incubate the denatured DAL tube (1000 µl) for **2 minutes.**

8.14 After the incubation, invert DAL 1–2 times to mix and immediately place in an ice bath. Keep in ice water bath for **5 minutes.**

8.15 Use the following instructions to dilute the 10 pM DAL further to bring to the desired loading concentration. The final loading volume will be 600 µl.

8.15.1 Dilute the DAL to the desired concentration using the following examples (assumes a 5% phiX spike in)

8.15.2 **NOTE:** Usually a **6 pM** loading concentration provides optimal cluster densities if using an initial pooled library concentration of ~1.2 ng/µl. However, final loading concentrations may be adjusted as needed.

Final Loading Concentration	5 pM	6 pM	8 pM	10 pM
10 pM Diluted Amplicon Library (DAL)	300 µl	360 µl	480 µl	570 µl
Pre-chilled HT1	270 µl	210 µl	90 µl	0 µl
phiX **12.5 pM**	30 µl (5%)	30 µl (5%)	30 µl (5%)	30 µl (5%)

8.15.3 Invert several times to mix and then briefly centrifuge.

8.15.4 Place the combined DAL/HT1/phiX on ice until you are ready to load your samples onto the MiSeq reagent cartridge.

7.9.1 Load the 600 µl DAL/HT1/phiX mix into a thawed MiSeq reagent cartridge.

7.9.2 Sequence your library as indicated in the "LP-312 Performing a MiSeq Next Generation Sequencing Run" protocol.

9.0 Related Procedures

9.1 LP-325 QIAxcel Sample Preparation and Quality Control Procedure

9.2 LP-518 Manual Procedure Illumina DNA Prep (Quarter volume reaction)

9.3 LP-515 DNA Quantification with Qubit dsDNA High Sensitivity

9.4 LP-312 Performing a MiSeq Next Generation Sequencing Run

10.0 References

10.0 Illumina MiSeq System "Denature and Dilute Libraries Guide" Document # 15039740_v10 https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-denature-dilute-libraries-guide-15039740-10.pdf

10.1 Biosafety in Microbiological and Biomedical Laboratories (BMBL), current edition (http://www.cdc.gov/labs/BMBL.html)