Preparing Libraries for Sequencing on the MiSeq®

FOR RESEARCH USE ONLY

ILLUMINA PROPRIETARY
Part # 15039740 Rev. D
October 2013

illumina®

Revision History

Part #	Revision	Date	Description of Change
15039740	D	October 2013	Added recommendation for low diversity libraries to dilute PhiX control libraries to the same concentration as denatured sample libraries.
15039740	C	August 2013	Added recommendation to use molecular biology-grade NaOH. Added recommended library denaturation and PhiX control protocols for use with MiSeq Reagent Kit v3. Removed loading samples library information. That information is now in the *MiSeq System User Guide (part # 15027617)*.
15039740	B	March 2013	Reduced PhiX recommendations for low diversity libraries from ≥ 25% to ≥ 5%. This change is possible when using RTA 1.17.28, or later, released with MCS v2.2. Corrected the resulting NaOH concentration for denatured 10 pM library to 1 mM. Updated instructions for combining prepared libraries and PhiX control to total 600 µl.
15039740	A	January 2013	Initial release.

Introduction

This guide explains steps to denature and dilute libraries after sample preparation to prepare them for sequencing on the MiSeq.

This guide also explains how to prepare an Illumina PhiX control and combine libraries with the PhiX control before loading them onto the MiSeq reagent cartridge.

NOTE

Denaturation and dilution is not necessary for all library types. If you are sequencing TruSeq Amplicon libraries (either Custom Amplicon or Cancer Panel) or Nextera XT libraries, ***do not*** perform this step. TruSeq Amplicon and Nextera XT protocols result in a ready-to-use normalized concentration of pooled libraries.

Required Consumables

The following consumables are required to prepare DNA libraries for sequencing on the MiSeq.

Consumable	Supplier
HT1 (Hybridization Buffer), thawed and pre-chilled	Illumina-supplied Provided in the MiSeq Reagent Kit
Illumina PhiX Control, Catalog # FC-110-3001	Illumina-supplied (Optional)
Stock 1.0 N NaOH, molecular biology-grade	General lab supplier
Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20	General lab supplier

Best Practices

- ***Always*** prepare freshly diluted NaOH for denaturing libraries for cluster generation. This step is essential to the denaturation process.
- To prevent small pipetting errors from affecting the final NaOH concentration, prepare at least 1 ml of freshly diluted 0.2 N NaOH.

NOTE

If you are planning to sequence indexed libraries, Illumina recommends that you pool the libraries before the denaturation and dilution step. For more information, see the associated sample preparation guide for the Illumina kit you use to prepare the library.

About Low Diversity Libraries

Low diversity libraries are libraries where a significant number of the reads have the same sequence. This lack of variation shifts the base composition because the reads are no longer random.

Low diversity can occur with some expression studies with > 25% one type of transcript, low plexity amplicon pools, adapter dimer, or bisulfite sequencing, for example. A higher concentration spike-in of PhiX helps balance the overall lack of sequence diversity.

NOTE

For proper focus, the MiSeq needs to detect signal in the C or T channel in the first cycle.

Prepare HT1

The tube of HT1 (Hybridization Buffer) is used to dilute denatured libraries before loading libraries onto the reagent cartridge for sequencing.

1. Remove the tube of HT1 (Hybridization Buffer) from -15°C to -25°C storage and set aside at room temperature to thaw.
2. When thawed, store at 2°C to 8°C until you are ready to dilute denatured libraries.

Prepare a Fresh Dilution of NaOH

CAUTION

Using freshly diluted NaOH is essential in denaturing libraries for cluster generation on the MiSeq.

1 Prepare 1 ml of 0.2 N NaOH by combining the following volumes in a microcentrifuge tube:
   - Laboratory-grade water (800 µl)
   - Stock 1.0 N NaOH (200 µl)

2 Invert the tube several times to mix.

NOTE

A fresh dilution of 0.2 N NaOH is required for the denaturation process in preparing sample DNA and preparing a PhiX control. After preparing the sample DNA, you can set aside remaining 0.2 N NaOH if you plan to prepare a PhiX control within the next **12 hours**. Otherwise, discard the remaining dilution of 0.2 N NaOH.

Denature and Dilute DNA

It is important that the concentration of NaOH is equal to 0.2 N in the denaturation solution and not more than 0.001 (1 mM) in the final solution after diluting with HT1.

NOTE
Higher concentrations of NaOH in the library inhibit library hybridization to the flow cell and decrease cluster density.

Table 1 Recommended Denaturation Protocols

Chemistry	Recommended Protocol
v3 reagents	4 nM library denaturation
v2 reagents	4 nM library denaturation
	2 nM library denaturation

To make sure that NaOH concentration does not exceed 1 mM in the final solution, use one of the following denaturation and dilution protocols most appropriate for your library:

- **4 nM library denaturation**
  - Requires a 4 nM library.
  - Supports high library concentrations (10–20 pM).
  - Results in a 20 pM DNA solution in 1 mM NaOH.
- **2 nM library denaturation**
  - Uses a 2 nM library.
  - Results in a 10 pM DNA solution in 1 mM NaOH.

Denature DNA for 4 nM Library

1. Combine the following volumes of sample DNA and freshly diluted 0.2 N NaOH in a microcentrifuge tube:
   - 4 nM sample DNA (5 µl)
   - 0.2 N NaOH (5 µl)
2. Discard the remaining dilution of 0.2 N NaOH or set aside to prepare a PhiX control within the next 12 hours.
3. Vortex briefly to mix the sample solution, and then centrifuge the sample solution to 280 × g for 1 minute.

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

5 Add the following volume of pre-chilled HT1 to the tube containing denatured DNA:
- Denatured DNA (10 µl)
- Pre-chilled HT1 (990 µl)

The result is a 20 pM denatured library in 1 mM NaOH.

6 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.

Denature DNA for 2 nM Library

1 Combine the following volumes of sample DNA and freshly diluted 0.2 N NaOH in a microcentrifuge tube:
- 2 nM sample DNA (5 µl)
- 0.2 N NaOH (5 µl)

2 Discard the remaining dilution of 0.2 N NaOH or set aside to prepare a PhiX control within the next 12 hours.

3 Vortex briefly to mix the sample solution, and then centrifuge the sample solution to 280 × g for 1 minute.

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

5 Add the following volume of pre-chilled HT1 to the tube containing denatured DNA:
   - Denatured DNA (10 µl)
   - Pre-chilled HT1 (990 µl)

   The result is a 10 pM denatured library in 1 mM NaOH.

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

Dilute Denatured DNA for 2 nM Library

Use the following instructions to dilute the 10 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
10 pM denatured DNA	360 µl	480 µl	600 µl
Pre-chilled HT1	240 µl	120 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.

Prepare PhiX Control

Use the following instructions to prepare the 10 nM PhiX library to 20 pM. If you are using the v2 kit, the 20 pM sample is further diluted to 12.5 pM.

1. Combine the following volumes to dilute the PhiX library to 4 nM:
   - 10 nM PhiX library (2 µl)
   - 10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20 (3 µl)
2. If not prepared within the last **12 hours**, prepare a fresh dilution of 0.2 N NaOH.

Denature PhiX Control

1. Combine the following volumes of 4 nM PhiX library and freshly diluted 0.2 N NaOH in a microcentrifuge tube:
   - 4 nM PhiX library (5 µl)
   - 0.2 N NaOH (5 µl)
2. Vortex briefly to mix the 2 nM PhiX library solution.
3. Centrifuge the template solution to 280 × g for 1 minute.
4. Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.
5. Add the following volume of pre-chilled HT1 to the tube containing denatured PhiX library to result in a 20 pM PhiX library.
   - Denatured PhiX library (10 µl)
   - Pre-chilled HT1 (990 µl)

NOTE

You can store the denatured 20 pM PhiX library up to 3 weeks at -15° to -25°C. After 3 weeks, cluster numbers tend to decrease.

Dilute Denatured PhiX Control

NOTE
You can store the denatured 20 pM PhiX library up to 3 weeks at -15° to -25°C. After 3 weeks, cluster numbers tend to decrease.

NOTE
For low diversity libraries, Illumina recommends that you dilute your PhiX control library to the same concentration as your denatured sample library. This recommendation applies to MiSeq v2 and v3 reagents.

If you are using MiSeq Reagent Kit v3, use your PhiX control at a 20 pM concentration. No further dilution is required.

1 Dilute the denatured 20 pM PhiX library as follows.

Chemistry	Final Concentration	Volume for 20 pM denatured PhiX	Pre-chilled HT1
v2 reagents	12.5 pM	375 µl	225 µl

2 Invert several times to mix the solution.

3 Discard the remaining dilution of 0.2 N NaOH.

Combine Sample Library and PhiX Control

Illumina recommends a low-concentration PhiX control spike-in at 1% for most libraries. For low diversity libraries, increase the PhiX control spike-in to at least 5%.

NOTE

For Low Diversity Libraries— The recommended PhiX control spike-in of ≥ 5% is possible with RTA v1.17.28, which is bundled with MCS v2.2. If you are using an older version of the software, Illumina recommends using ≥ 25% PhiX control spike-in.

1 Combine the following volumes of denatured PhiX control library and your denatured sample library.

	Most Libraries (1%)	Low Diversity Libraries (≥ 5%)
Denatured and diluted PhiX control	6 µl	30 µl
Denatured and diluted sample library	594 µl	570 µl

2 Set the combined sample library and PhiX control aside on ice until you are ready to load it onto the MiSeq reagent cartridge.

Next Steps

After denaturing and diluting your libraries and preparing the optional PhiX control, you are ready to set up the sequencing run. See the *MiSeq System User Guide (part # 15027617)*.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 2 Illumina General Contact Information

Illumina Website	www.illumina.com
Email	techsupport@illumina.com

Table 3 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at www.illumina.com/msds.

Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support, select a product, then click **Documentation & Literature**.

Illumina
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com