Preparing DNA Libraries for Sequencing on the MiSeq®

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ILLUMINA PROPRIETARY
Part # 15039740 Rev. A
January 2013

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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 prior to loading them onto the MiSeq reagent cartridge.

NOTE
This Process Does Not Apply to 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	Illumina-supplied (Optional)
Stock 1.0 N NaOH	User-supplied
Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20	User-supplied

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 freshly diluted NaOH in a volume of 1 ml of 0.2 N NaOH.

About Low Diversity Libraries

Low diversity libraries are libraries where a significant number of the reads have the same sequence. This 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 helps balance the overall lack of sequence diversity.

To optimize performance on the MiSeq and to obtain higher quality data, Illumina recommends sequencing samples with high diversity and avoiding monotemplate stretches during sequencing. Low diversity can occur with the following libraries, for example:

- Libraries derived from RNA with more than 25% of the reads from a single transcript
- Pools of one or a few amplicons
- Libraries with initial cycle indexing

When sequencing PCR amplicons, bisulfite converted samples, 16S rRNA libraries, or other samples where diversity is low, one approach is to concatamerize the amplicons and fragment to create sequencing diversity. Other options include indexing your libraries and sequencing them with other diverse libraries, or pooling together different amplicons, ensuring that the index read has diversity. For recommendations on index read diversity, see low plexity pooling guidelines in the *Nextera DNA Sample Preparation Guide* or the *TruSeq Custom Amplicon Library Preparation Guide*.

NOTE

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

Prepare a Fresh Dilution of NaOH

CAUTION
Using freshly diluted NaOH is essential in order to completely denature samples 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 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 will inhibit library hybridization to the flow cell and decrease cluster density.

To ensure 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 and dilution**
  - Requires a 4 nM library.
  - Supports high library concentrations (> 10 pM).
  - Results in a 1 ml of a 20 pM DNA solution in 1 mM NaOH.
- **2 nM library denaturation and dilution**
  - Uses a 2 nM library.
  - Results in a 1 ml of a 10 pM DNA solution in 1 mM NaOH.

NOTE
If a loading concentration > 10 pM is required, increase the 2 nM library to 4 nM using a speed vac, for example, and then follow the denaturation and dilution protocol for a 4 nM library.

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 xg 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)

This results in 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

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 denatured DNA	180 µl	240 µl	300 µl	360 µl	450 µl
Pre-chilled HT1	420 µl	360 µl	300 µl	240 µl	150 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 xg 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)

This results in a 10 pM denatured library in 2 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 further dilute the 10 pM DNA 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 denature and dilute the 10 nM PhiX library to 12.5 pM. This should result in a cluster density of 1000–1200 K/mm².

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 xg 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

1. Dilute the denatured 20 pM PhiX library to 12.5 pM as follows:
   - 20 pM denatured PhiX library (375 µl)
   - Pre-chilled HT1 (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 metagenomics or low diversity libraries, increase the PhiX control spike-in to at least 25%.

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

	Most Libraries (1%)	Metagenomics or Low Diversity Libraries (≥ 25%)
Denatured and diluted PhiX control	10 µl	250 µl
Denatured and diluted sample library	990 µl	750 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.

Load Sample Libraries onto Cartridge

Make sure that the MiSeq reagent cartridge is fully thawed and prepared as described in the *MiSeq System User Guide*, Part # 15027617.

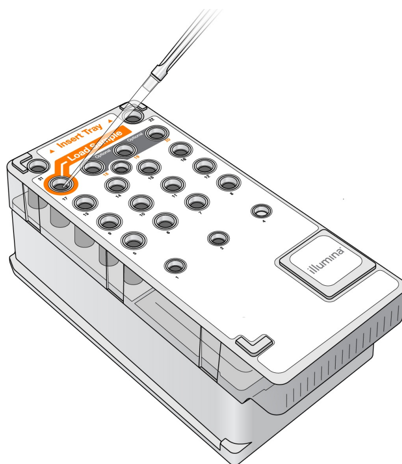
When the reagent cartridge is ready for use, load the prepared libraries onto the cartridge.

1 Using a separate, clean, and empty 1 ml pipette tip, pierce the foil seal over the reservoir labeled **Load Samples**.

NOTE
Do not pierce any other reagent positions. Other reagent positions are pierced automatically during the sequencing run.

2 Pipette 600 µl of prepared libraries into the **Load Samples** reservoir. Avoid touching the foil seal as you dispense the sample.

Figure 1 Load Libraries



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

Notes

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 1 Illumina General Contact Information

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

Table 2 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

Additional product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support and select a product. A MyIllumina login is required. To register for a MyIllumina account, please visit my.illumina.com/Account/Register.

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