

MiSeq System

Denature and Dilute Libraries Guide

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Overview

This guide explains steps to denature and dilute prepared libraries for sequencing on the Illumina® MiSeq® system.

This guide includes instructions for preparing a PhiX library for use as a sequencing control.

Loading Volume and Concentration

This procedure denatures and dilutes libraries to a final volume of 600 µl. The recommended loading concentration varies depending on the version of reagents used for the sequencing run.

Chemistry	Recommended Loading Concentration
MiSeq Reagent Kit v3	Supports 6–20 pM loading concentration. Requires at least a 4 nM library before diluting and denaturing.
MiSeq Reagent Kit v2	Supports 6–10 pM loading concentration.

In practice, loading concentration can vary depending on library preparation and quantification methods.

Protocol Variations

Follow the appropriate denature and dilute protocol depending on the normalization procedure used during library prep.

- } **Standard normalization**—Libraries are normalized using standard library quantification and quality control procedures recommended in the library prep documentation. For these libraries, follow **Protocol A**. See Protocol A: Standard Normalization Method on page 5.
- } **Bead-based normalization**—Libraries are normalized using a bead-based procedure described in the library prep documentation for methods that support bead-based normalization. For these libraries, follow **Protocol B**. See Protocol B: Bead-Based Normalization Method on page 7.

Consumables and Equipment

Consumables

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

Consumable	Supplier
HT1 (Hybridization Buffer), thawed and prechilled	Illumina, Provided in the MiSeq Reagent Kit
Illumina PhiX Control, Catalog # FC-110-3001	Illumina (Optional)
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

Equipment

The following equipment is used to denature libraries that have been normalized using a bead-based method.

Equipment	Supplier
Hybex Microsample Incubator	SciGene, catalog # 1057-30-O (115 V), or equivalent SciGene, catalog # 1057-30-2 (230 V), or equivalent
Block for 1.5 ml microcentrifuge tubes	SciGene, catalog # 1057-34-0, or equivalent

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 NaOH.
- } For best results, begin thawing the reagent cartridge before denaturing and diluting libraries. For instructions, see the **MiSeq System User Guide (part # 15027617)**.

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 low diversity libraries, dilute your PhiX control library to the same concentration as your denatured library.

Protocol A: Standard Normalization Method

Use protocol A to denature and dilute libraries that have been normalized using standard library quantification and quality control procedures recommended in the library prep documentation.

Follow the steps most appropriate for your library and the version of MiSeq reagent kit you are using.

Chemistry	Compatible Denature and Dilute Steps
MiSeq Reagent Kit v3	4 nM library —Results in a 6–20 pM loading concentration.
MiSeq Reagent Kit v2	4 nM library —Results in a 6–20 pM loading concentration. 2 nM library —Results in a 6–10 pM loading concentration.

The denaturation steps described in this guide ensure that the concentration of NaOH is not more than 0.001 (1 mM) in the final solution after diluting with HT1. Higher concentrations of NaOH in the library inhibit library hybridization to the flow cell and decrease cluster density.

Prepare Reagents

Prepare a Fresh Dilution of NaOH

- Combine the following volumes in a microcentrifuge tube.
 - } Laboratory-grade water (800 μ l)
 - } Stock 1.0 N NaOH (200 μ l)
 The result is 1 ml of 0.2 N NaOH.
- Invert the tube several times to mix.



NOTE

Use the fresh dilution within **12 hours**.

Prepare HT1

- Remove HT1 from -25°C to -15°C storage and thaw at room temperature.
- Store at 2°C to 8°C until you are ready to dilute denatured libraries.

Denature a 4 nM Library

- Combine the following volumes in a microcentrifuge tube.
 - } 4 nM library (5 μ l)
 - } 0.2 N NaOH (5 μ l)
- Vortex briefly and then centrifuge at $280 \times g$ for 1 minute.
- Incubate for 5 minutes at room temperature.
- Add 990 μ l prechilled HT1 to the tube containing denatured library.
The result is 1 ml of a 20 pM denatured library.

Dilute Denatured 20 pM Library

- 1 Dilute to the desired concentration using the following volumes.

Concentration	6 pM	8 pM	10 pM	12 pM	15 pM	20 pM
20 pM library	180 µl	240 µl	300 µl	360 µl	450 µl	600 µl
Prechilled HT1	420 µl	360 µl	300 µl	240 µl	150 µl	0 µl

- 2 Invert to mix and then pulse centrifuge.
- 3 To add a PhiX control, proceed to [Denature and Dilute PhiX Control](#) on page 8. Otherwise, see [Next Steps](#) on page 10.

Denature a 2 nM Library

- 1 Combine the following volumes in a microcentrifuge tube.
 - } 2 nM library (5 µl)
 - } 0.2 N NaOH (5 µl)
- 2 Vortex briefly and then centrifuge at $280 \times g$ for 1 minute.
- 3 Incubate for 5 minutes at room temperature.
- 4 Add 990 µl prechilled HT1 to the tube containing denatured library.
The result is 1 ml of a 10 pM denatured library.

Dilute Denatured 10 pM Library

- 1 Dilute to the desired concentration using the following volumes.

Concentration	6 pM	8 pM	10 pM
10 pM library	360 µl	480 µl	600 µl
Prechilled HT1	240 µl	120 µl	0 µl

- 2 Invert to mix and then pulse centrifuge.
- 3 To add a PhiX control, proceed to [Denature and Dilute PhiX Control](#) on page 8. Otherwise, see [Next Steps](#) on page 10.

Protocol B: Bead-Based Normalization Method

Use protocol B to denature and dilute libraries that have been normalized and pooled using a bead-based procedure described in the library prep documentation for methods that support bead-based normalization.

Bead-based normalization procedures can be variable. The actual volume of library varies depending upon library type and experience.

Prepare HT1

- 1 Remove HT1 from -25°C to -15°C storage and thaw at room temperature.
- 2 Store at 2°C to 8°C until you are ready to dilute denatured libraries.

Prepare Incubator

- 1 Preheat the incubator to 98°C.

Dilute Library to Loading Concentration

- 1 Combine the following volumes of pooled libraries and prechilled HT1 in a microcentrifuge tube.

Amplicon Library Pool	Prechilled HT1
6 µl	594 µl
7 µl	593 µl
8 µl	592 µl
9 µl	591 µl
10 µl	590 µl
Nextera XT Library Pool	Prechilled HT1
24 µl	576 µl

The total volume is 600 µl.

- 2 Vortex briefly and then centrifuge at $280 \times g$ for 1 minute.

Denature Diluted Library

- 1 Place the tube on the preheated incubator for 2 minutes.
- 2 Immediately cool on ice.
- 3 Leave on ice for 5 minutes.
- 4 To add a PhiX control, proceed to Denature and Dilute PhiX Control on page 8. Otherwise, see Next Steps on page 10.

Denature and Dilute PhiX Control

Use the following procedure to denature and dilute a PhiX library for use as a sequencing control.

Follow the steps appropriate for the version of MiSeq reagent kit you are using.

Chemistry	Final PhiX Concentration
MiSeq Reagent Kit v3	Dilute the denatured PhiX control to 20 pM, which produces an optimal cluster density using v3 reagents.
MiSeq Reagent Kit v2	Dilute the denatured PhiX control to 12.5 pM, which produces an optimal cluster density using v2 reagents.

Dilute PhiX to 4 nM

- 1 Combine the following volumes in a microcentrifuge tube.
 - } 10 nM PhiX library (2 μ l)
 - } 10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20 (3 μ l)
- 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 in a microcentrifuge tube.
 - } 4 nM PhiX library (5 μ l)
 - } 0.2 N NaOH (5 μ l)
- 2 Vortex briefly to mix.
- 3 Centrifuge at $280 \times g$ for 1 minute.
- 4 Incubate at room temperature for 5 minutes.

Dilute Denatured PhiX to 20 pM

- 1 Add prechilled HT1 to the denatured PhiX library.
 - } Denatured PhiX library (10 μ l)
 - } Prechilled HT1 (990 μ l)The result is 1 ml of a 20 pM PhiX library.
- 2 Invert to mix.



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 to 12.5 pM

If you are using MiSeq Reagent Kit v3, no further dilution is required.

- 1 Add prechilled HT1 to the denatured PhiX library.
 - } 20 pM denatured PhiX library (375 μ l)
 - } Prechilled HT1 (225 μ l)The result is 600 μ l of a 12.5 pM PhiX library.

- 2 Invert to mix.

Combine Library and PhiX Control

For most libraries, use a low-concentration PhiX control spike-in of 1% as a sequencing control. For low diversity libraries, increase the PhiX control spike-in to at least 5%.

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

	Most Libraries (1% Spike-In)	Low-Diversity Libraries (≥ 5% Spike-In)
Denatured and diluted PhiX	6 µl	30 µl
Denatured and diluted library (from protocol A or protocol B)	594 µl	570 µl

- 2 Set aside on ice until you are ready to load it onto the reagent cartridge.



NOTE
Actual PhiX percentage varies depending upon the quality and quantity of the library pool.

Next Steps

After denaturing and diluting your libraries and preparing the optional PhiX control, you are ready to load libraries onto the reagent cartridge and set up the sequencing run. See the [MiSeq System User Guide \(part # 15027617\)](#).

Revision History

Document	Date	Description of Change
Document # 15039740 v01	January 2016	Added procedure for denaturing and diluting libraries that have been normalized using a bead-based procedure. Organized procedures as Protocol A and Protocol B.
Part # 15039740 Rev. D	November 2013	Added recommendation for low diversity libraries to dilute PhiX control libraries to the same concentration as denatured sample libraries.
Part # 15039740 Rev. 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 <i>MiSeq System User Guide</i> (part # 15027617).
Part # 15039740 Rev. B	March 2013	Reduced PhiX recommendations for low diversity libraries from $\geq 25\%$ to $\geq 5\%$. 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 μ l.
Part # 15039740 Rev. A	January 2013	Initial release.

Notes

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 1 Illumina General Contact Information

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	Japan	0800.111.5011
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
Belgium	0800.81102	Norway	800.16836
China	400.635.9898	Singapore	1.800.579.2745
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	Taiwan	00806651752
Hong Kong	800960230	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000
Italy	800.874909		

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.



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