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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 also 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 MiSeq Reagent Kit used for the sequencing run. In practice, loading concentration can vary depending on library preparation and quantification methods.

Chemistry	Recommended Final 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.

Protocol Variations

Follow the appropriate denature and dilute protocol depending on the 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 4.
- 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 6.
- AmpliSeq™ for Illumina normalization—For all libraries prepared using the standard AmpliSeq for Illumina workflow, follow Protocol C. See *Protocol C: AmpliSeq for Illumina Panels Normalization Method* on page 7.
- AmpliSeq Library Equalizer™ for Illumina normalization—For all libraries prepared using the AmpliSeq Library Equalizer for Illumina workflow, follow Protocol D. See *Protocol D: AmpliSeq Library Equalizer for Illumina Normalization Method* on page 9.

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.

For example, low diversity can occur with some expression studies with > 25% of one type of transcript, low-plexity amplicon pools, adapter dimer, or bisulfite sequencing. 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.

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
[Optional] Illumina PhiX Control	Illumina, catalog # FC-110-3001
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
Tris-HCl, pH 7.0	General lab supplier
[Protocol C] Low TE	Illumina, Provided in the AmpliSeq Library PLUS kit

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

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. Loading concentration can also vary depending on library type and quantification methods.

For the Nextera™ DNA Flex Library Prep Kit, see dilute and denature directions in the *Nextera DNA Flex Library Prep Reference Guide (document # 1000000025416)*.

For the TruSight® Cardio Sequencing Kit, see dilute and denature directions in the *TruSight Cardio Sequencing Kit Reference Guide (document # 15063774)*.

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.

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The denaturation steps described in this guide make sure 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

1 Combine the following volumes in a microcentrifuge tube.
   - Laboratory-grade water (800 µl)
   - Stock 1.0 N NaOH (200 µl)

   The result is 1 ml of 0.2 N NaOH.
2 Invert the tube several times to mix.

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

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.

Denature a 4 nM Library

1 Combine the following volumes in a microcentrifuge tube.
   - 4 nM library (5 µl)
   - 0.2 N NaOH (5 µl)
2 Vortex briefly and then centrifuge at 280 × g for 1 minute.
3 Incubate at room temperature for 5 minutes.
4 Add 990 µl 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 10. Otherwise, see *Next Steps* on page 12.

Denature a 2 nM Library

1 Combine the following volumes in a microcentrifuge tube.
   - 2 nM library (5 µl)

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- 0.2 N NaOH (5 µl)

2 Vortex briefly and then centrifuge at 280 × g for 1 minute.

3 Incubate at room temperature for 5 minutes.

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 10. Otherwise, see *Next Steps* on page 12.

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. Loading concentration can also vary depending on library type and quantification methods.

For TruSight HLA Sequencing Kits, see dilute and denature directions in the *TruSight HLA v1 Sequencing Kit Reference Guide (document # 15056536)* or *TruSight HLA v2 Sequencing Kit Reference Guide (document # 1000000010159)*.

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.
The total volume is 600 µl. If cluster density results are too high or low, adjust the dilution ratio.
Check *BBN Loading Concentration Exceptions* on page 12 to see if your kit requires loading volumes that are different from general amplicon recommendations.

Table 1 General Amplicon Recommendations

Library Pool	Prechilled HT1	Chemistry
6 µl	594 µl	MiSeq Reagent Kit v3 or v2
7 µl	593 µl	MiSeq Reagent Kit v3 or v2
8 µl	592 µl	MiSeq Reagent Kit v3 or v2
9 µl	591 µl	MiSeq Reagent Kit v3 or v2
10 µl	590 µl	MiSeq Reagent Kit v3 or v2

2 Vortex briefly and then centrifuge at 280 × 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 10. Otherwise, see *Next Steps* on page 12.

Protocol C: AmpliSeq for Illumina Panels Normalization Method

Use protocol C to denature and dilute libraries prepared using the standard AmpliSeq for Illumina workflow. Final loading concentration and volume vary depending on library preparation and quantification methods. For information about the number of libraries supported per sequencing run, use the Illumina support website to refer to the AmpliSeq for Illumina support page for your panel.

Prepare Reagents

Prepare a Fresh Dilution of NaOH

1 Combine the following volumes in a microcentrifuge tube:
- Laboratory-grade water (800 µl)
- Stock 1.0 N NaOH (200 µl)

The result is 1 ml of 0.2 N NaOH.

2 Invert the tube several times to mix.

NOTE

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

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 Low TE

1 If frozen, remove Low TE from -25°C to -15°C storage and thaw at room temperature.

2 Store thawed Low TE at room temperature until you are ready to dilute libraries.

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Dilute Libraries

1 In a new 96-well LoBind PCR plate, dilute each library to 2 nM using Low TE.

Pool Libraries

1 Transfer equal volumes of each 2 nM library from the plate to a 1.5 mL LoBind tube.
If applicable, make sure to use separate tubes for DNA and RNA libraries.

2 Vortex each tube to mix.

3 Centrifuge each tube briefly.

4 If DNA and RNA libraries are to be grouped in a single sequencing run, combine the DNA and RNA library pools at the following ratio of DNA to RNA:

Panel	DNA to RNA ratio
AmpliSeq for Illumina Myeloid Panel	8:1
AmpliSeq for Illumina Childhood Cancer Panel	5:1
AmpliSeq for Illumina Focus Panel	7:3
AmpliSeq for Illumina Comprehensive Panel v3	25:1

5 After combining the pools, vortex tube to mix and then centrifuge briefly.

Denature Libraries

1 Combine the following volumes of library and freshly diluted 0.2 N NaOH in a microcentrifuge tube.

Reagent	**Volume (µl)**
Pooled libraries	10
0.2 N NaOH	10

2 Vortex briefly and then centrifuge briefly.

3 Incubate at room temperature for 5 minutes.

4 Add 10 µl 200 mM Tris-HCl, pH 7.0 to the tube containing 2 nM pooled libraries.

5 Vortex briefly and then centrifuge briefly.

Dilute Denatured Libraries to 20 pM

1 Add 970 µl prechilled HT1 to the tube of 2 nM denatured library pool.
The result is a 20 pM denatured library.

2 Vortex briefly and then centrifuge briefly.

3 Place the 20 pM libraries on ice until you are ready to proceed to final dilution.

Dilute Libraries to Final Loading Concentration

1 Use prechilled HT1 to dilute the denatured 20 pM library solution to 7–9 pM at a final volume of 600 µl.

2 Invert to mix and then centrifuge briefly.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C.

Protocol D: AmpliSeq Library Equalizer for Illumina Normalization Method

Use protocol D to denature and dilute libraries prepared using the AmpliSeq Library Equalizer for Illumina workflow. Libraries prepared using the AmpliSeq Library Equalizer for Illumina workflow are normalized to a starting concentration ready for sample pooling. For information about the number of libraries supported per sequencing run, use the Illumina support website to refer to the AmpliSeq for Illumina support page for your panel.

Prepare Reagents

Prepare a Fresh Dilution of NaOH

1. Combine the following volumes in a microcentrifuge tube:
   - Laboratory-grade water (800 µl)
   - Stock 1.0 N NaOH (200 µl)

   The result is 1 ml of 0.2 N NaOH.
2. Invert the tube several times to mix.

NOTE

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

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.

Pool Libraries

1. Transfer equal volumes of each library from the plate to a 1.5 mL LoBind tube.
   If applicable, make sure to use separate tubes for DNA and RNA libraries.
2. Vortex each tube to mix.
3. Centrifuge each tube briefly.
4. If DNA and RNA libraries are to be grouped in a single sequencing run, combine the DNA and RNA library pools at the following ratio of DNA to RNA:

Panel	DNA to RNA ratio
AmpliSeq for Illumina Myeloid Panel	8:1
AmpliSeq for Illumina Childhood Cancer Panel	5:1
AmpliSeq for Illumina Focus Panel	7:3
AmpliSeq for Illumina Comprehensive Panel v3	25:1

5. After combining the pools, vortex tube to mix and then centrifuge briefly.

Denature Libraries

1. Combine the following volumes of library and freshly diluted 0.2 N NaOH in a microcentrifuge tube.

Reagent	Volume (µl)
Pooled libraries	10
0.2 N NaOH	10

2 Vortex briefly and then centrifuge briefly.

3 Incubate at room temperature for 5 minutes.

4 Add 10 µl 200 mM Tris-HCl, pH 7.0 to the tube containing pooled libraries.

5 Vortex briefly and then centrifuge briefly.

Dilute Denatured Libraries

1 Add 970 µl prechilled HT1 to the tube of denatured library pool.

2 Vortex briefly and then centrifuge briefly.

3 Place the libraries on ice until you are ready to proceed to final dilution.

Dilute Libraries to Final Loading Concentration

1 Combine the following volumes to dilute the denatured library solution to the final loading concentration:
- Denatured library (385 µl)
- HT1 (215 µl)

2 Invert to mix and then centrifuge briefly.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C.

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 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 in a microcentrifuge tube.
- 4 nM PhiX library (5 µl)

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- 0.2 N NaOH (5 µl)

2 Vortex briefly to mix.

3 Centrifuge at 280 × 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 µl)
- Prechilled HT1 (990 µl)

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°C 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 µl)
- Prechilled HT1 (225 µl)

The result is 600 µl 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, B, C, or D)	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.

Supplemental Information

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BBN Loading Concentration Exceptions

Table 2 Nextera XT DNA

Library Pool	Prechilled HT1	Chemistry
24 µl	576 µl	MiSeq Reagent Kit v3 and v2

NOTE

24 µl is a suggested starting volume for Nextera XT DNA.

Table 3 TruSight Myeloid Sequencing Panel

Library Pool	Prechilled HT1	Chemistry
20 µl	580 µl	MiSeq Reagent Kit v3
6 µl	594 µl	MiSeq Reagent Kit v2

Table 4 TruSeq® Custom Amplicon v1.5

Library Pool	Prechilled HT1	Chemistry
20 µl	580 µl	MiSeq Reagent Kit v3
6 µl	594 µl	MiSeq Reagent Kit v2

Table 5 TruSeq Custom Amplicon Low Input Kit

Library Pool	Prechilled HT1	Chemistry
7 µl	593 µl	MiSeq Reagent Kit v3 or v2
8 µl	592 µl	MiSeq Reagent Kit v3 or v2
9 µl	591 µl	MiSeq Reagent Kit v3 or v2
10 µl	590 µl	MiSeq Reagent Kit v3 or v2

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)*.

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Revision History

Document	Date	Description of Change
Document # 15039740 v10	February 2019	Replaced Suggested Final Loading Concentration table in Protocol C with a single suggested concentration range.
Document # 15039740 v09	November 2018	Fixed AmpliSeq for Illumina Myeloid Panel pooling ratio in Protocol D.
Document # 15039740 v08	November 2018	Fixed AmpliSeq for Illumina Myeloid Panel pooling ratio in Protocol C. Added AmpliSeq for Illumina Childhood Cancer Research Assay Panel pooling ratio.
Document # 15039740 v07	October 2018	Added Protocol D for denaturing and diluting libraries prepared using the AmpliSeq Library Equalizer for Illumina workflow.
Document # 15039740 v06	July 2018	Added pooling ratio for AmpliSeq Myeloid Panel for Illumina.
Document # 15039740 v05	May 2018	Removed caution against using PhiX with Protocol C.
Document # 15039740 v04	April 2018	Added Protocol C for denaturing and diluting AmpliSeq for Illumina Panels.
Document # 15039740 v03	December 2017	Added recommendation in Protocol A to reference the Nextera DNA Flex Library Prep Reference Guide when working with the Nextera DNA Flex Library Prep Kit.
Document # 15039740 v02	February 2017	Added loading concentration recommendations for TruSeq Myeloid Sequencing Panel, TruSeq Custom Amplicon v1.5, and TruSeq Custom Amplicon Low Input Sequencing Kit.
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 *MiSeq System User Guide (part # 15027617)*.
Part # 15039740 Rev. 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.
Part # 15039740 Rev. A	January 2013	Initial release.

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Email:	techsupport@illumina.com

Illumina Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
Australia	+1.800.775.688	
Austria	+43 800006249	+43 19286540
Belgium	+32 80077160	+32 34002973
China	400.066.5835	
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Italy	+39 800985513	+39 236003759
Japan	0800.111.5011	
Netherlands	+31 8000222493	+31 207132960
New Zealand	0800.451.650	
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Taiwan	00806651752	
United Kingdom	+44 8000126019	+44 2073057197
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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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Illumina
5200 Illumina Way
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

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