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MiniSeq System

Denature and Dilute Libraries Guide

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Revision History

Document	Date	Description of Change
Document # 1000000002697 v09	April 2021	Updated loading concentration information for clarity and to include information for both standard and rapid kits.
Document # 1000000002697 v08	September 2020	Updated loading concentration information to be inclusive of rapid kits.
Document # 1000000002697 v07	February 2019	Replaced Suggested Final Loading Concentration table in Protocol C with a single suggested concentration range.
Document # 1000000002697 v06	November 2018	Fixed AmpliSeq for Illumina Myeloid Panel pooling ratio in Protocol D.
Document # 1000000002697 v05	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 # 1000000002697 v04	October 2018	Added Protocol D for denaturing and diluting libraries prepared using the AmpliSeq Library Equalizer for Illumina workflow.
Document # 1000000002697 v03	July 2018	Added pooling ratio for AmpliSeq Myeloid Panel for Illumina.
Document # 1000000002697 v02	May 2018	Removed caution against using PhiX with Protocol C.

Document	Date	Description of Change
Document # 1000000002697 v01	April 2018	Added Protocol C for denaturing and diluting AmpliSeq for Illumina Panels.
Document # 1000000002697 v00	January 2016	Initial release.

Table of Contents

Overview

This guide explains how to denature and dilute prepared libraries for sequencing on the Illumina® MiniSeq™ system.

This guide includes instructions for preparing a PhiX library for the following purposes:

- **For a control**—Prepare a PhiX library to combine with prepared libraries for use as a sequencing control. See *Denature and Dilute PhiX Control* on page 11.
- **For troubleshooting**—Prepare a PhiX library for a PhiX-only sequencing run for troubleshooting purposes. See *Prepare PhiX for a Troubleshooting Run* on page 12.

Loading Volume and Concentration

This procedure denatures and dilutes libraries to a final loading volume of 500 µl at a recommended concentration of 1.4 pM for standard kits and 1.6 pM for rapid kits. 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 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 3.
- **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 5.
- **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 6.
- **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 at a pH > 12.5 to denature 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 freshly diluted NaOH.
- For best results, begin thawing the reagent cartridge before denaturing and diluting libraries. For instructions, see the *MiniSeq System Guide (document # 1000000002695)*.

Consumables and Equipment

Consumables

The following consumables are required to denature and dilute libraries and prepare a PhiX control.

Consumables	Supplier
HT1	Component of the MiniSeq Kit
[Protocol C] Low TE	Illumina, Provided in the AmpliSeq Library PLUS kit

User-Supplied Consumables	Supplier
1 N NaOH, molecular biology grade	General lab supplier
200 mM Tris-HCl, pH 7.0	General lab supplier

The following additional consumables are required to prepare a PhiX control.

Consumables	Kit Name
PhiX, 10 nM RSB (Resuspension Buffer)	Illumina, catalog # FC-110-3002

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
Heat 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.

Prepare Reagents

Prepare a Fresh Dilution of NaOH

1. Combine the following volumes in a microcentrifuge tube.
   - Laboratory-grade water (900 µl)
   - Stock 1.0 N NaOH (100 µl)

   The total volume is 1 ml 0.1 N NaOH.
2. Invert the tube several times to mix.

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

Prepare HT1

1. Remove the tube of Hybridization Buffer from -25°C to -15°C storage and thaw at room temperature.
2. When thawed, store at 2°C to 8°C until you are ready to dilute denatured libraries.
3. Vortex briefly before use.

Prepare RSB

In place of RSB, you can use 10 mM Tris-HCl, pH 8.5 with 0.1% Tween 20.

1. Remove the tube of RSB from -25°C to -15°C storage and thaw at room temperature.
2. When thawed, store at 2°C to 8°C until you are ready to dilute libraries.

Create a Normalized Library Pool

If your libraries have not yet been normalized and pooled, use the following instructions to normalize to 10 nM and pool libraries. To load libraries onto the MiniSeq flow cell, libraries have to be combined into a single pool.

If your libraries have already been normalized and pooled, proceed to the Dilute library to 1nM step.

Create a Set of Normalized Libraries at 10 nM

1. Transfer 10 µl of each library to a corresponding well in a new MIDI or PCR plate.
2. Based on the concentration determined by the quantification method recommended in the library prep guide, use the following equation to dilute each library to 10 nM with RSB.

$$x\ \mu l = \frac{(10\ \mu l)(y\ \text{nM})}{10\ \text{nM}} - 10\ \mu l$$

   In this equation, *y* denotes the concentration of the individual library and *x* denotes the volume of RSB.

   If individual libraries are less than 10 nM, normalize to a concentration as low as 1 nM.

3. Gently pipette to mix.
   Depending on the concentration of each library, the final volume can vary from 10 µl to 400 µl.

Create a 10 nM Library Pool

1. Add 10 µl of each 10 nM library to a new microcentrifuge tube.
   The final volume of the 10 nM library pool varies depending on the number of libraries pooled.
2. [Optional] Store the remainder of 10 nM libraries at -25°C to -15°C.

Dilute Library to 1 nM

1. Based on library concentration, transfer library to a new microcentrifuge tube and add RSB.

Library Pool Concentration	Library Volume	RSB Volume
10 nM	10 µl	90 µl
4 nM	25 µl	75 µl
2 nM	50 µl	50 µl

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

Denature Library

1. Combine the following volumes in a microcentrifuge tube.
   - 1 nM library (5 µl)
   - 0.1 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 5 µl 200 mM Tris-HCl, pH 7.0.

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

Typically, the final solution can contain no more than 1 mM NaOH after diluting with Hybridization Buffer. However, introducing 200 mM Tris-HCl ensures that the NaOH is fully hydrolyzed in the final solution. As a result, template hybridization is not affected even when the final NaOH concentration is greater than 1 mM.

Dilute Library to Loading Concentration

1. Add 985 µl of prechilled Hybridization Buffer to the tube of denatured library.
   The total volume is 1 ml at 5 pM.
2. Vortex briefly and then centrifuge at 280 × g for 1 minute.
3. Dilute to the desired concentration using the following volumes.

	Standard Kit	Rapid Kit
Final Concentration	1.4 pM	1.6 pM
5 pM denatured library pool	140 µl	160 µl
Prechilled HT1	360 µl	340 µl

4. Vortex briefly and then centrifuge at 280 × g for 1 minute.
5. If you plan to add a PhiX control, proceed to *Dilute Library to Loading Concentration* on page 5. 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. Depending upon library type and experience, 2–5 µl of library produces optimal results.

Prepare HT1

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

3. Vortex briefly before use.

Prepare Incubator

1. Preheat the incubator to 98°C.

Dilute Library to Loading Concentration

1. Combine the following volumes of pooled libraries and prechilled Hybridization Buffer in a microcentrifuge tube.

Library Pool	Prechilled Hybridization Buffer
2 µl	998 µl
3 µl	997 µl
4 µl	996 µl
5 µl	995 µl

   The total volume is 1 ml.
2. Vortex briefly and then centrifuge at 280 × g for 1 minute.
3. Transfer 250 µl diluted library to a new microcentrifuge tube.
4. Add 250 µl prechilled Hybridization Buffer.
5. 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. If you plan to add a PhiX control, proceed to *Denature and Dilute PhiX Control* on page 11. 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 0.2 N NaOH.
2. Invert the tube several times to mix.

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. 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.

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

Panel	DNA to RNA ratio
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 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 1.1–1.9 pM at a final volume of 500 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 0.2 N NaOH.
2. Invert the tube several times to mix.

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 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 (28 µl)
   - HT1 (472 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

Dilute PhiX to 4 nM

1. Thaw a tube of 10 nM PhiX stock.
2. Combine the following volumes in a microcentrifuge tube.
   - 10 nM PhiX (10 µl)
   - RSB (15 µl)

   The total volume is 25 µl at 4 nM.
3. Vortex briefly and then pulse centrifuge.

[Optional] Store the 4 nM PhiX at -25°C to -15°C for up to 3 months.

Denature PhiX

1. Combine the following volumes in a microcentrifuge tube.
   - 4 nM PhiX (5 µl)
   - 0.1 N NaOH (5 µl)
2. Vortex briefly and then pulse centrifuge.
3. Incubate at room temperature for 5 minutes.
4. Add 5 µl 200 mM Tris-HCl, pH 7.0.
5. Vortex briefly and then centrifuge at 280 × g for 1 minute.

Dilute Denatured PhiX to Loading Concentration

1. Add 985 µl of prechilled Hybridization Buffer to the tube of denatured PhiX library.
   The total volume is 1 ml at 20 pM.
2. Vortex briefly and then centrifuge at 280 × g for 1 minute.
3. Dilute to the desired concentration using the following volumes.

	Standard Kit	Rapid Kit
Final Concentration	1.4 pM	1.6 pM
20 pM denatured PhiX	35 µl	40 µl
Prechilled HT1	465 µl	460 µl

4. Vortex briefly and then centrifuge at 280 × g for 1 minute.
5. Set aside on ice until you are ready to load the library onto the reagent cartridge.

[Optional] Store the denatured PhiX at -25°C to -15°C for up to two weeks. After two weeks, cluster numbers tend to decrease.

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 equal concentrations of the following volumes of denatured PhiX control and denatured library.

	Most Libraries (1% Spike-In)	Low-Diversity Libaries (≥10% Spike-In)
Denatured and diluted PhiX	5 µl	50 µl
Denatured and diluted library (from protocol A, B, C, or D)	495 µl	450 µl

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

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 a thawed reagent cartridge and set up the sequencing run. For complete instructions, see the *MiniSeq System Guide (document # 1000000002695)*.

Visit the MiniSeq support page on the Illumina support website for access to documentation, software downloads, frequently asked questions, and online training.

Prepare PhiX for a Troubleshooting Run

Use the following procedure to denature and dilute a PhiX library for use as a PhiX-only sequencing run. Performing a PhiX-only run is helpful in confirming instrument performance or for troubleshooting purposes. A PhiX-only run requires 100% PhiX library at recommended volumes and loading concentration.

Before proceeding, prepare reagents as described in *Prepare Reagents* on page 3.

Dilute PhiX to 4 nM

1. Thaw a tube of 10 nM PhiX stock.
2. Combine the following volumes in a microcentrifuge tube.
   - 10 nM PhiX (10 µl)
   - RSB (15 µl)

   The total volume is 25 µl at 4 nM.
3. Vortex briefly and then pulse centrifuge.

[Optional] Store the 4 nM PhiX at -25°C to -15°C for up to 3 months.

Denature PhiX

1. Combine the following volumes in a microcentrifuge tube.
   - 4 nM PhiX (5 µl)
   - 0.1 N NaOH (5 µl)
2. Vortex briefly and then pulse centrifuge.
3. Incubate at room temperature for 5 minutes.
4. Add 5 µl 200 mM Tris-HCl, pH 7.0.
5. Vortex briefly and then centrifuge at 280 × g for 1 minute.

Dilute Denatured PhiX Library to Loading Concentration

1. Add 985 µl of prechilled Hybridization Buffer to the tube of denatured PhiX library.
   The total volume is 1 ml at 20 pM.
2. Vortex briefly and then centrifuge at 280 × g for 1 minute.
3. Dilute to the desired concentration using the following volumes.

	Standard Kit	Rapid Kit
Final Concentration	1.4 pM	1.6 pM
20 pM denatured PhiX	35 µl	40 µl
Prechilled HT1	465 µl	460 µl

4. Vortex briefly and then centrifuge at 280 × g for 1 minute.
5. Set aside on ice until you are ready to load the library onto the reagent cartridge.

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