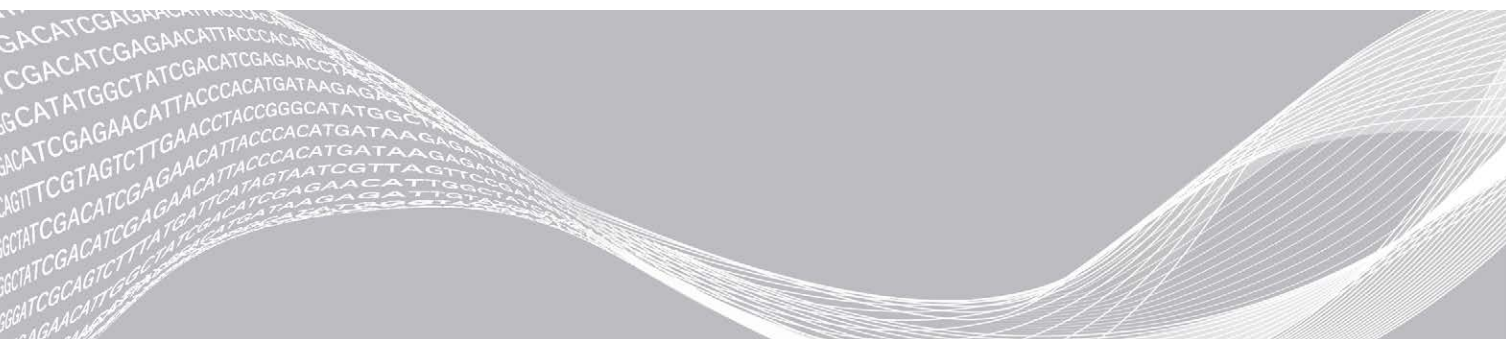


# NextSeq System

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## Overview

This guide explains how to denature and dilute prepared libraries for sequencing on the Illumina® NextSeq™ 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. PhiX control protocols start [on page 15](#).
- ▶ **For troubleshooting**—Prepare a PhiX library for a PhiX-only sequencing run for troubleshooting purposes. See [Prepare PhiX for a Troubleshooting Run on page 19](#).

## Loading Volume and Concentration

This procedure denatures and dilutes libraries to a final loading volume of 1.3 ml at a recommended concentration of 1.8 pM for high output kits and 1.5 pM for mid output 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 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](#).
  - ▶ For TruSight™ Tumor 170 libraries, follow [Protocol E: TruSight Tumor 170 Library Denaturation and Dilution Method on page 10](#).
  - ▶ For TruSight Oncology 500 libraries, follow [Protocol F: TruSight Oncology 500 Library Denaturation and Dilution Method on page 12](#).
- ▶ **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

- ▶ To prevent small pipetting errors from affecting the final NaOH concentration, prepare at least 1 ml freshly diluted NaOH.
- ▶ For best results, begin thawing reagents before denaturing and diluting libraries. For instructions, see the system guide for your instrument.

## Consumables and Equipment

### Consumables

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

Consumables	Supplier
HT1 (Hybridization Buffer)	Component of the NextSeq 500/550 Kit
<b>[Protocol C]</b> Low TE	Illumina, Provided in the AmpliSeq Library PLUS kit

User-Supplied Consumables	Supplier
1 N NaOH, molecular biology-grade	General lab supplier
<b>[Protocol A–D]</b> 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
<b>[Protocol E and F]</b> HP3 (2 N NaOH)	Illumina, provided in library prep kit contents

## 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.



### NOTE

Typically, it is important that not more than 1 mM NaOH is in the final solution after diluting with HT1. 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 if the final NaOH concentration after diluting with HT1 is greater than 1 mM.

## Prepare Reagents

### Prepare a Fresh Dilution of NaOH

- Combine the following volumes in a microcentrifuge tube:
  - ▶ Laboratory-grade water (800  $\mu$ l)
  - ▶ Stock 1.0 N NaOH (200  $\mu$ 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

- 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 RSB



### NOTE

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 Store thawed RSB at 2°C to 8°C until you are ready to dilute libraries.

## Denature Libraries

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

Starting Library Concentration	Library	0.2 N NaOH
4 nM	5 µl	5 µl
2 nM	10 µl	10 µl
1 nM	20 µl	20 µl
0.5 nM	40 µl	40 µl

- 2 Vortex briefly and then centrifuge at 280 × g for 1 minute.
- 3 Incubate at room temperature for 5 minutes.
- 4 Add the following volume of 200 mM Tris-HCl, pH 7.

Starting Library Concentration	200 mM Tris-HCl, pH 7
4 nM	5 µl
2 nM	10 µl
1 nM	20 µl
0.5 nM	40 µl

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

## Dilute Denatured Libraries to 20 pM

- 1 Add the following volume of prechilled HT1 to the tube of denatured libraries.

Starting Library Concentration	Prechilled HT1
4 nM	985 µl
2 nM	970 µl
1 nM	940 µl
0.5 nM	880 µl

The result is a 20 pM denatured library.

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

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

## Dilute Libraries to Loading Concentration

### High Output Kits

- 1 Dilute the denatured 20 pM library solution to 1.8 pM as follows.
  - ▶ Denatured library solution (117  $\mu$ l)
  - ▶ Prechilled HT1 (1183  $\mu$ l)The total volume is 1.3 ml at 1.8 pM.
- 2 Invert to mix and then pulse centrifuge.
- 3 If you plan to add a PhiX control, proceed to *Denature and Dilute PhiX Control Using Protocols A–D on page 15*. Otherwise, see *Next Steps on page 19*.

### Mid Output Kits

- 1 Dilute the denatured 20 pM library solution to 1.5 pM as follows.
  - ▶ Denatured library solution (97  $\mu$ l)
  - ▶ Prechilled HT1 (1203  $\mu$ l)The total volume is 1.3 ml at 1.5 pM.
- 2 Invert to mix and then pulse centrifuge.
- 3 If you plan to add a PhiX control, proceed to *Denature and Dilute PhiX Control Using Protocols A–D on page 15*. Otherwise, see *Next Steps on page 19*.

## 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.

For TruSight Tumor 170 libraries, follow *Protocol E: TruSight Tumor 170 Library Denaturation and Dilution Method on page 10*.

For TruSight Oncology 500 libraries, follow *Protocol F: TruSight Oncology 500 Library Denaturation and Dilution Method on page 12*.

Bead-based normalization procedures can be variable. Depending upon library type and experience, 2–5  $\mu$ l library produces optimal results.

### 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 Hybridization Buffer in a microcentrifuge tube.

Library Pool	Prechilled Hybridization Buffer
2 $\mu$ l	998 $\mu$ l
3 $\mu$ l	997 $\mu$ l
4 $\mu$ l	996 $\mu$ l
5 $\mu$ l	995 $\mu$ l

The total volume is 1 ml.

- 2 Vortex briefly and then centrifuge at  $280 \times g$  for 1 minute.
- 3 Transfer 750  $\mu$ l diluted library to a new microcentrifuge tube.
- 4 Add 750  $\mu$ l prechilled Hybridization Buffer.
- 5 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 If you plan to add a PhiX control, proceed to *Denature and Dilute PhiX Control Using Protocols A–D* on page 15. Otherwise, see *Next Steps* on page 19.

## 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  $\mu$ l)
  - ▶ Stock 1.0 N NaOH (200  $\mu$ l)
 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  $-25^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  storage and thaw at room temperature.
- 2 Store at  $2^{\circ}\text{C}$  to  $8^{\circ}\text{C}$  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.

## 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 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 1.1–1.9 pM at a final volume of 1.3 ml.
- 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 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 (95 μl)
  - ▶ HT1 (1205 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 E: TruSight Tumor 170 Library Denaturation and Dilution Method

Use protocol E to denature and dilute TruSight Tumor 170 libraries.

Use the following procedures to achieve optimal coverage:

- ▶ Sequence 16 libraries (eight DNA and eight RNA) per run with a NextSeq High Output flow cell to achieve maximum coverage for each library.
- ▶ If you are sequencing DNA libraries only, you can sequence up to 10 libraries.
- ▶ If you are sequencing RNA libraries only, you can sequence up to 16 libraries.
- ▶ Contact Illumina Technical Support if you are sequencing other combinations of DNA and RNA libraries.

## Prepare HT1

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

## Prepare Heat Block

- 1 Preheat the heat block to 96°C.

## Denature Libraries

- 1 Incubate each pooled library tube in a heat block at 96°C for 2 minutes.
- 2 Invert each tube two times to mix.
- 3 Centrifuge briefly, and then place on ice for 5 minutes.

### SAFE STOPPING POINT

If you are stopping, store denatured libraries at -25°C to -15°C for up to 30 days. To use frozen library pools, repeat *Denature Libraries on page 11* to redenate, mix, and cool tubes before proceeding to the next step.

## Dilute Libraries

Choose one of the following dilution procedures to produce a denatured library solution. If you are sequencing the same number of DNA and RNA libraries, pool at a 4:1 ratio of DNA to RNA. If you are sequencing an unequal number of libraries (for example, 7 DNA + 3 RNA), contact Illumina Technical Support.

### Sequence DNA and RNA Libraries Simultaneously

- 1 Transfer 20 µl denatured DNA library to a new, screw-top microcentrifuge tube.
- 2 Add 5 µl denatured RNA library to the tube.
- 3 Add 475 µl HT1 buffer to the tube to make a 1:20 dilution.
- 4 Vortex to mix, and then centrifuge briefly.

### Sequence DNA Libraries

- 1 Transfer 10 µl denatured DNA library to a new, screw-top microcentrifuge tube.
- 2 Add 190 µl HT1 buffer to the tube to make a 1:20 dilution.
- 3 Vortex to mix, and then centrifuge briefly.

### Sequence RNA Libraries

- 1 Transfer 10 µl denatured RNA library to a new, screw-top microcentrifuge tube.
- 2 Add 190 µl HT1 buffer to the tube to make a 1:20 dilution.
- 3 Vortex to mix, and then centrifuge briefly.

## Dilute Denatured Libraries to Final Loading Concentration

- 1 Transfer 40 µl denatured library solution into a new, snap-cap microcentrifuge tube.
- 2 Add 1360 µl HT1 buffer to the tube.
- 3 Vortex to mix, and then centrifuge briefly.
- 4 If you plan to add a PhiX control, proceed to *Denature and Dilute PhiX Control Using Protocol E on page 17*. Otherwise, see *Next Steps on page 19*.

## Protocol F: TruSight Oncology 500 Library Denaturation and Dilution

### Method

Use protocol F to pool, denature, and dilute libraries prepared using the TruSight Oncology 500 workflow. Libraries prepared using the workflow are normalized to a starting concentration ready for sample pooling. TruSight Oncology 500 libraries that are RNA only require PhiX control to ensure sequencing quality. PhiX control is optional for TruSight Oncology 500 DNA only libraries or combined DNA and RNA libraries.

Use the following procedures to achieve optimal coverage:

- ▶ If sequencing DNA libraries only, sequence up to eight libraries.
- ▶ If sequencing DNA libraries and RNA libraries simultaneously, sequence up to eight DNA and eight RNA libraries.
- ▶ If sequencing RNA libraries only, sequence up to 16 libraries.

Select one of the following options to pool, denature, and dilute the libraries.

- ▶ Sequence DNA libraries only. See *Denature and Dilute DNA Libraries* on page 12.
- ▶ Sequence DNA libraries and RNA libraries simultaneously. See *Denature and Dilute DNA and RNA Libraries* on page 13.
- ▶ Sequence RNA libraries only. See *Denature and Dilute RNA Libraries* on page 14.

## Denature and Dilute DNA Libraries

### Prepare HT1

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

### Prepare Heat Block

- 1 Preheat the heat block to 96°C.

### Pool Libraries

- 1 Thaw DNA NL plate to room temperature. Pipette to mix and centrifuge.
- 2 Label a 1.5 ml screw top microcentrifuge tube PDL (Pooled DNA Libraries).
- 3 Transfer 10 µl of each normalized DNA library from the NL plate to the PDL tube.
- 4 Vortex the PDL tube to mix.
- 5 Centrifuge the PDL tube briefly.

### Denature Libraries

- 1 Incubate the PDL tube in a heat block at 96°C for 2 minutes.
- 2 Invert the PDL tube two times to mix.
- 3 Centrifuge briefly, and then place on ice for 5 minutes.

## SAFE STOPPING POINT

If you are stopping, store denatured libraries at -25°C to -15°C for up to 30 days. To use frozen library pools, repeat *Denature Libraries* on page 12 to redenature, mix, and cool tubes before proceeding to the next step.

## Dilute Libraries

- 1 Transfer 10 µl denatured PDL tube to a new, screw-top microcentrifuge tube.
- 2 Add 190 µl HT1 buffer to the tube to make a 1:20 dilution.
- 3 Vortex to mix, and then centrifuge briefly.

## Dilute Denatured Libraries to Final Loading Concentration

- 1 Transfer 40 µl denatured diluted library solution into a new, 2 ml screw top tube.
- 2 Add 1660 µl HT1 buffer to the tube.
- 3 Vortex to mix, and then centrifuge briefly.
- 4 Proceed to *Denature and Dilute PhiX Control Using Protocol F* on page 18.

## Denature and Dilute DNA and RNA Libraries

### Prepare HT1

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

### Prepare Heat Block

- 1 Preheat the heat block to 96°C.

### Pool Libraries

- 1 Thaw both DNA and RNA NL plates to room temperature. Pipette to mix and centrifuge.
- 2 Label a 1.5 ml screw top microcentrifuge tube PDL (Pooled DNA Libraries).
- 3 Transfer 10 µl of each normalized DNA library from the NL plate to the PDL tube.
- 4 Label a 1.5 ml screw top microcentrifuge tube PRL (Pooled RNA Libraries).
- 5 Transfer 10 µl of each normalized RNA library from the RNA NL plate to the PRL tube.
- 6 Vortex each tube to mix, and then centrifuge briefly.

### Denature Libraries

- 1 Incubate each pooled library tube in a heat block at 96°C for 2 minutes.
- 2 Invert each tube two times to mix.
- 3 Centrifuge briefly, and then place on ice for 5 minutes.

## SAFE STOPPING POINT

If you are stopping, store denatured libraries at -25°C to -15°C for up to 30 days. To use frozen library pools, repeat *Denature Libraries* on page 13 to redenature, mix, and cool tubes before proceeding to the next step.

## Dilute Libraries

- 1 Transfer 20 µl denatured DNA library (PDL) to a new, screw-top microcentrifuge tube.
- 2 Add 5 µl denatured RNA library (PRL) to the tube.
- 3 Add 475 µl HT1 buffer to the tube to make a 1:20 dilution.  
If you are sequencing the same number of DNA and RNA libraries, pool at a 4:1 ratio of DNA to RNA. If you are sequencing an unequal number of libraries, contact Illumina Technical Support.
- 4 Vortex to mix, and then centrifuge briefly.

## Dilute Denatured Libraries to Final Loading Concentration

- 1 Transfer 40 µl denatured diluted library solution into a new, 2 ml screw top tube.
- 2 Add 1660 µl HT1 buffer to the tube.
- 3 Vortex to mix, and then centrifuge briefly.
- 4 Proceed to *Denature and Dilute PhiX Control Using Protocol F* on page 18.

## Denature and Dilute RNA Libraries

### Prepare HT1

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

### Prepare Heat Block

- 1 Preheat the heat block to 96°C.

### Pool Libraries

- 1 Thaw RNA NL plate to room temperature. Pipette to mix and centrifuge.
- 2 Label a 1.5 ml screw top microcentrifuge tube PRL (Pooled RNA Libraries).
- 3 Transfer 10 µl of each normalized RNA library from the NL plate to the PRL tube.
- 4 Vortex the PRL tube to mix.
- 5 Centrifuge the PRL tube briefly.

### Denature Libraries

- 1 Incubate the PRL tube in a heat block at 96°C for 2 minutes.
- 2 Invert the PRL tube two times to mix.
- 3 Centrifuge briefly, and then place on ice for 5 minutes.

## SAFE STOPPING POINT

If you are stopping, store denatured libraries at -25°C to -15°C for up to 30 days. To use frozen library pools, repeat *Denature Libraries* on page 14 to redenate, mix, and cool tubes before proceeding to the next step.

## Dilute Libraries

- 1 Transfer 10 µl denatured PRL tube to a new, screw-top microcentrifuge tube.
- 2 Add 190 µl HT1 buffer to the tube to make a 1:20 dilution.
- 3 Vortex to mix, and then centrifuge briefly.

## Dilute Denatured Libraries to Final Loading Concentration

- 1 Transfer 40 µl denatured diluted library solution into a new, 2 ml screw top tube.
- 2 Add 1660 µl HT1 buffer to the tube.
- 3 Vortex to mix, and then centrifuge briefly.
- 4 Proceed to *Denature and Dilute PhiX Control Using Protocol F* on page 18.

## Denature and Dilute PhiX Control Using Protocols A–D

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

### Dilute PhiX to 4 nM

- 1 Thaw a tube of 10 nM PhiX stock (10 µl/tube).
- 2 Combine the following volumes in a microcentrifuge tube.
  - ▶ 10 nM PhiX (10 µl)
  - ▶ RSB (15 µl)The total volume is 25 µl of 4 nM PhiX.
- 3 Vortex briefly and then pulse centrifuge.



#### NOTE

**[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)
  - ▶ 0.2 N NaOH, freshly diluted (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

### High Output Kits

- 1 Add 985  $\mu$ l prechilled HT1 to the tube of denatured PhiX.  
The total volume is 1 ml at 20 pM.
- 2 Dilute the denatured 20 pM PhiX to 1.8 pM as follows.
  - ▶ Denatured PhiX (117  $\mu$ l)
  - ▶ Prechilled HT1 (1183  $\mu$ l)
 The total volume is 1.3 ml at 1.8 pM.
- 3 Invert to mix and then centrifuge at  $280 \times g$  for 1 minute.



#### NOTE

**[Optional]** Store the denatured 1.8 pM PhiX at  $-25^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  for up to 2 weeks. After 2 weeks, cluster numbers tend to decrease.

### Mid Output Kits

- 1 Add 985  $\mu$ l prechilled HT1 to the tube of denatured PhiX.  
The total volume is 1 ml at 20 pM.
- 2 Dilute the denatured 20 pM PhiX to 1.5 pM as follows.
  - ▶ Denatured PhiX (97  $\mu$ l)
  - ▶ Prechilled HT1 (1203  $\mu$ l)
 The total volume is 1.3 ml at 1.5 pM.
- 3 Invert to mix and then centrifuge at  $280 \times g$  for 1 minute.



#### NOTE

**[Optional]** Store the denatured 1.5 pM PhiX at  $-25^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  for up to 2 weeks. After 2 weeks, cluster numbers tend to decrease.

## Combine Library and PhiX Control

For most libraries use a low-concentration PhiX control spike-in at 1% as a sequencing control.

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

Library and Concentration (Using 1.5 pM PhiX for Mid Output Kits)	Volume
Denatured and diluted PhiX control at 1.5 pM	13 $\mu$ l
Denatured and diluted library (from protocol A, B, C, or D)	1287 $\mu$ l

Library and Concentration (Using 20 pM PhiX for Mid Output Kits)	Volume
Denatured and diluted PhiX control at 20 pM	1 $\mu$ l
Denatured and diluted library (from protocol A, B, C, or D)	1299 $\mu$ l

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



**NOTE**

The library and PhiX mixture provides a PhiX spike-in of 0.5%–2.0%. Actual PhiX percentage varies depending upon the quality and quantity of the library pool.

## Denature and Dilute PhiX Control Using Protocol E

Use the following procedure to denature and dilute a PhiX library for use as a sequencing control for TruSight Tumor 170 libraries.

### Prepare Reagents

#### Prepare HP3

- 1 Remove HP3 from 2°C to 8°C storage and bring to room temperature.

#### Prepare a Fresh Dilution of NaOH

- 1 Combine the following volumes in a microcentrifuge tube:
  - ▶ RNase/DNase-free water (950 µl)
  - ▶ HP3 (50 µl)
 The result is 1 ml of 0.1 N NaOH.
- 2 Invert the tube several times to mix.

**CAUTION**

Use the fresh dilution within **1 hour**.

### Prepare PhiX Control

#### Dilute PhiX to 2 nM

- 1 Thaw a tube of 10 nM PhiX stock (10 µl/tube).
- 2 Combine the following volumes in a microcentrifuge tube.
  - ▶ 10 nM PhiX (2 µl)
  - ▶ RSB (8 µl)
 The total volume is 10 µl of 2 nM PhiX.
- 3 Pipette up and down five times to mix.

#### Denature PhiX

- 1 Combine the following volumes in a microcentrifuge tube.
  - ▶ 2 nM PhiX (10 µl)
  - ▶ 0.1 N NaOH, freshly diluted (10 µl)
- 2 Vortex to mix, and then centrifuge briefly.
- 3 Incubate at room temperature for 5 minutes.

#### Dilute Denatured PhiX to Loading Concentration

- 1 Add 980 µl prechilled HT1 to the tube of denatured PhiX.

The total volume is 1 ml at 20 pM.

- 2 Invert to mix, and then centrifuge briefly.



#### NOTE

**[Optional]** Store the denatured 20 pM PhiX at -25°C to -15°C for up to 3 weeks as single-use 50 µl aliquots.

## Combine Library and PhiX Control

- 1 Combine the following volumes of denatured PhiX control and denatured library.
  - ▶ Denatured 20 pM PhiX control (2.5 µl)
  - ▶ Denatured library (1300 µl)
- 2 Vortex to mix, and then centrifuge briefly.
- 3 Set aside on ice until you are ready to load it onto the reagent cartridge.

## Denature and Dilute PhiX Control Using Protocol F

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

### Prepare Reagents

#### Prepare HP3

- 1 Remove HP3 from 2°C to 8°C storage and bring to room temperature.

#### Prepare a Fresh Dilution of NaOH

- 1 Combine the following volumes in a microcentrifuge tube:
  - ▶ RNase/DNase-free water (190 µl)
  - ▶ HP3 (10 µl)

The result is 200 µl 0.1 N NaOH.
- 2 Invert the tube several times to mix.



#### CAUTION

Use the fresh dilution within **1 hour**.

### Prepare PhiX Control

#### Dilute PhiX to 2 nM

- 1 Thaw a tube of 10 nM PhiX stock (10 µl/tube).
- 2 Combine the following volumes in a microcentrifuge tube.
  - ▶ 10 nM PhiX (2 µl)
  - ▶ RSB (8 µl)

The total volume is 10 µl of 2 nM PhiX.
- 3 Pipette up and down five times to mix.

## Denature PhiX

- 1 Combine the following volumes in a microcentrifuge tube.
  - ▶ 2 nM PhiX (10  $\mu$ l)
  - ▶ 0.1 N NaOH, freshly diluted (10  $\mu$ l)
- 2 Vortex to mix, and then centrifuge briefly.
- 3 Incubate at room temperature for 5 minutes.

## Dilute Denatured PhiX to Loading Concentration

- 1 Add 980  $\mu$ l prechilled HT1 to the tube of denatured PhiX.  
The total volume is 1 ml at 20 pM.
- 2 Invert to mix, and then centrifuge briefly.



### NOTE

**[Optional]** Store the denatured 20 pM PhiX at -25°C to -15°C for up to 3 weeks as single-use 50  $\mu$ l aliquots.

## Combine Library and PhiX Control (DNA Only or DNA and RNA)

- 1 Combine the following volumes in a microcentrifuge tube.
  - ▶ Denatured 20 pM PhiX control (2.5  $\mu$ l)
  - ▶ Denatured TruSight Oncology 500 library (1700  $\mu$ l)
- 2 Vortex to mix, and then centrifuge briefly.
- 3 Set aside on ice until you are ready to load it onto the reagent cartridge.

## Combine Library and PhiX Control (RNA Only)

- 1 Combine the following volumes in a microcentrifuge tube.
  - ▶ Denatured 20 pM PhiX control (16.7  $\mu$ l)
  - ▶ Denatured TruSight Oncology 500 library (1646  $\mu$ l)
- 2 Vortex to mix, and then centrifuge briefly.
- 3 Set aside on ice until you are ready to load it onto the reagent cartridge.

## 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 *NextSeq 500 System Guide* (document # 15046563) or *NextSeq 550 System Guide* (document # 15069765).

## 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 18*.

## Dilute PhiX to 4 nM

- 1 Thaw a tube of 10 nM PhiX stock (10  $\mu$ l/tube).
- 2 Combine the following volumes in a microcentrifuge tube.
  - ▶ 10 nM PhiX (10  $\mu$ l)
  - ▶ RSB (15  $\mu$ l)The total volume is 25  $\mu$ l of 4 nM PhiX.
- 3 Vortex briefly and then pulse centrifuge.



### NOTE

[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  $\mu$ l)
  - ▶ 0.2 N NaOH, freshly diluted (5  $\mu$ l)
- 2 Vortex briefly, and then pulse centrifuge.
- 3 Incubate at room temperature for 5 minutes.
- 4 Add 5  $\mu$ l 200 mM Tris-HCl, pH 7.0.
- 5 Vortex briefly, and then centrifuge at 280  $\times$  g for 1 minute.

## Dilute Denatured PhiX to Loading Concentration

- 1 Add 985  $\mu$ l prechilled HT1 to the tube of denatured PhiX.  
The total volume is 1 ml at 20 pM.
- 2 Dilute the denatured 20 pM PhiX to 1.8 pM as follows.
  - ▶ Denatured PhiX (117  $\mu$ l)
  - ▶ Prechilled HT1 (1183  $\mu$ l)The total volume is 1.3 ml at 1.8 pM.
- 3 Invert to mix and then centrifuge at 280  $\times$  g for 1 minute.
- 4 Set aside on ice until you are ready to load the library onto the reagent cartridge.

## Revision History

Document	Date	Description of Change
Document # 15048776 v13	November 2019	Added the following information for TruSight Oncology 500: <ul style="list-style-type: none"> <li>Guidelines on the number of libraries and possible DNA/RNA combinations per sequencing run.</li> <li>Clarification to select one of three options to pool, denature, and dilute libraries</li> </ul>
Document # 15048776 v12	October 2019	Added a new RNA only protocol for denaturing and diluting TruSight Oncology 500 libraries. Combined common topics in denaturation and dilution method for protocol F. For TruSight Oncology 500, removed guidelines on the number of libraries and possible DNA/RNA combinations per sequencing run and moved the information to the <a href="#">TruSight Oncology 500 support pages</a> .
Document # 15048776 v11	April 2019	Added a new protocol for denaturing and diluting TruSight Oncology 500 DNA and RNA libraries. Replaced instances of incubator with heat block in Protocols E and F. Added heat block and thermal cycler to Equipment list. Updated Denature and Dilute PhiX Control procedures to more clearly identify the compatible protocol.
Document # 15048776 v10	February 2019	Replaced Suggested Final Loading Concentration table in Protocol C with a single suggested concentration range.
Document # 15048776 v09	December 2018	Added a new protocol for denaturing and diluting TruSight Tumor 170 libraries. Added a new procedure for denaturing and diluting PhiX for TruSight Tumor 170. Added a new protocol for pooling, denaturing, and diluting TruSight Oncology 500 libraries. Added a new procedure for denaturing and diluting PhiX for TruSight Oncology 500.
Document # 15048776 v08	November 2018	Fixed AmpliSeq for Illumina Myeloid Panel pooling ratio in Protocol D.
Document # 15048776 v07	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 # 15048776 v06	October 2018	Added Protocol D for denaturing and diluting libraries prepared using the AmpliSeq Library Equalizer for Illumina workflow.
Document # 15048776 v05	July 2018	Added pooling ratio for AmpliSeq Myeloid Panel for Illumina.
Document # 15048776 v04	May 2018	Added note regarding mid output kit loading concentration. Added information on high and mid output kits. Removed caution against using PhiX with Protocol C.
Document # 15048776 v03	April 2018	Added Protocol C for denaturing and diluting AmpliSeq for Illumina Panels.
Document # 15048776 v02	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. Add instructions to dilute PhiX to 1.8 pM for use as a control.

Document	Date	Description of Change
Document # 15048776 v01	October 2015	Removed extra vortex and centrifuge steps from PhiX preparation instructions. Removed instructions for using NCS v1.2 software.
Part # 15048776 Rev. E	May 2015	Changed title to the NextSeq System Denature and Dilute Libraries Guide. This guide applies to the NextSeq 500 and NextSeq 550 systems.
Part # 15048776 Rev. D	October 2014	Corrected library volume to 2995 $\mu$ l when combining libraries with a PhiX spike-in and when using NCS v1.2. Added information about performing a PhiX-only run for troubleshooting purposes.
Part # 15048776 Rev. C	September 2014	Updated URL for Safety Data Sheets (SDS) to <a href="http://support.illumina.com/sds.html">support.illumina.com/sds.html</a> . Updated NextSeq product markings from <sup>TM</sup> to <sup>®</sup> .
Part # 15048776 Rev. B	August 2014	Added instructions for preparing a library loading concentration of 1.8 pM, and reduced loading volume from of 1.3 ml. This change requires NCS v1.3. Corrected volumes for denaturing and diluting a 0.5 nM library. Updated URL for Safety Data Sheets (SDS) to <a href="http://support.illumina.com/sds.ilmn">support.illumina.com/sds.ilmn</a> .
Part # 15048776 Rev. A	January 2014	Initial release.

## Technical Assistance

For technical assistance, contact Illumina Technical Support.

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

Product documentation—Available for download from [support.illumina.com](http://support.illumina.com).







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