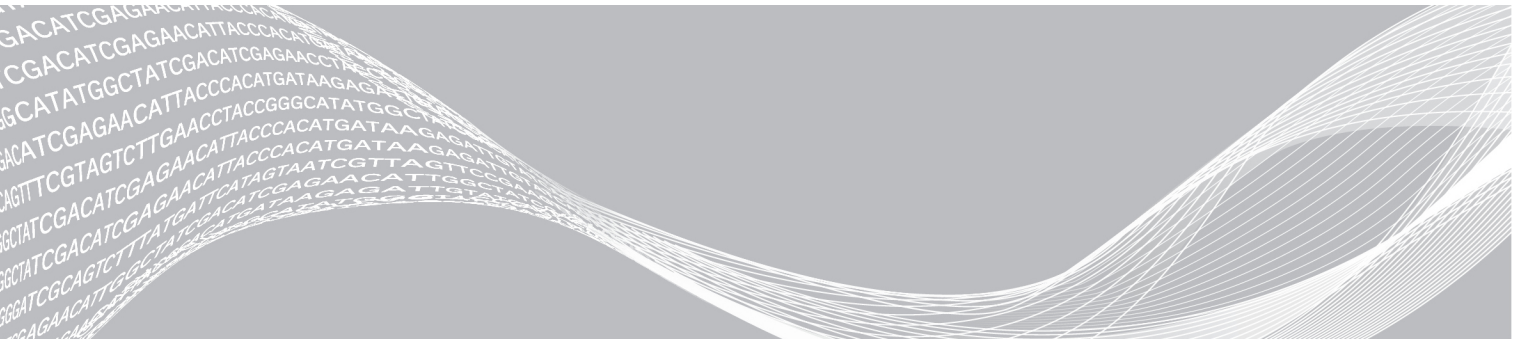
illumina®

NovaSeq 6000

Denature and Dilute Libraries Guide



Document # 1000000106351 v03

ILLUMINA PROPRIETARY

November 2020

Overview

This guide explains how to denature and dilute prepared libraries for sequencing on the Illumina® NovaSeq 6000™ system.

This guide is intended to be used in conjunction with the NovaSeq 6000 System Guide (document # 1000000019358).

Library Guidelines

All instructions apply to supported library prep methods and assume an insert size typical for supported NovaSeq 6000 applications.

- For best results, pool and denature libraries for immediate sequencing.
- Dilute the library to a loading concentration appropriate for the application. A loading concentration that is too low or too high negatively impacts the percentage of clusters passing filter (%PF). A low library concentration increases sequencing duplicates. An overly high library concentration depresses %PF.
- Achieving optimal %PF requires accurate library quantification and proper quality control. For recommendations, see the documentation for your library prep kit.
- For Xp protocols, load an empty library tube into position #8 of the cluster cartridge before you set up the sequencing run. The empty library tube is used to prepare the conditioning mix before distribution to the flow cell. The conditioning mix helps boost clustering efficiency for sequencing.

Protocol Variations

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

- Standard loading (Protocol A)—Libraries are normalized using standard library quantification and quality control procedures recommended in the library prep documentation. For these libraries, follow *Protocol A: Pool and Denature Libraries for Sequencing (Standard Loading)* on page 5.
- Xp loading (Protocol B)—Libraries are normalized using standard library quantification and quality control procedures recommended in the library prep documentation. For these libraries, follow *Protocol B: Pool and Denature Libraries for Sequencing (Xp Loading)* on page 9.
- TruSight Oncology 500 ctDNA library (Standard loading - Protocol C)—For TruSight Oncology 500 ctDNA libraries using standard loading, follow *Protocol C: TruSight Oncology 500 ctDNA Library Denaturation and Dilution Method (Standard Loading)* on page 12.
- TruSight Oncology 500 ctDNA library (Xp loading - Protocol D)—For TruSight Oncology 500 ctDNA libraries using Xp loading, follow *Protocol D: TruSight Oncology 500 ctDNA Library Denaturation and Dilution Method (Xp Loading)* on page 15.
- TruSight Oncology 500 HT library (Standard loading - Protocol E)—For TruSight Oncology 500 HT libraries using standard loading, follow *Protocol E: TruSight Oncology 500 HT Library Denaturation and Dilution Method (Standard Loading)* on page 18.
- TruSight Oncology 500 HT library (Xp loading - Protocol F)—For TruSight Oncology 500 HT libraries using Xp loading, follow *Protocol F: TruSight Oncology 500 HT Library Denaturation and Dilution Method (Xp Loading)* on page 23.

Best Practices

- For best results, begin thawing the SBS and cluster cartridges before denaturing and diluting libraries. For instructions, see the *NovaSeq 6000 System Guide (document # 1000000019358)*.

Consumables and Equipment

Consumables

The following consumables are required to denature and dilute libraries.

Consumables	Supplier	Purpose
[Protocol A and B] 1 N NaOH	General lab supplier	Diluting to 0.2 N for denaturing libraries.
[Protocol A and B] 10 mM Tris-HCl, pH 8.5	General lab supplier	Diluting libraries and an optional PhiX control before denaturation.
[Protocol A and B] 400 mM Tris-HCl, pH 8.0	General lab supplier	Neutralizing libraries and an optional PhiX control after denaturation.
[Protocol C, D, E, and F] 1 M Tris-HCl, pH 8.0	General lab supplier	Neutralizing libraries and an optional PhiX control after denaturation.
[Protocol C, D, E, and F] RNase/DNase-free water	General lab supplier	Diluting NaOH for denaturing libraries. Diluting Tween 20 and sodium hypochlorite for a maintenance wash.
Disposable gloves, powder-free	General lab supplier	General purpose.
Microcentrifuge tube, 1.5 ml	VWR, catalog # 20170-038, or equivalent	Combining volumes when diluting NaOH and library.
Pipette tips, 20 µl	General lab supplier	Pipetting for diluting and loading libraries.
Pipette tips, 200 µl	General lab supplier	Pipetting for diluting and loading libraries.
[Protocol A and B] Water, laboratory-grade	General lab supplier	Diluting NaOH for denaturing libraries. Diluting Tween 20 and sodium hypochlorite for a maintenance wash.
[NovaSeq XP workflow] One of the following kits: • NovaSeq XP 2-Lane Kit • NovaSeq XP 4-Lane Kit	Illumina: • Catalog # 20021664 • Catalog # 20021665	Manually loading libraries onto a flow cell: • Two-lane kit for SP, S1, and S2 flow cells • Four-lane kit for S4 flow cells
[NovaSeq XP workflow] One of the following kits: • NovaSeq XP 2-Lane Kit v1.5 • NovaSeq XP 4-Lane Kit v1.5	Illumina: • Catalog # 20043130 • Catalog # 20043131	Manually loading libraries onto a flow cell: • Two-lane kit for SP, S1, and S2 flow cells • Four-lane kit for S4 flow cells
[NovaSeq XP workflow] 0.5 ml and 1.7 ml tubes	General lab supplier	Required for ExAmp mixing.
[NovaSeq XP workflow] [Optional] One of the following manifold packs: • NovaSeq XP 2-Lane Manifold Pack • NovaSeq XP 4-Lane Manifold Pack	Illumina: • Catalog # 20021666 • Catalog # 20021667	Spare NovaSeq Xp manifolds for manually loading libraries onto a flow cell.
[Optional] PhiX Control v3	Illumina, catalog # FC-110-3001	Spiking in PhiX control.

The following consumables for denaturing and diluting libraries and PhiX are provided in the TruSight Oncology 500 ctDNA Library Prep Kit and the TruSight Oncology 500 HT Library Prep Kit.

Consumables	Purpose
RSB	For diluting libraries and diluting and denaturing optional PhiX control.
HP3	2 N NaOH for denaturing optional PhiX control.

Equipment

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

Equipment	Supplier
[Protocol C, D, E and F] Heat block for 1.5 ml microcentrifuge tubes	General lab supplier

Protocol A: Pool and Denature Libraries for Sequencing (Standard Loading)

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.

For Xp loading, proceed to *Protocol B: Pool and Denature Libraries for Sequencing (Xp Loading)* on page 9.

For TSO500 ctDNA libraries, proceed to *Protocol C: TruSight Oncology 500 ctDNA Library Denaturation and Dilution Method (Standard Loading)* on page 12 or *Protocol D: TruSight Oncology 500 ctDNA Library Denaturation and Dilution Method (Xp Loading)* on page 15.

For TSO500 HT libraries, proceed to *Protocol E: TruSight Oncology 500 HT Library Denaturation and Dilution Method (Standard Loading)* on page 18 or *Protocol F: TruSight Oncology 500 HT Library Denaturation and Dilution Method (Xp Loading)* on page 23.

Create a Normalized Library Pool

Use the following instructions to normalize libraries to the appropriate concentration and then pool. Libraries sequenced on the same flow cell must be combined into a single normalized pool.

1 Refer to the following table for the typical number of reads and recommended plexity by application and flow cell type.

Table 1 Recommended Library Pool Plexity

Application	Flow Cell Type	Paired-End Reads Passing Filter per Flow Cell (B)	Libraries per Lane
Human Genomes	SP	1.3–1.6	~2
	S1	2.6–3.2	~4
	S2	6.6–8.2	~10
	S4	16–20	~24
Exomes	SP	1.3–1.6	~20
	S1	2.6–3.2	~40
	S2	6.6–8.2	~100
	S4	16–20	~250

Application	Flow Cell Type	Paired-End Reads Passing Filter per Flow Cell (B)	Libraries per Lane
Transcriptomes	SP	1.3–1.6	~16
	S1	2.6–3.2	~32
	S2	6.6–8.2	~82
	S4	16–20	~200

Normalize Libraries for Pooling

1 Determine the required pooled library concentration based on the desired final loading concentration. See *Recommended Loading Concentrations* on page 6.

Final Loading Concentration (pM)	Pooled Library Concentration (nM)
100	0.50
150	0.75
200	1
250	1.25
300	1.50
350	1.75
400	2
450	2.25
500	2.50

2 Normalize libraries to the desired pooled library concentration using 10 mM Tris-HCl, pH 8.5. For assistance diluting libraries to the appropriate concentration, see the Pooling Calculator on the Illumina website.

Recommended Loading Concentrations

The optimal DNA loading concentration depends on the library type and insert size. The following table provides DNA loading concentrations that are recommended based on Illumina libraries with insert sizes that are ≤ 450 bp. Load libraries with smaller insert sizes at the lower end of the recommended range. For libraries > 450 bp, higher loading concentrations might be necessary.

NOTE

For libraries generated from non-Illumina library prep methods, you may need to perform a titration of your specific library type initially to obtain optimal seeding concentration to yield best %PF. When optimal loading concentration is determined, it should be applicable for identical library types moving forward.

Table 2 Recommended Loading Concentrations for Standard Workflow (Software Version 1.1, or later)

Library Type	Final Loading Concentration (pM)	Pooled Loading Concentration (nM)
PhiX[1]	250	1.25
Illumina DNA PCR-free library pool	400–600[2]	2–3[2]
TruSeq DNA PCR-free library pool	175–350	0.875–1.75
DNA PCR-amplified library pool	300–600	1.5–3.0
Single Cell[3]	250–500	1.25–2.5

[1] For a PhiX-only run.

[2] Calculated based on 450 bp as the median insert size, 660 g/mol as the DNA mass, and ssQubit concentration values.

[3] Single Cell has been verified for the Xp workflow only.

If you have optimized a final loading concentration for HiSeq™ X, HiSeq™ 4000, or HiSeq™ 3000, use 1.5× that concentration for NovaSeq 6000. For example, if your final loading concentration for HiSeq X is 200 pM, use 300 pM for NovaSeq 6000.

Pool Normalized Libraries and Add Optional PhiX Control

1 Combine the appropriate volume of each normalized library in a new microcentrifuge tube to result in one of the following final volumes:

Mode	Final Volume (µl)
SP/S1	100
S2	150
S4	310

For example, for a six-plex library pool and S2 mode, combine 25 µl of each library that has been normalized to the same concentration. Or, for a four-plex library pool and S1 mode, combine 25 µl of each normalized nondenatured library.

2 [Optional] Store remaining *unpooled* libraries at -25°C to -15°C.

3 [Optional] Spike-in 1% nondenatured PhiX as follows.

a Dilute 10 nM PhiX to 2.5 nM using 10 mM Tris-HCl, pH 8.5.

b Add the appropriate volume of nondenatured 2.5 nM PhiX to the tube of nondenatured library pool.

Mode	Nondenatured 2.5 nM PhiX (µl)	Nondenatured Library Pool (µl)
SP/S1	0.6	100
S2	0.9	150
S4	1.9	310

When spiking in PhiX, 1% is the recommended amount for well-balanced libraries. Low-diversity libraries can require more. To use a PhiX control with low-diversity libraries, contact Illumina Technical Support for guidance.

Prepare a Fresh Dilution of NaOH

Prepare a ***fresh*** dilution of 0.2 N NaOH to denature libraries for sequencing. To prevent small pipetting errors from affecting the final NaOH concentration, extra volume is prepared.

CAUTION

Freshly diluted 0.2 N NaOH is essential to the denaturation process. Improper denaturation can reduce yield.

1 Combine the following volumes in a microcentrifuge tube to dilute 1 N NaOH to 0.2 N:

Table 3 SP/S1/S2 Mode

Reagent	Volume for One Flow Cell (µl)	Volume for Two Flow Cells (µl)
Laboratory-grade water	40	80
Stock 1 N NaOH	10	20

These volumes result in 50 µl 0.2 N NaOH for one flow cell or 100 µl 0.2 N NaOH for two flow cells.

Table 4 S4 Mode

Reagent	Volume for One Flow Cell (µl)	Volume for Two Flow Cells (µl)
Laboratory-grade water	80	160
Stock 1 N NaOH	20	40

These volumes result in 100 µl 0.2 N NaOH for one flow cell or 200 µl 0.2 N NaOH for two flow cells.

2 Invert several times to mix, or vortex thoroughly. Keep the tube capped and use within *12 hours*.

Denature Library Pool and Optional PhiX Control

1 Add 0.2 N NaOH to the tube of nondenatured library pool and optional PhiX as follows.

Flow Cell	0.2 N NaOH	Nondenatured Library Pool (µl)	Resulting Volume
SP/S1	25	100	125 µl, or 125.6 µl with PhiX
S2	37	150	187 µl, or 187.9 µl with PhiX
S4	77	310	387 µl, or 388.9 µl with PhiX

2 Cap and then vortex briefly.

3 Centrifuge at 280 × g for up to 1 minute.

4 Incubate at room temperature for 8 minutes to denature.

5 Add 400 mM Tris-HCl, pH 8.0 as follows to neutralize.

Mode	400 mM Tris-HCl, pH 8.0 (µl)	Resulting Volume
SP/S1	25	150 µl, or 150.6 µl with PhiX
S2	38	225 µl, or 225.9 µl with PhiX
S4	78	465 µl, or 466.9 µl with PhiX

6 Cap and then vortex briefly.

7 Centrifuge at 280 × g for up to 1 minute.

8 Transfer the full volume of denatured library or denatured library and PhiX to the library tube provided with the NovaSeq 6000 Reagent Kit.

9 Immediately proceed to loading the library tube into the cluster cartridge and setting up the run. The reagent cartridges, including the library tube, must be loaded onto the instrument within *30 minutes*.

10 [Optional] If you cannot immediately proceed, cap the library tube and store at -25°C to -15°C for up to 3 weeks. Do not refreeze after thawing.

CAUTION

Store the library tube only if necessary. Long-term storage at -25°C to -15°C can increase duplicates, which decreases yield.

NOTE

After denaturing and diluting your libraries and preparing the optional PhiX control, proceed to *Prepare SBS and Cluster Cartridges* in the Standard Workflow section of the *NovaSeq 6000 System Guide (document # 1000000019358)*.

Protocol B: Pool and Denature Libraries for Sequencing (Xp Loading)

Use Protocol B to denature and dilute libraries that have been normalized using standard library quantification and quality control procedures recommended in the library prep documentation. For addressable lane loading, see the NovaSeq Xp Workflow chapter in the NovaSeq 6000 System Guide (document # 1000000019358).

For Standard loading, proceed to *Protocol A: Pool and Denature Libraries for Sequencing (Standard Loading)* on page 5.

For TSO500 ctDNA libraries, proceed to *Protocol C: TruSight Oncology 500 ctDNA Library Denaturation and Dilution Method (Standard Loading)* on page 12 or *Protocol D: TruSight Oncology 500 ctDNA Library Denaturation and Dilution Method (Xp Loading)* on page 15.

For TSO500 HT libraries, proceed to *Protocol E: TruSight Oncology 500 HT Library Denaturation and Dilution Method (Standard Loading)* on page 18 or *Protocol F: TruSight Oncology 500 HT Library Denaturation and Dilution Method (Xp Loading)* on page 23.

Create a Normalized Library Pool

Use the following instructions to normalize libraries to the appropriate concentration and then pool. Libraries sequenced on the same lane must be combined into a single pool. The total volume per lane of each normalized pool is shown in the following table. If the same pool is sequenced across more than one lane, multiply the value from Table 5 by the number of lanes.

Table 5 Total Volume of Pooled Library

Mode	Total Volume of Pool Per Lane (µl)
SP/S1	18
S2	22
S4	30

For the Xp workflow, the data output is obtained for each lane, as opposed to all the lanes in aggregate for the Standard workflow. As a result, library pools for the Xp Workflow contain fewer libraries compared to the Standard workflow.

Refer to the following table for the typical number of reads and recommended plexity by application and flow cell type.

Table 6 Recommended Library Pool Plexity

Application	Flow Cell Type	Paired-End Reads Passing Filter per Lane (B)	Libraries per Lane
Human Genomes	SP	.65–.8	1
	S1	1.3–1.6	~2
	S2	3.3–4.1	~5
	S4	4.0–5.0	~6
Exomes	SP	.65–.8	~10
	S1	1.3–1.6	~20
	S2	3.3–4.1	~50
	S4	4.0–5.0	~62

Application	Flow Cell Type	Paired-End Reads Passing Filter per Lane (B)	Libraries per Lane
Transcriptomes	SP	.65–.8	~8
	S1	1.3–1.6	~16
	S2	3.3–4.1	~41
	S4	4.0–5.0	~50

Normalize Libraries for Pooling

1 Determine the required pooled library concentration based on the desired final loading concentration. See *Recommended Loading Concentrations* on page 10.

Final Loading Concentration (pM)	Pooled Library Concentration (nM)
100	0.5
150	0.75
200	1.0
250	1.25
300	1.5
350	1.75
400	2.0
450	2.25
500	2.5

2 Normalize libraries to the desired pooled library loading concentration using 10 mM Tris-HCl, pH 8.5. For assistance diluting libraries to the appropriate concentration, see the Pooling Calculator at support.illumina.com/help/pooling-calculator/pooling-calculator.html.

Recommended Loading Concentrations

The optimal DNA loading concentration depends on the library type and insert size. The following table provides DNA loading concentrations that are recommended based on Illumina libraries with insert sizes that are ≤ 450 bp. Load libraries with smaller insert sizes at the lower end of the recommended range. For libraries > 450 bp, higher loading concentrations might be necessary.

Table 7 Recommended Loading Concentrations

Library Type	Final Loading Concentration (pM)	Pooled Loading Concentration (nM)
PhiX[1]	100	0.5
Illumina DNA PCR-free library pool	300–400[2]	1.5-2.0[2]
TruSeq DNA PCR-free library pool	115–235	0.575–1.175
DNA PCR-amplified library pool	200–400	1.0–2.0
Single Cell	175–275	.875–1.375

[1] For a PhiX-only run.

[2] Calculated based on 450 bp as the median insert size, 660 g/mol as the DNA mass, and ssQubit concentration values.

If you have optimized the loading concentration for HiSeq™ X, HiSeq™ 4000, or HiSeq™ 3000, use about the same concentration for the NovaSeq XP workflow. If you have optimized the loading concentration for the NovaSeq Standard workflow, use about 1/3 less for the NovaSeq XP workflow.

NOTE

Libraries might need to be titrated to obtain optimal seeding concentration. When the optimal loading concentration is determined, it is applicable for identical library types.

Pool Normalized Libraries and Add Optional PhiX Control

1 Combine the appropriate volume of each normalized library in a new microcentrifuge tube to result in the appropriate final volumes per lane.

Mode	Total Volume of Pool Per Lane (µl)
SP/S1	18
S2	22
S4	30

For example, for a six-plex library pool and S4 mode, combine 5 µl of each library that has been normalized to the same concentration.

2 [Optional] Store remaining *unpooled* libraries at -25°C to -15°C.

3 [Optional] Spike-in 1% nondenatured PhiX as follows.

a Dilute 10 nM PhiX to 0.25 nM using 10 mM Tris-HCl, pH 8.5.
b Add the appropriate volume of PhiX to the tube of nondenatured library pool.

Mode	Nondenatured 0.25 nM PhiX (µl)	Nondenatured Library Pool (µl)
SP/S1	0.7	18
S2	0.8	22
S4	1.1	30

When spiking in PhiX, 1% is the recommended amount for well-balanced libraries. Low-diversity libraries can require more. To use a PhiX control with low-diversity libraries, contact Illumina Technical Support for guidance.

Prepare a Fresh Dilution of NaOH

Prepare a *fresh* dilution of 0.2 N NaOH to denature libraries for sequencing. To minimize pipetting errors that could affect the final NaOH concentration, prepare at least 30 µl diluted NaOH per flow cell. For a dual flow cell run, prepare 60 µl diluted NaOH.

CAUTION

Freshly diluted 0.2 N NaOH is essential to the denaturation process. Improper denaturation can reduce yield.

1 For one flow cell, combine the following volumes in a microcentrifuge tube to dilute 1 N NaOH to 0.2 N.
- Laboratory-grade water (24 µl)
- Stock 1 N NaOH (6 µl)

These volumes result in 30 µl 0.2 N NaOH. For two flow cells, double the volumes.

2 Invert several times to mix, or vortex thoroughly. Keep the tube capped and use within *12 hours*.

Denature Library Pool and Optional PhiX Control

1 Add 0.2 N NaOH to the tube of nondenatured library pool and optional PhiX as follows.

Mode	0.2 N NaOH (µl)	Nondenatured Library Pool (µl)	Resulting Volume
SP/S1	4.0	18.0	22.0 µl, or 22.7 µl with PhiX
S2	5.0	22.0	27.0 µl, or 27.8 µl with PhiX
S4	7.0	30.0	37.0 µl, or 38.1 µl with PhiX

2 Cap and then vortex briefly.

3 Centrifuge at a maximum of 280 × g for up to 1 minute.

4 Incubate at room temperature for 8 minutes to denature.

5 Add 400 mM Tris-HCl, pH 8.0 to neutralize as follows.

Mode	400 mM Tris-HCl, pH 8.0 (µl)	Resulting Volume
SP/S1	5.0	27.0 µl, or 27.7 µl with PhiX
S2	6.0	33.0 µl, or 33.8 µl with PhiX
S4	8.0	45.0 µl, or 46.1 µl with PhiX

6 Cap and then vortex briefly.

7 Centrifuge at a maximum of 280 × g for up to 1 minute.

8 Keep denatured libraries on ice until ready to add the ExAmp master mix.

9 [Optional] If you cannot proceed immediately, cap the tube and store at -25°C to -15°C for up to three weeks. Do not refreeze after thawing.

CAUTION

Store denatured library pools only if necessary. Long-term storage can increase duplicates, which decrease yield.

NOTE

After denaturing and diluting your libraries and preparing the optional PhiX control, proceed to *Prepare the Flow Cell and Dock* in the Xp Workflow section of the *NovaSeq 6000 System Guide (document # 1000000019358)*.

Protocol C: TruSight Oncology 500 ctDNA Library Denaturation and Dilution Method (Standard Loading)

The NovaSeq Standard workflow for TruSight Oncology 500 ctDNA libraries is used for denaturing and diluting libraries intended for loading on to the NovaSeq 6000 system. For addressable lane loading, see the NovaSeq Xp Workflow chapter in the *NovaSeq 6000 System Guide (document # 1000000019358)*. Libraries prepared using the TruSight Oncology 500 ctDNA workflow are normalized to a starting concentration that is ready for sample pooling.

Use Protocol C if sequencing TSO500 ctDNA libraries in S2 or S4 mode. You can sequence up to eight libraries per S2 flow cell and up to 16 libraries per S4 flow cell.

For Xp loading, proceed to *Protocol D: TruSight Oncology 500 ctDNA Library Denaturation and Dilution Method (Xp Loading)* on page 15.

Prepare PhiX Control [Optional]

Preparation

1 Remove RSB from 2°C to 8°C or -25°C to -15°C storage, and bring to room temperature.

2 Thaw a tube of 10 nM PhiX (10 µl/tube).

3 Label a microcentrifuge tube dHP3 (diluted HP3).

4 Label a microcentrifuge tube dTris (diluted Tris-HCl).

5 Label a microcentrifuge tube dPhiX (diluted PhiX).

Prepare a Fresh Dilution of NaOH

1 Vortex HP3 to mix, and then centrifuge briefly.

2 Combine the following volumes in the dHP3 tube.
- RNase/DNase-free water (32.5 µl)
- HP3 (7.5 µl)

3 Vortex dHP3 to mix, and then centrifuge briefly.

Prepare a Fresh Dilution of Tris-HCl

1 Combine the following volumes in the dTris tube.
- RNase/DNase-free water (25.0 µl)
- 1 M Tris-HCl, pH 8.0 (15.0 µl)

2 Vortex dTris to mix, and then centrifuge briefly.

Dilute PhiX

1 Vortex RSB to mix.

2 Vortex PhiX control to mix, and then centrifuge briefly.

3 Combine the following volumes in the dPhiX tube.
- RSB (2.0 µl)
- PhiX control (6.0 µl)

4 Vortex dPhiX tube to mix, and then centrifuge briefly.

5 [Optional] dPhiX can be stored at -25°C to -15°C for up to 3 months.

Denature PhiX

1 Add 8 µl dHP3 to the dPhiX tube.

2 Discard the dHP3 tube.

3 Vortex the dPhiX tube to mix, and then centrifuge briefly.

4 Incubate at room temperature for 5 minutes.

5 Immediately add 8 µl dTris to the dPhiX tube to neutralize the reaction.

6 Discard the dTris tube.

7 Vortex to mix, and then centrifuge briefly.
The final concentration of PhiX is 2.5 nM.

8 [Optional] Store denatured 2.5 nM PhiX at -25°C to -15°C for up to 2 weeks.

Pool Normalized Libraries

Preparation

Visit the TruSight Oncology 500 ctDNA support page on the Illumina website for more information on the supported number of samples per pool per flow cell.

1 If the Normalized Library (NL) plate was stored, thaw to room temperature, and then centrifuge the plate at 280 × g for 1 minute.

2 Preheat the heat block to 96°C.

3 Prepare an ice bucket.

Procedure

1 Set a pipette to 30 µl, and then gently pipette to mix the libraries in the NL plate five times.
- Use fresh tips for each library.

For optimal library sequencing performance, make sure to mix libraries as directed.

2 Label a 1.5 ml screw-top microcentrifuge tube PDL (Pooled DNA Libraries).

3 Transfer equal volumes of each normalized DNA library from the NL plate to the PDL tube to result in one of the following volumes:

Mode	Recommended Pool Volume (µl)
S2	100
S4	200

For example, for an eight-plex library pool and S2 mode, combine 12.5 µl of each library that has been normalized to the same concentration.

4 Vortex the PDL tube to mix.

5 Centrifuge the PDL tube briefly.

Denature Normalized Libraries

1 Incubate PDL tube in a heat block at 96°C for 2 minutes.

2 Immediately place on ice for 5 minutes.

3 Vortex PDL tube to mix, and then centrifuge briefly.

4 Place PDL tube on ice.

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, thaw the tubes and repeat *Denature Normalized Libraries* on page 14 before proceeding to the next step.

Dilute Libraries and Add Optional PhiX Control

Prepare RSB

1 Remove RSB from 2°C to 8°C or -25°C to -15°C storage, and bring to room temperature.

Prepare Denatured 2.5 nM PhiX

1 If the denatured PhiX was stored, remove the denatured 2.5 nM PhiX from -25°C to -15°C storage, and thaw at room temperature.

2 Vortex to mix, and then centrifuge briefly.

Dilute Libraries

1 Label a new 1.5 ml microcentrifuge tube DIL1 (Dilution 1).

2 Vortex the PDL tube to mix.

3 Centrifuge the PDL tube briefly.

4 Add the appropriate volume of PDL and RSB to the DIL1 tube as follows.

Mode	PDL (µl)	RSB (µl)	Resulting Volume (µl)
S2	65	160	**225**
S4	134	331	**465**

5 [Optional] Add the appropriate volume of denatured 2.5 nM PhiX to the DIL1 tube as follows.

Mode	2.5 nM PhiX (µl)	Resulting Volume (µl)
S2	0.9	**225.9**
S4	1.9	**466.9**

6 Vortex the DIL1 tube to mix.

7 Centrifuge the DIL1 tube briefly.

8 Transfer the full volume of DIL1 to the library tube provided with the NovaSeq 6000 reagent kit.

9 Immediately proceed to *Prepare SBS and Cluster Cartridges* in the Standard Workflow section of the *NovaSeq 6000 System Guide (document # 1000000019358)*.
The reagent cartridges, including the library tube, must be loaded onto the instrument within ***30 minutes.***

Protocol D: TruSight Oncology 500 ctDNA Library Denaturation and Dilution Method (Xp Loading)

The NovaSeq Xp workflow for TruSight Oncology 500 ctDNA libraries is used for denaturing and diluting libraries intended for addressable loading onto the NovaSeq 6000 system. Libraries prepared using the TruSight Oncology 500 ctDNA workflow are normalized to a starting concentration that is ready for sample pooling. For addressable lane loading see the NovaSeq Xp Workflow chapter in the *NovaSeq 6000 System Guide (document # 1000000019358)*.

Use Protocol D if sequencing TSO500 ctDNA libraries in S4 mode for addressable lane loading. You can sequence up to six libraries per lane.

For Standard loading, proceed to *Protocol C: TruSight Oncology 500 ctDNA Library Denaturation and Dilution Method (Standard Loading)* on page 12.

Prepare PhiX Control [Optional]

Preparation

1 Remove RSB from 2°C to 8°C or -25°C to -15°C storage, and bring to room temperature.

2 Thaw a tube of 10 nM PhiX (10 µl/tube).

3 Label a microcentrifuge tube dHP3 (diluted HP3).

4 Label a microcentrifuge tube dTris (diluted Tris-HCl).

5 Label a microcentrifuge tube dPhiX (diluted PhiX).

Prepare a Fresh Dilution of NaOH

1 Vortex HP3 to mix, and then centrifuge briefly.

2 Combine the following volumes in the dHP3 tube.
- RNase/DNase-free water (32.5 µl)
- HP3 (7.5 µl)

3 Vortex dHP3 to mix, and then centrifuge briefly.

Prepare a Fresh Dilution of Tris-HCl

1 Combine the following volumes in the dTris tube.
- RNase/DNase-free water (25.0 µl)
- 1 M Tris-HCl, pH 8.0 (15.0 µl)

2 Vortex dTris to mix, and then centrifuge briefly.

Dilute PhiX

1 Vortex RSB to mix.

2 Vortex PhiX control to mix, and then centrifuge briefly.

3 Combine the following volumes in the dPhiX tube.
- RSB (2.0 µl)
- PhiX control (6.0 µl)

4 Vortex dPhiX tube to mix, and then centrifuge briefly.

5 [Optional] dPhiX can be stored at -25°C to -15°C for up to 3 months.

Denature PhiX

1 Add 8 µl dHP3 to the dPhiX tube.

2 Discard the dHP3 tube.

3 Vortex the dPhiX tube to mix, and then centrifuge briefly.

4 Incubate at room temperature for 5 minutes.

5 Immediately add 8 µl dTris to the dPhiX tube to neutralize the reaction.

6 Discard the dTris tube.

7 Vortex to mix, and then centrifuge briefly.

8 Add 216 µl RSB to the diluted and denatured PhiX solution.

9 Vortex to mix, and then centrifuge briefly.
The final concentration of PhiX is 0.25 nM.

10 [Optional] Store denatured 0.25 nM PhiX at -25°C to -15°C for up to 2 weeks.

Pool Normalized Libraries

Preparation

Visit the TruSight Oncology 500 ctDNA support page on the Illumina website for more information on the supported number of samples per pool per flow cell.

1 If the Normalized Library (NL) plate was stored, thaw to room temperature, and then centrifuge the plate at 280 × g for 1 minute.

2 Preheat the heat block to 96°C.

3 Prepare an ice bucket.

Procedure

1 Set a pipette to 30 µl, and then gently pipette to mix the libraries in the NL plate five times.
- Use fresh tips for each library.

For optimal library sequencing performance, make sure to mix libraries as directed.

2 Label a 1.5 ml screw-top microcentrifuge tube PDL_L1 (Pooled DNA Libraries_Lane 1). Repeat for any additional lanes (see Table 8).

Table 8 PDL Tube Naming Convention

Flow Cell	Lane 1	Lane 2	Lane 3	Lane 4
S4	PDL_L1	PDL_L2	PDL_L3	PDL_L4

3 Transfer 5 µl of each normalized DNA library from the NL plate to the PDL tube, and then repeat for each additional lane.

4 Vortex each PDL tube to mix.

5 Centrifuge each PDL tube briefly.

Denature Normalized Libraries

1 Incubate each PDL tube in a heat block at 96°C for 2 minutes.

2 Immediately place on ice for 5 minutes.

3 Vortex each PDL tube to mix, and then centrifuge briefly.

4 Place PDL tubes on ice.

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, thaw the tubes and repeat *Denature Normalized Libraries* on page 17 before proceeding to the next step.

Dilute Libraries and Add Optional PhiX Control

Prepare RSB

1 Remove RSB from 2°C to 8°C or -25°C to -15°C storage, and bring to room temperature.

Prepare Denatured 0.25 nM PhiX

1 If the denatured PhiX was stored, remove the denatured 0.25 nM PhiX from -25°C to -15°C storage, and thaw at room temperature.

2 Vortex to mix, and then centrifuge briefly.

Dilute Libraries

1 Label a new 1.5 ml screw-top microcentrifuge tube DIL1_L1 (Dilution 1_Lane 1). Repeat for any additional lanes (see Table 9).

Table 9 DIL1 Tube Naming Convention

Flow Cell	Lane 1	Lane 2	Lane 3	Lane 4
S4	DIL1_L1	DIL1_L2	DIL1_L3	DIL1_L4

2 Vortex the PDL tubes to mix.

3 Centrifuge the PDL tubes briefly.

4 Transfer the appropriate volume of PDL and RSB to each DIL1 tube as follows.

Mode	PDL (µl)	RSB (µl)	Resulting Volume (µl)
S4	6.8	38.2	**45**

5 [Optional] Add the appropriate volume of denatured 0.25 nM PhiX to each DIL1 tube as follows.

Mode	0.25 nM PhiX (µl)	Resulting Volume (µl)
S4	1.1	**46.1**

6 Vortex the DIL1 tubes to mix.

7 Centrifuge the DIL1 tubes briefly.

8 After denaturing and diluting your libraries and preparing the optional PhiX control, proceed to *Prepare the Flow Cell and Dock* in the Xp Workflow section of the *NovaSeq 6000 System Guide (document # 1000000019358)*.

Protocol E: TruSight Oncology 500 HT Library Denaturation and Dilution Method (Standard Loading)

The NovaSeq Standard workflow for TruSight Oncology 500 HT libraries is used for denaturing and diluting libraries intended for loading on to the NovaSeq 6000 system. For standard loading, see the NovaSeq Standard Workflow chapter in the *NovaSeq 6000 System Guide (document # 1000000019358)*. Libraries prepared using the TruSight Oncology 500 HT workflow are normalized to a starting concentration that is ready for sample pooling.

Use Protocol E if sequencing TSO500 HT libraries in SP, S1, S2, or S4 mode with Standard loading. You can sequence up to 16 samples per SP flow cell, 32 samples per S1 flow cell, 72 samples per S2 flow cell, and up to 192 samples per S4 flow cell.

Visit the TruSight Oncology 500 HT support page on the Illumina website for more information on the supported number of samples per pool per flow cell.

For Xp loading, proceed to *Protocol F: TruSight Oncology 500 HT Library Denaturation and Dilution Method (Xp Loading)* on page 23.

Prepare PhiX Control [Optional]

Preparation

1 Remove RSB from 2°C to 8°C or -25°C to -15°C storage, and bring to room temperature.

2 Thaw a tube of 10 nM PhiX (10 µl/tube).

3 Label a microcentrifuge tube dHP3 (diluted HP3).

4 Label a microcentrifuge tube dTris (diluted Tris-HCl).

5 Label a microcentrifuge tube dPhiX (diluted PhiX).

Prepare a Fresh Dilution of NaOH

1 Vortex HP3 to mix, and then centrifuge briefly.

2 Combine the following volumes in the dHP3 tube.
- RNase/DNase-free water (32.5 µl)
- HP3 (7.5 µl)

3 Vortex dHP3 to mix, and then centrifuge briefly.

Prepare a Fresh Dilution of Tris-HCl

1 Combine the following volumes in the dTris tube.
- RNase/DNase-free water (25.0 µl)
- 1 M Tris-HCl, pH 8.0 (15.0 µl)

2 Vortex dTris to mix, and then centrifuge briefly.

Dilute PhiX

1 Vortex RSB to mix.

2 Vortex PhiX control to mix, and then centrifuge briefly.

3 Combine the following volumes in the dPhiX tube.
- RSB (2.0 µl)
- PhiX control (6.0 µl)

4 Vortex dPhiX tube to mix, and then centrifuge briefly.

5 [Optional] dPhiX can be stored at -25°C to -15°C for up to 3 months.

Denature PhiX

1 Add 8 µl dHP3 to the dPhiX tube.

2 Discard the dHP3 tube.

3 Vortex the dPhiX tube to mix, and then centrifuge briefly.

4 Incubate at room temperature for 5 minutes.

5 Immediately add 8 µl dTris to the dPhiX tube to neutralize the reaction.

6 Discard the dTris tube.

7 Vortex to mix, and then centrifuge briefly.
The final concentration of PhiX is 2.5 nM.

8 [Optional] Store denatured 2.5 nM PhiX at -25°C to -15°C for up to 2 weeks.

Pool Normalized Libraries

Preparation

Visit the TruSight Oncology 500 HT support page on the Illumina website for more information on the supported number of samples per pool per flow cell.

1 If the Normalized Library (NL) plate was stored, thaw to room temperature, and then centrifuge the plate at 280 × g for 1 minute.

2 Preheat the heat block to 96°C.

3 Prepare an ice bucket.

Procedure

1 Set a multichannel pipette to 30 µl, and then gently pipette to mix the libraries in the NL plate five times.
- Use fresh tips for each library.

Library sequencing performance is diminished if libraries are not sufficiently mixed prior to pooling.

2 Select one of the following options to pool the normalized libraries:
- To sequence libraries derived from RNA samples and DNA samples simultaneously, see *Pool RNA and DNA* on page 20.
- To sequence libraries derived from DNA samples only, see *Pool DNA Only* on page 21.

Pool RNA and DNA

1 Label a 1.5 ml screw cap microcentrifuge tube PRL (Pooled RNA Libraries).
- If pooling more than 40 RNA (cDNA) libraries, label an additional 1.5 ml screw cap microcentrifuge tube TPRL (Transferred Pooled RNA Libraries).

2 Label a 1.5 ml screw cap microcentrifuge tube PDL (Pooled DNA Libraries).
- If pooling more than 40 DNA libraries, label an additional 1.5 ml screw cap microcentrifuge tube TPDL (Transferred Pooled DNA Libraries).

3 Transfer 5 µl of each normalized RNA library from the NL plate to the PRL tube.

4 Transfer 5 µl of each normalized DNA library from the NL plate to the PDL tube.

5 Vortex each tube to mix.

6 Centrifuge each tube briefly.

7 If the PRL tube contains more than 40 RNA libraries, transfer 200 µl from the PRL tube to the TPRL tube, and then discard the PRL tube.

8 If the PDL tube contains more than 40 DNA libraries, transfer 200 µl from the PDL tube to the TPDL tube, and then discard the PDL tube.

9 Proceed to *Denature Normalized Libraries* on page 21.

Pool DNA Only

1 Label a 1.5 ml screw cap microcentrifuge tube PDL (Pooled DNA Libraries).
   - If pooling more than 40 DNA libraries, label an additional 1.5 ml screw cap microcentrifuge tube TPDL (Transferred Pooled DNA Libraries).

2 Transfer 5 µl of each normalized DNA library from the NL plate to the PDL tube.

3 Vortex the PDL tube to mix.

4 Centrifuge the PDL tube briefly.

5 If the PDL tube contains more than 40 DNA libraries, transfer 200 µl from the PDL tube to the TPDL tube, and then discard the PDL tube.

Denature Normalized Libraries

1 Vortex and centrifuge each of the following tubes briefly.
   - PRL (≤ 40 RNA libraries) or TPRL (> 40 RNA libraries)
   - PDL (≤ 40 DNA libraries) or TPDL (> 40 DNA libraries)

2 Incubate in a heat block at 96°C for 2 minutes.

3 Immediately place on ice for 5 minutes.

4 Vortex each tube to mix, and then centrifuge briefly.

5 Place tubes on ice.

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, thaw the tubes and repeat *Denature Normalized Libraries* on page 26 before proceeding to the next step.

Dilute Libraries and Add Optional PhiX Control

Prepare RSB

1 Remove RSB from 2°C to 8°C or -25°C to -15°C storage, and bring to room temperature.

Prepare Denatured 2.5 nM PhiX

1 If the denatured PhiX was stored, remove the denatured 2.5 nM PhiX from -25°C to -15°C storage and thaw to room temperature.

2 Vortex to mix, and then centrifuge briefly.

Dilute Libraries

1 Label a new 1.5 ml microcentrifuge tube DIL1 (Dilution 1).

2 Select one of the following options to dilute your libraries:
- To sequence libraries derived from RNA samples and DNA samples simultaneously, see *Dilute RNA and DNA Libraries* on page 22.
- To sequence libraries derived from DNA samples only, see *Dilute DNA Libraries Only* on page 23.

Dilute RNA and DNA Libraries

1 Vortex and centrifuge each of the following types of tubes briefly:
- PRL (≤ 40 RNA libraries) or TPRL (> 40 RNA libraries)
- PDL (≤ 40 DNA libraries) or TPDL (> 40 DNA libraries)

2 Transfer the appropriate volume of denatured PRL or TPRL to the DIL1 tube.

Mode	PRL or TPRL (µl)
SP/S1	10.4
S2	15.6
S4	32.2

3 Transfer the appropriate volume of denatured PDL or TPDL to the DIL1 tube.

Mode	PDL or TPDL (µl)	Resulting Volume (µl)
SP/S1	41.6	52
S2	62.4	78
S4	128.8	161

4 Add the appropriate volume of RSB to the DIL1 tube.

Mode	RSB (µl)	Resulting Volume (µl)
SP/S1	98	150
S2	147	225
S4	304	465

5 [Optional] Add the appropriate volume of denatured 2.5 nM PhiX to the DIL1 tube.

Mode	2.5 nM PhiX (µl)	Resulting Volume (µl)
SP/S1	0.6	150.6
S2	0.9	225.9
S4	1.9	466.9

6 Vortex the DIL1 tube to mix.

7 Centrifuge the DIL1 tube briefly.

8 Transfer the full volume of DIL1 to the library tube provided with the NovaSeq 6000 reagent kit.

9 Immediately proceed to *Prepare SBS and Cluster Cartridges* in the Standard Workflow section of the *NovaSeq 6000 System Guide (document # 1000000019358)*.
The reagent cartridges, including the library tube, must be loaded onto the instrument within ***30 minutes.***

NOTE
Use 10 index cycles when sequencing TSO500 HT libraries.

Dilute DNA Libraries Only

1 Vortex the tube and centrifuge briefly.
- PDL (≤ 40 DNA libraries)
- TPDL (> 40 DNA libraries)

2 Add the appropriate volume of PDL or TPDL to the DIL1 tube.

Mode	PDL or TPDL (µl)
SP/S1	52
S2	78
S4	161

3 Add the appropriate volume of RSB to the DIL1 tube.

Mode	RSB (µl)	Resulting Volume (µl)
SP/S1	98	150
S2	147	225
S4	304	465

4 [Optional] Add the appropriate volume of denatured 2.5 nM PhiX to the DIL1 tube.

Mode	2.5 nM PhiX (µl)	Resulting Volume (µl)
SP/S1	0.6	150.6
S2	0.9	225.9
S4	1.9	466.9

5 Vortex the DIL1 tube to mix.

6 Centrifuge the DIL1 tube briefly.

7 Transfer the full volume of DIL1 to the library tube provided with the NovaSeq 6000 reagent kit.

8 Immediately proceed to *Prepare SBS and Cluster Cartridges* in the Standard Workflow section of the *NovaSeq 6000 System Guide (document # 1000000019358)*.
The reagent cartridges, including the library tube, must be loaded onto the instrument within ***30 minutes.***

NOTE

Use 10 index cycles when sequencing TSO500 HT libraries.

Protocol F: TruSight Oncology 500 HT Library Denaturation and Dilution Method (Xp Loading)

The NovaSeq Xp workflow for TruSight Oncology 500 HT libraries is used for denaturing and diluting libraries intended for loading on to the NovaSeq 6000 system. For addressable lane loading, see the NovaSeq Xp Workflow chapter in the *NovaSeq 6000 System Guide (document # 1000000019358)*. Libraries prepared using the TruSight Oncology 500 HT workflow are normalized to a starting concentration that is ready for sample pooling.

Use Protocol F if sequencing TSO500 HT libraries in SP, S1, S2, or S4 mode with Xp loading. You can sequence up to eight samples per lane on an SP flow cell, 16 samples per lane on an S1 flow cell, 36 samples per lane on an S2 flow cell, and 48 samples per lane on an S4 flow cell.

Visit the TruSight Oncology 500 HT support page on the Illumina website for more information on the supported number of samples per pool per flow cell.

For Standard loading, proceed to *Protocol E: TruSight Oncology 500 HT Library Denaturation and Dilution Method (Standard Loading)* on page 18.

Prepare PhiX Control [Optional]

Preparation

1 Remove RSB from 2°C to 8°C or -25°C to -15°C storage, and bring to room temperature.

2 Thaw a tube of 10 nM PhiX (10 µl/tube).

3 Label a microcentrifuge tube dHP3 (diluted HP3).

4 Label a microcentrifuge tube dTris (diluted Tris-HCl).

5 Label a microcentrifuge tube dPhiX (diluted PhiX).

Prepare a Fresh Dilution of NaOH

1 Vortex HP3 to mix, and then centrifuge briefly.

2 Combine the following volumes in the dHP3 tube.
- RNase/DNase-free water (32.5 µl)
- HP3 (7.5 µl)

3 Vortex dHP3 to mix, and then centrifuge briefly.

Prepare a Fresh Dilution of Tris-HCl

1 Combine the following volumes in the dTris tube.
- RNase/DNase-free water (25.0 µl)
- 1 M Tris-HCl, pH 8.0 (15.0 µl)

2 Vortex dTris to mix, and then centrifuge briefly.

Dilute PhiX

1 Vortex RSB to mix.

2 Vortex PhiX control to mix, and then centrifuge briefly.

3 Combine the following volumes in the dPhiX tube.
- RSB (2.0 µl)
- PhiX control (6.0 µl)

4 Vortex dPhiX tube to mix, and then centrifuge briefly.

5 [Optional] dPhiX can be stored at -25°C to -15°C for up to 3 months.

Denature PhiX

1 Add 8 µl dHP3 to the dPhiX tube.

2 Discard the dHP3 tube.

3 Vortex the dPhiX tube to mix, and then centrifuge briefly.

4 Incubate at room temperature for 5 minutes.

5 Immediately add 8 µl dTris to the dPhiX tube to neutralize the reaction.

6 Discard the dTris tube.

7 Vortex to mix, and then centrifuge briefly.

8 Add 216 µl RSB to the dPhiX tube.

9 Vortex to mix, and then centrifuge briefly.
The final concentration of PhiX is 0.25 nM.

10 [Optional] Store denatured 0.25 nM PhiX at -25°C to -15°C for up to 2 weeks.

Pool Normalized Libraries

Preparation

Visit the TruSight Oncology 500 HT support page on the Illumina website for more information on the supported number of samples per pool per flow cell.

1 If the Normalized Library (NL) plate was stored, thaw to room temperature, and then centrifuge the plate at 280 × g for 1 minute.

2 Preheat the heat block to 96°C.

3 Prepare an ice bucket.

Procedure

1 Set a multichannel pipette to 30 µl, and then gently pipette to mix the libraries in the NL plate five times.
- Use fresh tips for each library.

Library sequencing performance is diminished if libraries are not sufficiently mixed prior to pooling.

2 Select one of the following options to pool the normalized libraries.
- To sequence libraries derived from RNA samples and DNA samples simultaneously, see *Pool RNA and DNA* on page 25.
- To sequence libraries derived from DNA samples only, see *Pool DNA Only* on page 26.

NOTE

In the procedure, use the naming convention tables as a guide to labeling tubes. Make sure that the tubes you transfer to have the correct labeling for the corresponding flow cell lane.

Pool RNA and DNA

1 Label a 1.5 ml screw cap microcentrifuge tube PRL with the flow cell lane number. Repeat for any additional lanes. Use Table 10 as a guide.
- If pooling more than 40 RNA (cDNA) libraries, label an additional 1.5 ml screw cap microcentrifuge tube TPRL with the flow cell lane number. Repeat for any additional lanes.

Table 10 Naming Convention for RNA Tubes

Flow Cell	Lane 1	Lane 2	Lane 3	Lane 4
SP/S1	PRL_L1	PRL_L2	N/A	N/A
S2	PRL_L1	PRL_L2	N/A	N/A
S4	PRL_L1	PRL_L2	PRL_L3	PRL_L4

2 Label a 1.5 ml screw cap microcentrifuge tube PDL with the flow cell lane number. Repeat for any additional lanes. Use Table 11 as a guide.
   - If pooling more than 40 DNA libraries, label an additional 1.5 ml screw cap microcentrifuge tube TPDL with the flow cell lane number. Repeat for any additional lanes.

Table 11 Naming Convention for DNA Tubes

Flow Cell	Lane 1	Lane 2	Lane 3	Lane 4
SP/S1	PDL_L1	PDL_L2	N/A	N/A
S2	PDL_L1	PDL_L2	N/A	N/A
S4	PDL_L1	PDL_L2	PDL_L3	PDL_L4

3 Transfer 5 µl of each normalized RNA library from the NL plate to the PRL tube. Repeat for any additional lanes.

4 Transfer 5 µl of each normalized DNA library from the NL plate to the PDL tube. Repeat for any additional lanes.

5 Vortex each tube to mix.

6 Centrifuge each tube briefly.

7 If the PRL tube contains more than 40 RNA libraries, transfer 200 µl from the PRL tube to the TPRL tube, and then discard the PRL tube. Repeat for any additional lanes.

8 If the PDL tube contains more than 40 DNA libraries, transfer 200 µl from the PDL tube to the TPDL tube, and then discard the PDL tube. Repeat for any additional lanes.

9 Proceed to *Denature Normalized Libraries* on page 26.

Pool DNA Only

1 Label a 1.5 ml screw cap microcentrifuge tube PDL with the flow cell lane number. Repeat for any additional lanes.
Use Table 12 as a guide.

Table 12 Naming Convention for DNA Tubes

Flow Cell	Lane 1	Lane 2	Lane 3	Lane 4
SP/S1	PDL_L1	PDL_L2	N/A	N/A
S2	PDL_L1	PDL_L2	N/A	N/A
S4	PDL_L1	PDL_L2	PDL_L3	PDL_L4

   - If pooling more than 40 DNA libraries, label an additional 1.5 ml screw-top microcentrifuge tube TPRL (Transferred Pooled DNA Libraries) with the flow cell lane number. Repeat for any additional lanes.

2 Transfer 5 µl of each normalized DNA library from the NL plate to the PDL tube. Repeat for any additional lanes.

3 Vortex each tube to mix.

4 Centrifuge each tube briefly.

5 If the PDL tube contains more than 40 DNA libraries, transfer 200 µl from the PDL tube to the TPDL tube, and then discard the PDL tube. Repeat for any additional lanes.

Denature Normalized Libraries

1 Vortex and centrifuge each of the following briefly:

- PRL (≤ 40 RNA libraries) or TPRL (> 40 RNA libraries)
- PDL (≤ 40 DNA libraries) or TPDL (> 40 DNA libraries)

2 Incubate in a heat block at 96°C for 2 minutes.

3 Immediately place on ice for 5 minutes.

4 Vortex each tube to mix, and then centrifuge briefly.

5 Place tubes on ice.

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, thaw the tubes and repeat *Denature Normalized Libraries* on page 26 before proceeding to the next step.

Dilute Libraries and Add Optional PhiX Control

Prepare RSB

1 Remove RSB from 2°C to 8°C or -25°C to -15°C storage, and bring to room temperature.

Prepare Denatured 0.25 nM PhiX

1 If the denatured PhiX was stored, remove the denatured 0.25 nM PhiX from -25°C to -15°C storage and thaw to room temperature.

2 Vortex to mix, and then centrifuge briefly.

Dilute Libraries

1 Select one of the following options to dilute your libraries.
- To sequence libraries derived from RNA samples and DNA samples simultaneously, see *Dilute RNA and DNA Libraries* on page 27.
- To sequence libraries derived from DNA samples only, see *Dilute DNA Libraries Only* on page 28.

Dilute RNA and DNA Libraries

1 Label a new 1.5 ml screw cap microcentrifuge tube to combine PRL and PDL libraries. Repeat for any additional lanes. Use Table 13 as a guide.

Table 13 Naming Convention for combined PRL and PDL Tubes

Flow Cell	Lane 1	Lane 2	Lane 3	Lane 4
SP/S1	PRL+PDL_L1	PRL+PDL_L2	N/A	N/A
S2	PRL+PDL_L1	PRL+PDL_L2	N/A	N/A
S4	PRL+PDL_L1	PRL+PDL_L2	PRL+PDL_L3	PRL+PDL_L4

2 Vortex and centrifuge each of the following types of tubes briefly:
- PRL (≤ 40 RNA libraries) or TPRL (> 40 RNA libraries)
- PDL (≤ 40 DNA libraries) or TPDL (> 40 DNA libraries)

3 Transfer 5 µl of each PRL or TPRL tube into the corresponding PRL+PDL tube.

4 Transfer 20 µl of each PDL or TPDL tube into the corresponding PRL+PDL tube.

5 Vortex the PRL+PDL tubes to mix.

6 Centrifuge the PRL+PDL tubes briefly.

7 Label a new 1.5 ml screw cap microcentrifuge tube to dilute the combined PRL+PDL libraries. Repeat for any additional lanes. Use Table 14 as a guide.

Table 14 Naming Convention for DIL 1 Tubes

Flow Cell	Lane 1	Lane 2	Lane 3	Lane 4
SP/S1	DIL1_L1	DIL1_L2	N/A	N/A
S2	DIL1_L1	DIL1_L2	N/A	N/A
S4	DIL1_L1	DIL1_L2	DIL1_L3	DIL1_L4

8 Transfer the appropriate volume of combined PRL and PDL to each of the corresponding DIL1 tubes.

Flow Cell	PRL+PDL (µl)
SP/S1	4
S2	5
S4	6.8

9 Add the appropriate volume of RSB to each of the corresponding DIL1 tubes.

Flow Cell	RSB (µl)	Resulting Volume (µl)
SP/S1	23	27
S2	28	33
S4	38.2	45

10 [Optional] Add the appropriate volume of denatured 0.25 nM PhiX to each of the corresponding DIL1 tubes.

Flow Cell	0.25 nM PhiX (µl)	Resulting Volume (µl)
SP/S1	0.7	27.7
S2	0.8	33.8
S4	1.1	46.1

11 Vortex the DIL1 tubes to mix.

12 Centrifuge the DIL1 tubes briefly.

13 After denaturing and diluting your libraries and preparing the optional PhiX control, proceed to *Prepare the Flow Cell and Dock* in the Xp Workflow section of the *NovaSeq 6000 System Guide (document # 1000000019358)*.

NOTE

Use 10 index cycles when sequencing TSO500 HT libraries.

Dilute DNA Libraries Only

1 Label a new 1.5 ml screw cap microcentrifuge tube to dilute the PDL libraries. Repeat for any additional lanes. Use Table 15 as a guide.

Table 15 Naming convention for tubes

Flow Cell	Lane 1	Lane 2	Lane 3	Lane 4
SP/S1	DIL1_L1	DIL1_L2	N/A	N/A
S2	DIL1_L1	DIL1_L2	N/A	N/A
S4	DIL1_L1	DIL1_L2	DIL1_L3	DIL1_L4

2 Vortex and centrifuge the PDL or TPDL tubes briefly.

3 Transfer the appropriate volume of PDL or TPDL to each of the corresponding DIL1 tubes.

Flow Cell	PDL or TPDL (µl)
SP/S1	4
S2	5
S4	6.8

4 Add the appropriate volume of RSB to each of the corresponding DIL1 tubes.

Flow Cell	RSB (µl)	Resulting Volume (µl)
SP/S1	23	27
S2	28	33
S4	38.2	45

5 [Optional] Add the appropriate volume of denatured 0.25 nM PhiX to each of the corresponding DIL1 tubes.

Flow Cell	0.25 nM PhiX (µl)	Resulting Volume (µl)
SP/S1	0.7	27.7
S2	0.8	33.8
S4	1.1	46.1

6 Vortex the DIL1 tubes to mix.

7 Centrifuge the DIL1 tubes briefly.

8 After denaturing and diluting your libraries and preparing the optional PhiX control, proceed to *Prepare the Flow Cell and Dock* in the Xp Workflow section of the *NovaSeq 6000 System Guide (document # 1000000019358)*.

NOTE

Use 10 index cycles when sequencing TSO500 HT libraries.

Revision History

Document	Date	Description of Change
Document # 1000000106351 v03	November 2020	Added information on index cycles when sequencing TruSight Oncology 500 High Throughput Libraries.
Document # 1000000106351 v02	July 2020	Added information in support of the NovaSeq 6000 Reagent Kit v1.5.
Document # 1000000106351 v01	March 2020	Added Protocols E and F for TruSight Oncology 500 HT Standard and Xp loading methods.
Document # 1000000106351 v00	January 2020	Initial release.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
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	
Denmark	+45 80820183	+45 89871156
Finland	+358 800918363	+358 974790110
France	+33 805102193	+33 170770446
Germany	+49 8001014940	+49 8938035677
Hong Kong, China	800960230	
Ireland	+353 1800936608	+353 016950506
Italy	+39 800985513	+39 236003759
Japan	0800.111.5011	
Netherlands	+31 8000222493	+31 207132960
New Zealand	0800.451.650	
Norway	+47 800 16836	+47 21939693
Singapore	+1.800.579.2745	
South Korea	+82 80 234 5300	
Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
Taiwan, China	00806651752	
United Kingdom	+44 8000126019	+44 2073057197
Other countries	+44.1799.534000	

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

Product documentation—Available for download from support.illumina.com.

Document # 1000000106351 v03

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

For Research Use Only. Not for use in diagnostic procedures.

illumina®