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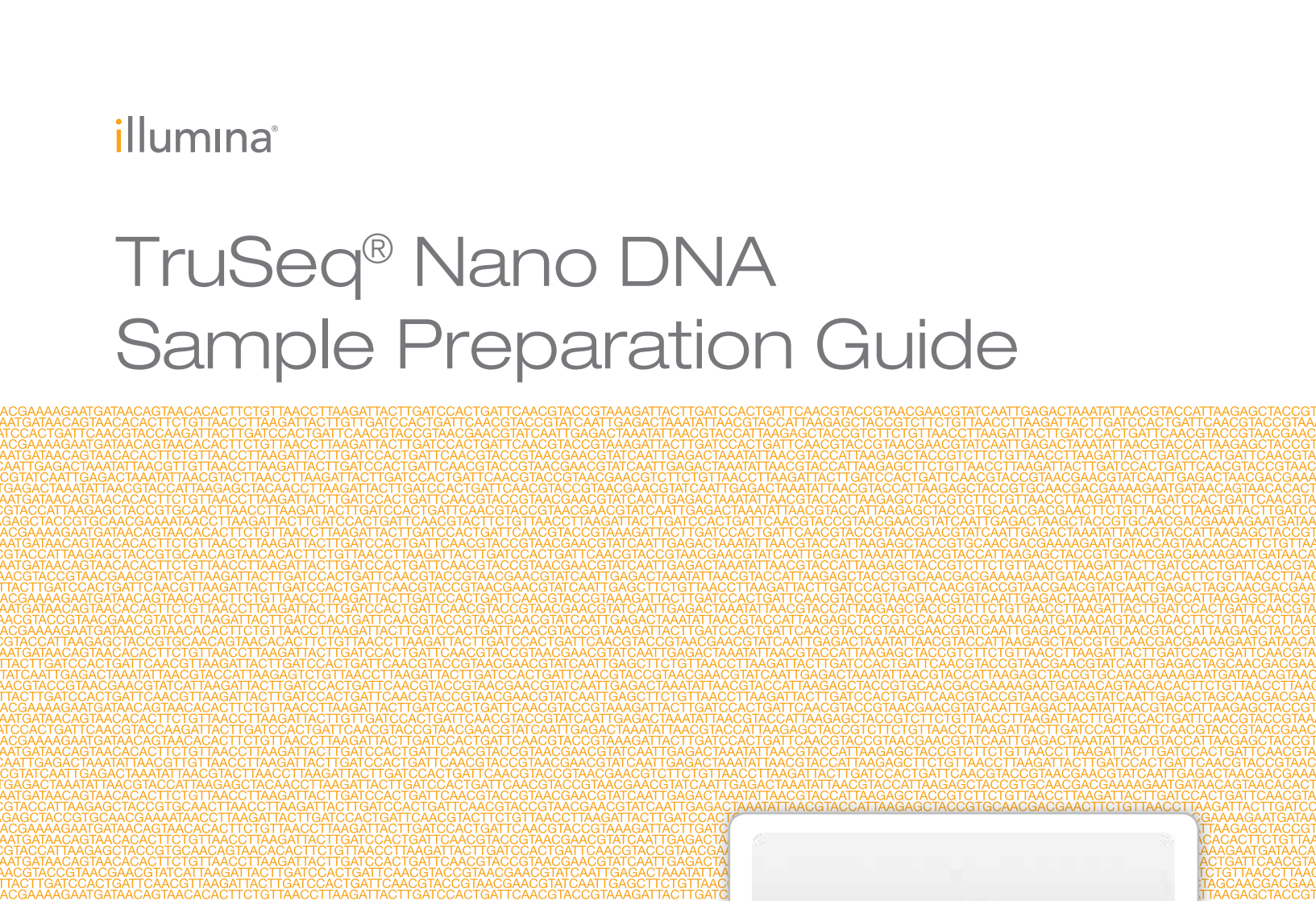
TruSeq® Nano DNA Sample Preparation Guide



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Revision History

Part #	Revision	Date	Description of Change
15041110	B	November 2013	• Renamed Incubate 1 IMP to Incubate IMP • Added recommended thermal cycler settings to *Consumables and Equipment*
15041110	A	May 2013	Initial Release

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Overview

Chapter 1

Introduction

This protocol explains how to prepare up to 96 pooled, indexed paired-end libraries of genomic DNA (gDNA) for subsequent cluster generation and DNA sequencing using the reagents provided in the Illumina® TruSeq® Nano DNA Sample Prep Kits (low-throughput (LT) and high-throughput (HT)). The goal of this protocol is to add adapter sequences onto the ends of DNA fragments to generate indexed single read or paired-end sequencing libraries.

The sample preparation protocol offers:

Streamlined Workflow

- Master-mixed reagents to reduce reagent containers and pipetting
- Universal adapter for preparation of single read, paired-end, and indexing

Optimized shearing for whole-genome resequencing with 350 bp and 550 bp insert size workflows

Bead-based size selection reagents included in each kit

Optimized workflows for processing low sample (LS) and high sample (HS) numbers in parallel

Compatibility with LT and HT kit configurations

High Throughput

- Adapter plate allows for simultaneous preparation of 96 dual-indexed DNA samples
- Volumes optimized for standard 96-well plate

Index Adapter Tags All Samples

- Additional adapters and primers not necessary
- Each TruSeq Nano DNA LT Sample Prep Kit contains adapter index tubes recommended for preparing up to 24 samples for sequencing. Together kits A and B allow for pooling up to 24 samples
- The TruSeq Nano DNA HT Sample Prep Kit contains a 96-well plate with 96 uniquely indexed adapter combinations designed for manual or automated preparation of 96 uniquely indexed samples

The protocol is compatible with single sample sequencing or lower indexing pooling levels.

Protocol Features

This guide documents the TruSeq Nano DNA Sample Preparation protocol using a TruSeq Nano DNA LT Sample Prep Kit or TruSeq Nano DNA HT Sample Prep Kit.

- Chapter 2 Low Sample (LS) Protocol explains how to perform the TruSeq Nano DNA Sample Preparation using the Low Sample Protocol
- Chapter 3 High Sample (HS) Protocol explains how to perform the TruSeq Nano DNA Sample Preparation using the High Sample Protocol

Equivalent results can be expected from either protocol, however the HS protocol can yield more consistent results between samples. Their distinguishing elements are as follows:

Table 1 Protocol Features

	Low Sample	High Sample
LT Kit - Number of samples processed at one time	≤ 24 with indexed adapter tubes*	> 24 with indexed adapter tubes*
HT Kit - Number of samples processed at one time	≤ 24 with indexed adapter plate	> 24 with indexed adapter plate
Plate Type	96-well 0.3 ml PCR	96-well HSP 96-well MIDI
Incubation Equipment	96-well thermal cycler	Microheating systems
Mixing Method	Pipetting	Microplate shaker

* Each TruSeq Nano DNA LT Sample Prep Kit contains enough reagents to prepare up to 24 samples. When used together, TruSeq Nano DNA LT Sample Prep Kits A and B allow for pooling up to 24 samples using the 12 different indices in each kit. Illumina does not recommend preparing more than 24 samples at a time using the LS protocol. An alternative to using the HS protocol for more than 24 samples is to perform separate library preparations to ensure robust performance.

The TruSeq Nano DNA Sample Preparation fragmentation process is optimized to obtain final libraries, with the following average insert size.

Table 2 Insert Size Options

Insert Size	350 bp	550 bp
Input DNA Per Sample	100 ng	200 ng
Recommended Read Length	≤ 2 x 101 bp	≤ 2 x 151 bp*

* Read lengths greater than 2 x 151 bp produce a significantly higher percentage of overlapping read-pairs.

DNA Input Recommendations

It is important to quantitate the input DNA and assess the DNA quality before performing TruSeq Nano DNA Sample Preparation.

Input DNA Quantitation

Follow these DNA input recommendations:

- Correct quantification of gDNA is essential.
- 100 ng input DNA is recommended for the 350 bp insert size workflow and 200 ng for the 550 bp insert size workflow.
- The ultimate success or failure of library preparation strongly depends on using an accurately quantified amount of input DNA.
- Illumina recommends using fluorometric based methods for quantification including Qubit or PicoGreen to provide accurate quantification of dsDNA. UV spectrophotometric-based methods, such as the Nanodrop, measure any nucleotides present in the sample including RNA, dsDNA, ssDNA, and free nucleotides, which can give an inaccurate measurement of gDNA.
- Use multiple methods of quantification to verify results.
- DNA quantification methods that rely on intercalating fluorescent dyes measure only double-stranded DNA and are less subject to the presence of excess nucleic acids.
  - These methods require the preparation of calibration curves and are highly sensitive to pipetting error.
  - Make sure that pipettes are correctly calibrated and are not used at the volume extremes of their performance specifications.

Assessing DNA Quality

Absorbance measurements at 260 nm are commonly used to assess DNA quality:

- The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity, and values of 1.8–2.0 are considered indicative of relatively pure DNA.
- The presence of RNA or small nucleic acid fragments, such as nucleotides, can compromise both absorbance measurements.
- Carefully collect gDNA samples to make sure that they are free of contaminants.

Positive Control

In-line controls are not provided in TruSeq Nano DNA Sample Preparation kits. Therefore, Illumina recommends using Coriell Human-1 DNA (NA 18507) or Promega Human Genomic DNA (G3041) as a positive control sample for this protocol.

Additional Resources

The following resources are available for TruSeq Nano DNA Sample Preparation protocol guidance and sample tracking. Access these and other resources on the Illumina website at support.illumina.com/sequencing/kits.ilmn. Then, select **TruSeq Nano DNA LT Sample Prep Kit Support** or **TruSeq Nano DNA HT Sample Prep Kit Support**.

Resource	Description
Training	Illustrates elements of the TruSeq Nano DNA Sample Preparation process. Viewing these videos is recommended for new and less experienced users before starting sample preparation. • Click **Training** on **TruSeq Nano DNA LT Sample Prep Kit Support** or • Click **Training** on **TruSeq Nano DNA HT Sample Prep Kit Support**
Best Practices	Provides best practices specific to this protocol. Review these best practices before starting sample preparation. Topics include: • Handling Liquids • Handling Master Mix Reagents • Handling Magnetic Beads • Avoiding Cross-Contamination • Potential DNA Contaminants • Temperature Considerations • Equipment • Click **Best Practices** on **TruSeq Nano DNA LT Sample Prep Kit Support** or • Click **Best Practices** on **TruSeq Nano DNA HT Sample Prep Kit Support**

Resource	Description
TruSeq Nano DNA Sample Preparation LS Protocol Experienced User Card and Lab Tracking Form (part # 15041111)	Provides LS protocol instructions, but with less detail than what is provided in this user guide. **New or less experienced users are advised to follow this user guide and not the EUC and LTF.** • Click **Documentation & Literature** on **TruSeq Nano DNA LT Sample Prep Kit Support** or • Click **Documentation & Literature** on **TruSeq Nano DNA HT Sample Prep Kit Support**
TruSeq Nano DNA Sample Preparation HS Protocol Experienced User Card and Lab Tracking Form (part # 15041112)	Provides HS protocol instructions, but with less detail than what is provided in this user guide. **New or less experienced users are advised to follow this user guide and not the EUC and LTF.** • Click **Documentation & Literature** on **TruSeq Nano DNA LT Sample Prep Kit Support** or • Click **Documentation & Literature** on **TruSeq Nano DNA HT Sample Prep Kit Support**
TruSeq Sample Preparation Pooling Guide (part # 15042173)	Provides TruSeq pooling guidelines for sample preparation. Review this guide before beginning library preparation. • Click **Documentation & Literature** on **TruSeq Nano DNA LT Sample Prep Kit Support** or • Click **Documentation & Literature** on **TruSeq Nano DNA HT Sample Prep Kit Support**
Illumina Experiment Manager (IEM)	Enables you to create and edit appropriate sample sheets for Illumina sequencers and analysis software and record parameters for your sample plate. To download the software: • Click **Downloads** on **TruSeq Nano DNA LT Sample Prep Kit Support** or • Click **Downloads** on **TruSeq Nano DNA HT Sample Prep Kit Support** To download the documentation: • Click **Documentation & Literature** on **TruSeq Nano DNA LT Sample Prep Kit Support** or • Click **Documentation & Literature** on **TruSeq Nano DNA HT Sample Prep Kit Support**

Low Sample (LS) Protocol

Chapter 2

Introduction

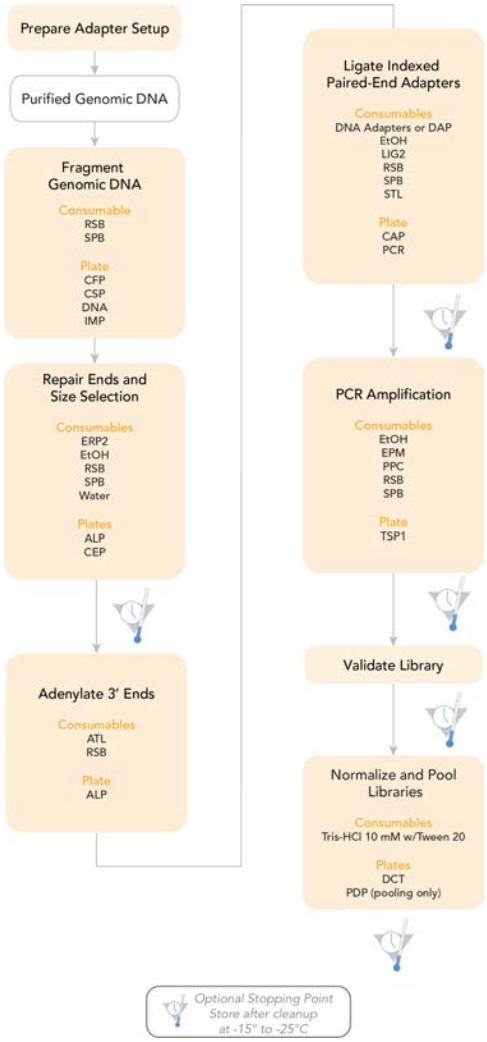
This chapter describes the TruSeq Nano DNA Sample Preparation LS protocol. This protocol is intended for preparing up to 24 samples at one time using either the LT or HT kit.

- Follow the protocol in the order described, using the specified volumes and incubation parameters.
- Review Best Practices before proceeding. See *Additional Resources* on page 6 for information on how to access TruSeq Nano DNA Sample Preparation Best Practices on the Illumina website.
- Review the *TruSeq Sample Preparation Pooling Guide (part # 15042173)* before proceeding. See *Additional Resources* on page 6 for information on how to download the guide from the Illumina website.
- Review Appendix A Supporting Information before proceeding, to confirm your kit contents and make sure that you have obtained all of the requisite equipment and consumables for the LS protocol.

Sample Prep Workflow

The following figure illustrates the processes of the TruSeq Nano DNA Sample Preparation LS protocol to prepare templates using indexed adapter tubes or a DAP.

Figure 1 TruSeq Nano DNA Sample Preparation LS Workflow



Prepare Adapter Setup

If you are pooling, record information about your samples before beginning library preparation for later use in data analysis.

- Use IEM to create and edit a sample sheet for Illumina sequencers and analysis software. See *Additional Resources* on page 6 for information on how to download IEM software and documentation from the Illumina website.
- Review planning steps in the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 6 for information on how to download the guide from the Illumina website.

If you are pooling using adapter index tubes, Illumina recommends arranging samples that will be combined into a common pool in the same row. Include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.

If you are pooling with the DAP, arrange samples that will be pooled together in the same orientation as the indices in the DAP.

Fragment DNA

This process describes how to optimally fragment the gDNA depending on the downstream application. Covaris shearing generates dsDNA fragments with 3' or 5' overhangs. The fragmentation process is optimized to obtain final libraries with the following average insert sizes:

Table 3 Insert Size Options

Insert Size	350 bp	550 bp
Input DNA Per Sample	100 ng	200 ng
Recommended Read Length	≤ 2 x 101 bp	≤ 2 x 151 bp*

* Read lengths greater than 2 x 151 bp produce a significantly higher percentage of overlapping read-pairs.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	-15°C to -25°C (2°C to 8°C after initial thaw)	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Barcode labels for: • CFP (Covaris Fragmentation Plate) • CSP (Clean Up Sheared DNA Plate) • DNA (DNA Plate) • IMP (Insert Modification Plate)	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR plates	4	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
Covaris tubes	1 per sample	15°C to 30°C	User
DNA samples	100 ng per sample for a 350 bp insert size or 200 ng per sample for a 550 bp insert size	-15°C to -25°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User

Preparation

- Review *DNA Input Recommendations* on page 4.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 6 for information on how to access TruSeq Nano DNA Sample Preparation Best Practices on the Illumina website.
- Remove the Sample Purification Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove one tube of Resuspension Buffer from -15°C to -25°C storage and thaw it at room temperature.

  NOTE
  The Resuspension Buffer can be stored at 2°C to 8°C after the initial thaw.

- Turn on the Covaris instrument and follow the manufacturer's guidelines to set up your instrument.
- Apply a CFP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a CSP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a DNA barcode label to a new 96-well 0.3 ml PCR plate.
- Apply an IMP barcode label to a new 96-well 0.3 ml PCR plate.

Make CFP

1 Illumina recommends quantifying gDNA samples using a fluorometric quantification method that uses dsDNA binding dyes.

2 Normalize the gDNA samples with Resuspension Buffer to one of the following in each well of the new 0.3 ml PCR plate labeled with the DNA barcode:
- 100 ng in a final volume of 52.5 µl for a 350 bp insert size
- 200 ng in a final volume of 52.5 µl for a 550 bp insert size

Fragment DNA

1 Shear one of the following amounts of gDNA sample by transferring 52.5 µl of each DNA sample from the DNA plate to a separate, new Covaris tube:
- 100 ng for a 350 bp insert size
- 200 ng for a 550 bp insert size

Use the wells of the new 0.3 ml PCR plate labeled with CFP barcode or another device to hold the Covaris tubes upright.

NOTE
Load the DNA sample into the Covaris tube slowly to avoid creating air bubbles. However, air bubbles might not be preventable.

2 Centrifuge the CFP plate at 600 × g for 5 seconds.

3 Fragment the DNA using the following settings:

Table 4 Covaris S220 Settings

Setting	350 bp Insert	550 bp Insert
Duty factor	5%	
Peak Incident Power	175 W	
Cycles per burst	200	
Duration	50 seconds	25 seconds
Mode	Frequency sweeping	
Temperature	5.5° to 6°C	

Table 5 Covaris M220 Settings

Setting	350 bp Insert	550 bp Insert
Duty factor	20%	
Peak Incident Power	50 W	
Cycles per burst	200	
Duration	65 seconds	45 seconds
Temperature	20°C	

NOTE
The Covaris M220 settings are optimized for use with the Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm.

Table 6 Covaris S2 and E210 Settings

Setting	350 bp Insert	550 bp Insert
Duty cycle	10%	
Intensity	5.0	2.0
Cycles per burst	200	
Duration	45 seconds	
Mode	Frequency sweeping	
Displayed Power	S2—23 W	S2—9 W
	E210—14 W	E210—7 W
Temperature	5.5° to 6°C	

4 Centrifuge the CFP plate at 600 × g for 5 seconds.

5 Transfer 50 µl of fragmented DNA from each Covaris tube in the CFP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CSP barcode, using a single channel pipette.

6 Proceed immediately to *Clean Up Fragmented DNA*.

Clean Up Fragmented DNA

1 Vortex the room temperature Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add 80 µl well-mixed Sample Purification Beads to each well of the CSP plate containing 50 µl of fragmented gDNA. Set a 200 µl pipette to 125 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. Illumina recommends the following:

- If using a single channel pipette, vortex the beads after processing four samples
- If using a multichannel pipette, vortex the beads after processing four columns
- If the beads are in a reagent reservoir, mix with a 1000 µl pipette.

NOTE
Keep the Sample Purification Beads tube at room temperature for later use in the protocol.

3 Incubate the CSP plate at room temperature for 5 minutes.

4 Place the CSP plate on the magnetic stand at room temperature for 8 minutes or until the liquid is clear.

5 Using a 200 µl single channel or multichannel pipette set to 125 µl, remove and discard 125 µl of the supernatant from each well of the CSP plate.

NOTE
Leave the CSP plate on the magnetic stand while performing the following steps 6–10.

6 With the CSP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

7 Incubate the CSP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

8 Repeat steps 6 and 7 one time for a total of two 80% EtOH washes.

9 With the CSP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Remove and discard any remaining EtOH with a 10 µl pipette.

10 With the CSP plate on the magnetic stand, add 62.5 µl Resuspension Buffer to each well of the plate.

11 Remove the CSP plate from the magnetic stand.

12 Resuspend the beads in each well of the CSP plate by repeatedly dispensing the Resuspension Buffer over the bead pellet until it is immersed in the solution. Gently pipette the entire volume up and down 10 times to mix thoroughly.

13 Incubate the CSP plate at room temperature for 2 minutes.

14 Place the CSP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

15 Transfer 60 µl of the clear supernatant from each well of the CSP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the IMP barcode. Take care not to disturb the beads.

NOTE

Make sure that you use a 0.3 ml PCR plate, because IMP plate volumes are greater than a 0.2 ml PCR plate. Final volumes during size selection are up to 260 µl per well.

16 Proceed immediately to *Perform End Repair and Size Selection* on page 19.

Perform End Repair and Size Selection

This process converts the overhangs resulting from fragmentation into blunt ends using End Repair Mix 2. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs. Following end repair, the appropriate library size is selected using different ratios of the Sample Purification Beads.

Consumables

Item	Quantity	Storage	Supplied By
End Repair Mix 2 (ERP2)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Barcode labels for: • ALP (Adapter Ligation Plate) • CEP (Clean Up End Repair Plate)	1 label per plate	15°C to 30°C	Illumina
15 ml conical tube (when processing > 6 samples at a time) or 1.7 ml microcentrifuge tube (when processing ≤ 6 samples at a time)	1	15°C to 30°C	User
96-well 0.3 ml PCR plates	2	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
Ice bucket	As needed	-15°C to -25°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User
PCR grade water	1 bottle	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	6	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	6	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the End Repair Mix 2 from -15°C to -25°C storage and thaw it at room temperature. Place the tube on ice.
- Remove the Sample Purification Beads and Resuspension Buffer from 2°C to 8°C storage and bring them to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 6 for information on how to access TruSeq Nano DNA Sample Preparation Best Practices on the Illumina website.
- Pre-program the thermal cycler with the following program and save as **ERP**:
  - Choose the thermal cycler pre-heat lid option and set to 100°C
  - 30°C for 30 minutes
  - Hold at 4°C
- Apply an ALP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a CEP barcode label to a new 96-well 0.3 ml PCR plate.

Make IMP

1 Centrifuge the thawed End Repair Mix 2 tube at 600 × g for 5 seconds.

2 Add 40 µl End Repair Mix 2 to each well of the IMP plate containing the fragmented DNA. Set a 200 µl pipette to 95 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

3 Seal the IMP plate with a Microseal 'B' adhesive seal.

4 Return the End Repair Mix 2 tube to -15°C to -25°C storage.

Incubate IMP

1 Place the sealed IMP plate on the pre-programmed thermal cycler. Close the lid then select and run the **ERP** program.
   a Choose the thermal cycler pre-heat lid option and set to 100°C
   b 30°C for 30 minutes
   c Hold at 4°C

2 Remove the IMP plate from the thermal cycler when the program reaches 4°C.

Clean Up IMP and Size Selection

1 Remove the adhesive seal from the IMP plate.

Remove Large DNA Fragments

1 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add the Sample Purification Beads and PCR grade water to one of the following tubes, to create a diluted bead mixture of 160 µl per 100 µl of end-repaired sample:

- New 15 ml conical tube, when processing > 6 samples at a time
- New 1.7 ml microcentrifuge tube, when processing ≤ 6 samples at a time

Determine the volumes using the following formulas, which include 15% excess for multiple samples:

Table 7 Diluted Bead Mixture for a 350 bp Insert Size

	Formula	Example Amount per 12 samples	Your Calculation
Sample Purification Beads	# of samples X 109.25 µl	1311 µl	
PCR grade water	# of samples X 74.75 µl	897 µl	

Table 8 Diluted Bead Mixture for a 550 bp Insert Size

	Formula	Example Amount per 12 samples	Your Calculation
Sample Purification Beads	# of samples X 92 µl	1104 µl	
PCR grade water	# of samples X 92 µl	1104 µl	

3 Vortex the diluted bead mixture for 5 seconds to make sure that the beads are evenly dispersed.

4 Add 160 µl of the diluted bead mixture to each well of the IMP plate containing 100 µl of the end repaired sample. Set a 200 µl pipette to 200 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

NOTE

Aspirate the diluted bead mixture slowly and dispense it slowly due to the viscosity of the solution. Changes in the volume of the diluted bead mixture affect the insert size of your library.

NOTE
Vortex the diluted bead mixture frequently. Illumina recommends the following:
- If using a single channel pipette, vortex the mixture after processing four samples
- If using a multichannel pipette, vortex the mixture after processing four columns
- If the mixture is in a reagent reservoir, mix with a 1000 µl pipette.

5 Incubate the IMP plate at room temperature for 5 minutes.

6 Place the IMP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

7 Use a 200 µl single channel or multichannel pipette set to 125 µl to **transfer 125 µl of the supernatant, containing the DNA of interest, from each well of the IMP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CEP barcode**. Take care not to disturb the beads.

NOTE
Transfer, do not discard, the supernatant. It contains the DNA of interest.

8 Repeat step 7 one time. **Each CEP plate well now contains a total of 250 µl of DNA of interest.**

9 Discard the IMP plate containing the beads.

10 Discard any remaining diluted bead mixture.

Remove Small DNA Fragments

NOTE
In the following steps, use **undiluted** Sample Purification Beads.

1 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add 30 µl **undiluted** Sample Purification Beads to each well of the CEP plate containing 250 µl of the supernatant with the DNA of interest. Set a 200 µl pipette to 200 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

NOTE
Aspirate the Sample Purification Beads slowly and dispense them slowly due to the viscosity of the solution. Changes in the volume of the bead mixture affect the insert size of your library.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. Illumina recommends the following:

- If using a single channel pipette, vortex the beads after processing four samples
- If using a multichannel pipette, vortex the beads after processing four columns
- If the beads are in a reagent reservoir, mix with a 1000 µl pipette.

3 Incubate the CEP plate at room temperature for 5 minutes.

4 Place the CEP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

5 Using a 200 µl single channel or multichannel pipette set to 138 µl, remove and discard 138 µl of the supernatant from each well of the CEP plate. Take care not to disturb the beads.

6 Repeat step 5 one time, removing and discarding a total of 276 µl of the supernatant from each well.

NOTE
Leave the CEP plate on the magnetic stand while performing the following steps 7–11.

7 With the CEP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

8 Incubate the CEP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

9 Repeat steps 7 and 8 one time for a total of two 80% EtOH washes.

10 With the CEP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Remove and discard any remaining EtOH with a 10 µl pipette.

11 With the CEP plate on the magnetic stand, add 20 µl Resuspension Buffer to each well of the plate.

12 Remove the CEP plate from the magnetic stand.

13 Resuspend the beads in each well of the CEP plate by repeatedly dispensing the Resuspension Buffer over the bead pellet until it is immersed in the solution. Gently pipette the entire volume up and down 10 times to mix thoroughly.

14 Incubate the CEP plate at room temperature for 2 minutes.

15 Place the CEP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

16 Transfer 17.5 µl of the clear supernatant from each well of the CEP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the ALP barcode.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Adenylate 3′ Ends* on page 26, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to 7 days.

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

Item	Quantity	Storage	Supplied By
A-Tailing Mix (ATL)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	2	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	2	15°C to 30°C	User

Preparation

- Remove the A-Tailing Mix from -15°C to -25°C storage and thaw it at room temperature:
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the ALP plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up IMP and Size Selection* on page 21.
  - Let it thaw at room temperature.

- Centrifuge the thawed ALP plate at 280 × g for 1 minute.
- Remove the adhesive seal from the ALP plate.

▸ Pre-program the thermal cycler with the following program and save as **ATAIL70**:

- Choose the pre-heat lid option and set to 100°C
- 37°C for 30 minutes
- 70°C for 5 minutes
- 4°C for 5 minutes
- Hold at 4°C

Add ATL

1. Centrifuge the thawed A-Tailing Mix tube at 600 × g for 5 seconds.
2. Add 12.5 µl thawed A-Tailing Mix to each well of the ALP plate. Set a 20 µl pipette to 20 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.
3. Seal the ALP plate with a Microseal 'B' adhesive seal.
4. Return the A-Tailing Mix tube to -15° to -25°C storage.

Incubate 1 ALP

1. Place the sealed ALP plate, containing 30 µl of each sample, on the pre-programmed thermal cycler. Close the lid, then select and run the **ATAIL70** program.
   a. Choose the pre-heat lid option and set to 100°C
   b. 37°C for 30 minutes
   c. 70°C for 5 minutes
   d. 4°C for 5 minutes
   e. Hold at 4°C
2. When the thermal cycler temperature has been at 4°C for 5 minutes, remove the ALP plate from the thermal cycler.
3. Centrifuge the ALP plate at 280 × g for 1 minute.
4. Proceed immediately to *Ligate Adapters* on page 28.

Ligate Adapters

This process ligates multiple indexing adapters to the ends of the DNA fragments, preparing them for hybridization onto a flow cell.

Consumables

Item	Quantity	Storage	Supplied By

Choose from the following depending on the kit you are using:
• TruSeq Nano DNA LT Sample Prep Kit contents:
  • DNA Adapter Indices (AD001–AD016, AD018–AD023, AD025, AD027)
• TruSeq Nano DNA HT Sample Prep Kit contents:
  • DAP (DNA Adapter Plate)
	1 tube of each index being used, per column of 8 reactions
	or
	1 DAP
		-15°C to -25°C	Illumina

Ligation Mix 2 (LIG2)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Stop Ligation Buffer (STL)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina

Item	Quantity	Storage	Supplied By
Barcode labels for: • CAP (Clean Up ALP Plate) • DAP (DNA Adapter Plate) (if using the HT kit) • PCR (Polymerase Chain Reaction Plate)	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR plates	2	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	800 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	3–27	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	3–27	15°C to 30°C	User

Preparation

- Remove the following from -15°C to -25°C storage and thaw them at room temperature:
  - Appropriate DNA Adapter tubes (depending on the DNA Adapter Indices being used) or the DAP.

NOTE

- Review the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 6 for information on how to download the guide from the Illumina website.
- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that are going to be combined into a common pool in the same row. Also, include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
- When indexing libraries with the DAP, arrange samples that will be pooled together in the same orientation as the indices in the DAP.

NOTE
When indexing libraries with the DAP:

- Review *Handling Adapter Plate* in the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 6 for information on how to download the guide from the Illumina website.
- Illumina recommends that the DAP does not undergo more than four freeze-thaw cycles. To maximize the use of the DAP, process more than 24 samples at a time. These samples can then be pooled in any supported configuration.

- Stop Ligation Buffer

NOTE
Do not remove the Ligation Mix 2 tube from -15°C to -25°C storage until instructed to do so in the procedures.

- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 6 for information on how to access TruSeq Nano DNA Sample Preparation Best Practices on the Illumina website.
- Remove the Sample Purification Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-program the thermal cycler with the following program and save as **LIG**:
  - Choose the thermal cycler pre-heat lid option and set to 100°C
  - 30°C for 10 minutes
  - Hold at 4°C
- Apply a CAP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a PCR barcode label to a new 96-well 0.3 ml PCR plate.

Add LIG

1 Do one of the following:
- If using DNA Adapter tubes, centrifuge the thawed tubes at 600 × g for 5 seconds.

- If using a DAP:
  - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to make sure that they all are thawed.
  - Remove the adapter plate tape seal.
  - Centrifuge the plate at 280 × g for 1 minute to collect all of the adapter to the bottom of the well.
  - Remove the plastic cover. Save the cover if you are not processing the entire plate at one time.
  - If it is the first time using this DAP, apply the DAP barcode label to the plate.

2 Centrifuge the Stop Ligation Buffer tube at 600 × g for 5 seconds.

3 Immediately before use, remove the Ligation Mix 2 tube from -15°C to -25°C storage.

4 Remove the adhesive seal from the ALP plate.

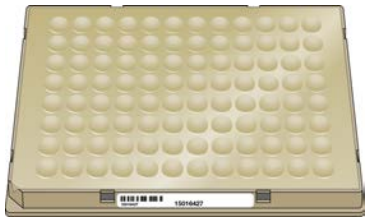
5 Add 2.5 µl Resuspension Buffer to each well of the ALP plate.

6 Add 2.5 µl Ligation Mix 2 to each well of the ALP plate.

7 Return the Ligation Mix 2 tube to -15°C to -25°C storage immediately after use.

8 Do one of the following:
- If using DNA Adapter tubes, add 2.5 µl thawed DNA Adapter Index to each well of the ALP plate. Set a 200 µl pipette to 35 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.
- If using a DAP:
  - Place the DAP on the benchtop so that the part number barcode, on the long side of the plate, is facing you and the clipped corner is on the lower left.

Figure 2 Correct DAP Orientation



- Do one of the following to pierce the foil seal:
  - If using the entire plate at one time, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously. Gently, but firmly, press the clean plate over the foil seal.
  - If using only part of the plate, use the bottom of a clean eight-tube strip, with caps attached, to pierce holes in the seals of the wells that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each row or column of adapters that will be used for ligation.
- Using an eight-tip multichannel pipette, transfer 2.5 µl thawed DNA Adapter from the DAP well to each well of the ALP plate. Set a 200 µl pipette to 35 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.

9 Seal the ALP plate with a Microseal 'B' adhesive seal.

10 Centrifuge the ALP plate at 280 × g for 1 minute.

Incubate 2 ALP

1 Place the sealed ALP plate, containing 37.5 µl of each sample, on the pre-programmed thermal cycler. Close the lid then select and run the **LIG** program.
   a Choose the thermal cycler pre-heat lid option and set to 100°C
   b 30°C for 10 minutes
   c Hold at 4°C

2 Remove the ALP plate from the thermal cycler when the program reaches 4°C.

Add STL

1 Remove the adhesive seal from the ALP plate.

2 Add 5 µl Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation. Set a 200 µl pipette to 40 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.

Clean Up ALP

1 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add 42.5 µl well-mixed Sample Purification Beads to each well of the ALP plate. Set a 200 µl pipette to 75 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. Illumina recommends the following:

- If using a single channel pipette, vortex the beads after processing four samples
- If using a multichannel pipette, vortex the beads after processing four columns
- If the beads are in a reagent reservoir, mix with a 1000 µl pipette.

3 Incubate the ALP plate at room temperature for 5 minutes.

4 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

5 Remove and discard 80 µl of the supernatant from each well of the ALP plate. Take care not to disturb the beads.

NOTE
Leave the ALP plate on the magnetic stand while performing the following steps 6–10.

6 With the ALP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

7 Incubate the ALP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

8 Repeat steps 6 and 7 one time for a total of two 80% EtOH washes.

9 With the ALP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Remove and discard any remaining EtOH with a 10 µl pipette.

10 With the ALP plate on the magnetic stand, add 52.5 µl Resuspension Buffer to each well of the plate.

11 Remove the ALP plate from the magnetic stand.

12 Resuspend the beads in each well of the ALP plate by repeatedly dispensing the Resuspension Buffer over the bead pellet until it is immersed in the solution. Gently pipette the entire volume up and down 10 times to mix thoroughly.

13 Incubate the ALP plate at room temperature for 2 minutes.

14 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

15 Transfer 50 µl of the clear supernatant from each well of the ALP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CAP barcode. Take care not to disturb the beads.

16 Vortex the Sample Purification Beads until they are well dispersed.

17 Add 50 µl mixed Sample Purification Beads to each well of the CAP plate for a second cleanup. Set a 200 µl pipette to 90 µl, and then gently pipette the entire volume up and down 10 times to mix thoroughly.

18 Incubate the CAP plate at room temperature for 5 minutes.

19 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

20 Remove and discard 95 µl of the supernatant from each well of the CAP plate. Take care not to disturb the beads.

NOTE
Leave the CAP plate on the magnetic stand while performing the following steps 21–25.

21 With the CAP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Take care not to disturb the beads.

22 Incubate the CAP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

23 Repeat steps 21 and 22 one time for a total of two 80% EtOH washes.

24 With the CAP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Remove and discard any remaining EtOH with a 10 µl pipette.

25 With the CAP plate on the magnetic stand, add 27.5 µl Resuspension Buffer to each well of the plate.

26 Remove the CAP plate from the magnetic stand.

27 Resuspend the beads in each well of the CAP plate by repeatedly dispensing the Resuspension Buffer over the bead pellet until it is immersed in the solution. Gently pipette the entire volume up and down 10 times to mix thoroughly.

28 Incubate the CAP plate at room temperature for 2 minutes.

29 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

30 Transfer 25 µl of the clear supernatant from each well of the CAP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the PCR barcode. Take care not to disturb the beads.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Enrich DNA Fragments* on page 36, you can safely stop the protocol here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to 7 days.

Enrich DNA Fragments

This process uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the amount of DNA in the library. The PCR is performed with a PCR Primer Cocktail that anneals to the ends of the adapters. Minimize the number of PCR cycles to avoid skewing the representation of the library.

NOTE
PCR enriches for fragments that have adapters ligated on both ends. Fragments with only one or no adapters on their ends are by-products of inefficiencies in the ligation reaction. Neither species can be used to make clusters. Fragments without any adapters cannot hybridize to surface-bound primers in the flow cell. Fragments with an adapter on only one end can hybridize to surface bound primers, but cannot form clusters.

Consumables

Item	Quantity	Storage	Supplied By
Enhanced PCR Mix (EPM)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina
PCR Primer Cocktail (PPC)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
TSP1 (Target Sample Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR plate	1	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- Remove the Enhanced PCR Mix and PCR Primer Cocktail from -15°C to -25°C storage and thaw them at room temperature.
- Centrifuge the thawed Enhanced PCR Mix and PCR Primer Cocktail tubes to 600 × g for 5 seconds.
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the Sample Purification Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 6 for information on how to access TruSeq Nano DNA Sample Preparation Best Practices on the Illumina website.
- Remove the PCR plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 32.
  - Let it thaw at room temperature.
  - Centrifuge the thawed PCR plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed PCR plate.

- Pre-program the thermal cycler with the following program and save as **PCRNano**:
  - Choose the pre-heat lid option and set to 100°C
  - 95°C for 3 minutes
  - 8 cycles of:
    - 98°C for 20 seconds
    - 60°C for 15 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 4°C

  NOTE
  Illumina recommends 8 cycles of PCR for robust protocol performance.

- Apply a TSP1 barcode label to a new 96-well 0.3 ml PCR plate.

Make PCR

The following procedure assumes the following amount of input DNA sample for library preparation and is designed to result in high library yields:

- 100 ng for a 350 bp insert size
- 200 ng for a 550 bp insert size

1. Add 5 µl thawed PCR Primer Cocktail to each well of the PCR plate.
2. Add 20 µl thawed Enhanced PCR Mix to each well of the PCR plate. Set a 200 µl pipette to 40 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.
3. Seal the PCR plate with a Microseal 'B' adhesive seal.

Amp PCR

1. Place the sealed PCR plate, containing 50 µl of each sample, on the pre-programmed thermal cycler. Close the lid, then select and run **PCRNano** to amplify the plate.
   a. Choose the pre-heat lid option and set to 100°C
   b. 95°C for 3 minutes
   c. 8 cycles of:
      — 98°C for 20 seconds
      — 60°C for 15 seconds
      — 72°C for 30 seconds
   d. 72°C for 5 minutes
   e. Hold at 4°C

Clean Up PCR

1. Centrifuge the PCR plate at 280 × g for 1 minute.
2. Remove the adhesive seal from the PCR plate.
3. Vortex the Sample Purification Beads until they are well dispersed.
4. Do one of the following, depending on the adapter type used:
   - If using the DNA Adapter tubes, add 50 µl mixed Sample Purification Beads to each well of the PCR plate containing 50 µl of the PCR amplified library. Set a 200 µl pipette to 90 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.
   - If using the DAP, add 47.5 µl mixed Sample Purification Beads to each well of the PCR plate containing 50 µl of the PCR amplified library. Set a 200 µl pipette to 90 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.
5. Incubate the PCR plate at room temperature for 5 minutes.
6. Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
7. Remove and discard 95 µl of the supernatant from each well of the PCR plate.

NOTE
Leave the PCR plate on the magnetic stand while performing the following 80% EtOH wash steps (8–10).

8 With the PCR plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

9 Incubate the PCR plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.

10 Repeat steps 8 and 9 one time for a total of two 80% EtOH washes.

11 With the PCR plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Remove and discard any remaining EtOH from each well of the PCR plate with a 10 µl pipette.

12 With the PCR plate on the magnetic stand, add 32.5 µl Resuspension Buffer to each well of the PCR plate.

13 Remove the PCR plate from the magnetic stand.

14 Resuspend the beads in each well of the PCR plate by repeatedly dispensing the Resuspension Buffer over the bead pellet until it is immersed in the solution. Gently pipette the entire volume up and down 10 times to mix thoroughly.

15 Incubate the PCR plate at room temperature for 2 minutes.

16 Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

17 Transfer 30 µl of the clear supernatant from each well of the PCR plate to the corresponding well of the new 0.3 ml PCR plate labeled with the TSP1 barcode.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Validate Library* on page 41, you can safely stop the protocol here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to 7 days.

Validate Library

Illumina recommends performing the following procedures for quality control analysis on your sample library and quantification of the DNA library templates.

Quantify Libraries

To achieve the highest data quality on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of a flow cell. Optimizing cluster densities requires accurate quantitation of DNA library templates. Quantify your libraries using a fluorometric quantification method that uses dsDNA binding dyes or qPCR.

NOTE

TruSeq Nano DNA Sample Prep library quantitation has been validated using the Eco Real-Time PCR System and KAPA Library Quantification Kit specified in the *Consumables and Equipment* on page 95. Follow the KAPA instructions with the KAPA standard. To calculate the library concentration in nM, perform the following insert size adjustment:

- For 350 bp libraries, use 470 bp for the average fragment length
- For 550 bp libraries, use 670 bp for the average fragment length

NOTE

You can download the *KAPA Library Quantification Kits for Illumina sequencing platforms Technical Data Sheet* from the Kapa Biosystems website (www.kapabiosystems.com).

[Optional] Quality Control

To verify the size of your fragments, check the template size distribution. Run samples on a Bioanalyzer for qualitative purposes only.

Do one of the following:

- If using a High Sensitivity DNA chip:
  - Prepare a 1:100 dilution of the DNA library with water.
  - Run 1 µl of the diluted DNA library on an Agilent Technologies 2100 Bioanalyzer.
- If using a DNA 7500 chip, run 1 µl of undiluted DNA library on an Agilent Technologies 2100 Bioanalyzer.

Figure 3 Example TruSeq Nano DNA Sample Preparation 350 bp Insert Library Distribution

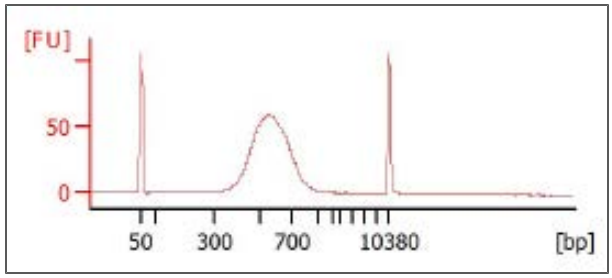
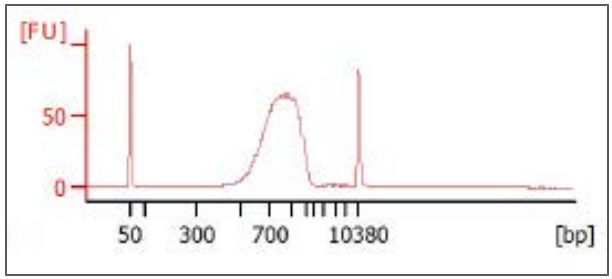


Figure 4 Example TruSeq Nano DNA Sample Preparation 550 bp Insert Library Distribution



Normalize and Pool Libraries

This process describes how to prepare DNA templates for cluster generation. Indexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. DNA libraries not intended for pooling are normalized to 10 nM in the DCT plate.

Consumables

Item	Quantity	Storage	Supplied By
Barcode labels for: • DCT (Diluted Cluster Template) • PDP (Pooled DCT Plate) (for pooling only)	1 label per plate	15°C to 30°C	Illumina
1.7 ml microcentrifuge tube (when processing > 48 samples at a time)	1	15°C to 30°C	User
96-well MIDI plates	2 (second plate for pooling only, if pooling > 40 samples)	15°C to 30°C	User
96-well 0.3 ml PCR plate (for pooling only, if pooling ≤ 40 samples)	1	15°C to 30°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User
Tris-HCl 10 mM, pH8.5 with 0.1% Tween 20	Enough to normalize the concentration of each sample library to 10 nM	15°C to 30°C	User

Preparation

- Remove the TSP1 plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 39.
  - Let it thaw at room temperature.
  - Centrifuge the thawed TSP1 plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.
- Apply a DCT barcode label to a new 96-well MIDI plate.
- [For pooling only] Apply a PDP barcode label to a new 96-well 0.3 ml PCR plate if pooling ≤ 40 samples or a 96-well MIDI plate if pooling > 40 samples.

Make DCT

1. Transfer 10 µl of sample library from each well of the TSP1 plate to the corresponding well of the new MIDI plate labeled with the DCT barcode.
2. Normalize the concentration of sample library in each well of the DCT plate to 10 nM using Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20.

   NOTE
   Depending on the yield quantification data of each sample library, the final volume in the DCT plate can vary from 10–400 µl.
3. Gently pipette the entire normalized sample library volume up and down 10 times to mix thoroughly.
4. Depending on the type of library you want to generate, do one of the following:
   - For non-pooled libraries, the protocol stops here. Do one of the following:
     - Proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina platform.
     - Seal the DCT plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C.
   - For pooled libraries, proceed to *Make PDP (for pooling only)*.

Make PDP (for pooling only)

NOTE
Do not make a PDP plate if you are not pooling samples.

1 Determine the number of samples to be combined together for each pool.

NOTE
Note the sample that is in each well, to avoid pooling two samples with the same index.

2 Do one of the following:

- If pooling 2–24 samples:
  - Transfer 10 µl of each normalized sample library to be pooled from the DCT plate to one well of the new 0.3 ml PCR plate labeled with the PDP barcode.

  The total volume in each well of the PDP plate is 10 X the number of combined sample libraries and 20–240 µl (2–24 libraries). For example, the volume for 2 samples is 20 µl, the volume for 12 samples is 120 µl, or the volume for 24 samples is 240 µl.
- If pooling 25–48 samples:
  - Using a multichannel pipette, transfer 5 µl of each normalized sample library in column 1 of the DCT plate to column 1 of the new 0.3 ml PCR or MIDI plate labeled with the PDP barcode.
  - Transfer 5 µl of each normalized sample library in column 2 of the DCT plate to column 1 of the PDP plate.
  - Repeat the transfer for as many times as there are remaining columns in the DCT plate. The result is a PDP plate with pooled samples in column 1. Gently pipette the entire volume of each well of column 1 up and down 10 times to mix thoroughly.
  - Combine the contents of each well of column 1 into well A2 of the PDP plate for the final pool.
- If pooling 49–96 samples:
  - Using a multichannel pipette, transfer 5 µl of each normalized sample library in column 1 of the DCT plate to column 1 of the new MIDI plate labeled with the PDP barcode.
  - Transfer 5 µl of each normalized sample library in column 2 of the DCT plate to column 1 of the PDP plate.
  - Repeat the transfer for as many times as there are remaining columns in the DCT plate. The result is a PDP plate with pooled samples in column 1. Gently pipette the entire volume of each well of column 1 up and down 10 times to mix thoroughly.
  - Combine the contents of each well of column 1 into a 1.7 ml microcentrifuge tube for the final pool.

3 Gently pipette the entire volume up and down 10 times to mix thoroughly.

4 Do one of the following:
- Proceed to cluster generation. For more information, see the user guide for your Illumina sequencer.
- Do one of the following, depending on the item that contains the final pool:
  - Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C.
  - Cap the 1.7 ml microcentrifuge tube and store at -15°C to -25°C.

High Sample (HS) Protocol

Chapter 3

Introduction

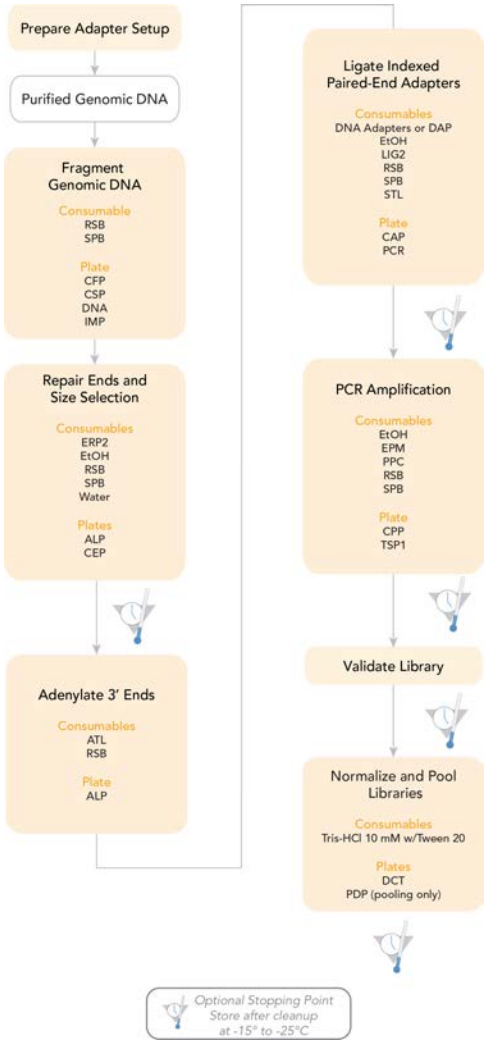
This chapter describes the TruSeq Nano DNA Sample Preparation HS protocol. This protocol is intended for preparing more than 24 samples at one time using either the LT or HT kit.

- Follow the protocols in the order described, using the specified volumes and incubation parameters.
- Review Best Practices before proceeding. See *Additional Resources* on page 6 for information on how to access TruSeq Nano DNA Sample Preparation Best Practices on the Illumina website.
- Review the *TruSeq Sample Preparation Pooling Guide (part # 15042173)* before proceeding. See *Additional Resources* on page 6 for information on how to download the guide from the Illumina website.
- Review Appendix A Supporting Information before proceeding, to confirm your kit contents and make sure that you have obtained all of the requisite equipment and consumables for the HS protocol.
- This HS protocol requires shaking and heating equipment to mix reagents and for incubation (see *User-Supplied Equipment - Additional Items for HS Processing* on page 98).

Sample Prep Workflow

The following figure illustrates the processes of the TruSeq Nano DNA Sample Preparation HS protocol to prepare templates using indexed adapter tubes or a DAP.

Figure 5 TruSeq Nano DNA Sample Preparation HS Workflow



Prepare Adapter Setup

If you are pooling, record information about your samples before beginning library preparation for later use in data analysis.

- Use IEM to create and edit a sample sheet for Illumina sequencers and analysis software. See *Additional Resources* on page 6 for information on how to download IEM software and documentation from the Illumina website.
- Review planning steps in the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 6 for information on how to download the guide from the Illumina website.

If you are pooling using adapter index tubes, Illumina recommends arranging samples that will be combined into a common pool in the same row. Include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.

If you are pooling with the DAP, arrange samples that will be pooled together in the same orientation as the indices in the DAP.

Fragment DNA

This process describes how to optimally fragment the gDNA depending on the downstream application. Covaris shearing generates dsDNA fragments with 3' or 5' overhangs. The fragmentation process is optimized to obtain final libraries with the following average insert sizes:

Table 9 Insert Size Options

Insert Size	350 bp	550 bp
Input DNA Per Sample	100 ng	200 ng
Recommended Read Length	≤ 2 x 101 bp	≤ 2 x 151 bp*

* Read lengths greater than 2 x 151 bp produce a significantly higher percentage of overlapping read-pairs.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	-15°C to -25°C (2°C to 8°C after initial thaw)	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Barcode labels for: • CFP (Covaris Fragmentation Plate) • CSP (Clean Up Sheared DNA Plate) • DNA (DNA Plate) • IMP (Insert Modification Plate)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate	1	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
96-well MIDI plates	3	15°C to 30°C	User
Covaris tubes	1 per sample	15°C to 30°C	User
DNA samples	100 ng per sample for a 350 bp insert size or 200 ng per sample for a 550 bp insert size	-15°C to -25°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User

Preparation

- Review *DNA Input Recommendations* on page 4.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 6 for information on how to access TruSeq Nano DNA Sample Preparation Best Practices on the Illumina website.
- Remove the Sample Purification Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove one tube of Resuspension Buffer from -15°C to -25°C storage and thaw it at room temperature.

  NOTE
  The Resuspension Buffer can be stored at 2°C to 8°C after the initial thaw.

- Turn on the Covaris instrument and follow the manufacturer's guidelines to set up your instrument.
- Calibrate the microplate shaker with a stroboscope and set it to 1800 rpm.
- Apply a CFP barcode label to a new 96-well HSP plate
- Apply a CSP barcode label to a new 96-well MIDI plate.
- Apply a DNA barcode label to a new 96-well MIDI plate.
- Apply an IMP barcode label to a new 96-well MIDI plate.

Make CFP

1 Illumina recommends quantifying gDNA samples using a fluorometric quantification method that uses dsDNA binding dyes.

2 Normalize the gDNA samples with Resuspension Buffer to one of the following in each well of the new 0.3 ml PCR plate labeled with the DNA barcode:
- 100 ng in a final volume of 52.5 µl for a 350 bp insert size
- 200 ng in a final volume of 52.5 µl for a 550 bp insert size

Fragment DNA

1 Shear one of the following amounts of gDNA sample by transferring 52.5 µl of each DNA sample from the DNA plate to a separate, new Covaris tube:
- 100 ng for a 350 bp insert size
- 200 ng for a 550 bp insert size

Use the wells of the new HSP plate labeled with CFP barcode or another device to hold the Covaris tubes upright.

NOTE
Load the DNA sample into the Covaris tube slowly to avoid creating air bubbles. However, air bubbles might not be preventable.

2 Centrifuge the CFP plate at 600 × g for 5 seconds.

3 Fragment the DNA using the following settings:

Table 10 Covaris S220 Settings

Setting	350 bp Insert	550 bp Insert
Duty factor	5%	
Peak Incident Power	175 W	
Cycles per burst	200	
Duration	50 seconds	25 seconds
Mode	Frequency sweeping	
Temperature	5.5° to 6°C	

Table 11 Covaris M220 Settings

Setting	350 bp Insert	550 bp Insert
Duty factor	20%	
Peak Incident Power	50 W	
Cycles per burst	200	
Duration	65 seconds	45 seconds
Temperature	20°C	

NOTE
The Covaris M220 settings are optimized for use with the Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm.

Table 12 Covaris S2 and E210 Settings

Setting	350 bp Insert	550 bp Insert
Duty cycle	10%	
Intensity	5.0	2.0
Cycles per burst	200	
Duration	45 seconds	
Mode	Frequency sweeping	
Displayed Power	S2—23 W	S2—9 W
	E210—14 W	E210—7 W
Temperature	5.5° to 6°C	

4 Centrifuge the CFP plate at 600 × g for 5 seconds.

5 Transfer 50 µl of fragmented DNA from each Covaris tube in the CFP plate to the corresponding well of the new MIDI plate labeled with the CSP barcode, using a single channel pipette.

6 Proceed immediately to *Clean Up Fragmented DNA*.

Clean Up Fragmented DNA

1 Vortex the room temperature Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add 80 µl well-mixed Sample Purification Beads to each well of the CSP plate containing 50 µl of fragmented gDNA. Mix thoroughly as follows:
   a Seal the CSP plate with a Microseal 'B' adhesive seal.
   b Shake the CSP plate on a microplate shaker at 1800 rpm for 2 minutes.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. Illumina recommends the following:
- If using a single channel pipette, vortex the beads after processing four samples
- If using a multichannel pipette, vortex the beads after processing four columns
- If the beads are in a reagent reservoir, mix with a 1000 µl pipette.

NOTE
Keep the Sample Purification Beads tube at room temperature for later use in the protocol.

3 Incubate the CSP plate at room temperature for 5 minutes.

4 Centrifuge the CSP plate at 280 × g for 1 minute.

5 Remove the adhesive seal from the CSP plate.

6 Place the CSP plate on the magnetic stand at room temperature for 8 minutes or until the liquid is clear.

7 Using a 200 µl single channel or multichannel pipette set to 125 µl, remove and discard 125 µl of the supernatant from each well of the CSP plate. Take care not to disturb the beads.

NOTE
Leave the CSP plate on the magnetic stand while performing the following steps 8–12.

8 With the CSP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

9 Incubate the CSP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

10 Repeat steps 8 and 9 one time for a total of two 80% EtOH washes.

11 With the CSP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Remove and discard any remaining EtOH with a 10 µl pipette.

12 With the CSP plate on the magnetic stand, add 62.5 µl Resuspension Buffer to each well of the plate.

13 Remove the CSP plate from the magnetic stand.

14 Mix thoroughly as follows:
   a Seal the CSP plate with a Microseal 'B' adhesive seal.
   b Shake the CSP plate on a microplate shaker at 1800 rpm for 2 minutes.

15 Incubate the CSP plate at room temperature for 2 minutes.

16 Centrifuge the CSP plate at 280 × g for 1 minute.

17 Remove the adhesive seal from the CSP plate.

18 Place the CSP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

19 Transfer 60 µl of the clear supernatant from each well of the CSP plate to the corresponding well of the new MIDI plate labeled with the IMP barcode. Take care not to disturb the beads.

20 Proceed immediately to *Perform End Repair and Size Selection* on page 57.

Perform End Repair and Size Selection

This process converts the overhangs resulting from fragmentation into blunt ends using End Repair Mix 2. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs. Following end repair, the appropriate library size is selected using different ratios of the Sample Purification Beads.

Consumables

Item	Quantity	Storage	Supplied By
End Repair Mix 2 (ERP2)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Barcode labels for: • ALP (Adapter Ligation Plate) • CEP (Clean Up End Repair Plate)	1 label per plate	15°C to 30°C	Illumina
15 ml conical tube (when processing > 6 samples at a time) or 1.7 ml microcentrifuge tube (when processing ≤ 6 samples at a time)	1	15°C to 30°C	User
96-well MIDI plates	2	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
Ice bucket	As needed	-15°C to -25°C	User
Microseal 'B' adhesive seals	5	15°C to 30°C	User
PCR grade water	1 bottle	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the End Repair Mix 2 from -15°C to -25°C storage and thaw it at room temperature. Place the tube on ice.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 6 for information on how to access TruSeq Nano DNA Sample Preparation Best Practices on the Illumina website.
- Make sure that the Sample Purification Beads and Resuspension Buffer are at room temperature.
- Pre-heat the microheating system to 30°C.
- Apply an ALP barcode label to a new 96-well MIDI plate.
- Apply a CEP barcode label to a new 96-well MIDI plate.

Make IMP

1. Centrifuge the thawed End Repair Mix 2 tube at 600 × g for 5 seconds.
2. Add 40 µl End Repair Mix 2 to each well of the IMP plate containing the fragmented DNA. Mix thoroughly as follows:
   a. Seal the IMP plate with a Microseal 'B' adhesive seal.
   b. Shake the IMP plate on a microplate shaker at 1800 rpm for 2 minutes.
3. Centrifuge the IMP plate at 280 × g for 1 minute.

4 Return the End Repair Mix 2 tube to -15°C to -25°C storage.

Incubate IMP

1 Place the sealed IMP plate on the pre-heated microheating system. Close the lid and incubate at 30°C for 30 minutes.

2 Remove the IMP plate from the microheating system and place the plate on ice until you are ready for the next step.

Clean Up IMP and Size Selection

1 Remove the adhesive seal from the IMP plate.

Remove Large DNA Fragments

1 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add the Sample Purification Beads and PCR grade water to one of the following tubes, to create a diluted bead mixture of 160 µl per 100 µl of end-repaired sample:

- New 15 ml conical tube, when processing > 6 samples at a time
- New 1.7 ml microcentrifuge tube, when processing ≤ 6 samples at a time

Determine the volumes using the following formulas, which include 15% excess for multiple samples:

Table 13 Diluted Bead Mixture for a 350 bp Insert Size

	Formula	Example Amount per 12 samples	Your Calculation
Sample Purification Beads	# of samples X 109.25 µl	1311 µl	
PCR grade water	# of samples X 74.75 µl	897 µl	

Table 14 Diluted Bead Mixture for a 550 bp Insert Size

	Formula	Example Amount per 12 samples	Your Calculation
Sample Purification Beads	# of samples X 92 µl	1104 µl	
PCR grade water	# of samples X 92 µl	1104 µl	

3 Vortex the diluted bead mixture for 5 seconds to make sure that the beads are evenly dispersed.

4 Add 160 µl of the diluted bead mixture to each well of the IMP plate containing 100 µl of the end repaired sample. Mix thoroughly as follows:

a Seal the IMP plate with a Microseal 'B' adhesive seal.

b Shake the IMP plate on a microplate shaker at 1800 rpm for 2 minutes.

NOTE

Aspirate the diluted bead mixture slowly and dispense it slowly due to the viscosity of the solution. Changes in the volume of the diluted bead mixture affect the insert size of your library.

NOTE
Vortex the diluted bead mixture frequently. Illumina recommends the following:
- If using a single channel pipette, vortex the mixture after processing four samples
- If using a multichannel pipette, vortex the mixture after processing four columns
- If the mixture is in a reagent reservoir, mix with a 1000 µl pipette.

5 Incubate the IMP plate at room temperature for 5 minutes.

6 Centrifuge the IMP plate at 280 × g for 1 minute.

7 Remove the adhesive seal from the IMP plate.

8 Place the IMP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

9 Use a 200 µl single channel or multichannel pipette set to 125 µl to **transfer 125 µl of the supernatant, containing the DNA of interest, from each well of the IMP plate to the corresponding well of the new MIDI plate labeled with the CEP barcode**. Take care not to disturb the beads.

NOTE
Transfer, do not discard, the supernatant. It contains the DNA of interest.

10 Repeat step 9 one time. **Each CEP plate well now contains a total of 250 µl of DNA of interest.**

11 Discard the IMP plate containing the beads.

12 Discard any remaining diluted bead mixture.

Remove Small DNA Fragments

NOTE
In the following steps, use **undiluted** Sample Purification Beads.

1 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

2 Add 30 µl **undiluted** Sample Purification Beads to each well of the CEP plate containing 250 µl of supernatant with the DNA of interest. Mix thoroughly as follows:
   a Seal the CEP plate with a Microseal 'B' adhesive seal.
   b Shake the CEP plate on a microplate shaker at 1800 rpm for 2 minutes.

NOTE
Aspirate the Sample Purification Beads slowly and dispense them slowly due to the viscosity of the solution. Changes in the volume of the bead mixture affect the insert size of your library.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. Illumina recommends the following:

- If using a single channel pipette, vortex the beads after processing four samples
- If using a multichannel pipette, vortex the beads after processing four columns
- If the beads are in a reagent reservoir, mix with a 1000 µl pipette.

3 Incubate the CEP plate at room temperature for 5 minutes.

4 Centrifuge the CEP plate at 280 × g for 1 minute.

5 Remove the adhesive seal from the CEP plate.

6 Place the CEP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

7 Using a 200 µl single channel or multichannel pipette set to 138 µl, remove and discard 138 µl of the supernatant from each well of the CEP plate. Take care not to disturb the beads.

8 Repeat step 7 one time, removing and discarding a total of 276 µl of supernatant from each well.

NOTE
Leave the CEP plate on the magnetic stand while performing the following steps 9–13.

9 With the CEP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

10 Incubate the CEP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

11 Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.

12 With the CEP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Remove and discard any remaining EtOH with a 10 µl pipette.

13 With the CEP plate on the magnetic stand, add 20 µl Resuspension Buffer to each well of the plate.

14 Remove the CEP plate from the magnetic stand.

15 Mix thoroughly as follows:
   a Seal the CEP plate with a Microseal 'B' adhesive seal.
   b Shake the CEP plate on a microplate shaker at 1800 rpm for 2 minutes.

16 Incubate the CEP plate at room temperature for 2 minutes.

17 Centrifuge the CEP plate at 280 × g for 1 minute.

18 Remove the adhesive seal from the CEP plate.

19 Place the CEP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

20 Transfer 17.5 µl of the clear supernatant from each well of the CEP plate to the corresponding well of the new MIDI plate labeled with the ALP barcode.

SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3' Ends* on page 64 immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to 7 days.

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

Item	Quantity	Storage	Supplied By
A-Tailing Mix (ATL)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Ice bucket	As needed	-15°C to -25°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	2	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	2	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the A-Tailing Mix from -15°C to -25°C storage and thaw it at room temperature:
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.

- Remove the ALP plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up IMP and Size Selection* on page 59.
  - Let it thaw at room temperature.
  - Centrifuge the thawed ALP plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the ALP plate.
- Pre-heat two microheating systems: system 1 to 37°C and system 2 to 70°C.

Add ATL

1. Centrifuge the thawed A-Tailing Mix tube at 600 × g for 5 seconds.
2. Add 12.5 µl thawed A-Tailing Mix to each well of the ALP plate. Mix thoroughly as follows:
   a. Seal the ALP plate with a Microseal 'B' adhesive seal.
   b. Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.
3. Centrifuge the ALP plate at 280 × g for 1 minute.
4. Return the A-Tailing Mix tube to -15° to -25°C storage.

Incubate 1 ALP

1. Place the sealed ALP plate on the pre-heated microheating system 1. Close the lid and incubate at 37°C for 30 minutes.
2. Immediately after the 37°C incubation, remove the ALP plate from system 1 and place the plate on the pre-heated microheating system 2. Close the lid and incubate at 70°C for 5 minutes.
3. Set the microheating system 1 to 30°C in preparation for *Ligate Adapters*.
4. Immediately remove the ALP plate from the microheating system 2 and place the plate on ice for 5 minutes.
5. Proceed immediately to *Ligate Adapters* on page 66.

Ligate Adapters

This process ligates indexing adapters to the ends of the DNA fragments, preparing them for hybridization onto a flow cell.

Consumables

Item	Quantity	Storage	Supplied By

Choose from the following depending on the kit you are using:
• TruSeq Nano DNA LT Sample Prep Kit contents:
  • DNA Adapter Indices (AD001–AD016, AD018–AD023, AD025, AD027)
• TruSeq Nano DNA HT Sample Prep Kit contents:
  • DAP (DNA Adapter Plate)
	1 tube of each index being used, per column of 8 reactions
	or
	1 DAP
		-15°C to -25°C	Illumina

Ligation Mix 2 (LIG2)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Stop Ligation Buffer (STL)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina

Item	Quantity	Storage	Supplied By
Barcode labels for: • CAP (Clean Up ALP Plate) • DAP (DNA Adapter Plate) (if using the HT kit) • PCR (Polymerase Chain Reaction Plate)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	800 µl per sample	15°C to 30°C	User
Ice bucket	As needed	-15°C to -25°C	User
Microseal 'B' adhesive seals	7	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	3–27	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	3–27	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the following from -15°C to -25°C storage and thaw them at room temperature:
  - Appropriate DNA Adapter tubes (depending on the DNA Adapter Indices being used) or the DAP.

NOTE

- Review the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 6 for information on how to download the guide from the Illumina website.
- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that are going to be combined into a common pool in the same row. Also, include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
- When indexing libraries with the DAP, arrange samples that will be pooled together in the same orientation as the indices in the DAP.

NOTE

When indexing libraries with the DAP:

- Review *Handling Adapter Plate* in the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 6 for information on how to download the guide from the Illumina website.
- Illumina recommends that the DAP does not undergo more than four freeze-thaw cycles. To maximize the use of the DAP, process more than 24 samples at a time. These samples can then be pooled in any supported configuration.

- Stop Ligation Buffer

NOTE

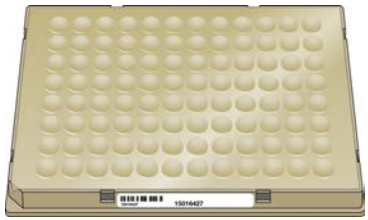
Do not remove the Ligation Mix 2 tube from -15°C to -25°C storage until instructed to do so in the procedures.

- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 6 for information on how to access TruSeq Nano DNA Sample Preparation Best Practices on the Illumina website.
- Remove the Sample Purification Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the microheating system 1 to 30°C.
- Apply a CAP barcode label to a new 96-well MIDI plate.
- Apply a PCR barcode label to a new 96-well HSP plate.

Add LIG

1. Do one of the following:
   - If using DNA Adapter tubes, centrifuge the thawed tubes at 600 × g for 5 seconds.
   - If using a DAP:
     - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to make sure that they all are thawed.
     - Remove the adapter plate tape seal.
     - Centrifuge the plate at 280 × g for 1 minute to collect all of the adapter to the bottom of the well.
     - Remove the plastic cover. Save the cover if you are not processing the entire plate at one time.
     - If it is the first time using this DAP, apply the DAP barcode label to the plate.
2. Centrifuge the Stop Ligation Buffer tube at 600 × g for 5 seconds.
3. Immediately before use, remove the Ligation Mix 2 tube from -15°C to -25°C storage.
4. Remove the adhesive seal from the ALP plate.
5. Add 2.5 µl Resuspension Buffer to each well of the ALP plate.
6. Add 2.5 µl Ligation Mix 2 to each well of the ALP plate.
7. Return the Ligation Mix 2 tube to -15°C to -25°C storage immediately after use.
8. Do one of the following:
   - If using DNA Adapter tubes, add 2.5 µl thawed DNA Adapter Index to each well of the ALP plate.
   - If using a DAP:
     - Place the DAP on the benchtop so that the part number barcode, on the long side of the plate, is facing you and the clipped corner is on the lower left.

Figure 6 Correct DAP Orientation



- Do one of the following to pierce the foil seal:
  - If using the entire plate at one time, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously. Gently, but firmly, press the clean plate over the foil seal.
  - If using only part of the plate, use the bottom of a clean eight-tube strip, with caps attached, to pierce holes in the seals of the wells that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each row or column of adapters that will be used for ligation.
- Using an eight-tip multichannel pipette, transfer 2.5 µl thawed DNA Adapter from the DAP well to each well of the ALP plate.

9 Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

10 Centrifuge the ALP plate at 280 × g for 1 minute.

Incubate 2 ALP

1 Incubate the ALP plate on the pre-heated microheating system, with the lid closed, at 30°C for 10 minutes.

2 Remove the ALP plate from the microheating system and place the plate on ice until you are ready for the next step.

Add STL

1 Remove the adhesive seal from the ALP plate.

2 Add 5 µl Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation mix. Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

3 Centrifuge the ALP plate at 280 × g for 1 minute.

Clean Up ALP

1 Remove the adhesive seal from the ALP plate.

2 Vortex the Sample Purification Beads for at least 1 minute or until they are well dispersed.

3 Add 42.5 µl well-mixed Sample Purification Beads to each well of the ALP plate. Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

NOTE
Vortex the Sample Purification Beads frequently to make sure that they are evenly distributed. Illumina recommends the following:
- If using a single channel pipette, vortex the beads after processing four samples
- If using a multichannel pipette, vortex the beads after processing four columns
- If the beads are in a reagent reservoir, mix with a 1000 µl pipette.

4 Incubate the ALP plate at room temperature for 5 minutes.

5 Centrifuge the ALP plate at 280 × g for 1 minute.

6 Remove the adhesive seal from the ALP plate.

7 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

8 Remove and discard 80 µl of the supernatant from each well of the ALP plate. Take care not to disturb the beads.

NOTE
Leave the ALP plate on the magnetic stand while performing the following steps 9–13.

9 With the ALP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

10 Incubate the ALP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

11 Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.

12 With the ALP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Remove and discard any remaining EtOH with a 10 µl pipette.

13 With the ALP plate on the magnetic stand, add 52.5 µl Resuspension Buffer to each well of the plate.

14 Remove the ALP plate from the magnetic stand.

15 Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

16 Incubate the ALP plate at room temperature for 2 minutes.

17 Centrifuge the ALP plate at 280 × g for 1 minute.

18 Remove the adhesive seal from the ALP plate.

19 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

20 Transfer 50 µl of the clear supernatant from each well of the ALP plate to the corresponding well of the new MIDI plate labeled with the CAP barcode. Take care not to disturb the beads.

21 Vortex the Sample Purification Beads until they are well dispersed.

22 Add 50 µl mixed Sample Purification Beads to each well of the CAP plate. Mix thoroughly as follows:
   a Seal the CAP plate with a Microseal 'B' adhesive seal.
   b Shake the CAP plate on a microplate shaker at 1800 rpm for 2 minutes.

23 Incubate the CAP plate at room temperature for 5 minutes.

24 Centrifuge the CAP plate at 280 × g for 1 minute.

25 Remove the adhesive seal from the CAP plate.

26 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

27 Remove and discard 95 µl of the supernatant from each well of the CAP plate. Take care not to disturb the beads.

NOTE
Leave the CAP plate on the magnetic stand while performing the following steps 28–32.

28 With the CAP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Take care not to disturb the beads.

29 Incubate the CAP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

30 Repeat steps 28 and 29 one time for a total of two 80% EtOH washes.

31 With the CAP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Remove and discard any remaining EtOH with a 10 µl pipette.

32 With the CAP plate on the magnetic stand, add 27.5 µl Resuspension Buffer to each well of the plate.

33 Remove the CAP plate from the magnetic stand.

34 Mix thoroughly as follows:
   a Seal the CAP plate with a Microseal 'B' adhesive seal.
   b Shake the CAP plate on a microplate shaker at 1800 rpm for 2 minutes.

35 Incubate the CAP plate at room temperature for 2 minutes.

36 Centrifuge the CAP plate at 280 × g for 1 minute.

37 Remove the adhesive seal from the CAP plate.

38 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

39 Transfer 25 µl of the clear supernatant from each well of the CAP plate to the corresponding well of the new HSP plate labeled with the PCR barcode. Take care not to disturb the beads.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Enrich DNA Fragments* on page 74, the protocol can be safely stopped here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to 7 days.

Enrich DNA Fragments

This process uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the amount of DNA in the library. The PCR is performed with a PCR Primer Cocktail that anneals to the ends of the adapters. Minimize the number of PCR cycles to avoid skewing the representation of the library.

NOTE
PCR enriches for fragments that have adapters ligated on both ends. Fragments with only one or no adapters on their ends are by-products of inefficiencies in the ligation reaction. Neither species can be used to make clusters. Fragments without any adapters cannot hybridize to surface-bound primers in the flow cell. Fragments with an adapter on only one end can hybridize to surface bound primers, but cannot form clusters.

Consumables

Item	Quantity	Storage	Supplied By
Enhanced PCR Mix (EPM)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina
PCR Primer Cocktail (PPC)	LT kit - 1 tube per 24 reactions or HT kit - 1 tube per 48 reactions	-15°C to -25°C	Illumina
Sample Purification Beads (SPB)	1 tube per 24 reactions	2°C to 8°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Barcode labels for: • CPP (Clean Up PCR Plate) • TSP1 (Target Sample Plate)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate	1	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
96-well MIDI plate	1	15°C to 30°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'A' film	1	15°C to 30°C	User
Microseal 'B' adhesive seals	3	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- Remove the Enhanced PCR Mix and PCR Primer Cocktail from -15°C to -25°C storage and thaw them at room temperature.
- Centrifuge the thawed Enhanced PCR Mix and PCR Primer Cocktail tubes to 600 × g for 5 seconds.
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the Sample Purification Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 6 for information on how to access TruSeq Nano DNA Sample Preparation Best Practices on the Illumina website.
- Remove the PCR plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 70.
  - Let it thaw at room temperature.
  - Centrifuge the thawed PCR plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed PCR plate.

- Pre-program the thermal cycler with the following program and save as **PCRNano**:
  - Choose the pre-heat lid option and set to 100°C
  - 95°C for 3 minutes
  - 8 cycles of:
    - 98°C for 20 seconds
    - 60°C for 15 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 4°C

  NOTE
  Illumina recommends 8 cycles of PCR for robust protocol performance.

- Apply a CPP barcode label to a new 96-well MIDI plate.
- Apply a TSP1 barcode label to a new 96-well HSP plate.

Make PCR

The following procedure assumes the following amount of input DNA sample for library preparation and is designed to result in high library yields:

- 100 ng for a 350 bp insert size
- 200 ng for a 550 bp insert size

1. Add 5 µl thawed PCR Primer Cocktail to each well of the PCR plate.
2. Add 20 µl thawed Enhanced PCR Mix to each well of the PCR plate.
   a. Seal the PCR plate with a Microseal 'A' film.

   WARNING
   Follow vendor instructions for applying Microseal "A" sealing films. Improper use could lead to inefficient sealing (evaporation of sample or cross-contamination) or too efficient sealing (parts of the seal remain in the well after removing the whole seal).

   b. Shake the PCR plate on a microplate shaker at 1600 rpm for 20 seconds.
3. Centrifuge the PCR plate at 280 × g for 1 minute.

Amp PCR

1. Place the sealed PCR plate, containing 50 µl of each sample, on the pre-programmed thermal cycler. Close the lid, then select and run **PCRNano** to amplify the plate.
   a. Choose the pre-heat lid option and set to 100°C
   b. 95°C for 3 minutes
   c. 8 cycles of:
      — 98°C for 20 seconds
      — 60°C for 15 seconds
      — 72°C for 30 seconds
   d. 72°C for 5 minutes
   e. Hold at 4°C

Clean Up PCR

1. Centrifuge the PCR plate at 280 × g for 1 minute.
2. Remove the adhesive seal from the PCR plate.
3. Vortex the Sample Purification Beads until they are well dispersed.
4. Do one of the following, depending on the adapter type used:
   - If using the DNA Adapter tubes, add 50 µl mixed Sample Purification Beads to each well of the new MIDI plate labeled with the CPP barcode.
   - If using the DAP, add 47.5 µl mixed Sample Purification Beads to each well of the new MIDI plate labeled with the CPP barcode.
5. Transfer the entire contents from each well of the PCR plate to the corresponding well of the CPP plate containing 50 µl mixed Sample Purification Beads. Mix thoroughly as follows:
   a. Seal the CPP plate with a Microseal 'B' adhesive seal.
   b. Shake the CPP plate on a microplate shaker at 1800 rpm for 2 minutes.
6. Incubate the CPP plate at room temperature for 5 minutes.
7. Centrifuge the CPP plate at 280 × g for 1 minute.
8. Remove the adhesive seal from the CPP plate.

9 Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

10 Remove and discard 95 µl of the supernatant from each well of the CPP plate.

NOTE
Leave the CPP plate on the magnetic stand while performing the following 80% EtOH wash steps (11–13).

11 With the CPP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.

12 Incubate the CPP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.

13 Repeat steps 11 and 12 one time for a total of two 80% EtOH washes.

14 With the CPP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes. Remove and discard any remaining EtOH from each well of the CPP plate with a 10 µl pipette.

15 With the CPP plate on the magnetic stand, add 32.5 µl Resuspension Buffer to each well of the CPP plate.

16 Remove the CPP plate from the magnetic stand.

17 Mix thoroughly as follows:
   a Seal the CPP plate with a Microseal 'B' adhesive seal.
   b Shake the CPP plate on a microplate shaker at 1800 rpm for 2 minutes.

18 Incubate the CPP plate at room temperature for 2 minutes.

19 Centrifuge the CPP plate at 280 × g for 1 minute.

20 Remove the adhesive seal from the CPP plate.

21 Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

22 Transfer 30 µl of the clear supernatant from each well of the CPP plate to the corresponding well of the new HSP plate labeled with the TSP1 barcode.

SAFE STOPPING POINT
If you do not plan to proceed immediately to *Validate Library* on page 79, you can safely stop the protocol here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to 7 days.

Validate Library

Illumina recommends performing the following procedures for quality control analysis on your sample library and quantification of the DNA library templates.

Quantify Libraries

To achieve the highest data quality on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of a flow cell. Optimizing cluster densities requires accurate quantitation of DNA library templates. Quantify your libraries using a fluorometric quantification method that uses dsDNA binding dyes or qPCR.

NOTE

TruSeq Nano DNA Sample Prep library quantitation has been validated using the Eco Real-Time PCR System and KAPA Library Quantification Kit specified in the *Consumables and Equipment* on page 95. Follow the KAPA instructions with the KAPA standard. To calculate the library concentration in nM, perform the following insert size adjustment:

- For 350 bp libraries, use 470 bp for the average fragment length
- For 550 bp libraries, use 670 bp for the average fragment length

NOTE

You can download the *KAPA Library Quantification Kits for Illumina sequencing platforms Technical Data Sheet* from the Kapa Biosystems website (www.kapabiosystems.com).

[Optional] Quality Control

To verify the size of your fragments, check the template size distribution. Run samples on a Bioanalyzer for qualitative purposes only.

Do one of the following:

- If using a High Sensitivity DNA chip:
  - Prepare a 1:100 dilution of the DNA library with water.
  - Run 1 µl of the diluted DNA library on an Agilent Technologies 2100 Bioanalyzer.
- If using a DNA 7500 chip, run 1 µl of undiluted DNA library on an Agilent Technologies 2100 Bioanalyzer.

Figure 7 Example TruSeq Nano DNA Sample Preparation 350 bp Insert Library Distribution

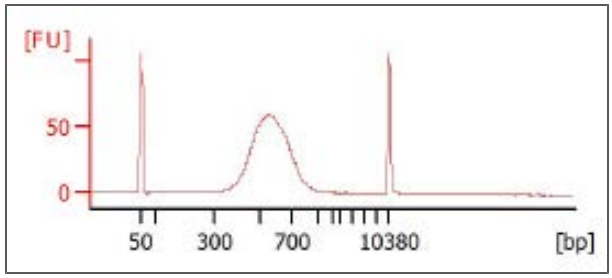
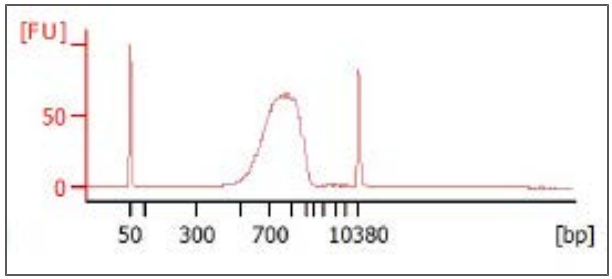


Figure 8 Example TruSeq Nano DNA Sample Preparation 550 bp Insert Library Distribution



Normalize and Pool Libraries

This process describes how to prepare DNA templates for cluster generation. Indexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. DNA libraries not intended for pooling are normalized to 10 nM in the DCT plate.

Consumables

Item	Quantity	Storage	Supplied By
Barcode labels for: • DCT (Diluted Cluster Template) • PDP (Pooled DCT Plate) (for pooling only)	1 label per plate	15°C to 30°C	Illumina
1.7 ml microcentrifuge tube (when processing > 48 samples at a time)	1	15°C to 30°C	User
96-well MIDI plates	2 (second plate for pooling only, if pooling > 40 samples)	15°C to 30°C	User
96-well 0.3 ml PCR plate (for pooling only, if pooling ≤ 40 samples)	1	15°C to 30°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User
Tris-HCl 10 mM, pH8.5 with 0.1% Tween 20	Enough to normalize the concentration of each sample library to 10 nM	15°C to 30°C	User

Preparation

- Remove the TSP1 plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 39.
  - Let it thaw at room temperature.
  - Centrifuge the thawed TSP1 plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.
- Apply a DCT barcode label to a new 96-well MIDI plate.
- [For pooling only] Apply a PDP barcode label to a new 96-well 0.3 ml PCR plate if pooling ≤ 40 samples or a 96-well MIDI plate if pooling > 40 samples.

Make DCT

1. Transfer 10 µl of sample library from each well of the TSP1 plate to the corresponding well of the new MIDI plate labeled with the DCT barcode.
2. Normalize the concentration of sample library in each well of the DCT plate to 10 nM using Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20.

   NOTE
   Depending on the yield quantification data of each sample library, the final volume in the DCT plate can vary from 10–400 µl.
3. Mix the DCT plate as follows:
   a. Seal the DCT plate with a Microseal 'B' adhesive seal.
   b. Shake the DCT plate on a microplate shaker at 1000 rpm for 2 minutes.
4. Centrifuge the DCT plate at 280 × g for 1 minute.
5. Remove the adhesive seal from the DCT plate.
6. Depending on the type of library you want to generate, do one of the following:
   - For non-pooled libraries, the protocol stops here. Do one of the following:
     - Proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina platform.
     - Seal the DCT plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C.
   - For pooled libraries, proceed to *Make PDP (for pooling only)*.

Make PDP (for pooling only)

NOTE
Do not make a PDP plate if you are not pooling samples.

1 Determine the number of samples to be combined together for each pool.

NOTE
Make a note of which sample goes into which well, to avoid pooling two samples with the same index.

2 Do one of the following:

- If pooling 2–24 samples:
  - Transfer 10 µl of each normalized sample library to be pooled from the DCT plate to one well of the new MIDI plate labeled with the PDP barcode.
  - The total volume in each well of the PDP plate is 10X the number of combined sample libraries and 20–240 µl (2–24 libraries). For example, the volume for 2 samples is 20 µl, the volume for 12 samples is 120 µl, or the volume for 24 samples is 240 µl.
- If pooling 25–48 samples:
  - Using a multichannel pipette, transfer 5 µl of each normalized sample library in column 1 from the DCT plate to column 1 of the new MIDI plate labeled with the PDP barcode.
  - Transfer 5 µl of each normalized sample library in column 2 of the DCT plate to column 1 of the PDP plate.
  - Repeat the transfer for as many times as there are remaining columns in the DCT plate. The result is a PDP plate with pooled samples in column 1. Mix the PDP plate as follows:
    - Seal the PDP plate with a Microseal 'B' adhesive seal.
    - Shake the PDP plate on a microplate shaker at 1800 rpm for 2 minutes.
  - Centrifuge the PDP plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the PDP plate.
  - Combine the contents of each well of column 1 into well A2 of the PDP plate for the final pool.

- If pooling 49–96 samples:
  - Using a multichannel pipette, transfer 5 µl of each normalized sample library in column 1 of the DCT plate to column 1 of the new MIDI plate labeled with the PDP barcode.
  - Transfer 5 µl of each normalized sample library in column 2 of the DCT plate to column 1 of the PDP plate.
  - Repeat the transfer for as many times as there are remaining columns in the DCT plate. The result is a PDP plate with pooled samples in column 1. Mix the PDP plate as follows:
    - Seal the PDP plate with a Microseal 'B' adhesive seal.
    - Shake the PDP plate on a microplate shaker at 1800 rpm for 2 minutes.
  - Centrifuge the PDP plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the PDP plate.
  - Combine the contents of each well of column 1 into a 1.7 ml microcentrifuge tube for the final pool.

3 Mix as follows, depending on the item that contains the final pool:
- For the PDP plate:
  - Seal the PDP plate with a Microseal 'B' adhesive seal.
  - Shake the PDP plate on a microplate shaker at 1800 rpm for 2 minutes.
  - Centrifuge the PDP plate at 280 × g for 1 minute.
- For the 1.7 ml microcentrifuge tube:
  - Cap the tube.
  - Vortex the tube to mix thoroughly.

4 Do one of the following:
- Proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina platform.
- Store the sealed PDP plate or capped 1.7 ml microcentrifuge tube at -15°C to -25°C.

Supporting Information

Appendix A

Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed your kit contents, and obtained all of the requisite consumables and equipment.

Acronyms

Table 15 TruSeq Nano DNA Sample Preparation Acronyms

Acronym	Definition
ALP	Adapter Ligation Plate
ATL	A-Tailing Mix
CAP	Clean Up ALP Plate
CEP	Clean Up End Repair Plate
CFP	Covaris Fragmentation Plate
CPP	Clean Up PCR Plate
CSP	Clean Up Sheared DNA Plate
DAP	DNA Adapter Plate
DCT	Diluted Cluster Template
DNA	Customer Sample DNA Plate
dsDNA	double-stranded DNA
EPM	Enhanced PCR Mix
ERP2	End Repair Mix 2
EUC	Experienced User Card
gDNA	genomic DNA
HSP	Hard-Shell Plate
HS	High Sample
HT	High Throughput

Acronym	Definition
IEM	Illumina Experiment Manager
IMP	Insert Modification Plate
LIG2	Ligation Mix 2
LS	Low Sample
LT	Low Throughput
LTF	Lab Tracking Form
PCR	Polymerase Chain Reaction
PDP	Pooled Dilution Plate
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
SPB	Sample Purification Beads
STL	Stop Ligation Buffer
TSP	Target Sample Plate

Kit Contents

Check to make sure that you have all of the reagents identified in this section before starting the TruSeq Nano DNA Sample Preparation protocol. The TruSeq Nano DNA LT Sample Prep Kits are available as Set A and B. Each TruSeq Nano DNA LT Sample Prep Kit contains enough reagents to prepare up to 24 samples. When used together, TruSeq Nano DNA LT Sample Prep Kits A and B allow for pooling up to 24 samples using the 12 different indices in each kit.

Table 16 TruSeq Nano DNA Sample Prep Kits

Kit Name	Catalog #	Number of Samples Supported	Number of Indices
TruSeq Nano DNA LT Sample Prep Kit - Set A	FC-121-4001	24	12
TruSeq Nano DNA LT Sample Prep Kit - Set B	FC-121-4002	24	12
TruSeq Nano DNA HT Sample Prep Kit	FC-121-4003	96	96

TruSeq Nano DNA LT Sample Prep Kit

The TruSeq Nano DNA LT Sample Prep Kit contains two boxes: a Set A or Set B box and an SP Beads box.

24 Samples - Set A or Set B Box

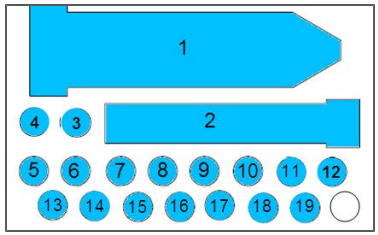
You receive either box A or B with the kit depending on the set you ordered. These boxes also contain plate barcode labels.

Store at -15°C to -25°C

These boxes are shipped on dry ice. As soon as you receive them, store the following components at -15°C to -25°C.

Set A

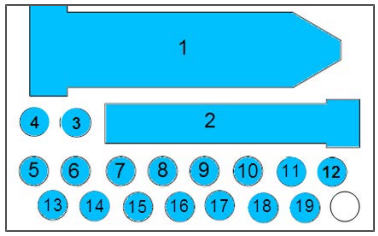
Figure 9 TruSeq Nano DNA LT Sample Prep Kit, 24 Samples-Set A (Box 1 of 2), part # 15041757



Slot	Reagent	Part #	Description
1	RSB	15026770	Resuspension Buffer
2	ERP2	15036418	End Repair Mix 2
3	ATL	15012495	A-Tailing Mix
4	LIG2	15036183	Ligation Mix 2
5	STL	15012546	Stop Ligation Buffer
6	PPC	15031748	PCR Primer Cocktail
7	EPM	15041700	Enhanced PCR Mix
8	AD002	15026621	DNA Adapter Index 2
9	AD004	15026623	DNA Adapter Index 4
10	AD005	15026624	DNA Adapter Index 5
11	AD006	15026625	DNA Adapter Index 6
12	AD007	15026627	DNA Adapter Index 7
13	AD012	15026632	DNA Adapter Index 12
14	AD013	15024641	DNA Adapter Index 13
15	AD014	15024642	DNA Adapter Index 14
16	AD015	15024643	DNA Adapter Index 15
17	AD016	15024644	DNA Adapter Index 16
18	AD018	15024646	DNA Adapter Index 18
19	AD019	15024647	DNA Adapter Index 19

Set B

Figure 10 TruSeq Nano DNA LT Sample Prep Kit, 24 Samples-Set B (Box 1 of 2), part # 15041759



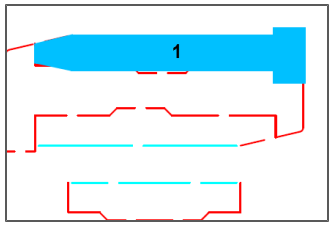
Slot	Reagent	Part #	Description
1	RSB	15026770	Resuspension Buffer
2	ERP2	15036418	End Repair Mix 2
3	ATL	15012495	A-Tailing Mix
4	LIG2	15036183	Ligation Mix 2
5	STL	15012546	Stop Ligation Buffer
6	PPC	15031748	PCR Primer Cocktail
7	EPM	15041700	Enhanced PCR Mix
8	AD001	15026620	DNA Adapter Index 1
9	AD003	15026622	DNA Adapter Index 3
10	AD008	15026628	DNA Adapter Index 8
11	AD009	15026629	DNA Adapter Index 9
12	AD010	15026630	DNA Adapter Index 10
13	AD011	15026631	DNA Adapter Index 11
14	AD020	15024648	DNA Adapter Index 20
15	AD021	15024649	DNA Adapter Index 21
16	AD022	15024650	DNA Adapter Index 22
17	AD023	15024651	DNA Adapter Index 23
18	AD025	15024653	DNA Adapter Index 25
19	AD027	15024654	DNA Adapter Index 27

24 Samples - SP Beads Box

Store at 2°C to 8°C

This box is shipped at 2°C to 8°C. As soon as you receive it, store the components at 2°C to 8°C.

Figure 11 TruSeq Nano DNA LT Sample Prep Kit, 24 Samples SP Beads (Box 2 of 2), part # 15041758



Slot	Reagent	Part #	Description
1	SPB	15041032	Sample Purification Beads

TruSeq Nano DNA HT Sample Prep Kit

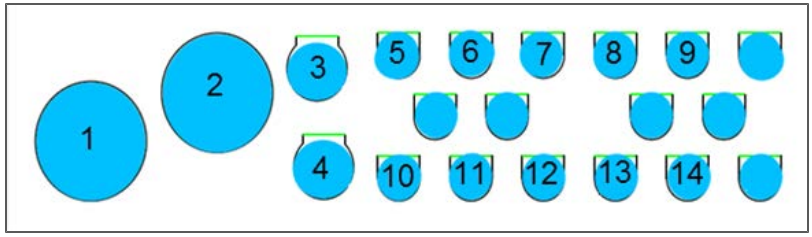
The TruSeq Nano DNA HT Sample Prep Kit contains three boxes: a core reagent box, an Adapter Plate box, and an SP Beads box.

96 Samples - Core Reagents Box

Store at -15°C to -25°C

This box is shipped on dry ice. As soon as you receive it, store the following components at -15°C to -25°C. This box also contains plate barcode labels.

Figure 12 TruSeq Nano DNA HT Sample Prep Kit, 96 Samples (Box 1 of 2), part # 15041877



Slot	Reagent	Part #	Description
1–2	RSB	15026770	Resuspension Buffer
3–4	ERP2	15036182	End Repair Mix 2
5–6	ATL	15012495	A-Tailing Mix
7–8	LIG2	15036184	Ligation Mix 2
9–10	STL	15012546	Stop Ligation Buffer
11–12	PPC	15031748	PCR Primer Cocktail
13–14	EPM	15041027	Enhanced PCR Mix

96 Samples - Adapter Plate Box

Store at -15°C to -25°C

This box is shipped on dry ice. As soon as you receive it, store the contents at -15°C to -25°C.

Figure 13 TruSeq Nano DNA HT Sample Prep Kit, 96, Adapter Plate Box, part # 15032317

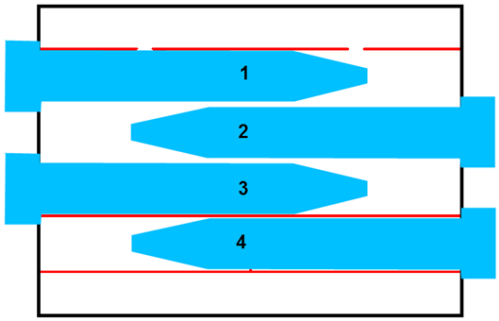
Slot	Reagent	Part #	Description
1	DAP	15016426	DNA Adapter Plate, 96plex

96 Samples - SP Beads Box

Store at 2°C to 8°C

This box is shipped at 2°C to 8°C. As soon as you receive it, store the components at 2°C to 8°C.

Figure 14 TruSeq Nano DNA HT Sample Prep Kit, 96 Samples SP Beads (Box 2 of 2), part # 15041878



Slot	Reagent	Part #	Description
1–4	SPB	15041032	Sample Purification Beads

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before starting the TruSeq Nano DNA Sample Preparation protocol. The requirement for some supplies is dependent upon the protocol performed (LS or HS) and these items are specified in separate tables.

NOTE
The TruSeq Nano DNA Sample Preparation protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Table 17 User-Supplied Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
15 ml conical tubes	General lab supplier
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
20 µl barrier pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier

Consumable	Supplier
96-well storage plates, round well, 0.8 ml ("MIDI" plate)	Fisher Scientific, part # AB-0859
Distilled water	General lab supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
[Optional] Fluorometric quantitation with dsDNA binding dye reagents	General lab supplier
Ice bucket	General lab supplier
[Optional] KAPA Library Quantification Kit - Illumina/Universal	KAPA Biosystems, part # KK4824
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
microTUBE AFA Fiber 6x16mm with • Crimp-Cap, or • Pre-Slit Snap-Cap (for use with Covaris M220)	Covaris, • part # 520052, or • part # 520045
PCR grade water	General lab supplier
RNaseZap (to decontaminate surfaces)	General lab supplier
RNase/DNase-free eight-tubes strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Tris-HCl 10 mM, pH 8.5	General lab supplier
Tween 20	Sigma-Aldrich, part # P7949

Table 18 User-Supplied Consumables - Additional Items for LS Processing

Consumable	Supplier
96-well 0.3 ml skirtless PCR plates, or Twin.tec 96-well PCR plates	E&K Scientific, part # 480096 Eppendorf, part # 951020303

Table 19 User-Supplied Consumables - Additional Items for HS Processing

Consumable	Supplier
Hard-Shell 96-well PCR Plates ("HSP" plate)	Bio-Rad, part # HSP-9601

Table 20 User-Supplied Equipment

Equipment	Supplier
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
[Optional] Agilent DNA 7500 Kit	Agilent, part # 5067-1506
[Optional] Agilent High Sensitivity DNA Kit	Agilent, part # 5067-4626
One of the following Covaris systems: • S2 • S220 • E210 • M220	Covaris M220, part # 500295 For all other models, contact Covaris
[Optional] Eco™ Real-Time PCR System	Illumina, catalog #: EC-100-1000 (110 V) EC-100-1001 (220 V)
[Optional] Fluorometer for quantitation with dsDNA binding dyes	General lab supplier
Magnetic stand-96	Life Technologies, catalog # AM10027
Microplate centrifuge	General lab supplier
Vortexer	General lab supplier

Table 21 User-Supplied Equipment - Additional Items for LS Processing

Equipment	Supplier
96-well thermal cycler (with heated lid) See *Thermal Cyclers* on page 99.	General lab supplier

Table 22 User-Supplied Equipment - Additional Items for HS Processing

Equipment	Supplier
High-Speed Microplate Shaker	VWR, catalog # 13500-890 (110 V/120 V) VWR, catalog # 14216-214 (230 V)
MIDI plate insert for heating system Note: Two inserts are recommended to support successive heating procedures.	Illumina, catalog # BD-60-601
Stroboscope	General lab supplier
SciGene TruTemp Heating System Note: Two systems are recommended to support successive heating procedures.	Illumina, catalog # SC-60-503 (115 V) Illumina, catalog # SC-60-504 (220 V)

Thermal Cyclers

The following table lists the recommended settings for the Illumina recommended thermal cycler, as well as other comparable models. If your lab has a thermal cycler that is not listed, validate the thermal cycler before performing the TruSeq Nano DNA Sample Preparation protocol.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad DNA Engine Tetrad 2	Calculated	Heated, constant at 100°C	Plate
MJ Research PTC-225 DNA Engine Tetrad	Calculated	Heated, constant at 100°C	Plate
Bio-Rad S1000	N/A	Heated, constant at 100°C	Plate

Indexed Adapter Sequences

This section details the indexed adapter sequences.

TruSeq Nano DNA LT Sample Prep Kit Indexed Adapter Sequences

The TruSeq Nano DNA LT Sample Prep Kit contains the following indexed adapter sequences.

NOTE

- The index numbering is not contiguous. There is no Index 17, 24, or 26.
- The base in parentheses () indicates the base for the seventh cycle and is not considered as part of the index sequence. Record the index in the sample sheet as only six bases. For indices 13 and above, the seventh base (in parentheses) might not be A, which is seen in the seventh cycle of the index read.
- For more information on the number of cycles used to sequence the index read, reference your instrument user guide.

Table 23 TruSeq Nano DNA LT Sample Prep Kit Set A Indexed Adapter Sequences

Adapter	Sequence	Adapter	Sequence
AD002	CGATGT(A)	AD013	AGTCAA(C)
AD004	TGACCA(A)	AD014	AGTTCC(G)
AD005	ACAGTG(A)	AD015	ATGTCA(G)
AD006	GCCAAT(A)	AD016	CCGTCC(C)
AD007	CAGATC(A)	AD018	GTCCGC(A)
AD012	CTTGTA(A)	AD019	GTGAAA(C)

Table 24 TruSeq Nano DNA LT Sample Prep Kit Set B Indexed Adapter Sequences

Adapter	Sequence	Adapter	Sequence
AD001	ATCACG(A)	AD020	GTGGCC(T)
AD003	TTAGGC(A)	AD021	GTTTCG(G)
AD008	ACTTGA(A)	AD022	CGTACG(T)
AD009	GATCAG(A)	AD023	GAGTGG(A)
AD010	TAGCTT(A)	AD025	ACTGAT(A)
AD011	GGCTAC(A)	AD027	ATTCCT(T)

TruSeq Nano DNA HT Sample Prep Kit Indexed Adapter Sequences

The DAP in the TruSeq Nano DNA HT Sample Prep Kit contains the following indexed adapter sequences:

NOTE

The Index recorded in the sample sheet is the full 8 bases and 8 bases are sequenced per indexed read.

Table 25 TruSeq Nano DNA HT Sample Prep Kit Indexed Adapter 1 Sequences

Adapter	Sequence	Adapter	Sequence
D701	ATTACTCG	D707	CTGAAGCT
D702	TCCGGAGA	D708	TAATGCGC
D703	CGCTCATT	D709	CGGCTATG
D704	GAGATTCC	D710	TCCGCGAA
D705	ATTCAGAA	D711	TCTCGCGC
D706	GAATTCGT	D712	AGCGATAG

Table 26 TruSeq Nano DNA HT Sample Prep Kit Indexed Adapter 2 Sequences

Adapter	Sequence	Adapter	Sequence
D501	TATAGCCT	D505	AGGCGAAG
D502	ATAGAGGC	D506	TAATCTTA
D503	CCTATCCT	D507	CAGGACGT
D504	GGCTCTGA	D508	GTACTGAC

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Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 27 Illumina General Contact Information

Illumina Website	www.illumina.com
Email	techsupport@illumina.com

Table 28 Illumina Customer Support Telephone Numbers

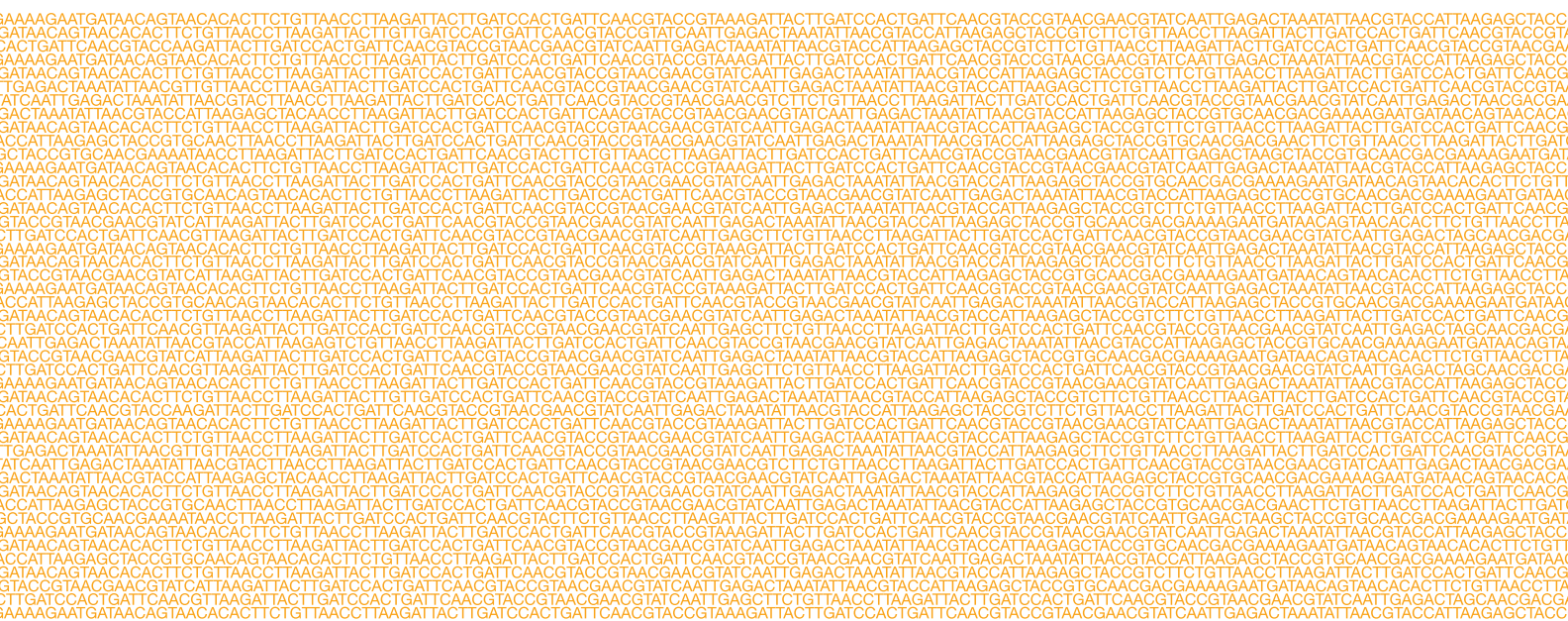
Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

Safety Data Sheets

Safety data sheets (SDSs) are available on the Illumina website at www.illumina.com/msds.

Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support, select a product, then click **Documentation & Literature**.



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