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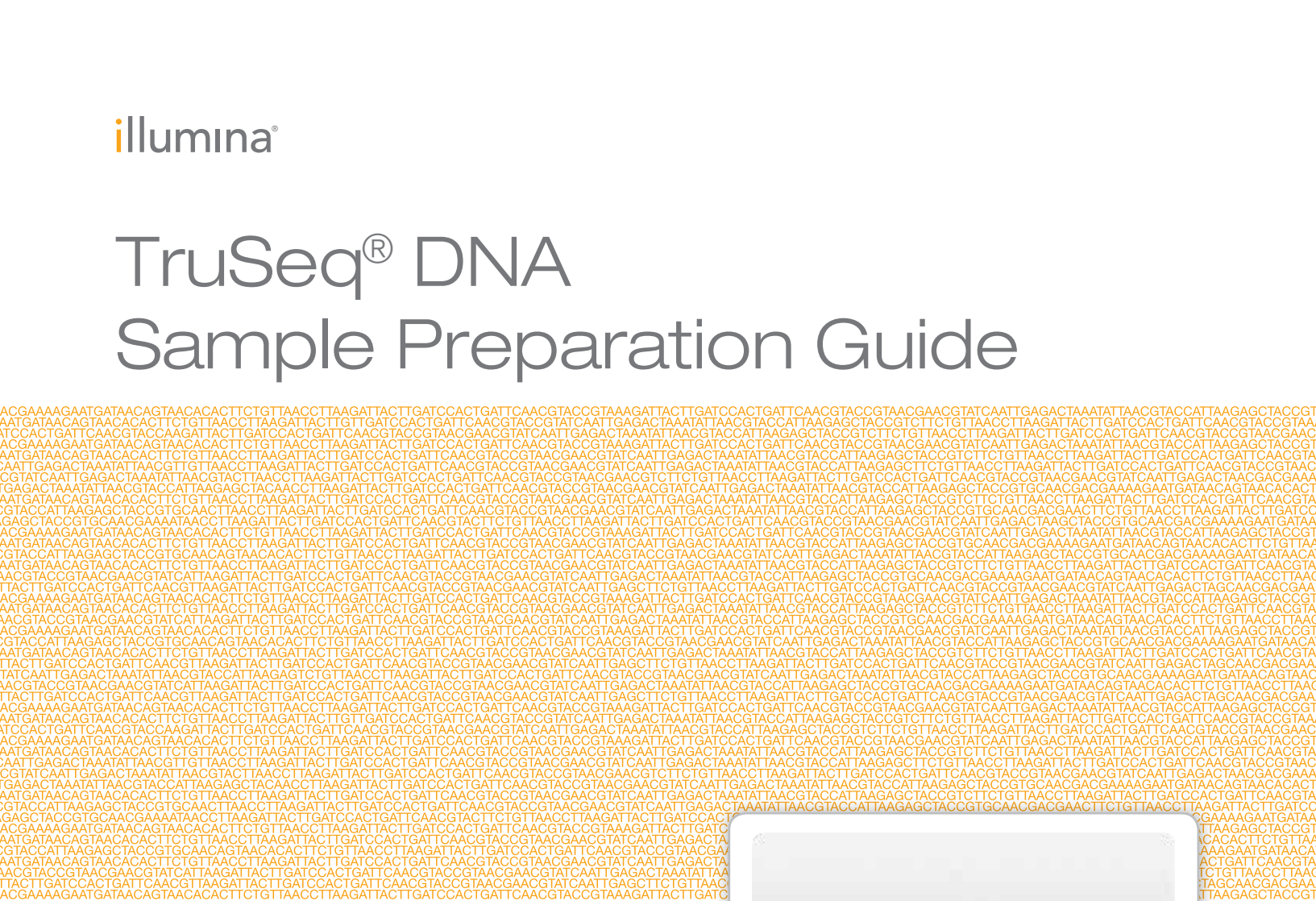
TruSeq® DNA Sample Preparation Guide



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Part # 15026486 Rev. C
July 2012

Revision History

Part #	Revision	Date	Description of Change
15026486	C	July 2012	• Added reagent volume table to *Usage Guidelines* • Revised *Tracking Tools* documentation download information • Removed detailed Sample Sheet description from *Tracking Tools* • Added instructions for which assay to select when using the Illumina Experiment Manager • Updated Dual-Indexed Sequencing Platform Compatibility table

15026486	B	April 2012	• *TruSeq DNA Sample Prep v2 Kit* renamed *TruSeq DNA LT Sample Prep Kit*
			• New *TruSeq DNA HT Sample Prep Kit* containing enough reagents for 96 samples and a DNA Adapter Plate (DAP). Information on the new DAP is in included in the following sections:
			  • *Usage Guidelines*
			  • *Indexed Adapter Sequences*
			  • *Adapter Options*
			  • *Pooling Guidelines*
			  • *Ligate Adapters* procedures
			  • *Enrich DNA Fragments* procedures
			• *Low Throughput (LT)* protocol renamed *Low Sample (LS)* protocol
			• *High Throughput (HT)* protocol renamed *High Sample (HS)* protocol
			• *TruSeq DNA Sample Prep v2* documentation renamed *TruSeq DNA Sample Prep* to encompass both the LT and HT kits and their protocols
			• Removed *TruSeq DNA Sample Prep* guide catalog number
			• Guide content emphasizes the gel-free option is not used for standard (whole-genome) sequencing and TruSeq Enrichment refers to additional kits used for sequencing targeted regions
			• *Best Practices* section changes:
			  • Renamed *AMPure XP Handling* to *Handling Magnetic Beads*
			  • Clarified *Usage Guidelines*
			  • Added *Adapter Option*, *Pooling Guidelines (for low-plexity index combinations)*, and *Equipment* sections

Part #	Revision	Date	Description of Change

15026486	B (continued)	April 2012	• *Tracking Tools* section changes:
			  • Removed sample sheet format guidelines and direct reader to sequencing analysis software user guide for detailed sample sheet guidelines
			  • *Illumina Experiment Manager* introduced
			• Added kit box A or B to Adapter Index Sequences table
			• *Kit Contents* section changes:
			  • Corrected TruSeq DNA LT Sample Prep Kit catalog numbers
			  • Added number of indices to top level kit table
			  • TruSeq DNA LT Sample Prep Kit part numbers added
			  • PCR Primer Cocktail part number changed
			  • New TruSeq DNA HT Sample Prep Kit included
			• *User-Supplied Equipment* section changes:
			  • Revised dark reader transilluminator catalog number
			  • Revised Magnetic stand-96 supplier to Life Technologies
			  • Updated recommended Covaris equipment with currently available products and revised system settings in Fragment DNA procedures
			• Removed instructions throughout protocol to "take care not to disturb the beads" and "change the tip after each sample", instead adding a note in the introduction section of each protocol to review the *Best Practices* on page 11
			• Removed instructions throughout LS protocol to adjust pipette to a specified volume before mixing.
			• Removed steps in the procedures to pre-heat the thermal cycler lid and thermal cycler program details, since thermal cycler programming and lid heating instructions are provided in the preparation section of each procedure
			• Changed "multiplexed" to "indexed" throughout documentation to refer to both dual-indexing and single-indexing
			• Revised *Ligate Adapters* procedures to include the new DAP component of the *TruSeq DNA HT Sample Prep Kit*
			• Specified using the 12-well comb included with the recommended gel system in *Purify Ligation Products* procedures

Part #	Revision	Date	Description of Change
15026486	B (continued)	April 2012	• *Enrich DNA Fragments*: • Removed "one tube each" following PMM and PPC specified in preparation steps, since 1 tube per 48 reactions is required • Named the thermal cycler program **PCR** • *Make PCR* (LS protocol) - mixing step modified to adjust pipette to 50 µl • *Clean Up PCR*- AMPure XP Beads quantity differs if using adapter index tubes or a DAP • *Make PDP* - Updated pooling procedures to include 2–96 libraries
15026486	A	August 2011	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® DNA Sample Preparation 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

Gel-free option for TruSeq Enrichment with optimized AMPure XP bead conditions

Optimized shearing for whole-genome resequencing

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

Compatibility with low-throughput (LT) and high-throughput (HT) kit configurations

High Throughput

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

Advanced Troubleshooting

- Process control checks built-in for quality control

Index Adapter Tags All Samples

- Additional adapters and primers not necessary
- Enables indexing earlier in the process
- The TruSeq DNA LT Sample Prep Kit contains adapter index tubes recommended for preparing and pooling 24 or fewer samples for sequencing
- The TruSeq 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

NOTE

- TruSeq Enrichment refers to the Illumina TruSeq Exome Enrichment and TruSeq Custom Enrichment Kits that can be used following TruSeq DNA Sample Prep to prepare the library for sequencing targeted regions. For more information, see the *TruSeq Enrichment Guide*.
- The gel-free method is ***not*** an option when preparing libraries for standard (whole-genome) sequencing.

The protocol is compatible with no indexing or a lower indexing pooling level. The libraries generated do not require PCR amplification to enable cluster generation, although PCR is recommended in the standard protocol to robustly meet the yield requirements of most standard applications.

Audience and Purpose

This guide documents the sample preparation protocol using the Illumina TruSeq DNA LT Sample Prep Kit or TruSeq DNA HT Sample Prep Kit.

- Chapter 3 Low Sample (LS) Protocol explains how to perform the TruSeq DNA Sample Preparation using the Low Sample (LS) Protocol
- Chapter 4 High Sample (HS) Protocol explains how to perform the TruSeq DNA Sample Preparation using the High Sample (HS) Protocol

Equivalent results can be expected from either protocol and their distinguishing elements are as follows:

Table 1 Protocol Features

	Low Sample	High Sample
LT Kit - Number of samples processed at one time	≤ 48 with indexed adapter tubes	> 48 with indexed adapter tubes
HT Kit - Number of samples processed at one time	≤ 48 with indexed adapter plate	> 48 with indexed adapter plate
Plate Type	96-well 0.3 ml PCR 96-well MIDI	96-well HSP 96-well MIDI
Incubation Equipment	96-well thermal cycler	96-well thermal cycler Microheating system
Mixing Method	Pipetting	Micro plate shaker

Illumina recommends the following kit, sample number, and protocol combinations:

Table 2 Kit and Sample Number Recommendations

Number of Samples Processed At One Time	Kit Recommended
<24	LT
24–48	LT or HT
>48	HT

Table 3 Kit and Protocol Recommendations

Kit	Number of Samples Supported	Number of Samples Processed At One Time	Protocol
LT	48	≤48	LS
		>48	HS
HT	96	≤48	LS
		>48	HS

The TruSeq DNA Sample Prep fragmentation process is optimized to obtain final libraries, with the following differences

Table 4 Fragmentation Method Options

	Whole-genome Resequencing	TruSeq Enrichment	
	Gel Method	Gel-free Method	Gel Method
Covaris Shearing Duration	40 seconds	120 seconds	
Insert Size	300–400 bp	100–900 bp	200–300 bp

NOTE

- TruSeq Enrichment refers to the Illumina TruSeq Exome Enrichment and TruSeq Custom Enrichment Kits that can be used following TruSeq DNA Sample Prep to prepare the library for sequencing targeted regions. For more information, see the *TruSeq Enrichment Guide*.
- The gel-free method is ***not*** an option when preparing libraries for standard (whole-genome) sequencing.

Getting Started

Chapter 2

Introduction

This chapter explains standard operating procedures and precautions for performing TruSeq DNA Sample Preparation. You will also find lists of standard equipment and consumables.

The sample preparation protocols described in the rest of this guide assume that you are familiar with the contents of this chapter, have implemented all the recommendations, and have obtained all of the requisite equipment and consumables.

Acronyms

Table 5 TruSeq DNA Sample Preparation Acronyms

Acronym	Definition
ALP	Adapter Ligation Plate
ATL	A-Tailing Mix
CAP	Clean Up ALP Plate
CFP	Covaris Fragmentation Plate
CPP	Clean Up PCR Plate
CTA	A-Tailing Control
CTE	End Repair Control
CTL	Ligation Control
DAP	DNA Adapter Plate
DCT	Diluted Cluster Template
DNA	Customer Sample DNA Plate
dsDNA	double-stranded DNA
ERP	End Repair Mix
EUC	Experienced User Card
gDNA	genomic DNA
HSP	Hardshell Plate
HS	High Sample
HT	High Throughput

Acronym	Definition
IMP	Insert Modification Plate
LIG	Ligation Mix
LS	Low Sample
LT	Low Throughput
LTF	Lab Tracking Form
PCR	Polymerase Chain Reaction
PDP	Pooled Dilution Plate
PMM	PCR Master Mix
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
SSP	Size Separate Plate
STL	Stop Ligation Buffer
TSP	Target Sample Plate

Best Practices

When preparing gDNA libraries for sequencing, you should always adhere to good molecular biology practices. Read through the entire protocol prior to starting, to ensure all of the required materials are available and your equipment is programmed and ready to use.

NOTE

For more information, see the *TruSeq Sample Preparation Best Practices and Troubleshooting Guide* which you can download from the Illumina website at http://www.illumina.com. Go to the TruSeq DNA Sample Preparation support page and click the **Documentation & Literature** tab. A MyIllumina account is required.

Handling Liquids

Good liquid handling measures are essential, particularly when quantifying libraries or diluting concentrated libraries for making clusters.

- Small differences in volumes (±0.5 µl) can sometimes give rise to very large differences in cluster numbers (~100,000).
- Small volume pipetting can be a source of potential error in protocols that require generation of standard curves, such as qPCR, or those that require small but precise volumes, such as the Agilent Bioanalyzer.
- If small volumes are unavoidable, then due diligence should be taken to make sure that pipettes are correctly calibrated.
- Make sure that pipettes are not used at the volume extremes of their performance specifications.
- Care should be taken with solutions of high molecular weight double-stranded DNA (dsDNA). These can be viscous and not evenly dispersed, resulting in aliquot measurements that are not representative of the true concentration of the solution.
- To minimize pipetting errors, especially with small volume enzyme additions, prepare the reagents for multiple samples simultaneously. As a result, pipette once from the reagent tubes with a larger volume, rather than many times with small volumes. This will allow you to aliquot in a single pipetting movement to individual samples and standardize across multiple samples.

Handling Master Mix Reagents

When handling the master mix reagents:

- Minimize freeze-thaw cycles. If you do not intend to consume the reagents in one use, dispense the reagent into aliquots after the initial thaw and refreeze the aliquots in order to avoid excessive freeze-thaw cycles. However, if you aliquot, you might not have enough reagents for the full number of reactions over multiple uses.
- Add reagents in the order indicated and avoid making master-mixes containing the in-line controls.
- Take care while adding the A-Tailing Mix (ATL) and Ligation Mix (LIG) due to the viscosity of the reagents.

Handling Magnetic Beads

Follow appropriate handling methods when working AMPure XP Beads:

NOTE
Cleanup procedures have only been validated using the 96-well plates and the magnetic stand specified in the Consumables and Equipment list. Comparable performance is not guaranteed when using a microcentrifuge tube or other formats, or other magnets.

- Prior to use, allow the beads to come to room temperature.
- Do not reuse beads. Always add fresh beads when performing these procedures.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous.
- **When performing the LS protocol**:
  - After adding the beads to the reaction, mix the solution gently and thoroughly by pipetting up and down 10 times, making sure the liquid comes in contact with the beads and that the beads are resuspended homogeneously.
  - Pipetting with the tips at the bottom of the well and not pipetting the entire volume of the solution helps prevent the solution from foaming. Excessive foaming leads to sample loss, because the foam is not transferred out of the plate efficiently.
- **When performing the HS protocol**, after adding the beads to the reaction, seal the plate and shake the plate on a microplate shaker at 1,800 rpm for 2 minutes.

Repeat, if necessary, until the color of the mixture appears homogeneous after mixing.

- Take care to minimize bead loss which can impact final yields.
- Change the tips for each sample.
- Let the mixed samples incubate for 15 minutes at room temperature for maximum recovery.
- When aspirating the cleared solution from the reaction plate and wash step, it is important to keep the plate on the magnetic stand and to not disturb the separated magnetic beads. Aspirate slowly to prevent the beads from sliding down the sides of the wells and into the pipette tips.
- To prevent the carryover of beads after elution, approximately 2.5 µl of supernatant are left when the eluates are removed from the bead pellet.
- Prepare fresh 80% ethanol. Ethanol tends to absorb water from the air, therefore, fresh 80% ethanol should be prepared for optimal results.
- Be sure to remove all of the ethanol from the bottom of the wells, as it can contain residual contaminants.
- Keep the reaction plate on the magnetic stand and let it air-dry at room temperature to prevent potential bead loss due to electrostatic forces. Allow for the complete evaporation of residual ethanol, as the presence of ethanol will impact the performance of the subsequent reactions. Illumina recommends at least 15 minutes drying time, but a longer drying time might be required. Remaining ethanol can be removed with a 20 µl pipette.
- Use the Resuspension Buffer (RSB) for DNA elution.
- Avoid over drying the beads, which can impact final yields.
- **When performing the LS protocol**, resuspend the dried pellets using a single channel or multichannel pipette.
- **When performing the HS protocol**, resuspend the dried pellets by shaking.
- When removing and discarding supernatant from the wells, use a single channel or multichannel pipette and take care not to disturb the beads.
- To maximize sample recovery during elution, incubate the sample/bead mix for 2 minutes at room temperature before placing the samples onto the magnet.

Avoiding Cross-Contamination

Practice the following to avoid cross-contamination:

- Open only one adapter tube at a time.

- Clean the bottom of the 96-well PCR plate or eight-tube strip used to pierce the foil seal of a DNA Adapter Plate (DAP) with a sterile 70% Ethanol wipe.
- Pipette carefully to avoid spillage.
- Clean pipettes and change gloves between handling different adapter stocks.
- Clean work surfaces thoroughly before and after the procedure.

Potential DNA Contaminants

Avoid potential DNA contaminants:

- Incorrect DNA quantitation can result from DNA contamination, for example, interference from superfluous nucleic acids in a sample (e.g., RNA, small nucleic acid fragments, nucleotides, single-stranded DNA), excess proteins, or other contaminating materials.
- DNA quality can also affect the quantity of usable DNA in a sample. For example, if the DNA is damaged (e.g., heavily nicked or containing extensive apurinic/apyrimidinic sites), many of the fragments generated may fail during library preparation.
- High molecular weight dsDNA derived from host genomes can also interfere with accurate quantitation. For example, bacterial artificial chromosomes (BACs) and other bacterially-derived plasmids usually contain a small percentage of the chromosomal DNA from the host cells, despite the best purification efforts. These sequences might ultimately give rise to unwanted clusters on a flow cell lane. However, this contamination can be accurately quantified by analyzing aligned reads generated during sequencing against known bacterial sequences and subtracting these out. High molecular weight contamination can also be estimated prior to library preparation using qPCR assays designed to target unique chromosomal markers.

Temperature Considerations

Temperature is an important consideration for making gDNA libraries:

- Keep libraries at temperatures ≤37°C.
- Place reagents on ice after thawing at room temperature.
- Avoid elevated temperatures, particularly in the steps preceding the adapter ligation.

- When processing more than 48 samples manually, Illumina recommends processing the plate on a bed of ice whenever possible, especially during the enzymatic steps (when using the End Repair Mix, A-Tailing Mix, and Ligation Mix). A large number of samples processed at room temperature may result in uneven catalytic activity, which can lead to reduced quality of the end product.
- DNA fragments that have a high AT content are more likely to denature into single strands than GC-rich fragments, which can result in an increased probability of creating a bias in the sequencing coverage.
- Take care not to denature the library prior to the agarose gel electrophoresis process, because single-stranded DNA has a different migration rate.

Usage Guidelines

Illumina recommends these guidelines as the most efficient lab setup and pipetting process when performing the procedures specified in Chapter 3 Low Sample (LS) Protocol and Chapter 4 High Sample (HS) Protocol.

NOTE
The TruSeq DNA LT Sample Prep Kit contains enough of each reagent to prepare 48 samples at one time and the TruSeq DNA HT Sample Prep Kit contains enough reagent to prepare 96 samples at one time. If an alternate lab setup and pipetting process is used, Illumina cannot guarantee that there will be enough of every reagent for the full number of samples.

NOTE
When using multichannel pipettes, take care to pipette accurately into the wells, as variations in volume will affect the sample preparation. Change tips after each sample.

Reference the following table to determine the required reagent volume per sample for these guidelines.

Table 6 TruSeq DNA Sample Prep Reagent Volumes

Reagent	Description	Volume per Sample (µl)
AD0XX or DAP	DNA Adapter tube or DNA Adapter Plate	2.5
ATL	A-Tailing Mix	12.5
CTA	A-Tailing Control	2.5

Reagent	Description	Volume per Sample (µl)
CTE	End Repair Control	10
CTL	Ligation Control	2.5
ERP	End Repair Mix	40
LIG	Ligation Mix	2.5
PMM	PCR Master Mix	25
PPC	PCR Primer Cocktail	5
STL	Stop Ligation Buffer	5

Preparing More Than 24 Samples

When preparing more than 24 samples, follow these guidelines as you perform each procedure in the protocol. Use a multichannel pipette with eight tips to perform all transfers from the reagent vessel to the sample plate.

Sample Distribution

Distribute each sample into a separate column of the plate. Use the appropriate plate for the protocol being performed:

- LS protocol - 96-well 0.3 ml PCR plate
- HS protocol - 96-well MIDI plate and 96-well HSP plate

NOTE

Illumina highly recommends using the Illumina Experiment Manager and reviewing the low-plex pooling guidelines in the *Normalize and Pool Libraries* procedures when setting up the sample plate for use with a DAP. Prepare each sample in the sample plate position that corresponds to the desired dual-indexed DNA adapter position in the DAP.

Reagents in Reservoirs

When each of the following reagents is required in the protocol, distribute each into a separate multichannel reagent reservoir as follows:

- 80% Ethanol
- AMPure XP Beads
- Resuspension Buffer

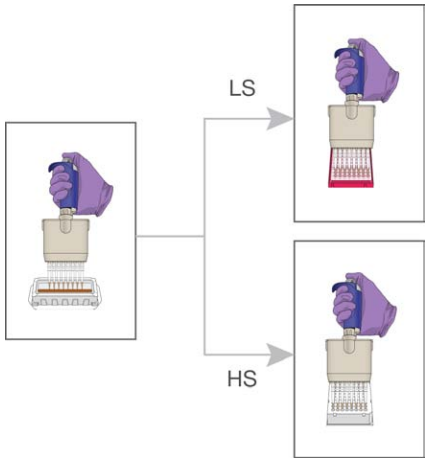
1 Determine the volume needed for each of the above reagents using the equation (# of samples x volume per sample) + 600 µl dead volume. Reference Table 6 for the required reagent volume per sample.

2 Fill a separate multichannel reagent reservoir with the determined amount of each reagent.

When each of the above reagents is required in the protocol, distribute each to the sample plate as follows:

1 Using an eight tip multichannel pipette, transfer the reagent in the reservoir to the samples in the plate as follows, while holding the pipette vertically. Reference Table 6 for the required reagent volume per sample.

Figure 1 Transfer Reagent from Reservoir to Sample Plate with 24 or More Samples



a Pipette the required reagent volume per sample from the reservoir.
b Add the reagent to column 1 of the sample plate. Change the tips.
c Pipette the required reagent volume per sample from the reservoir.
d Add the reagent to column 2 of the sample plate. Change the tips.
e Repeat as needed for each column containing a sample.

Reagents in Strip Tubes

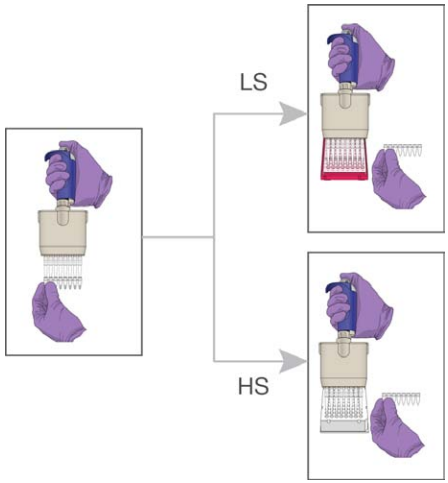
When the remaining reagents not mentioned above, except the adapters, are required in the protocol, distribute each evenly across eight wells of an eight-tube strip. Add an

allowance of 5 µl for dead volume per well.

When each reagent in an eight-tube strip is required in the protocol, distribute each to the sample plate as follows:

1 Using an eight tip multichannel pipette, transfer the reagent in the eight-tube strip to the samples in the plate as follows, while holding the pipette vertically. Reference Table 6 for the required reagent volume per sample.

Figure 2 Transfer Reagent from Strip Tube to Sample Plate with 24 or More Samples



a Pipette the reagent from the eight strip wells.
b Add the reagent to column 1 of the sample plate. Change the tips.
c Pipette the reagent from the eight strip wells.
d Add the reagent to column 2 of the sample plate. Change the tips.
e Repeat as needed for each column containing a sample.

Index Adapters

When using DNA index adapter tubes, do one of the following:

- Add 2.5 µl of the appropriate/desired adapter index individually to each well of the plate containing a sample, using a single channel pipette.
- Using an eight-tube strip:
  - Distribute the index adapters into the wells of an eight-tube strip, with a different adapter in each well.

- Add 2.5 µl of the appropriate/desired adapter index from the well of the eight-tube strip to each well of the plate containing a sample, using a multichannel pipette.

When using a DAP, see *Handling Adapter Plate* on page 42.

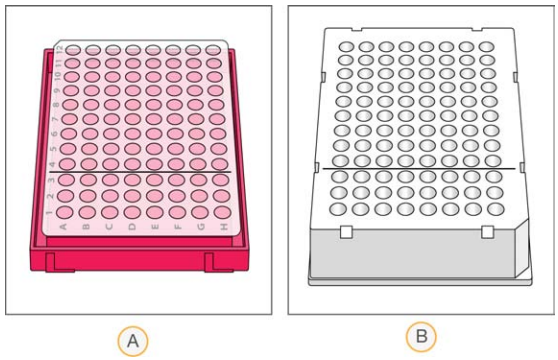
Preparing 12–24 Samples

When preparing 12–24 samples, follow these guidelines as you perform each procedure in the protocol. Use a multichannel pipette with three tips to perform all transfers from the reagent vessel to the sample plate.

Sample Distribution

Distribute the 12–24 samples into three columns and four to eight rows (e.g., four rows per 12 samples) of the plate. Draw a line on the plate to visually separate the three columns. Use the appropriate plate for the protocol being performed.

Figure 3 Draw Line on Plate



A 96-well 0.3 ml PCR plate (LS Protocol)
B 96-well MIDI plate and 96-well HSP plate (HS Protocol)

Reagents in Reservoirs

When each of the following reagents is required in the protocol, distribute each into a separate multichannel reagent reservoir as follows:

- 80% Ethanol
- AMPure XP Beads
- Resuspension Buffer

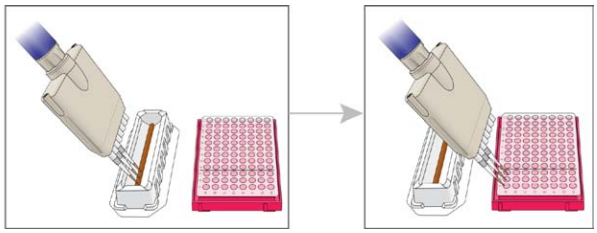
1 Determine the volume needed using the equation (# of samples x volume per sample) + 600 µl dead volume. Reference Table 6 for the required reagent volume per sample.

2 Fill a separate multichannel reagent reservoir with the determined amount of each reagent.

When each of the above reagents is required in the protocol, distribute each to the sample plate as follows:

1 Using a multichannel pipette with three tips, transfer the reagent in the reservoir to the samples in the plate as follows, while holding the pipette vertically. Reference Table 6 for the required reagent volume per sample.

Figure 4 Transfer Reagent from Reservoir to Sample Plate with 12–24 Samples



a Pipette the required reagent volume per sample from the reservoir.
b Add the reagent to row 1 of the sample plate. Change the tips.
c Pipette the required reagent volume per sample from the reservoir.
d Add the reagent to row 2 of the sample plate. Change the tips.
e Repeat as needed for each row containing a sample.

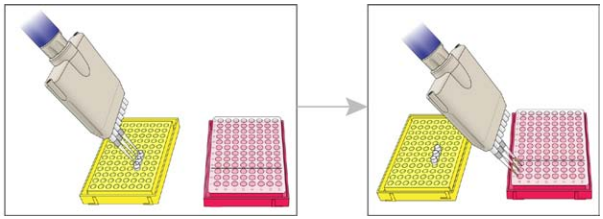
Reagents in Strip Tubes

When the remaining reagents not mentioned above, except the adapters, are required in the protocol, distribute each evenly across the three wells of an eight-tube strip. Add an allowance of 5 µl for dead volume per well.

When each reagent in an eight-tube strip is required in the protocol, distribute each to the sample plate as follows:

1 Using a multichannel pipette with three tips, transfer the reagent in the eight-tube strip to the samples in the plate as follows, while holding the pipette vertically. Reference Table 6 for the required reagent volume per sample.

Figure 5 Transfer Reagent from Strip Tube to Sample Plate with 12–24 Samples



a Pipette the reagent from the three strip wells.
b Add the reagent to row 1 of the sample plate. Change the tips.
c Pipette the reagent from the three strip wells.
d Add the reagent to row 2 of the sample plate. Change the tips.
e Repeat as needed for each row containing a sample.

Index Adapter Tubes

When DNA index adapter tubes are used, add 2.5 µl of the appropriate/desired adapter index individually to each well of the plate containing a sample, using a single channel pipette.

Preparing Less Than 12 Samples

When preparing less than 12 samples, follow these guidelines as you perform each procedure in the protocol:

- Add each reagent individually to the samples using a single channel pipette.
- If planning more than three freeze-thaw cycles, aliquot the reagents equally into six separate vessels.

Equipment

Review the programming instructions for your thermal cycler user guide to ensure that it is programmed appropriately using the heated lid function.

DNA Input Recommendations

It is important to quantitate the input DNA and assess the DNA quality prior to performingTruSeq DNA Sample Preparation.

Input DNA Quantitation

Follow these DNA input recommendations:

- Correct quantification of gDNA is essential.
- 1 µg input DNA is recommended.
- 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 PicoGreen or Qubit to provide accurate quantification of dsDNA. UV-spec based methods, such as the Nanodrop, will 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 validate results.
- DNA quantification methods that rely on intercalating fluorescent dyes measure only double-stranded DNA and are less subject to 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.
  - Both absorbance measurements can be compromised by the presence of RNA or small nucleic acid fragments such as nucleotides.
  - gDNA samples should be carefully collected to make sure that they are free of contaminants.

- Gel electrophoresis is a powerful means for revealing the condition (including the presence or absence) of DNA in a sample.
  - Where possible or necessary, a gel should be run to assess the condition of the DNA sample.
    - Impurities, such as detergents or proteins, can be revealed by visible smearing of DNA bands in the gel.
  - RNA, which interferes with 260 nm readings, is often visible at the bottom of a gel.
  - A ladder or smear below a band of interest might indicate nicking or other damage to DNA.

In-Line Control DNA

The End Repair Control, A-Tailing Control, and Ligation Control reagents contain DNA fragments used as controls for the enzymatic activities of the End Repair Mix, A-Tailing Mix, and Ligation Mix, respectively. Each reagent contains dsDNA fragments designed to report the success or failure of a specific enzymatic activity used in the library preparation process. Readout is determined by sequencing. If the sequence of an in-line control appears in the final sequencing data viewed in the Sequence Analysis Viewer (SAV), it indicates that its corresponding step was successful. If it does not, or if it appears in substantially diminished numbers, it indicates the step failed. The controls are intended for troubleshooting and are useful for identifying the specific mode of failure, but are uninformative in cases where sequencing data is not generated from a library.

NOTE
The use of these controls is optional and they can be replaced with the same volume of Resuspension Buffer.

The control molecules work through the design of their ends. Controls are added to the reactions just prior to their corresponding step in the protocol. Their end structures match those of a DNA molecule that has not gone through the step. If the step is successful, the control molecule will be modified to participate in downstream reactions of library generation and resulting in sequencing data. If the step fails, the control molecule will not go forward in the process and no sequencing data will be generated. Using 1 µg of starting material, the controls yield approximately 0.2% of clusters, although this can vary based on library yield.

Table 7 In-Line Control Functions

Reagent	Function	Control	Structure of Control DNA Ends
End Repair Mix	End repair: Generate blunt ended fragments by 3'–>5' exonuclease and polymerase activities	End Repair Control 1*	5' overhang at one end, 3' overhang at other end
End Repair Mix	End repair: Add 5'-phosphate groups needed for downstream ligation	End Repair Control 2*	Blunt with 5'-OH group

Reagent	Function	Control	Structure of Control DNA Ends
A-Tailing Mix	A-tailing: Make fragments compatible with adapters and prevent self-ligation by adding a 3'-A overhang	A-Tailing Control	Blunt with 5'-phosphate group
Ligation Mix	Ligation: Join adapters to inserts	Ligation Control	Single-base 3' 'A' base overhang

*End Repair Control 1 and End Repair Control 2 are separate controls included in the End Repair Control reagent

The control reagents can be used for a variety of library insert sizes. Each is provided in ladders ranging from approximately 150–850 bp in 100 bp increments. Each control molecule has a unique DNA sequence, indicating both its function and size. The RTA software (version 1.9 and higher) recognizes these sequences and isolates the control sequences from the main body of sequencing reads and reports their counts per lane in the controls tab of the RTA status.html page. For more information regarding the control read-out in the SAV, see the *Sequence Analysis Viewer User Guide*.

Tracking Tools

Illumina provides the following tools for sample tracking and guidance in the lab:

- **Experienced User Card (EUC)** to guide you through the protocol, but with less detail than provided in this user guide. **New or less experienced users are strongly advised to follow this user guide and not the EUC.**
- **Lab Tracking Form (LTF)** to record information about library preparation such as operator name, sample and index information, start and stop times, reagent lot numbers, and barcodes.
  - Create a copy of the lab tracking form for each time you perform this protocol to prepare a library for sequencing.
  - Use it online and save it electronically or print it and fill it out manually.

  NOTE
  You can download the above TruSeq DNA Sample Preparation documents from the Illumina website at http://www.illumina.com. Go to the TruSeq DNA Sample Preparation support page and click the **Documentation & Literature** tab. A MyIllumina account is required.

- **Illumina Experiment Manager (IEM)** to create your sample sheet using a wizard-based application. The sample sheet is used to record information about your samples for later use in data analysis. The IEM guides you through the steps to create your sample sheet based on the analysis workflow for your run. The IEM provides a feature for recording parameters for your sample plate, such as sample ID, dual indices, and other parameters applicable to your 96-well plate.
  - When prompted to select an Assay in IEM, choose:
    - **TruSeq LT** if you are using the TruSeq DNA LT Sample Prep Kit
    - **TruSeq HT** if you are using the TruSeq DNA HT Sample Prep Kit

  NOTE
  IEM can be run on any Windows platform. You can download it from the Illumina website at http://www.illumina.com. A MyIllumina account is required.

Kit Contents

Check to make sure that you have all of the reagents identified in this section before proceeding. The 48 samples kits are available as Set A and B, which differ in the indices provided and together allow for pooling of up to 24 samples.

Table 8 TruSeq DNA Sample Preparation Kits

Kit Name	Catalog #	Number of Samples Supported	Number of Indices
TruSeq DNA LT Sample Prep Kit - Set A	FC-121-2001	48	12
TruSeq DNA LT Sample Prep Kit - Set B	FC-121-2002	48	12
TruSeq DNA HT Sample Prep Kit	FC-121-2003	96	96

TruSeq DNA LT Sample Prep Kit

The TruSeq DNA LT Sample Prep Kit contains two boxes: a Set A or Set B box and a PCR box.

48 Samples - Set A Box or Set B Box

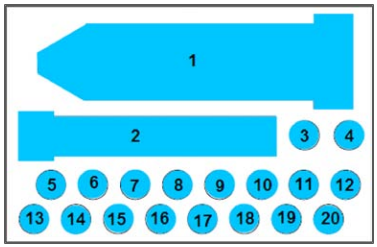
You will receive either box A or B with the kit depending on the set ordered. These boxes also contain plate barcode labels.

Store at -15° to -25°C

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

Set A

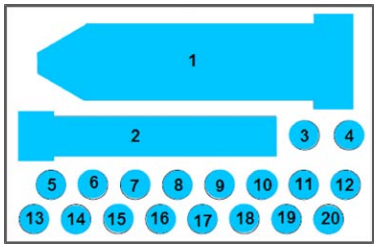
Figure 6 TruSeq DNA LT Sample Prep Kit, 48 Samples-Set A Box, part # 15025064



Slot	Reagent	Part #	Description
1	RSB	15026770	Resuspension Buffer
2	ERP	15012494	End Repair Mix
3	ATL	15012495	A-Tailing Mix
4	LIG	15026773	Ligation Mix
5	CTE	15026774	End Repair Control
6	CTA	15026775	A-Tailing Control
7	CTL	15026776	Ligation Control
8	STL	15012546	Stop Ligation Buffer
9	AR002	15026621	DNA Adapter Index 2
10	AR004	15026623	DNA Adapter Index 4
11	AR005	15026624	DNA Adapter Index 5
12	AR006	15026625	DNA Adapter Index 6
13	AR007	15026627	DNA Adapter Index 7
14	AR012	15026632	DNA Adapter Index 12
15	AR013	15024641	DNA Adapter Index 13
16	AR014	15024642	DNA Adapter Index 14
17	AR015	15024643	DNA Adapter Index 15
18	AR016	15024644	DNA Adapter Index 16
19	AR018	15024646	DNA Adapter Index 18
20	AR019	11324647	DNA Adapter Index 19

Set B

Figure 7 TruSeq DNA LT Sample Prep Kit, 48 Samples-Set B Box, part # 15025065



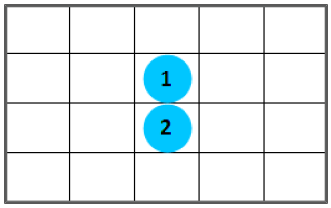
Slot	Reagent	Part #	Description
1	RSB	15026770	Resuspension Buffer
2	ERP	15012494	End Repair Mix
3	ATL	15012495	A-Tailing Mix
4	LIG	15026773	Ligation Mix
5	CTE	15026774	End Repair Control
6	CTA	15026775	A-Tailing Control
7	CTL	15026776	Ligation Control
8	STL	15012546	Stop Ligation Buffer
9	AD001	15026620	DNA Adapter Index 1
10	AD003	15026622	DNA Adapter Index 3
11	AD008	15026628	DNA Adapter Index 8
12	AD009	15026629	DNA Adapter Index 9
13	AD010	15026630	DNA Adapter Index 10
14	AD011	15026631	DNA Adapter Index 11
15	AD020	15024648	DNA Adapter Index 20
16	AD021	15024649	DNA Adapter Index 21
17	AD022	15024650	DNA Adapter Index 22
18	AD023	15024651	DNA Adapter Index 23
19	AD025	15024653	DNA Adapter Index 25
20	AD027	11324654	DNA Adapter Index 27

48 Samples - PCR Box

Store at -15° to -25°C

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

Figure 8 TruSeq DNA LT Sample Prep Kit, 48 Samples-PCR Box, part # 15027084



Slot	Reagent	Part #	Description
1	PMM	15026785	PCR Master Mix
2	PPC	15031748	PCR Primer Cocktail

TruSeq DNA HT Sample Prep Kit

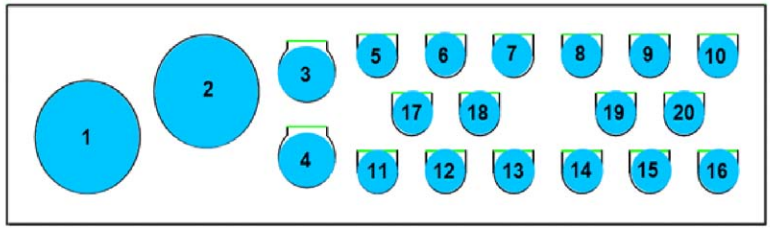
The TruSeq DNA HT Sample Prep Kit contains two boxes: a Core-PCR box and an Adapter Plate box.

96 Samples - Core-PCR Box

Store at -15° to -25°C

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

Figure 9 TruSeq DNA HT Sample Prep Kit, 96 Samples - Core-PCR Box, part # 15032316



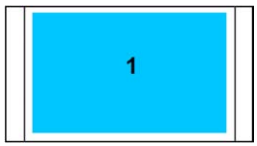
Slot	Reagent	Part #	Description
1–2	RSB	15026770	Resuspension Buffer
3–4	ERP	15012494	End Repair Mix
5–6	ATL	15012495	A-Tailing Mix
7–8	LIG	15026773	Ligation Mix
9–10	CTE	15026774	End Repair Control
11–12	CTA	15026775	A-Tailing Control
13–14	CTL	15026776	Ligation Control
15–16	STL	15012546	Stop Ligation Buffer
17–18	PMM	15026785	PCR Master Mix
19–20	PPC	15031748	PCR Primer Cocktail

96 Samples- Adapter Plate Box

Store at -15° to -25°C

This box is shipped on dry ice. As soon as you receive it, store the contents at -15° to ı25°C.

Figure 10 TruSeq DNA HT Sample Prep Kit, 96 Samples - Adapter Plate Box, part # 15032317



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

Consumables and Equipment

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

Table 9 User-Supplied Consumables

Consumable	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
6X gel loading dye	BioLabs, catalog # B7021S
50 X TAE buffer	Bio-Rad, part # 161-0743

Consumable	Supplier
96-well storage plates, round well, 0.8 ml ("MIDI" plate)	Fisher Scientific, part # AB-0859
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
BenchTop 100 bp DNA ladder	Promega, part # G829B
Certified low-range ultra agarose	Bio-Rad, part # 161-3107
Clean scalpels	General lab supplier
MicroTube (6x16mm), AFA fiber with crimp-cap	Covaris, part # 520052
Distilled water	General lab supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma Aldrich, part # E7023
Microseal 'B' adhesive seals	BioRad, part # MSB-1001
MinElute Gel Extraction Kit	QIAGEN, part# 28604
Paired-End Sample Prep Kit (Optional -for alternative fragmentation by nebulization only)	Illumina, 10 samples catalog # PE-102-1001 40 samples, catalog # PE-102-1002
PCR grade water (for gel-free method)	General lab supplier
QIAquick PCR Purification Kit (Optional - for alternative fragmentation by nebulization only)	QIAGEN, part # 28104

Consumable	Supplier
Qubit dsDNA BR Assay Kit	Life Technologies 100 assays, catalog # Q32850 500 assays, catalog # Q32853
Qubit assay tubes or Axygen PCR-05-C tubes	Life Technologies, catalog # Q32856 or VWR, part # 10011-830
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
RNase/DNase-free 8-well PCR strip tubes and caps	General lab supplier
RNase/DNase zapper (to decontaminate surfaces)	General lab supplier
SyBr Gold Nucleic acid gel stain	Invitrogen, part # S11494
Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20	General lab supplier
Tween 20	Sigma, part # P7949
Ultra pure water	General lab supplier

Table 10 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 11 User-Supplied Consumables - Additional Items for HS Processing

Consumable	Supplier
Microseal 96-well PCR plates ("HSP" plate)	Bio-Rad, part # HSP-9601
Microseal 'A' film	Bio-Rad, part # MSA-5001

Table 12 User-Supplied Equipment

Equipment	Supplier
96-well thermal cycler (with heated lid)	General lab supplier
Covaris S220 or Covaris E220	Covaris, part # SE-501-1001 or Covaris, part # SE-501-1002
Dark reader transilluminator	Clare Chemical Research, part # DR195M
Electrophoresis power supply	General lab supplier
Magnetic stand-96	Life Technologies, catalog # AM10027
Microplate centrifuge	General lab supplier
Qubit 2.0 Fluorometer	Life Technologies, catalog # Q32866 http://products.invitrogen.com/ivgn/product/Q32866
Thermo Scientific Owl B2 EasyCast Mini Gel System	(US) Thermo Scientific, part # B2, or Fisher Scientific, part # 09-528-110B (Other Regions) Fisher Scientific, part # OWL-130-101J B
Vortexer	General lab supplier

Table 13 User-Supplied Equipment - Additional Items for HS Processing

Consumable	Supplier
High Speed Micro Plate Shaker	VWR, catalog # 13500-890 (110V/120V) VWR, catalog # 14216-214 (230V)
MIDI plate insert for heating system	Illumina, catalog # BD-60-601
Stroboscope	General lab supplier
Tru Temp Microheating System	Illumina, catalog # SC-60-503 (115V) Illumina, catalog # SC-60-504 (220V)

Indexed Adapter Sequences

This section details the indexed adapter sequences.

TruSeq DNA LT Sample Prep Kit Indexed Adapter Sequences

The TruSeq DNA LT Sample Prep Kit contains the following the indexed adapter sequences. The set (A or B) containing the adapter is also specified.

NOTE

- The index numbering is not contiguous. Index 17, 24, and 26 are skipped.
- The base in parentheses () indicates the base for the seventh cycle and is not considered as part of the index sequence. The index should be recorded in the sample sheet as only six bases. For indexes 13 and above, the seventh base (in parentheses) might not be A, and this will be seen in the seventh cycle of the index read.

Table 14 TruSeq DNA LT Sample Prep Kit Indexed Adapter Sequences

Adapter	Sequence	Set		Adapter	Sequence	Set
AD001	ATCACG(A)	B		AD013	AGTCAA(C)	A
AD002	CGATGT(A)	A		AD014	AGTTCC(G)	A
AD003	TTAGGC(A)	B		AD015	ATGTCA(G)	A
AD004	TGACCA(A)	A		AD016	CCGTCC(C)	A
AD005	ACAGTG(A)	A		AD018	GTCCGC(A)	A
AD006	GCCAAT(A)	A		AD019	GTGAAA(C)	A
AD007	CAGATC(A)	A		AD020	GTGGCC(T)	B
AD008	ACTTGA(A)	B		AD021	GTTTCG(G)	B

Adapter	Sequence	Set		Adapter	Sequence	Set
AD009	GATCAG(A)	B		AD022	CGTACG(T)	B
AD010	TAGCTT(A)	B		AD023	GAGTGG(A)	B
AD011	GGCTAC(A)	B		AD025	ACTGAT(A)	B
AD012	CTTGTA(A)	A		AD027	ATTCCT(T)	B

TruSeq DNA HT Sample Prep Kit Indexed Adapter Sequences

The DAP in the TruSeq DNA HT Sample Prep Kit contains the following the 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 15 TruSeq DNA HT Sample Prep Kit Indexed Adapter Sequences

Indexed Adapter 1	Sequence	Indexed Adapter 2	Sequence
D701	ATTACTCG	D501	TATAGCCT
D702	TCCGGAGA	D502	ATAGAGGC
D703	CGCTCATT	D503	CCTATCCT
D704	GAGATTCC	D504	GGCTCTGA
D705	ATTCAGAA	D505	AGGCGAAG
D706	GAATTCGT	D506	TAATCTTA
D707	CTGAAGCT	D507	CAGGACGT
D708	TAATGCGC	D508	GTACTGAC

Indexed Adapter 1	Sequence	Indexed Adapter 2	Sequence
D709	CGGCTATG		
D710	TCCGCGAA		
D711	TCTCGCGC		
D712	AGCGATAG		

Adapter Options

Illumina provides two methods for indexing samples to perform pooled sequencing, using either DNA Adapter Index tubes or a DAP.

Adapter Tubes

The TruSeq DNA LT Sample Prep Kit contains DNA Adapter Index tubes that can be used to perform pooled sequencing.

- Each tube contains a unique single 6 base index adapter on the P7 strand and contains enough reagent for eight reactions.
- Samples prepared with these adapters can be sequenced on any Illumina sequencing platform using the 7 cycle Single Index Recipe.

For more information on pooling guidelines when using adapter index tubes, see *Adapter Tube Pooling Guidelines* on page 45.

For more information on sequencing samples prepared using the TruSeq DNA LT Sample Prep Kit, see your sequencing platform user guide.

Adapter Plate

The TruSeq DNA HT Sample Prep Kit contains a DAP, which is a 96-well plate containing 96 uniquely indexed adapter combinations designed for manual or automated preparation of 96 uniquely indexed samples.

- Each well of the plate is single-use and the plate can undergo up to 4 freeze/thaw cycles.
- The DNA adapters provided in this plate are dual-indexed, meaning that each adapter contains two indices. These are referred to as Index 1(i7), an 8 base Index on the P7 strand, and Index 2(i5), an 8 base Index on the P5 strand.
- There are 12 Index 1 sequences (D701-D712) arrayed across the columns and 8 Index 2 sequences (D501-D508) arrayed down the rows, to generate 96 uniquely dual-indexed adapter combinations in the plate.
- If compatible, samples prepared with these adapters can be sequenced on an Illumina sequencing platform using the dual-indexed recipes for dual indexing or the 8 cycle single-indexed recipe for single indexing.

For more information on pooling guidelines when using the DAP, see *Adapter Plate Pooling Guidelines* on page 46.

For more information on sequencing samples prepared using the TruSeq DNA HT Sample Prep Kit, see your sequencing platform user guide.

Table 16 Dual-Indexed Sequencing Platform Compatibility

Platform	Compatibility
MiSeq®	Full compatibility
HiSeq®	• Requires TruSeq Dual Index Sequencing Primer Box, Single Read for dual-indexed sequencing on a single-read flow cell.[a] • Requires HCS 1.5/RTA 1.13 or later • Process with OLB 1.9.3 or later if offline base call is needed • Process with CASAVA 1.8.2 or later
Genome Analyzer™	• Requires TruSeq Dual Index Sequencing Primer Box, Single Read for dual-indexed sequencing on a single-read flow cell.[a] • Requires SCS 2.10/RTA 1.13 or later • Process with OLB 1.9.4 or later if offline base call is needed • Process with CASAVA 1.8.2 or later

a. Not required for sequencing on paired-end flow cells.

Pooling Preparation with Adapter Plate

The TruSeq DNA HT Sample Prep Kit contains a DAP and enables preparation of up to 96 libraries with unique dual indexes.

Figure 11 DAP Dual-Indexed Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	D701-D501	D702-D501	D703-D501	D704-D501	D705-D501	D706-D501	D707-D501	D708-D501	D709-D501	D710-D501	D711-D501	D712-D501
B	D701-D502	D702-D502	D703-D502	D704-D502	D705-D502	D706-D502	D707-D502	D708-D502	D709-D502	D710-D502	D711-D502	D712-D502
C	D701-D503	D702-D503	D703-D503	D704-D503	D705-D503	D706-D503	D707-D503	D708-D503	D709-D503	D710-D503	D711-D503	D712-D503
D	D701-D504	D702-D504	D703-D504	D704-D504	D705-D504	D706-D504	D707-D504	D708-D504	D709-D504	D710-D504	D711-D504	D712-D504
E	D701-D505	D702-D505	D703-D505	D704-D505	D705-D505	D706-D505	D707-D505	D708-D505	D709-D505	D710-D505	D711-D505	D712-D505
F	D701-D506	D702-D506	D703-D506	D704-D506	D705-D506	D706-D506	D707-D506	D708-D506	D709-D506	D710-D506	D711-D506	D712-D506
G	D701-D507	D702-D507	D703-D507	D704-D507	D705-D507	D706-D507	D707-D507	D708-D507	D709-D507	D710-D507	D711-D507	D712-D507
H	D701-D508	D702-D508	D703-D508	D704-D508	D705-D508	D706-D508	D707-D508	D708-D508	D709-D508	D710-D508	D711-D508	D712-D508

When less than the full set of 96 libraries are pooled and sequenced, it is extremely important that libraries with compatible index combinations are used in the indexed pool. Illumina strongly recommends the following planning steps before beginning library preparation:

1 Determine the number of libraries that will be pooled for sequencing.

2 Ensure that the pool contains the required index combinations, as described in *Adapter Plate Pooling Guidelines* on page 46. Select the DNA index adapters based on the same guidelines.

3 Use the Illumina Experiment Manager to create a sample sheet which will be used during the sequencing run. This step also identifies any incorrect index combinations, allowing re-design before library preparation starts. For more information, see *Tracking Tools* on page 26.

4 Use the Lab Tracking Form or sample plate generator from the Illumina Experiment Manager to specify the layout of all sample plates in 96-well plate format for compatibility with the 96-well DAP. Arrange samples that will be pooled together in the same orientation as the indices in the DAP. For more information, see *Tracking Tools* on page 26.

Handling Adapter Plate

The DAP is designed for use in the TruSeq DNA Sample Prep high sample protocol.

- The DAP is single-use for each well.

- Illumina recommends that the DAP does not undergo more than 4 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.

Prepare Adapter Plate

Prepare the DAP for use as follows:

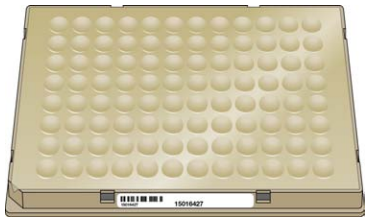
1. Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to ensure that they all are completely thawed.
2. Remove the adapter plate tape seal.
3. Centrifuge the plate at 280 xg for 1 minute to collect all of the adapter to the bottom of the well.
4. Remove the plastic cover and save the cover if you are not processing the entire plate at once.
5. Apply the DAP barcode label to the DAP.
   If using only part of the DAP, it may be useful to use a lab pen to mark on the foil seal the adapter wells being used. When doing so, be careful not to pierce the foil seal.

Pierce Adapter Plate Seal

Pierce the DAP foil seal as follows:

1. 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 located on the lower left.

   Figure 12 Correct DAP Orientation

2. Do one of the following:

  - If using the entire plate at once, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously by gently but firmly pressing 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 desired columns that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each column of adapters that will be used for ligation.
- Once the foil seal has been pierced for a well, Illumina does not recommend reusing the dual-indexed adapter from that well in future sample preparations.

Pipette Adapter Plate

Pipette the adapters from the DAP into the ligation reaction as follows, while keeping the plate in the same orientation:

1. Using an 8-tip multichannel pipette, transfer the thawed adapter from the DAP well to each well of the sample plate.
2. Change pipette tips between wells of the DAP. This is critical to avoid cross-contamination between wells.
3. Aspirate each dual-indexed adapter by column.
4. Discard the tips after pipetting into the ligation reaction.

Adapter Plate Storage

If not all adapter wells are used in a single experiment (< 96 samples), the plate can be stored for future use of unused wells as follows:

1. Wipe the foil seal covering unused wells with a sterile 70% Ethanol wipe.
2. Allow the foil seal to dry.
3. Put the plastic cover that came with the DAP back on the plate.
4. Store at -15° to -25°C.

NOTE

Do not reseal the plate with a disposable seal. This will rip the original foil seal when the disposable seal is removed for future uses.

Pooling Guidelines

Illumina uses a green laser to sequence G/T and a red laser to sequence A/C. At each cycle at least one of two nucleotides for each color channel needs to be read to ensure proper image registration. It is important to maintain color balance for each base of the index read being sequenced, otherwise index read sequencing could fail due to registration failure. Follow these low plex pooling guidelines, depending on the TruSeq DNA Sample Prep kit you are using.

Adapter Tube Pooling Guidelines

When using the index adapter tubes from the TruSeq DNA LT Sample Prep Kit, follow these pooling guidelines for single-indexed sequencing. The TruSeq DNA LT Sample Prep Kit Set A and B, each contain 12 unique index adapter tubes. When designing low-plexity index pools for single-indexed sequencing, always use at least two unique and compatible barcodes for each index sequenced. The following table describes possible pooling strategies for 2–4 samples generated with the adapter index tubes in each set.

- For 5–11plex pools, use 4-plex options with any other available adapters
- Not all color-balanced pools are listed. Check the color balance of such user-designed pools using the Illumina Experiment Manager's sample sheet generator.

Table 17 Single-Indexed Pooling Strategies for 2–4 Samples

Plexity	Option	Set A Only	Set B Only
2	1	AD006 and AD012	Not recommended
	2	AD005 and AD019	
3	1	AD002 and AD007 and AD019	AD001 and AD010 and AD020
	2	AD005 and AD006 and AD015	AD003 and AD009 and AD025
	3	2-plex options with any other adapter	AD008 and AD011 and AD022
4	1	AD005 and AD006 and AD012 and AD019	AD001 and AD008 and AD010 and AD011
	2	AD002 and AD004 and AD007 and AD016	AD003 and AD009 and AD022 and AD027
	3	3-plex options with any other adapter	3-plex options with any other adapter

For more information on the Single-Indexed Sequencing workflow, see the Illumina HiSeq, HiScan®, and Genome Analyzer user guides.

Adapter Plate Pooling Guidelines

When using the the DAP from the TruSeq DNA HT Sample Prep Kit, follow these pooling guidelines. In addition, please review *Handling Adapter Plate* on page 42 and *Pooling Preparation with Adapter Plate* on page 41.

Single-Indexed Sequencing

Follow the single-indexed sequencing workflow when pooling 12 or fewer samples. When designing low plexity index pools, always use at least two unique and compatible barcodes for each index sequenced. The following figures illustrate possible pooling strategies for 2–12 samples generated with the DAP.

- Color balanced pools are shaded light gray with green wells.
- For 5-plex pools, dark gray wells are not used for pooled sequencing. They are available for individual sequencing.
- For 7–11plex pools, combine any of the 2–6plex pools.
- Not all color-balanced pools are illustrated. Check the color balance of such user-designed pools using the Illumina Experiment Manager's sample sheet generator.

For more information on the single-indexed sequencing workflow, see the Illumina HiSeq, HiScan, and Genome Analyzer user guides.

Figure 13 Single-Indexed–2-plex

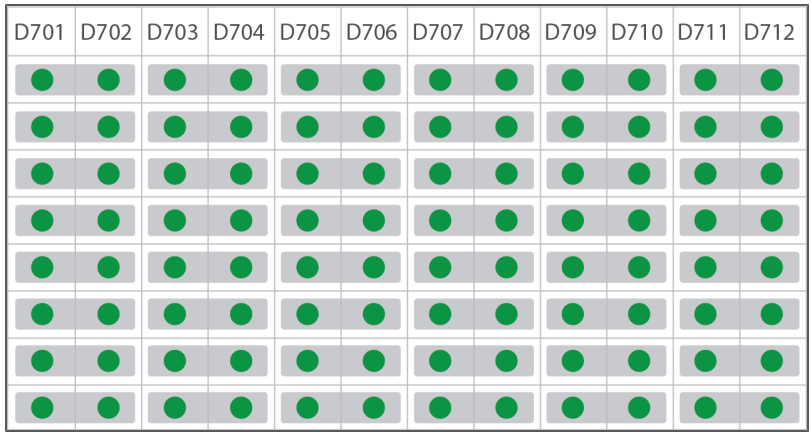


Figure 14 Single-Indexed–3-plex

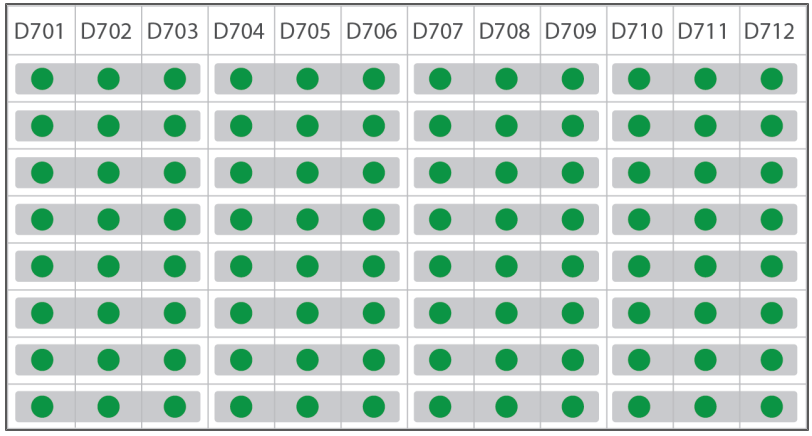


Figure 15 Single-Indexed–4-plex

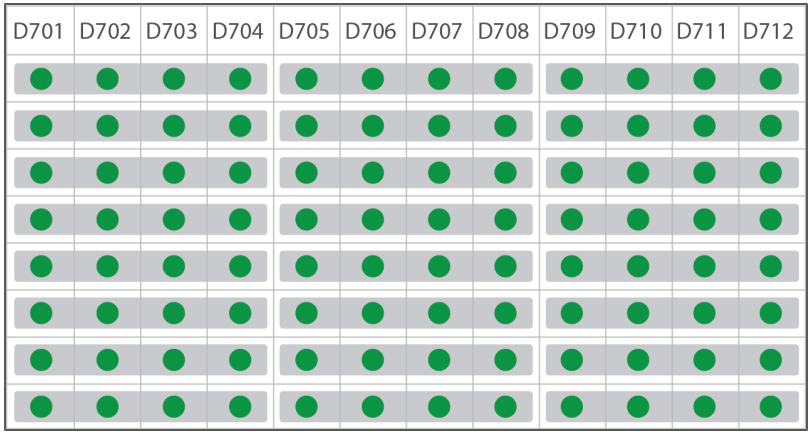


Figure 16 Single-Indexed–5-plex

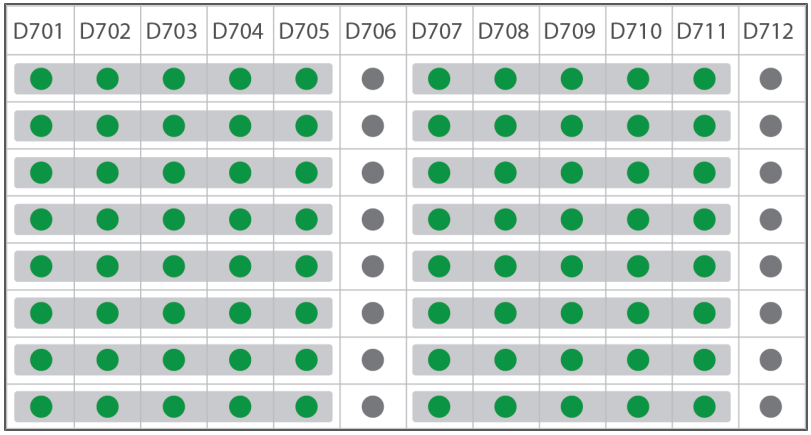


Figure 17 Single-Indexed–6-plex

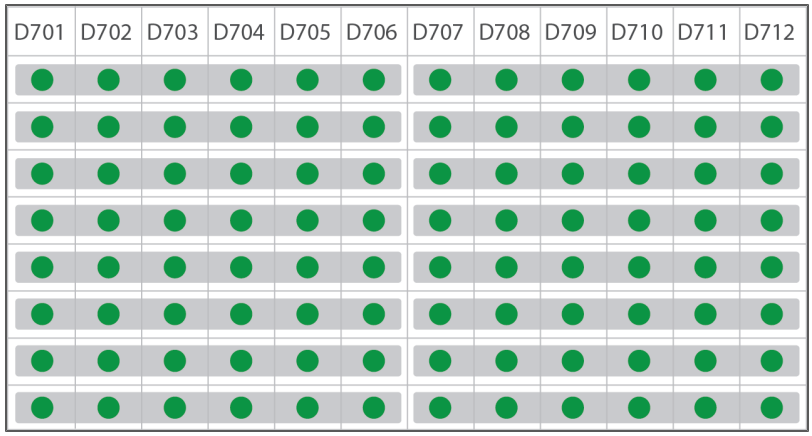
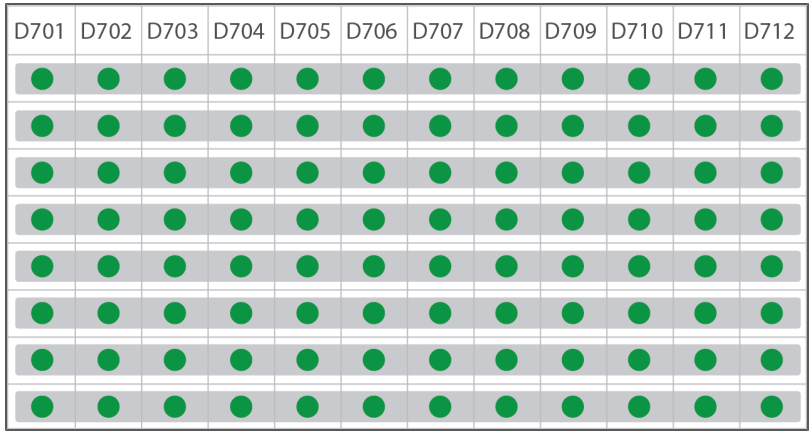


Figure 18 Single-Indexed–12-plex



Dual-Indexed Sequencing

Follow the dual-indexed sequencing workflow when pooling more than 12 samples. When designing the low-plexity index pools, always use at least two unique and compatible barcodes for each index sequenced. The following figures illustrate possible pooling strategies for 2–16 samples generated with the DAP.

- Color balanced pools are shaded light gray with green wells. The 2-plex pools are diagonal and shaded in light or dark gray with green wells.
- Odd numbered pools display dark gray wells that are not used for pooled sequencing. They are available for individual sequencing.
- Not all color-balanced pools are illustrated. Check the color balance of such user-designed pools using the Illumina Experiment Manager's sample sheet generator.

For more information on the dual-indexed sequencing workflow, see the Illumina HiSeq, HiScan, Genome Analyzer, and MiSeq user guides.

Figure 19 Dual-Indexed–2-plex

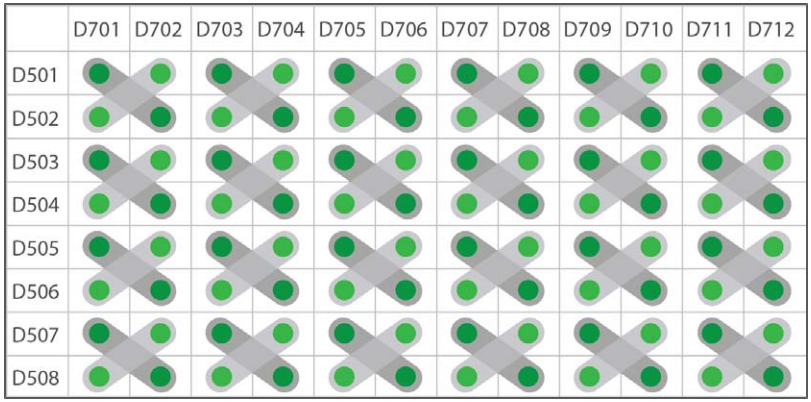


Figure 20 Dual-Indexed–3-plex

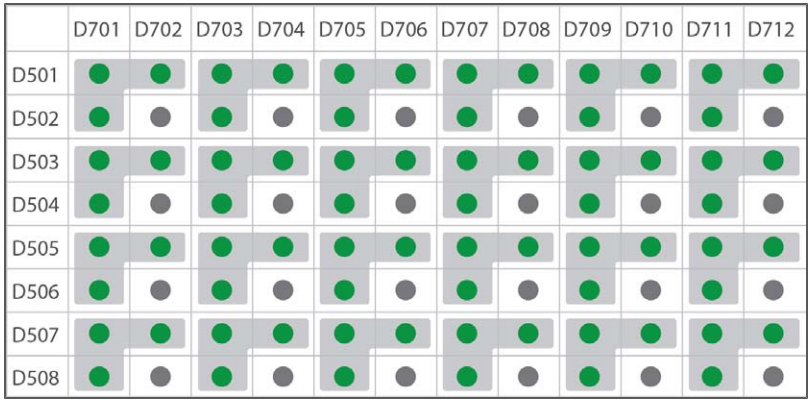


Figure 21 Dual-Indexed–4-plex

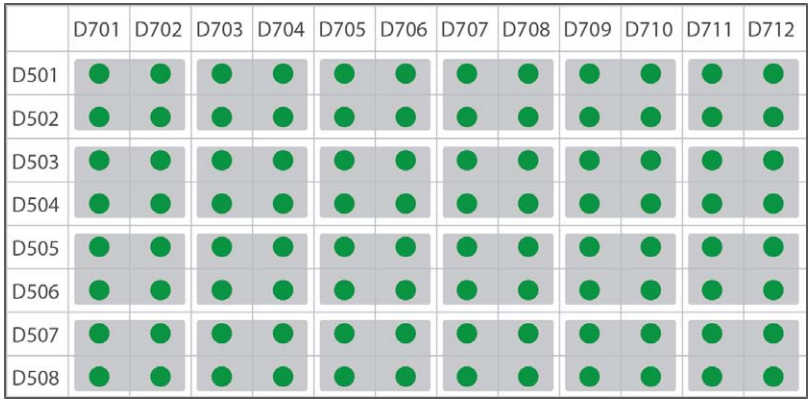


Figure 22 Dual-Indexed–5-plex

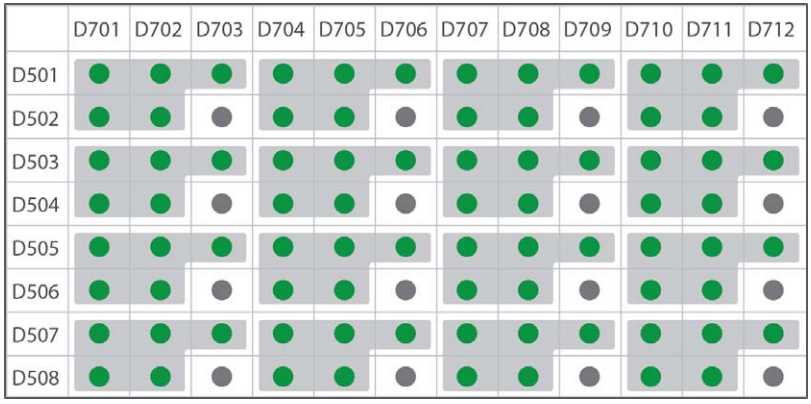


Figure 23 Dual-Indexed–6-plex

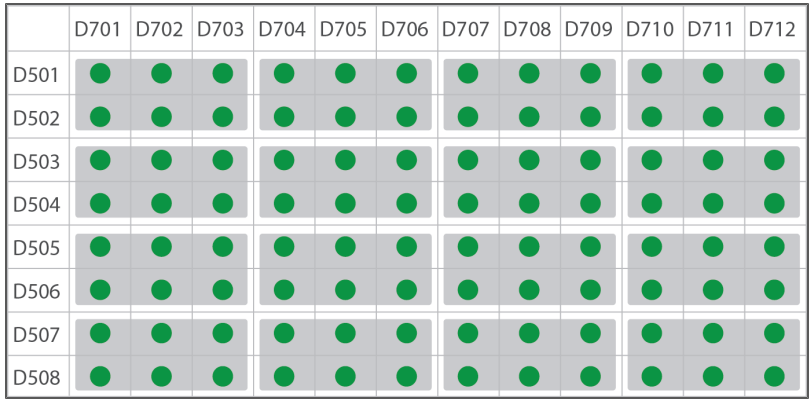


Figure 24 Dual-Indexed–7-plex

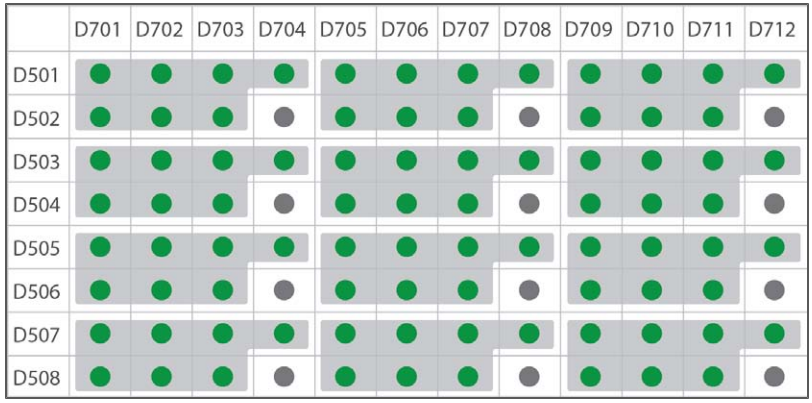


Figure 25 Dual-Indexed–8-plex, Option 1

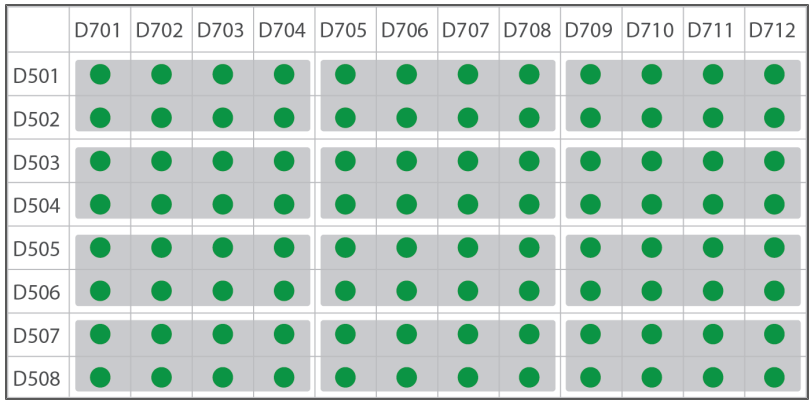


Figure 26 Dual-Indexed–8-plex, Option 2

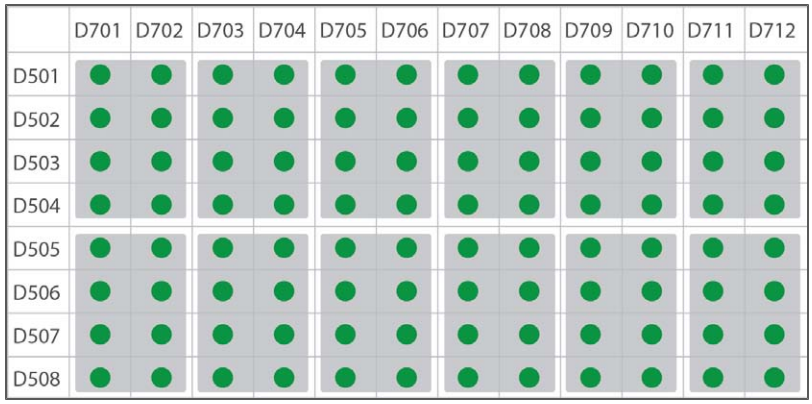


Figure 27 Dual-Indexed–12-plex

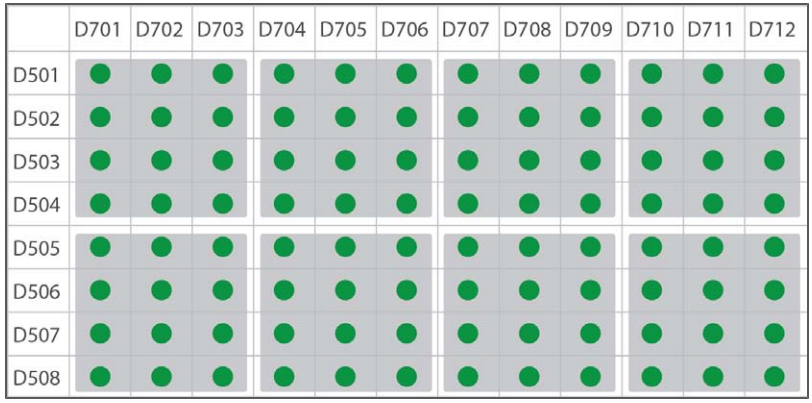
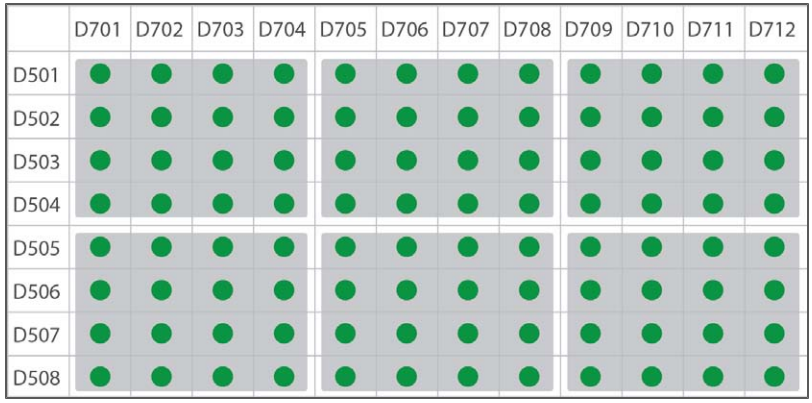


Figure 28 Dual-Indexed–16-plex



Low Sample (LS) Protocol

Chapter 3

Introduction

This chapter describes the TruSeq DNA Sample Preparation low sample (LS) protocol. Illumina recommends the following kit, sample number, and protocol combinations:

Table 18 Kit and Sample Number Recommendations

Number of Samples Processed At One Time	Kit Recommended
<24	LT
24–48	LT or HT
>48	HT

Table 19 Kit and Protocol Recommendations

Kit	Number of Samples Supported	Number of Samples Processed At One Time	Protocol
LT	48	≤48	LS
		>48	HS
HT	96	≤48	LS
		>48	HS

- Review *Best Practices* on page 11 before proceeding.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample preparation. For more information, see *Tracking Tools* on page 26.
- If you are pooling using adapter index tubes, record information about your samples before beginning library preparation for later use in data analysis. For more

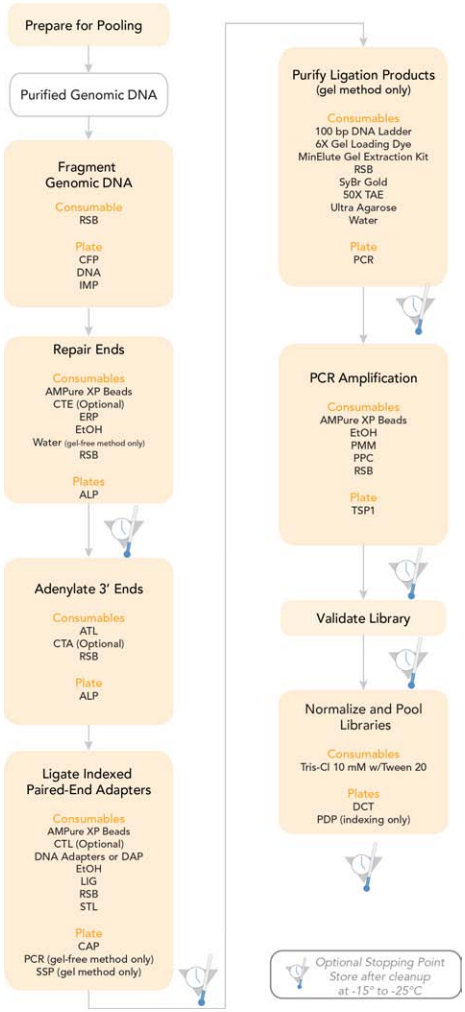
information, see *Tracking Tools* on page 26. Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.

- If you are pooling with the DAP, please review the planning steps in *Pooling Preparation with Adapter Plate* on page 41 before beginning library preparation.

Sample Prep Workflow

The following figure illustrates the processes of the TruSeq DNA Sample Preparation LS protocol to prepare templates using 24 indexed adapter tubes or a DAP.

Figure 29 TruSeq DNA Sample Preparation LS Workflow



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 described below was optimized to obtain final libraries with the following differences:

Table 20 Fragmentation Method Options

	Whole-genome Resequencing	TruSeq Enrichment	
	Gel Method	Gel-free Method	Gel Method
Covaris Shearing Duration	40 seconds	120 seconds	
Insert Size	300–400 bp	100–900 bp	200–300 bp

NOTE

If fragmenting using a nebulization technique, skip this procedure and perform the Appendix A Alternate Fragmentation Protocols. The nebulization procedures have only been validated for whole-genome resequencing or enrichment with the gel-method.

Calculate the amount of DNA to be fragmented based on 1 µg input DNA for each sample.

Illumina-Supplied Consumables

- Resuspension Buffer (RSB) (1 tube)
- CFP (Covaris Fragmentation Plate) barcode label
- DNA (DNA Plate) barcode label
- IMP (Insert Modification Plate) barcode label

User-Supplied Consumables

- 96-well 0.3 ml PCR plates (2)
- Covaris Tubes
- DNA samples

Preparation

- Review *DNA Input Recommendations* on page 22.
- Remove one tube of Resuspension Buffer from -15° to -25°C storage and thaw it at room temperature.

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

- Turn on the Covaris instrument at least 30 minutes before starting.
  - Following the manufacturer's instructions, de-gas and pre-chill the water to a temperature of 3° to 6°C. You can start the fragmentation procedure at 6°C.
- Apply a CFP barcode label to the Covaris tube plate.
- Apply a DNA barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a IMP barcode label to a new 96-well 0.3 ml PCR plate.

Make CFP

1. Illumina recommends to quantify gDNA samples using a fluorometric-based method such as Qubit or PicoGreen.
2. Illumina recommends to normalize the gDNA samples to a final volume of 55 µl at 20 ng/µl into each well of the new 0.3 ml PCR plate labeled with the DNA barcode.

Fragment DNA

1. Shear 1 µg of gDNA sample by transferring 52.5 µl of each DNA sample from the DNA plate to each Covaris tube in the new 0.3 ml PCR plate labeled with CFP barcode.

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

2. Fragment the DNA using the following settings:

Table 21 Covaris S220 or Covaris E220 Settings

Setting	Whole-genome Resequencing	TruSeq Enrichment
Duty factor	10%	10%
Peak Incident Power	175	175
Cycles per burst	200	200
Duration	40 seconds	2 x 60 seconds (120 seconds total)
Mode	Frequency sweeping	Frequency sweeping
Temperature	5.5° to 6°C	5.5° to 6°C

Table 22 Covaris S2 or E210 Settings

Setting	Whole-genome Resequencing	TruSeq Enrichment
Duty cycle	10%	10%
Intensity	5.0	5.0
Cycles per burst	200	200
Duration	40 seconds	2 x 60 seconds (120 seconds total)
Mode	Frequency sweeping	Frequency sweeping
Displayed Power	Covaris S2 - 23W Covaris E210 - 18W	Covaris S2 - 23W Covaris E210 - 18W
Temperature	5.5° to 6°C	5.5° to 6°C

3 Seal the Covaris tubes and centrifuge to 600 xg for 1 minute.

4 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 IMP barcode using a single channel pipette.

NOTE

- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate 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.

Perform End Repair

This process converts the overhangs resulting from fragmentation into blunt ends using an End Repair Mix. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the polymerase activity fills in the 5' overhangs.

Illumina-Supplied Consumables

- (Optional) End Repair Control (CTE) (1 tube per 48 reactions)
- End Repair Mix (ERP) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)
- ALP (Adapter Ligation Plate) barcode label

User-Supplied Consumables

- 96-well 0.3 ml PCR plate
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seal
- PCR Grade Water (for gel-free method for enrichment only)
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - End Repair Control
  - End Repair Mix

  NOTE
  The use of the End Repair Control is optional and it can be replaced with the same volume of Resuspension Buffer.
- Review *Handling Magnetic Beads* on page 12.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the thermal cycler to 30°C.
- Choose the thermal cycler pre-heat lid option and set to 100°C

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

Make IMP

1. Do one of the following:
   - If using the in-line control reagent:
     - Centrifuge the thawed End Repair Control tube to 600 xg for 5 seconds.
     - Add 10 µl of thawed End Repair Control to each well of the IMP plate that contains 50 µl of fragmented DNA.
   - If not using the in-line control reagent, add 10 µl of Resuspension Buffer to each well of the IMP plate that contains 50 µl of fragmented DNA.
2. Add 40 µl of End Repair Mix to each well of the IMP plate containing the fragmented DNA. Gently pipette the entire volume up and down 10 times to mix thoroughly.
3. Seal the IMP plate with a Microseal 'B' adhesive seal.

Incubate 1 IMP

1. Place the sealed IMP plate on the pre-heated thermal cycler. Close the lid and incubate at 30°C for 30 minutes.
2. Remove the IMP plate from the thermal cycler.

Clean Up IMP

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 12 when working with AMPure XP Beads.

1. Remove the adhesive seal from the IMP plate.
2. Vortex the AMPure XP Beads until they are well dispersed.
3. Do one of the following:
   - If using the gel-free method:
     - Determine the amount of AMPure XP beads and PCR grade water needed to combine to prepare a diluted bead mixture:
       AMPure XP beads: # of samples X 160 µl x 0.85 = µl AMPure XP beads. For

example, 1.632 ml of AMPure XP beads are needed for 12 samples.
PCR grade water: # of samples X 160 µl x 0.15 = µl PCR grade water. For example, 288 µl of PCR grade water is needed for 12 samples.

- Add 160 µl of the diluted bead mixture to each well of the IMP plate containing 100 µl of End Repair Mix. Gently pipette the entire volume up and down 10 times to mix thoroughly.

- If using the gel method, add 160 µl well-mixed AMPure XP Beads to each well of the IMP plate containing 100 µl of End Repair Mix. Gently pipette the entire volume up and down 10 times to mix thoroughly.

4 Incubate the IMP plate at room temperature for 15 minutes.

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

6 Using a 200 µl single channel or multichannel pipette set to 127.5 µl, remove and discard 127.5 µl of the supernatant from each well of the IMP plate.

7 Repeat step 6 once.

NOTE

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

8 With the IMP plate on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well with a sample without disturbing the beads.

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

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

11 Let the IMP plate stand at room temperature for 15 minutes to dry, then remove the plate from the magnetic stand.

12 Resuspend the dried pellet in each well with 17.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

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

15 Transfer 15 µl of the clear supernatant from each well of the IMP 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 to *Adenylate 3′ Ends* on page 67 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° to -25°C for up to seven 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.

Illumina-Supplied Consumables

- (Optional) A-Tailing Control (CTA) (1 tube per 48 reactions)
- A-Tailing Mix (ATL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)

User-Supplied Consumables

- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - A-Tailing Mix
  - A-Tailing Control

  NOTE
  The use of the A-Tailing Control is optional and it can be replaced with the same volume of Resuspension Buffer.
- Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up IMP* on page 64 and let stand to thaw at room temperature.
  - Centrifuge the thawed ALP plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the ALP plate.
- Pre-heat the thermal cycler to 37°C.
- Choose the thermal cycler pre-heat lid option and set to 100°C

Add ATL

1. Do one of the following:
   - If using the in-line control reagent, add 2.5 µl of thawed A-Tailing Control to each well of the ALP plate.
   - If not using the in-line control reagent, add 2.5 µl of Resuspension Buffer to each well of the ALP plate.
2. Add 12.5 µl of thawed A-Tailing Mix to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
3. Seal the ALP plate with a Microseal 'B' adhesive seal.

Incubate 1 ALP

1. Place the sealed ALP plate on the pre-heated thermal cycler. Close the lid and incubate at 37°C for 30 minutes.
2. Immediately remove the ALP plate from the thermal cycler, then proceed immediately to *Ligate Adapters* on page 69.

Ligate Adapters

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

Illumina-Supplied Consumables

- Ligation Mix (LIG) (1 tube per 48 reactions)
- Choose from the following depending on the kit you are using:
  - TruSeq DNA LT Sample Prep Kit contents:
    - DNA Adapter Indices (AD001–AD016, AD018–AD023, AD025, AD027) (1 tube per column of 8 reactions, depending on the DNA Adapter Indices being used)
  - TruSeq DNA HT Sample Prep Kit contents:
    - DAP (DNA Adapter Plate)
- (Optional) Ligation Control (CTL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)
- Stop Ligation Buffer (STL) (1 tube per 48 reactions)
- CAP (Clean Up ALP Plate) barcode label
- DAP (DNA Adapter Plate) barcode label (if using the HT kit)
- PCR (Polymerase Chain Reaction) barcode label (for gel-free method only)
- SSP (Size Separate Plate) barcode label (for gel method only)

User-Supplied Consumables

- 96-well 0.3 ml PCR plates (2)
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seals
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)

Preparation

- Remove the following from -15° 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.
  - If using the DAP, review *Handling Adapter Plate* on page 42.
- Stop Ligation Buffer

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

- Remove the Ligation Control from -15° to -25°C storage and thaw it at room temperature.

NOTE
The use of the Ligation Control is optional and it can be replaced with the same volume of Resuspension Buffer.

- Review *Handling Magnetic Beads* on page 12.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the thermal cycler to 30°C.
- Choose the thermal cycler pre-heat lid option and set to 100°C
- Apply a CAP barcode label to a new 96-well 0.3 ml PCR plate.
- Do one of the following:
  - If using the gel-free method, apply a PCR barcode label to a new 96-well 0.3 ml PCR plate.
  - If using the gel method, apply a SSP barcode label to a new 96-well 0.3 ml PCR plate.

NOTE
- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate 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.

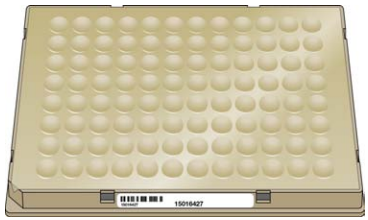
NOTE
Illumina recommends that the DAP does not undergo more than 4 freeze/thaw cycles. To maximize the use of the DAP, process more than 24 samples at a time. These samples can then be pooled in any supported configuration.

Add LIG

1. Do one of the following:
   - If using DNA Adapter tubes, centrifuge the appropriate/desired thawed tubes to 600 xg for 5 seconds.
   - If using a DAP:
     - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to ensure that they all are completely thawed.
     - Remove the adapter plate tape seal.
     - Centrifuge the plate at 280 xg for 1 minute to collect all of the adapter to the bottom of the well.
     - Remove the plastic cover and save the cover if you are not processing the entire plate at once.
     - If this is the first time using this DAP, apply the DAP barcode label to the plate.
2. Centrifuge the Ligation Control (if using Ligation Control) and Stop Ligation Buffer tubes to 600 xg for 5 seconds.
3. Immediately before use, remove the Ligation Mix tube from -15° to -25°C storage.
4. Remove the adhesive seal from the ALP plate.
5. Do one of the following:
   - If using the in-line control reagent, add 2.5 µl of thawed Ligation Control to each well of the ALP plate.
   - If not using the in-line control reagent, add 2.5 µl of Resuspension Buffer to each well of the ALP plate.
6. Add 2.5 µl of Ligation Mix to each well of the ALP plate.
7. Return the Ligation Mix tube back to -15° to -25°C storage immediately after use.
8. Do one of the following:
   - If using DNA Adapter tubes, add 2.5 µl of the appropriate/desired thawed DNA Adapter Index to each well of the ALP plate. 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 located on the lower left.

Figure 30 Correct DAP Orientation



- Do one of the following to pierce the foil seal:
  - If using the entire plate at once, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously by gently but firmly pressing 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 desired columns that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each column of adapters that will be used for ligation.
- Using an 8-tip multichannel pipette, transfer 2.5 µl of the appropriate/desired thawed DNA Adapter from the DAP well to each well of the ALP plate. 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 to 280 xg for 1 minute.

Incubate 2 ALP

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

2 Remove the ALP plate from the thermal cycler.

Add STL

1 Remove the adhesive seal from the ALP plate.

2 Add 5 µl of Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation. Gently pipette the entire volume up and down 10 times to mix thoroughly.

Clean Up ALP

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 12 when working with AMPure XP Beads.

1 Vortex the AMPure XP Beads until they are well dispersed, then add 42.5 µl of mixed AMPure XP Beads to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

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

4 Remove and discard 80 µl of the supernatant from each well of the ALP plate.

NOTE
Leave the ALP plate on the magnetic stand while performing the following 80% EtOH wash steps (5–7).

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

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

7 Repeat steps 5 and 6 once for a total of two 80% EtOH washes.

8 While keeping the ALP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes and then remove the plate from the magnetic stand.

9 Resuspend the dried pellet in each well with 52.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

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

12 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.

13 Vortex the AMPure XP Beads until they are well dispersed, then add 50 µl of mixed AMPure XP Beads to each well of the CAP plate for a second clean up. Gently pipette the entire volume up and down 10 times to mix thoroughly.

14 Incubate the CAP plate at room temperature for 15 minutes.

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

16 Remove and discard 95 µl of the supernatant from each well of the CAP plate.

NOTE
Leave the CAP plate on the magnetic stand while performing the following 80% EtOH wash steps (17–19)

17 With the CAP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

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

19 Repeat steps 17 and 18 once for a total of two 80% EtOH washes.

20 While keeping the CAP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes and then remove the plate from the magnetic stand.

21 Resuspend the dried pellet in each well with 22.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

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

24 Do one of the following:

- If using the gel-free method:
  - Transfer 20 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.
  - Proceed to *Enrich DNA Fragments* on page 80.
- If using the gel method:

- Transfer 20 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 SSP barcode.
- Proceed to *Purify Ligation Products (gel method only)* on page 76.

SAFE STOPPING POINT

If you do not plan to proceed to *Enrich DNA Fragments* on page 80 or *Purify Ligation Products (gel method only)* on page 76 immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR or SSP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Purify Ligation Products (gel method only)

This process is only performed when using the gel method. If you are running the gel-free method in preparation for the TruSeq Enrichment protocol, proceed to *Enrich DNA Fragments* on page 80.

NOTE

- TruSeq Enrichment refers to the Illumina TruSeq Exome Enrichment and TruSeq Custom Enrichment Kits that can be used following TruSeq DNA Sample Prep to prepare the library for sequencing targeted regions. For more information, see the *TruSeq Enrichment Guide*.
- The gel-free method is ***not*** an option when preparing libraries for standard (whole-genome) sequencing.

This process purifies the products of the ligation reaction on a gel and removes unligated adapters, as well as any adapters that might have ligated to one another, and selects a size-range of sequencing library appropriate for cluster generation.

Illumina suggests the following gel insert size targets and slice locations. The gel slice locations account for the length of the adapter sequences flanking the inserts. For other applications, other size ranges might be desired and the cut size adjusted accordingly.

Table 23 Size Selection Options

	Whole-genome Resequencing	TruSeq Enrichment
Insert Size Target	300–400 bp[a]	200–300 bp
3 mm Slice Location	400–500 bp	300–400 bp

a. +/- 1 standard deviation of 20 bp, i.e, a < 20% variance for read lengths of 2 × 75 bp or shorter

Illumina-Supplied Consumables

- PCR (Polymerase Chain Reaction Plate) barcode label
- Resuspension Buffer (RSB) (1 tube)

User-Supplied Consumables

- 50 X TAE Buffer
- 96-well 0.3 ml PCR plate
- BenchTop 100 bp DNA Ladder

- Clean Scalpels
- Certified Low-range Ultra Agarose
- Distilled Water
- 6X Gel Loading Dye
- MinElute Gel Extraction Kit
- SyBr Gold Nucleic Acid Gel Stain

NOTE
Illumina strongly recommends using the user-supplied consumables specified. Any deviation from these materials can result in incorrect size-excision or require additional user optimization.

Preparation

- Prepare 1X TAE buffer (> 1 L)
- Apply a PCR barcode label to a new 96-well 0.3 ml PCR plate.
- Remove the SSP plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 73 and let stand to thaw at room temperature.
  - Centrifuge the thawed SSP plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed SSP plate.
- Clean the tray, the comb, and the gel tank with ethanol and rinse them thoroughly with deionized water to avoid cross contamination.

NOTE
Use the 12-well comb included with the recommended gel system.

Size Separate SSP

1. Prepare a 150 ml, 2% agarose with SyBr Gold gel using 1 X TAE Buffer as follows:
   a. Add 3 g of agarose powder in 150 ml of 1X TAE buffer.
   b. Microwave the gel buffer until the agarose powder is completely dissolved.
   c. Cool the gel buffer on the bench for 5 minutes, and then add 15 µl of SyBr Gold. Swirl to mix.
   d. Pour the entire gel buffer to the gel tray.

NOTE
The final concentration of SyBr Gold should be 1X in the agarose gel buffer.

WARNING
It is very important to pre-stain your gel with SyBr Gold. When using other staining dyes or staining the gel after running, the DNA will migrate more slowly than the ladder. This will result in cutting out the wrong size fragments.

2 Remove the adhesive seal from the thawed SSP plate.

3 Add 4 µl of 6X Gel Loading Dye to each well of the SSP plate.

4 Add 17 µl Resuspension Buffer and 4 µl of 6X Gel Loading Dye to 3 µl of DNA ladder.

WARNING
Do not to overload the DNA ladder. Without clear and distinct bands, it is difficult to excise the correct fragment size. Also, an overloaded ladder might run faster than the DNA sample library.

5 When the agarose gel is set, put it in the gel electrophoresis unit and fill the tank with 1X TAE Buffer to the maximum fill mark.
Dimensions recommended for the electrophoresis unit;
12 cm x 14 cm (W x L), 800 ml buffer volume

6 Load all of the ladder solution onto one lane of the gel.

7 Load the samples from each well of the SSP plate onto the other lanes of the gel, leaving a gap of at least one empty lane between samples and ladders.

NOTE
Flanking the library on both sides with ladders can make the library excision easier.

NOTE
When handling multiple samples, to avoid the risk of cross-contamination between libraries, leave a gap of at least one empty lane between samples and use ladders on the first and last well of the gel to help locate the gel area to be excised.

8 Run the gel at 120 V constant voltage for 120 minutes.

9 View the gel on a Dark Reader transilluminator.

10 Do one of the following:

- For whole-genome resequencing, excise a band from the gel spanning the width of the lane and ranging in size from 400-500 bp using a clean scalpel. Use the DNA ladder as a guide.
- For enrichment, excise a band from the gel spanning the width of the lane and ranging in size from 300-400 bp using a clean scalpel. Use the DNA ladder as a guide. For more information, see the *TruSeq Enrichment Guide*.

NOTE
Cutting a band between 400–500 bp will result in an insert size of approximately 300–400 bp, accounting for the size of the adapters. Adapters add approximately 120 bp to each fragment. The sequencing read length should be considered when cutting fragment sizes. Sequencing reads that over-reach into the adapter will cause chimeric reads, unalignable to the reference sequence.

NOTE
Use a clean scalpel per sample to avoid sample cross-contamination.

Size Separate Gel

1. Follow the instructions in the MinElute Gel Extraction Kit to purify each sample. Incubate the gel slices in the QG solution at room temperature (not at 50°C as instructed) until the gel slices have completely dissolved, while vortexing every 2 minutes.
2. Follow the instructions in the MinElute Gel Extraction Kit to purify on one MinElute spin column, eluting in 25 µl of QIAGEN EB.
3. Transfer 20 µl of each sample from the MinElute collection tube to the new 0.3 ml PCR plate labeled with the PCR barcode using a single channel pipette.

SAFE STOPPING POINT
If you do not plan to proceed to *Enrich DNA Fragments* on page 80 immediately, 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° to -25°C for up to seven 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. The number of PCR cycles should be minimized 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, as fragments without any adapters cannot hybridize to surface-bound primers in the flow cell, and fragments with an adapter on only one end can hybridize to surface bound primers but cannot form clusters.

Illumina-Supplied Consumables

- PCR Master Mix (PMM) (1 tube per 48 reactions)
- PCR Primer Cocktail (PPC) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)
- TSP1 (Target Sample Plate) barcode label

User-Supplied Consumables

- 96-well 0.3 ml PCR plate
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seals
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)

Preparation

- Remove the PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature. When thawed, keep the tubes on ice.
- Centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- Review *Handling Magnetic Beads* on page 12.

- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the PCR plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 73 or *Size Separate Gel* on page 79 and let stand to thaw at room temperature.
  - Centrifuge the thawed PCR plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed PCR plate.
- Pre-program the thermal cycler with the following program and save as **PCR**:
  - Choose the pre-heat lid option and set to 100°C
  - 98°C for 30 seconds
  - 10 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 10°C

NOTE
Illumina recommends 10 cycles of PCR for robust protocol performance. However, to optimize yield versus cycle number, a titration of PCR cycles can also be performed.

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

Make PCR

The following procedure assumes 1 µg of input DNA to library preparation and is designed to result in high library yields.

1. Add 5 µl of thawed PCR Primer Cocktail to each well of the PCR plate.
2. Add 25 µl of thawed PCR Master Mix to each well of the PCR plate. 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 on the pre-programmed thermal cycler. Close the lid and select **PCR** to amplify the plate.

Clean Up PCR

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 12 when working with AMPure XP Beads.

1 Remove the adhesive seal from the PCR plate.

2 Vortex the AMPure XP Beads until they are well dispersed, then do one of the following:
- If using the DNA Adapter tubes, add 50 µl of the mixed AMPure XP Beads to each well of the PCR plate containing 50 µl of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- If using the DAP, add 47.5 µl of the mixed AMPure XP Beads to each well of the PCR plate containing 50 µl of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

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

5 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 (6–8).

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

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

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

9 While keeping the PCR plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes and then remove the plate from the magnetic stand.

10 Resuspend the dried pellet in each well with 32.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

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

13 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.

14 Do one of the following:

- If performing whole-genome resequencing, proceed to *Validate Library* on page 84.
- If performing enrichment, proceed to the *TruSeq Enrichment Guide* for instructions on how to quantify and qualify your library.

SAFE STOPPING POINT

If you do not plan to proceed to *Validate Library* on page 84 or TruSeq Enrichment immediately, the protocol can be safely stopped here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Validate Library

Illumina recommends performing the following procedures for quality control analysis on your whole-genome resequencing sample library and quantification of the DNA library templates. If performing enrichment, proceed directly to the *TruSeq Enrichment Guide* for instructions on how to quantify and qualify your library.

Quantify Libraries

In order to achieve the highest quality of data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of every flow cell. This requires accurate quantitation of DNA library templates. Quantify your libraries using qPCR according to the Illumina *Sequencing Library qPCR Quantification Guide*.

Quality Control (Optional)

To verify the size of your PCR enriched fragments, check the template size distribution by running an aliquot of the DNA library on a gel or on a Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip or DNA 1000 chip. Running samples on a Bioanalyzer should be used for qualitative purposes only.

- If validating by gel, load 10% of the volume of the library on a gel and check that the size range is as expected: a narrow smear similar in size to the DNA excised from the gel after the ligation.
- If using the Agilent Bioanalyzer with a High Sensitivity DNA chip, make a 1:50 dilution of the library using water and load 1 µl of the diluted library on the Agilent High Sensitivity DNA chip.
- If using the Agilent Bioanalyzer with a DNA 1000 chip, load 1 µl of the library on the Agilent DNA 1000 chip.

Figure 31 Example of DNA Library Size Distribution for Whole-Genome Resequencing

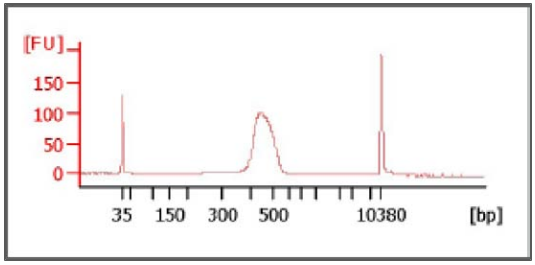
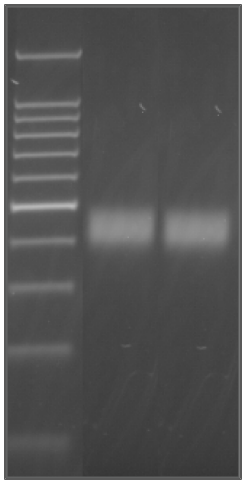


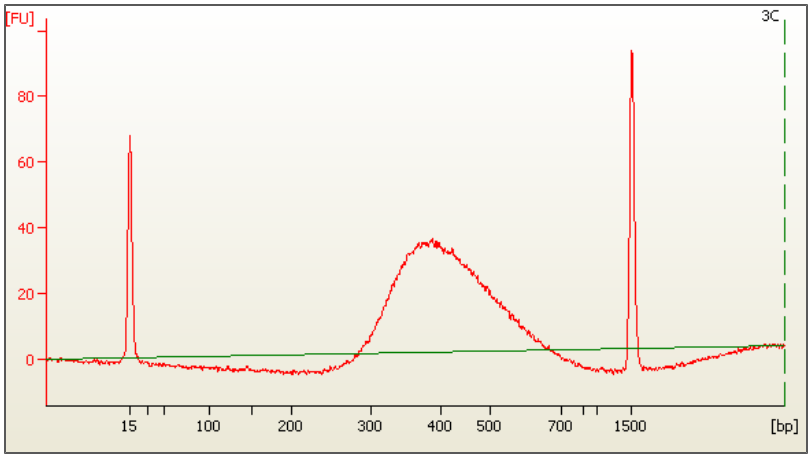
Figure 32 DNA PCR Product



NOTE

For a gel size selected library, if the DNA is not a narrow smear, but is comprised of a long smear of several hundred base pairs, or contains an intense 126 bp fragment (adapter-dimer), then another purification step is recommended. Repeat *Purify Ligation Products (gel method only)* on page 76.

Figure 33 Example of DNA Library Size Distribution with the Gel-Free Method



Normalize and Pool Libraries

This process describes how to prepare DNA templates that will be applied to 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 indexing are normalized to 10 nM in the DCT plate without pooling.

Illumina-Supplied Consumables

- DCT (Diluted Cluster Template) barcode label
- PDP (Pooled DCT Plate) barcode label (for indexing only)

User-Supplied Consumables

- 96-well 0.3 ml PCR plate (for indexing only)
- 96-well MIDI plate
- Microseal 'B' Adhesive seals
- Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20

Preparation

- Apply a DCT barcode label to a new 96-well MIDI plate.
- Apply a PDP barcode label to a new 96-well 0.3 ml PCR plate (for indexing only).
- Remove the TSP1 plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 82, and let stand to thaw at room temperature.
  - Centrifuge the thawed TSP1 plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.

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 DCT plate to 10 nM using Tris-Cl 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-indexed libraries, the protocol stops here. Do one of the following:
  - Proceed to cluster generation. For more information, see the *Illumina Cluster Generation User Guide*.
  - Seal the DCT plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.
- For indexed libraries, proceed to Make PDP.

Make PDP (for indexing only)

NOTE
Do not make a PDP plate if there is no pooling.

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

NOTE
Keep track 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 0.3 ml PCR plate labeled with the PDP barcode.

  The total volume in each well of the PDP plate should be 10X the number of combined sample libraries and will be 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 25–96 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 from 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 will be 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.

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 *Illumina Cluster Generation User Guide*.
- Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.

High Sample (HS) Protocol

Chapter 4

Introduction

This chapter describes the TruSeq DNA Sample Preparation high sample (HS) protocol. Illumina recommends the following kit, sample number, and protocol combinations:

Table 24 Kit and Sample Number Recommendations

Number of Samples Processed At One Time	Kit Recommended
<24	LT
24–48	LT or HT
>48	HT

Table 25 Kit and Protocol Recommendations

Kit	Number of Samples Supported	Number of Samples Processed At One Time	Protocol
LT	48	≤48	LS
		>48	HS
HT	96	≤48	LS
		>48	HS

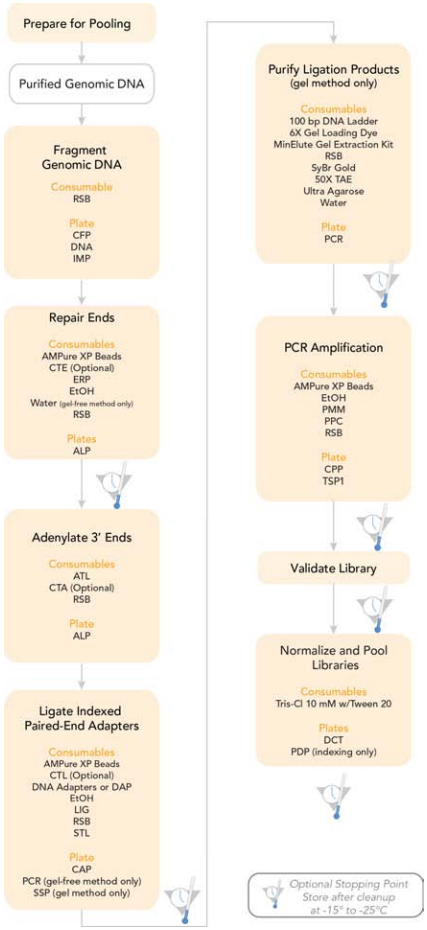
- Review *Best Practices* on page 11 before proceeding.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- This HS protocol requires shaking and heating equipment to mix reagents and for incubation (see *Consumables and Equipment* on page 32).
- For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample preparation. For more information, see *Tracking Tools* on page 26.

- If you are pooling using adapter index tubes, record information about your samples before beginning library preparation for later use in data analysis. For more information, see *Tracking Tools* on page 26. Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
- If you are pooling with the DAP, review the planning steps in *Pooling Preparation with Adapter Plate* on page 41 before beginning library preparation.

Sample Prep Workflow

The following figure illustrates the processes of the TruSeq DNA Sample Preparation HS protocol to prepare templates using 24 indexed adapter tubes or a DAP.

Figure 34 TruSeq DNA Sample Preparation HS Workflow



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 described below was optimized to obtain final libraries with the following differences:

Table 26 Fragmentation Method Options

	Whole-genome Resequencing	TruSeq Enrichment	
	Gel Method	Gel-free Method	Gel Method
Covaris Shearing Duration	40 seconds	120 seconds	
Insert Size	300–400 bp	100–900 bp	200–300 bp

NOTE

If fragmenting using a nebulization technique, skip this procedure and perform the Appendix A Alternate Fragmentation Protocols. The nebulization procedures have only been validated for whole-genome resequencing or enrichment with the gel-method.

Calculate the amount of DNA to be fragmented based on 1 µg input DNA for each sample.

Illumina-Supplied Consumables

- Resuspension Buffer (RSB) (1 tube)
- CFP (Covaris Fragmentation Plate) barcode label
- DNA (DNA Plate) barcode label
- IMP (Insert Modification Plate) barcode label

User-Supplied Consumables

- 96-well MIDI plates (2)
- Covaris Tubes
- DNA samples

Preparation

- Review *DNA Input Recommendations* on page 22.
- Remove one tube of Resuspension Buffer from -15° to -25°C storage and thaw it at room temperature.

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

- Turn on the Covaris instrument at least 30 minutes before starting.
  - Following the manufacturer's instructions, de-gas and pre-chill the water to a temperature of 3° to 6°C. You can start the fragmentation procedure at 6°C.
- Apply a CFP barcode label to the Covaris tube plate.
- Apply a DNA barcode label to a new 96-well MIDI plate.
- Apply a IMP barcode label to a new 96-well MIDI plate.

Make CFP

1. Illumina recommends to quantify gDNA samples using a fluorometric-based method such as Qubit or PicoGreen.
2. Illumina recommends to normalize the gDNA samples to a final volume of 55 µl at 20 ng/µl into each well of the new MIDI plate labeled with the DNA barcode.

Fragment DNA

1. Shear 1 µg of gDNA sample by transferring 52.5 µl of each DNA sample from the DNA plate to each Covaris tube in the new HSP plate labeled with CFP barcode.

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

2. Fragment the DNA using the following settings:

Table 27 Covaris S220 or Covaris E220 Settings

Setting	Whole-genome Resequencing	TruSeq Enrichment
Duty factor	10%	10%
Peak Incident Power	175	175
Cycles per burst	200	200
Duration	40 seconds	2 x 60 seconds (120 seconds total)
Mode	Frequency sweeping	Frequency sweeping
Temperature	5.5° to 6°C	5.5° to 6°C

Table 28 Covaris S2 or E210 Settings

Setting	Whole-genome Resequencing	TruSeq Enrichment
Duty cycle	10%	10%
Intensity	5.0	5.0
Cycles per burst	200	200
Duration	40 seconds	2 x 60 seconds (120 seconds total)
Mode	Frequency sweeping	Frequency sweeping
Displayed Power	Covaris S2 - 23W Covaris E210 - 18W	Covaris S2 - 23W Covaris E210 - 18W
Temperature	5.5° to 6°C	5.5° to 6°C

3 Seal the Covaris tubes and centrifuge to 600 xg for 1 minute.

4 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 IMP barcode using a single channel pipette.

NOTE

- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate 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.

Perform End Repair

This process converts the overhangs resulting from fragmentation into blunt ends using an End Repair Mix. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the polymerase activity fills in the 5' overhangs.

Illumina-Supplied Consumables

- (Optional) End Repair Control (CTE) (1 tube per 48 reactions)
- End Repair Mix (ERP) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)
- ALP (Adapter Ligation Plate) barcode label

User-Supplied Consumables

- 96-well MIDI plate
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seal
- PCR Grade Water (for gel-free method only)
- RNase/DNase-free Reagent Reservoirs
- RNase/DNase-free Strip Tubes and Caps

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - End Repair Control
  - End Repair Mix

  NOTE
  The use of the End Repair Control is optional and it can be replaced with the same volume of Resuspension Buffer.
- Review *Handling Magnetic Beads* on page 12.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the microheating system to 30°C.
- Calibrate the microplate shaker with a stroboscope and set it to 1,800 rpm.

- Apply a ALP barcode label to a new 96-well MIDI plate.

Make IMP

1. Do one of the following:
   - If using the in-line control reagent:
     - Centrifuge the thawed End Repair Control tube to 600 xg for 5 seconds.
     - Add 10 µl of thawed End Repair Control to each well of the IMP plate that contains 50 µl of fragmented DNA.
   - If not using the in-line control reagent, add 10 µl of Resuspension Buffer to each well of the IMP plate that contains 50 µl of fragmented DNA.
2. Add 40 µl of End Repair Mix 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 1,800 rpm for 2 minutes.
   c. Centrifuge the IMP plate to 280 xg for 1 minute.

Incubate 1 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.

Clean Up IMP

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 12 when working with AMPure XP Beads.

1. Remove the adhesive seal from the IMP plate.
2. Vortex the AMPure XP Beads until they are well dispersed.
3. Do one of the following:
   - If using the gel-free method for enrichment:
     - Determine the amount of AMPure XP beads and PCR grade water needed to combine to prepare a diluted bead mixture:
       AMPure XP beads: # of samples X 160 µl x 0.85 = µl AMPure XP beads. For

example, 6.528 ml of AMPure XP beads are needed for 48 samples.
PCR grade water: # of samples X 160 µl x 0.15 = µl PCR grade water. For example, 1.152 ml of PCR grade water is needed for 48 samples.

- Add 160 µl of the diluted bead mixture to each well of the IMP plate containing 100 µl of End Repair Mix.

- If using the gel method, add 160 µl well-mixed AMPure XP Beads to each well of the IMP plate containing 100 µl of End Repair Mix.

4 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 1,800 rpm for 2 minutes.

5 Incubate the IMP plate at room temperature for 15 minutes.

6 Place the IMP plate on the magnetic stand at room temperature for 15 minutes or until the liquid appears clear.

7 Remove the adhesive seal from the IMP plate.

8 Using a 200 µl single channel or multichannel pipette set to 127.5 µl, remove and discard 127.5 µl of the supernatant from each well of the IMP plate.

9 Repeat step 8 once.

NOTE

Leave the IMP plate on the magnetic stand while performing the following 80% EtOH wash steps (10–12).

10 With the IMP plate on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well with a sample without disturbing the beads.

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

12 Repeat steps 10 and 11 once for a total of two 80% EtOH washes.

13 Let the IMP plate stand at room temperature for 15 minutes to dry, then remove the plate from the magnetic stand.

14 Resuspend the dried pellet in each well with 17.5 µl Resuspension Buffer. 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 1,800 rpm for 2 minutes.

c Centrifuge the IMP plate to 280 xg for 1 minute.

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

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

17 Remove the adhesive seal from the IMP plate.

18 Transfer 15 µl of the clear supernatant from each well of the IMP 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 103 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° to -25°C for up to seven 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.

Illumina-Supplied Consumables

- (Optional) A-Tailing Control (CTA) (1 tube per 48 reactions)
- A-Tailing Mix (ATL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)

User-Supplied Consumables

- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs
- RNase/DNase-free Strip Tubes and Caps

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - A-Tailing Mix
  - A-Tailing Control

  NOTE
  The use of the A-Tailing Control is optional and it can be replaced with the same volume of Resuspension Buffer.

- Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up IMP* on page 100 and let stand to thaw at room temperature.
  - Centrifuge the thawed ALP plate to 280 xg for 1 minute
  - Remove the adhesive seal from the ALP plate.
- Pre-heat the microheating system to 37°C.

Add ATL

1 Do one of the following:
- If using the in-line control reagent, add 2.5 µl of thawed A-Tailing Control to each well of the ALP plate.
- If not using the in-line control reagent, add 2.5 µl of Resuspension Buffer to each well of the ALP plate.

2 Add 12.5 µl of 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 1,800 rpm for 2 minutes.
   c Centrifuge the ALP plate to 280 xg for 1 minute.

Incubate 1 ALP

1 Place the sealed ALP plate on the pre-heated microheating system. Close the lid and incubate at 37°C for 30 minutes.

2 Immediately remove the ALP plate from the microheating system, then proceed immediately to *Ligate Adapters* on page 105.

Ligate Adapters

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

Illumina-Supplied Consumables

- Ligation Mix (LIG) (1 tube per 48 reactions)
- Choose from the following depending on the kit you are using:
  - TruSeq DNA LT Sample Prep Kit contents:
    - DNA Adapter Indices (AD001–AD016, AD018–AD023, AD025, AD027) (1 tube per column of 8 reactions, depending on the DNA Adapter Indices being used)
  - TruSeq DNA HT Sample Prep Kit contents:
    - DAP (DNA Adapter Plate)
- (Optional) Ligation Control (CTL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)
- Stop Ligation Buffer (STL) (1 tube per 48 reactions)
- CAP (Clean Up ALP Plate) barcode label
- DAP (DNA Adapter Plate) barcode label (if using the HT kit)
- PCR (Polymerase Chain Reaction) barcode label (for gel-free method only)
- SSP (Size Separate Plate) barcode label (for gel method only)

User-Supplied Consumables

- 96-well MIDI plate
- 96-well HSP plate
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seals
- RNase/DNase-free Reagent Reservoirs
- RNase/DNase-free Strip Tubes and Caps

Preparation

- Remove the following from -15° 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.
  - If using the DAP, review *Handling Adapter Plate* on page 42.
- Stop Ligation Buffer

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

- Remove the Ligation Control from -15° to -25°C storage and thaw it at room temperature.

NOTE
The use of the Ligation Control is optional and it can be replaced with the same volume of Resuspension Buffer.

- Review *Handling Magnetic Beads* on page 12.
- Remove the AMPure XP beads from 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.
- Do one of the following:
  - If using the gel-free method for enrichment, apply a PCR barcode label to a new 96-well HSP plate.
  - If using the gel method, apply a SSP barcode label to a new 96-well HSP plate.

NOTE
- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate 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.

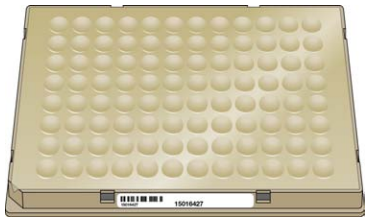
NOTE
Illumina recommends that the DAP does not undergo more than 4 freeze/thaw cycles. To maximize the use of the DAP, process more than 24 samples at a time. These samples can then be pooled in any supported configuration.

Add LIG

1. Do one of the following:
   - If using DNA Adapter tubes, centrifuge the appropriate/desired thawed tubes to 600 xg for 5 seconds.
   - If using a DAP:
     - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to ensure that they all are completely thawed.
     - Remove the adapter plate tape seal.
     - Centrifuge the plate at 280 xg for 1 minute to collect all of the adapter to the bottom of the well.
     - Remove the plastic cover and save the cover if you are not processing the entire plate at once.
     - If this is the first time using this DAP, apply the DAP barcode label to the plate.
2. Centrifuge the Ligation Control (if using Ligation Control) and Stop Ligation Buffer tubes to 600 xg for 5 seconds.
3. Immediately before use, remove the Ligation Mix tube from -15° to -25°C storage.
4. Remove the adhesive seal from the ALP plate.
5. Do one of the following:
   - If using the in-line control reagent, add 2.5 µl of thawed Ligation Control to each well of the ALP plate.
   - If not using the in-line control reagent, add 2.5 µl of Resuspension Buffer to each well of the ALP plate.
6. Add 2.5 µl of Ligation Mix to each well of the ALP plate.
7. Return the Ligation Mix tube back to -15° to -25°C storage immediately after use.
8. Do one of the following:
   - If using DNA Adapter tubes, add 2.5 µl of the appropriate/desired 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 located on the lower left.

Figure 35 Correct DAP Orientation



— Do one of the following to pierce the foil seal:
  — If using the entire plate at once, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously by gently but firmly pressing 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 desired columns that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each column of adapters that will be used for ligation.
— Using an 8-tip multichannel pipette, transfer 2.5 µl of the appropriate/desired 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 1,800 rpm for 2 minutes.
  c Centrifuge the ALP plate to 280 xg 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.

Add STL

1. Remove the adhesive seal from the ALP plate.
2. Add 5 µl of 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 1,800 rpm for 2 minutes.
   c. Centrifuge the ALP plate to 280 xg for 1 minute.

Clean Up ALP

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 12 when working with AMPure XP Beads.

1. Remove the adhesive seal from the ALP plate.
2. Vortex the AMPure XP Beads until they are well dispersed, then add 42.5 µl of mixed AMPure XP 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 1,800 rpm for 2 minutes.
3. Incubate the ALP plate at room temperature for 15 minutes.
4. Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.
5. Remove the adhesive seal from the ALP plate.
6. Remove and discard 80 µl of the supernatant from each well of the ALP plate.

NOTE
Leave the ALP plate on the magnetic stand while performing the following 80% EtOH wash steps (7–9).

7. With the ALP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.
8. Incubate the ALP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.
9. Repeat steps 7 and 8 once for a total of two 80% EtOH washes.

10 While keeping the ALP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes.

11 Resuspend the dried pellet in each well with 52.5 µl Resuspension Buffer. 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 1,800 rpm for 2 minutes.

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

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

14 Remove the adhesive seal from the ALP plate.

15 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.

16 Vortex the AMPure XP Beads until they are well dispersed, then add 50 µl of mixed AMPure XP 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 1,800 rpm for 2 minutes.

17 Incubate the CAP plate at room temperature for 15 minutes.

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

19 Remove the adhesive seal from the CAP plate.

20 Remove and discard 95 µl of the supernatant from each well of the CAP plate.

NOTE
Leave the CAP plate on the magnetic stand while performing the following 80% EtOH wash steps (21–23)

21 With the CAP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

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

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

24 While keeping the CAP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes.

25 Resuspend the dried pellet in each well with 22.5 µl Resuspension Buffer. 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 1,800 rpm for 2 minutes.

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

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

28 Remove the adhesive seal from the CAP plate.

29 Do one of the following:

- If using the gel-free method for enrichment:
  - Transfer 20 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.
  - Proceed to *Enrich DNA Fragments* on page 116.
- If using the gel method:
  - Transfer 20 µl of the clear supernatant from each well of the CAP plate to the corresponding well of the new HSP plate labeled with the SSP barcode.
  - Proceed to *Purify Ligation Products (gel method only)* on page 112.

SAFE STOPPING POINT

If you do not plan to proceed to *Enrich DNA Fragments* on page 116 or *Purify Ligation Products (gel method only)* on page 112 immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR or SSP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Purify Ligation Products (gel method only)

This process is only performed when using the gel method. If you are running the gel-free method in preparation for the TruSeq Enrichment protocol, proceed to *Enrich DNA Fragments* on page 116.

NOTE

- TruSeq Enrichment refers to the Illumina TruSeq Exome Enrichment and TruSeq Custom Enrichment Kits that can be used following TruSeq DNA Sample Prep to prepare the library for sequencing targeted regions. For more information, see the *TruSeq Enrichment Guide*.
- The gel-free method is ***not*** an option when preparing libraries for standard (whole-genome) sequencing.

This process purifies the products of the ligation reaction on a gel and removes unligated adapters, as well as any adapters that might have ligated to one another, and selects a size-range of sequencing library appropriate for cluster generation.

Illumina suggests the following gel insert size targets and slice locations. The gel slice locations account for the length of the adapter sequences flanking the inserts. For other applications, other size ranges might be desired and the cut size adjusted accordingly.

Table 29 Size Selection Options

	Whole-genome Resequencing	TruSeq Enrichment
Insert Size Target	300–400 bp[a]	200–300 bp
3 mm Slice Location	400–500 bp	300–400 bp

a. +/- 1 standard deviation of 20 bp, i.e, a < 20% variance for read lengths of 2 × 75 bp or shorter

Illumina-Supplied Consumables

- PCR (Polymerase Chain Reaction Plate) barcode label
- Resuspension Buffer (RSB) (1 tube)

User-Supplied Consumables

- 50 X TAE Buffer
- 96-well HSP plate
- BenchTop 100 bp DNA Ladder

- Clean Scalpels
- Certified Low-range Ultra Agarose
- Distilled Water
- 6X Gel Loading Dye
- MinElute Gel Extraction Kit
- SyBr Gold Nucleic Acid Gel Stain

NOTE
Illumina strongly recommends using the user-supplied consumables specified. Any deviation from these materials can result in incorrect size-excision or require additional user optimization

Preparation

- Prepare 1X TAE buffer (> 1 L)
- Apply a PCR barcode label to a new 96-well HSP plate.
- Remove the SSP plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 109 and let stand to thaw at room temperature.
  - Centrifuge the thawed SSP plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed SSP plate.
- Clean the tray, the comb, and the gel tank with ethanol and rinse them thoroughly with deionized water to avoid cross contamination.

NOTE
Use the 12-well comb included with the recommended gel system.

Size Separate SSP

1. Prepare a 150 ml, 2% agarose with SyBr Gold gel using 1 X TAE Buffer as follows:
   a. Add 3 g of agarose powder in 150 ml of 1X TAE buffer.
   b. Microwave the gel buffer until the agarose powder is completely dissolved.
   c. Cool the gel buffer on the bench for 5 minutes, and then add 15 µl of SyBr Gold. Swirl to mix.
   d. Pour the entire gel buffer to the gel tray.

NOTE
The final concentration of SyBr Gold should be 1X in the agarose gel buffer.

WARNING
It is very important to pre-stain your gel with SyBr Gold. When using other staining dyes or staining the gel after running, the DNA will migrate more slowly than the ladder. This will result in cutting out the wrong size fragments.

2 Remove the adhesive seal from the thawed SSP plate.

3 Add 4 µl of 6X Gel Loading Dye to each well of the SSP plate.

4 Add 17 µl Resuspension Buffer and 4 µl of 6X Gel Loading Dye to 3 µl of DNA ladder.

WARNING
Do not to overload the DNA ladder. Without clear and distinct bands, it is difficult to excise the correct fragment size. Also, an overloaded ladder might run faster than the DNA sample library.

5 When the agarose gel is set, put it in the gel electrophoresis unit and fill the tank with 1X TAE Buffer to the maximum fill mark.
Dimensions recommended for the electrophoresis unit;
12 cm x 14 cm (W x L), 800 ml buffer volume

6 Load all of the ladder solution onto one lane of the gel.

7 Load the samples from each well of the SSP plate onto the other lanes of the gel, leaving a gap of at least one empty lane between samples and ladders.

NOTE
Flanking the library on both sides with ladders can make the library excision easier.

NOTE
When handling multiple samples, to avoid the risk of cross-contamination between libraries, leave a gap of at least one empty lane between samples and use ladders on the first and last well of the gel to help locate the gel area to be excised.

8 Run the gel at 120 V constant voltage for 120 minutes.

9 View the gel on a Dark Reader transilluminator.

10 Do one of the following:

- For whole-genome resequencing, excise a band from the gel spanning the width of the lane and ranging in size from 400-500 bp using a clean scalpel. Use the DNA ladder as a guide.
- For enrichment, excise a band from the gel spanning the width of the lane and ranging in size from 300-400 bp using a clean scalpel. Use the DNA ladder as a guide. For more information, see the *TruSeq Enrichment Guide*.

NOTE
Cutting a band between 400–500 bp will result in an insert size of approximately 300–400 bp, accounting for the size of the adapters. Adapters add approximately 120 bp to each fragment. The sequencing read length should be considered when cutting fragment sizes. Sequencing reads that over-reach into the adapter will cause chimeric reads, unalignable to the reference sequence.

NOTE
Use a clean scalpel per sample to avoid sample cross-contamination.

Size Separate Gel

1. Follow the instructions in the MinElute Gel Extraction Kit to purify each sample. Incubate the gel slices in the QG solution at room temperature (not at 50°C as instructed) until the gel slices have completely dissolved, while vortexing every 2 minutes.
2. Follow the instructions in the MinElute Gel Extraction Kit to purify on one MinElute spin column, eluting in 25 µl of QIAGEN EB.
3. Transfer 20 µl of each sample from the MinElute collection tube to the new HSP plate labeled with the PCR barcode using a single channel pipette.

SAFE STOPPING POINT
If you do not plan to proceed to *Enrich DNA Fragments* on page 116 immediately, 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° to -25°C for up to seven 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. The number of PCR cycles should be minimized 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, as fragments without any adapters cannot hybridize to surface-bound primers in the flow cell, and fragments with an adapter on only one end can hybridize to surface bound primers but cannot form clusters.

Illumina-Supplied Consumables

- PCR Master Mix (PMM) (1 tube per 48 reactions)
- PCR Primer Cocktail (PPC) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)
- CPP (Clean Up PCR Plate) barcode label
- TSP1 (Target Sample Plate) barcode label

User-Supplied Consumables

- 96-well MIDI plate
- 96-well HSP plate
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'A' Film
- Microseal 'B' Adhesive Seals
- RNase/DNase-free Reagent Reservoirs
- RNase/DNase-free Strip Tubes and Caps

Preparation

- Remove the PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature. When thawed, keep the tubes on ice.

- Centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- Review *Handling Magnetic Beads* on page 12.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the PCR plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 109 or *Size Separate Gel* on page 115 and let stand to thaw at room temperature.
  - Centrifuge the thawed PCR plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed PCR plate.
- Pre-program the thermal cycler with the following program and save as **PCR**:
  - Choose the pre-heat lid option and set to 100°C
  - 98°C for 30 seconds
  - 10 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 10°C

NOTE
Illumina recommends 10 cycles of PCR for robust protocol performance. However, to optimize yield versus cycle number, a titration of PCR cycles can also be performed.

- 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 1 µg of input DNA to library preparation and is designed to result in high library yields.

1. Add 5 µl of thawed PCR Primer Cocktail to each well of the PCR plate.
2. Add 25 µl of thawed PCR Master Mix to each well of the PCR plate. Mix thoroughly as follows:
   a. Seal the PCR plate with a Microseal 'A' film.

WARNING
Follow the vendor's 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 1,600 rpm for 20 seconds.

c Centrifuge the PCR plate to 280 xg for 1 minute.

Amp PCR

1 Place the sealed PCR plate on the pre-programmed thermal cycler. Close the lid and select **PCR** to amplify the plate.

Clean Up PCR

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 12 when working with AMPure XP Beads.

1 Remove the adhesive seal from the PCR plate.

2 Vortex the AMPure XP Beads until they are well dispersed, then do one of the following:
- If using the DNA Adapter tubes, add 50 µl of the mixed AMPure XP Beads to each well of the new MIDI plate labeled with the CPP barcode.
- If using the DAP, add 47.5 µl of the mixed AMPure XP Beads to each well of the new MIDI plate labeled with the CPP barcode.

3 Transfer the entire contents from each well of the PCR plate to the corresponding well of the CPP plate containing 50 µl of mixed AMPure XP 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 1,800 rpm for 2 minutes.

4 Incubate the CPP plate at room temperature for 15 minutes.

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

6 Remove the adhesive seal from the CPP plate.

7 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 (8–10).

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

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

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

11 While keeping the CPP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes.

12 Resuspend the dried pellet in each well with 32.5 µl Resuspension Buffer. 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 1,800 rpm for 2 minutes.

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

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

15 Remove the adhesive seal from the CPP plate.

16 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.

17 Do one of the following:

- If performing whole-genome resequencing, proceed to *Validate Library* on page 120.
- If performing enrichment, proceed to the *TruSeq Enrichment Guide* for instructions on how to quantify and qualify your library.

SAFE STOPPING POINT
If you do not plan to proceed to *Validate Library* on page 120 or TruSeq Enrichment immediately, the protocol can be safely stopped here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Validate Library

Illumina recommends performing the following procedures for quality control analysis on your whole-genome resequencing sample library and quantification of the DNA library templates. If performing enrichment, proceed directly to the *TruSeq Enrichment Guide* for instructions on how to quantify and qualify your library.

Quantify Libraries

In order to achieve the highest quality of data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of every flow cell. This requires accurate quantitation of DNA library templates. Quantify your libraries using qPCR according to the Illumina *Sequencing Library qPCR Quantification Guide*.

Quality Control (Optional)

To verify the size of your PCR enriched fragments, check the template size distribution by running an aliquot of the DNA library on a gel or on a Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip or DNA 1000 chip. Running samples on a Bioanalyzer should be used for qualitative purposes only.

- If validating by gel, load 10% of the volume of the library on a gel and check that the size range is as expected: a narrow smear similar in size to the DNA excised from the gel after the ligation.
- If using the Agilent Bioanalyzer with a High Sensitivity DNA chip, make a 1:50 dilution of the library using water and load 1 µl of the diluted library on the Agilent High Sensitivity DNA chip.
- If using the Agilent Bioanalyzer with a DNA 1000 chip, load 1 µl of the library on the Agilent DNA 1000 chip.

Figure 36 Example of DNA Library Size Distribution for Whole-Genome Resequencing

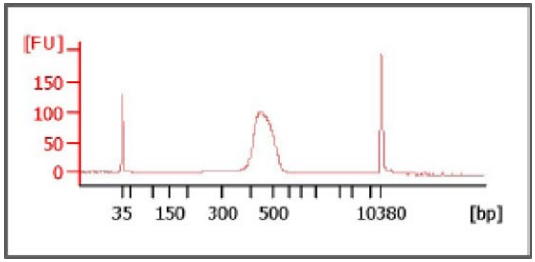
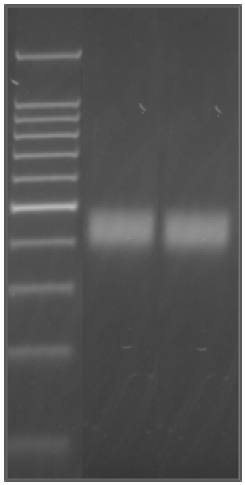


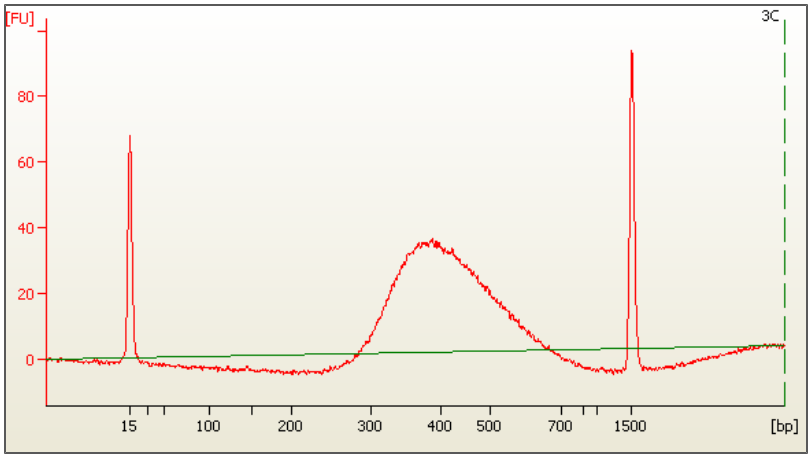
Figure 37 DNA PCR Product



NOTE

For a gel size selected library, if the DNA is not a narrow smear, but is comprised of a long smear of several hundred base pairs, or contains an intense 126 bp fragment (adapter-dimer), then another purification step is recommended. Repeat *Purify Ligation Products (gel method only)* on page 112.

Figure 38 Example of DNA Library Size Distribution with the Gel-Free Method



Normalize and Pool Libraries

This process describes how to prepare DNA templates that will be applied to 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 indexing are normalized to 10 nM in the DCT plate without pooling.

Illumina-Supplied Consumables

- DCT (Diluted Cluster Template) barcode label
- PDP (Pooled DCT Plate) barcode label (for indexing only)

User-Supplied Consumables

- 96-well HSP plate (for indexing only)
- 96-well MIDI plate
- Microseal 'B' Adhesive seals
- Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20

Preparation

- Apply a DCT barcode label to a new 96-well MIDI plate.
- Apply a PDP barcode label to a new 96-well HSP plate (for indexing only).
- Remove the TSP1 plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 118, and let stand to thaw at room temperature.
  - Centrifuge the thawed TSP1 plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.

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 DCT plate to 10 nM using Tris-Cl 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 1,000 rpm for 2 minutes.
   c Centrifuge the DCT plate to 280 xg for 1 minute.
   d Remove the adhesive seal from the DCT plate.

4 Depending on the type of library you want to generate, do one of the following:
   - For non-indexed libraries, the protocol stops here. Do one of the following:
     — Proceed to cluster generation. For more information, see the *Illumina Cluster Generation User Guide*.
     — Seal the DCT plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.
   - For indexed libraries, proceed to Make PDP.

Make PDP (for indexing only)

NOTE
Do not make a PDP plate if there is no pooling.

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

NOTE
Keep track 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 0.3 ml PCR plate labeled with the PDP barcode.

     The total volume in each well of the PDP plate should be 10X the number of combined sample libraries and will be 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 25–96 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 labeled with the PDP barcode.

- Transfer 5 µl of each normalized sample library in column 2 from 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 will be a PDP plate with pooled samples in column 1. Mix the PDP plate as follows:
  - Seal PDP plate with Microseal 'B' adhesive seal.
  - Shake PDP plate on microplate shaker at 1,800 rpm for 2 minutes.
- Combine the contents of each well of column 1 into well A2 of the PDP plate, for the final pool.

3 Mix the PDP plate as follows:

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

b Shake the PDP plate on a microplate shaker at 1,800 rpm for 2 minutes.

4 Do one of the following:

- Proceed to cluster generation. For more information, see the *Illumina Cluster Generation User Guide*.
- Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.

Alternate Fragmentation Protocols

Appendix A

Introduction

An alternative fragmentation method for TruSeq DNA Sample Preparation to the procedures described in *Fragment DNA* on page 59 for the LS protocol or *Fragment DNA* on page 95 for the HS protocol is using a nebulization technique, which breaks up DNA into pieces less than 800 bp in minutes using a disposable device. This process generates double-stranded DNA fragments containing 3' or 5' overhangs.

NOTE
These nebulization procedures have only been validated for whole-genome resequencing or enrichment with the gel-method.

Illumina-Supplied Consumables

- IMP (Insert Modification Plate) barcode label

User-Supplied Consumables

- 96-well 0.3 ml PCR plate (for LS protocol), or
- 96-well MIDI plate (for HS protocol)
- The following consumables are provided in the Paired-End Sample Preparation Kit:
  - Nebulizers (box of 10 nebulizers and vinyl accessory tubes)
  - Nebulization Buffer (7 ml)
  - TE Buffer
- QIAquick PCR Purification Kit
- Purified DNA (0.1–2 µg, 2 µg recommended)
  DNA should be as intact as possible, with an OD260/280 ratio of 1.8–2.0
- Compressed Air of at least 32 psi
  Do not use CO_2 which could alter the pH of the nebulizer buffer
- PVC tubing
  Dimensions: 1/4 inch ID, 3/8 inch OD, 1/16 inch wall, 1 meter length

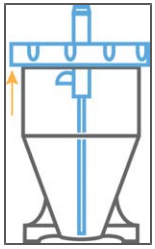
Procedure

The DNA sample to be processed should be highly pure, having an OD260/280 ratio of between 1.8 and 2.0, and should be as intact as possible.

NOTE
If you are not familiar with this shearing method, Illumina recommends that you test this procedure on test samples and practice assembling the nebulizer before proceeding with your sample DNA.

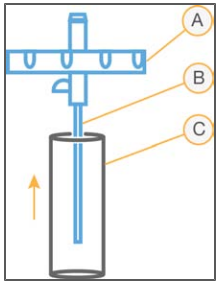
1 Remove a nebulizer from the plastic packaging and unscrew the blue lid.

Figure 39 Remove the Nebulizer Lid



2 Using gloves, remove a piece of vinyl tubing from the packaging and slip it over the central atomizer tube. Push it all the way to the inner surface of the blue lid.

Figure 40 Assemble the Nebulizer



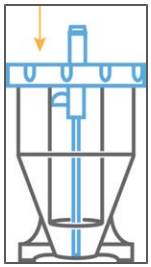
A Blue Lid
B Atomizer
C Vinyl Tubing

3 Add 0.1–2 µg of Purified DNA in a total volume of 50 µl of TE Buffer to the nebulizer.

4 Add 700 µl Nebulization Buffer to the DNA and mix well.

5 Screw the lid back on (finger-tight).

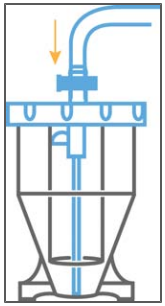
Figure 41 Replace the Nebulizer Lid



6 Chill the nebulizer containing the DNA solution on ice while performing the next step.

7 Connect the compressed air source to the inlet port on the top of the nebulizer with the PVC tubing, ensuring a tight fit.

Figure 42 Connect Compressed Air



8 Bury the nebulizer in an ice bucket and place it in a fume hood.

9 Use the regulator on the compressed air source to make sure the air is delivered at 32-35 psi.

10 Nebulize for 6 minutes. You might notice vapor rising from the nebulizer; this is normal. Also, the Nebulization Buffer might turn white or appear frozen.

11 Centrifuge the nebulizer at 450 xg for 2 minutes to collect the droplets from the side of the nebulizer. If necessary, use an old nebulizer as a counter-balance.

12 If a centrifuge is not available, then use 2 ml of the binding buffer (PB or PBI buffer) from the QIAquick PCR Purification Kit to rinse the sides of the nebulizer and collect the DNA solution at the base of the nebulizer.

13 Measure the recovered volume. Typically, you should recover 400–600 µl.

14 Follow the instructions in the QIAquick PCR Purification Kit to purify the sample solution and concentrate it on one QIAquick column, eluting in 50 µl of QIAGEN EB.

15 Transfer all of the 50 µl of fragmented DNA to each well of the new plate labeled with the IMP barcode using a single channel pipette.

16 Do one of the following:

- For LS processing, proceed to *Perform End Repair* on page 63.
- For HS processing, proceed to *Perform End Repair* on page 99.

SAFE STOPPING POINT

If you do not plan to proceed to Perform End Repair immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer. When proceeding, thaw the samples on ice.

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Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 30 Illumina General Contact Information

Illumina Website	http://www.illumina.com
Email	techsupport@illumina.com

Table 31 Illumina Customer Support Telephone Numbers

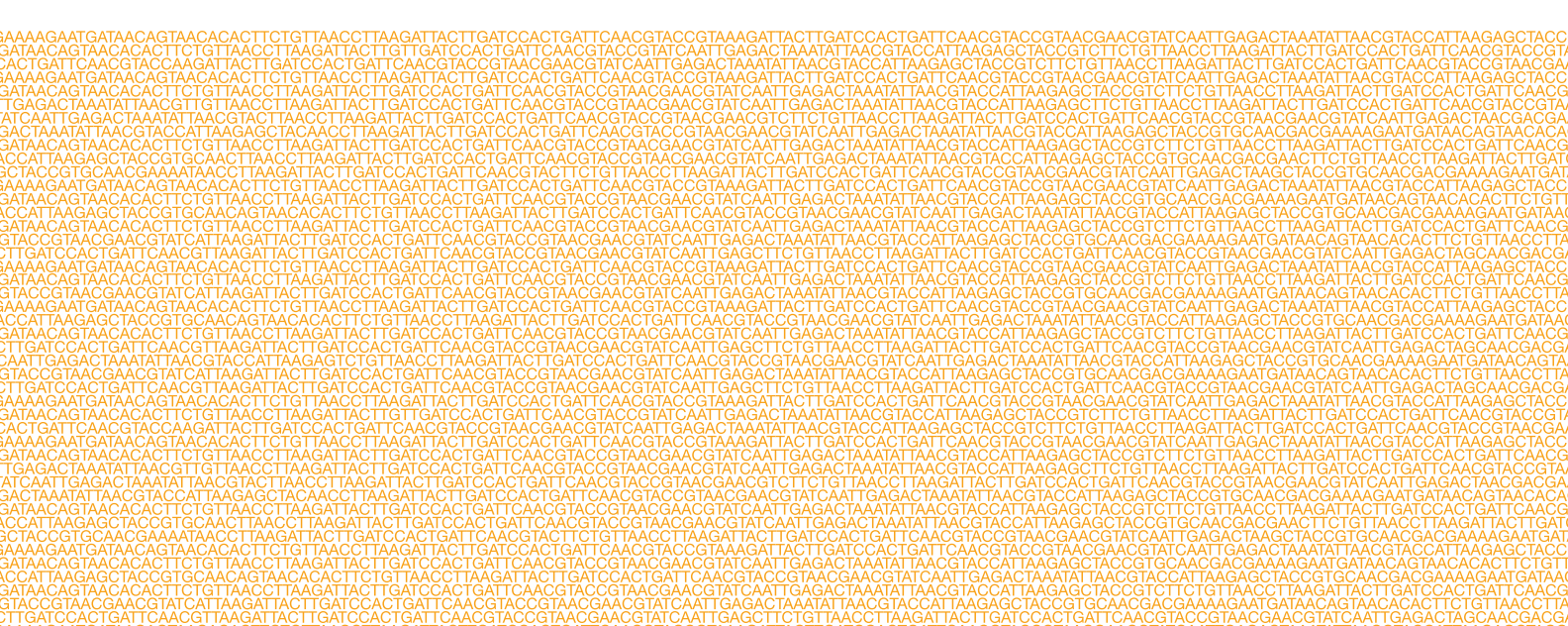
Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994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

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at http://www.illumina.com/msds.

Product Documentation

You can obtain PDFs of additional product documentation from the Illumina website. Go to http://www.illumina.com/support and select a product. To download documentation, you will be asked to log in to MyIllumina. After you log in, you can view or save the PDF. To register for a MyIllumina account, please visit https://my.illumina.com/Account/Register.



Illumina
Headquartered in San Diego, California, U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com