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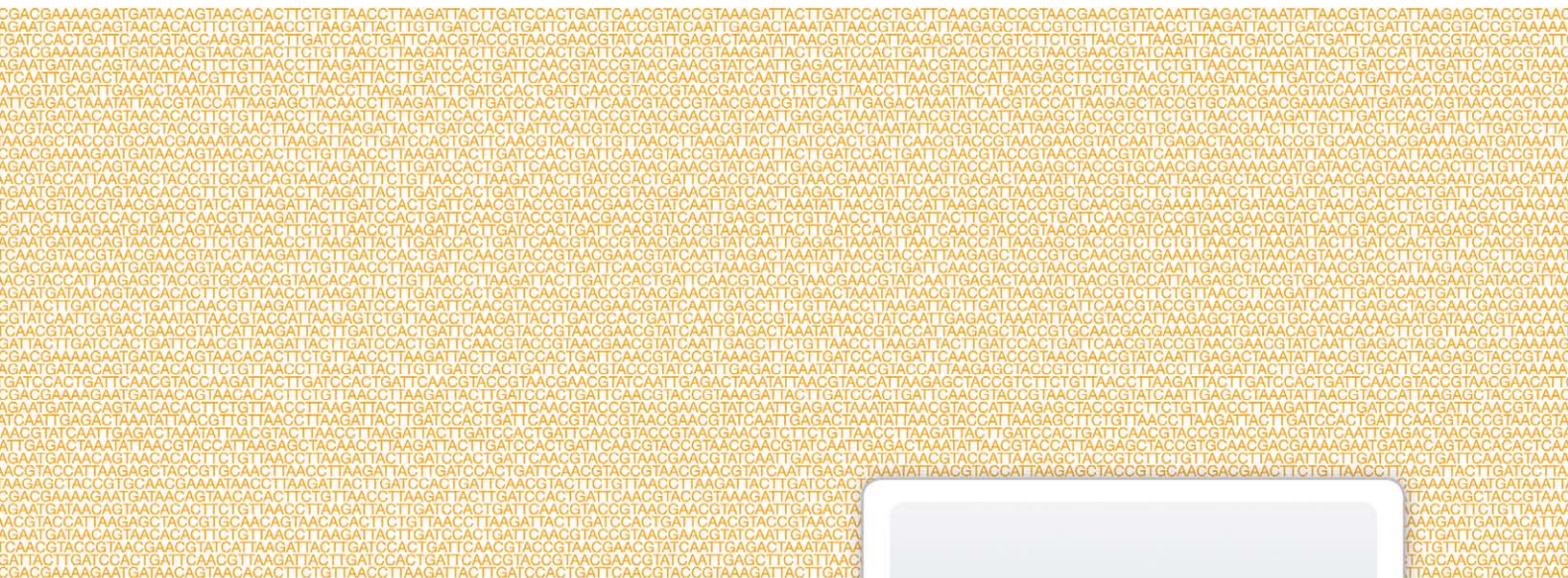
TruSeq™ DNA Sample Preparation v2 Guide



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Part #	Revision	Date	Description of Change
15026486	A	August 2011	Initial Release

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Overview

Chapter 1

Introduction

This protocol explains how to prepare 12 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 v2 Kit. The goal of this protocol is to add adapter sequences onto the ends of DNA fragments to generate multiplexed 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 multiplexing

Gel-free option for enrichment with optimized SPRI conditions

Optimized shearing for whole-genome resequencing

Higher Throughput

- Simultaneous preparation of 96 multiplexed DNA samples
- Volumes optimized for standard 96-well plate

Improved Troubleshooting

- Process control checks built-in for quality control

Universal Index Adapter Tags All Samples

- Additional adapters and primers not necessary
- Enables multiplexing earlier in the process

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 Sample Prep Kit v2.

- Chapter 3, explains how to perform the TruSeq DNA Sample Preparation v2 using the Low Throughput (LT) Protocol
- Chapter 4, explains how to perform the TruSeq DNA Sample Preparation v2 using the High Throughput (HT) Protocol

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

Table 1 Protocol Features

	Low Throughput	High Throughput
Number of Samples Processed	48 or fewer with indexed adapters	More than 48 with indexed adapters
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

The TruSeq DNA Sample Prep v2 fragmentation process is optimized to obtain final libraries, with the following differences

Table 2 Fragmentation Method Options

	Whole-genome Resequencing	TruSeq Exome 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

Getting Started

Chapter 2

Introduction

This chapter explains standard operating procedures and precautions for performing the TruSeq DNA Sample Preparation v2. 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 3 TruSeq DNA Sample Preparation v2 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
DCT	Diluted Cluster Template
dsDNA	double-stranded DNA
ERP	End Repair Mix
EUC	Experienced User Card
gDNA	genomic DNA
HSP	Hardshell Plate
HT	High Throughput
IMP	Insert Modification Plate
ISP	Intermediate Source Plate
LIG	Ligation Mix
LT	Low Throughput

Acronym	Definition
LTF	Lab Tracking Form
PCR	Polymerase Chain Reaction
PDP	Pooled Dilution Plate
PMM	PCR Master Mix
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
SSM	Second Strand Master Mix
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.

Liquid Handling

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 PicoGreen assays or 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 ensure that pipettes are correctly calibrated.
- Ensure 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 1 µl volumes. This will allow you to aliquot in a single pipetting movement to individual samples and standardize across multiple samples.

Master Mix Reagent Handling

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 may not have enough reagents for 48 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.

AMPure XP Handling

Following appropriate handling methods when working with Agencourt AMPure XP Beads:

NOTE
Cleanup procedures have only been verified using a 300 µl 96-well PCR or MIDI plate. Comparable performance is not guaranteed when using a microcentrifuge tube or other formats..

NOTE
Cleanup procedures have only been tested and validated using the magnetic stand specified in *Consumables and Equipment* on page 31 . Comparable performance is not guaranteed when using other magnets.

- Prior to use, allow the beads to come to room temperature.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous.
- **When performing the LT 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..
- **When performing the HT 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.
- For the wash steps, 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 may 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 may be required.
- Use the Resuspension Buffer (RSB) for DNA elution.
- Resuspend the dried pellets using a single channel or multichannel pipette.
- 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 DNA recovery during elution, incubate the DNA/bead mix for 2 minutes at room temperature before placing the samples onto the magnet.

Avoid Cross-Contamination

Practice the following to avoid cross-contaimination:

- Open only one adapter at the time.
- 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 may result from DNA contamination, for example, by 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 may 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), then many of these fragments 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 may 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 may also be estimated prior to library preparation using qPCR assays designed to target unique chromosomal markers.

Usage Guidelines

Illumina recommends these usage guidelines as the most efficient lab setup and pipetting process when performing the procedures specified in Chapter 3 Low-Throughput (LT) Protocol and Chapter 4 High-Throughput (HT) Protocol.

NOTE
The TruSeq DNA Sample Prep Kit v2 contains enough of each reagent to prepare 48 samples. If an alternate procedure is used, Illumina cannot guarantee that there will be enough of every reagent for 48 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.

Preparing 24 or More Samples

When preparing 24 or more samples, follow these reagent setup and distribution guidelines as you perform each procedure described in Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) 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:

- LT protocol - 0.3 ml PCR plate
- HT protocol - 96-well MIDI plate and 96-well HSP plate

Reagents in Reservoirs

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

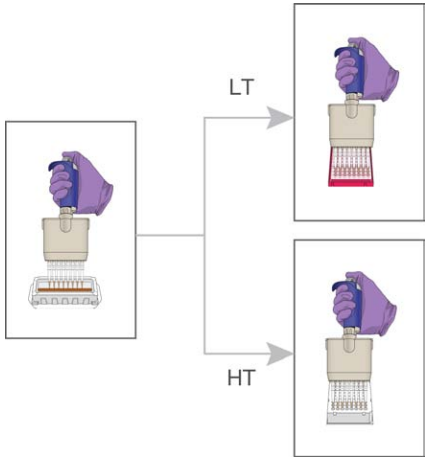
- 80% Ethanol
- AMPure XP Beads
- Resuspension Buffer

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 Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol 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, holding the pipette vertically. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol for the required reagent volume per sample.
   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.

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



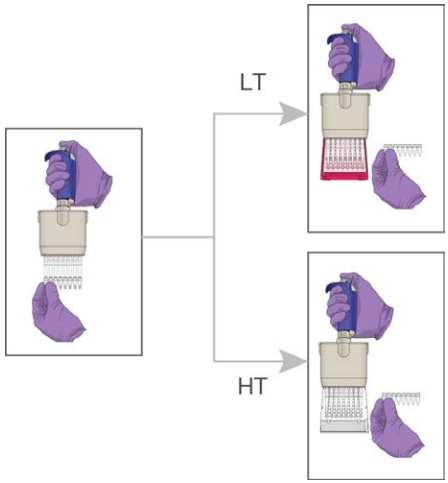
Reagents in Strip Tubes

When the remaining reagents not mentioned above, except the adapters, are required in the protocol, distribute each evenly across three wells of an 8-well strip tube. Add an allowance of 5 µl for dead volume per well.

When each reagent in an 8-well strip tube 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 8-well strip tube to the samples in the plate as follows, holding the pipette vertically. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol for the required reagent volume per sample.
   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.

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



Index Adapter Usage

When the index adapters are required in the protocol, 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 8-well strip tube:
  - Distribute the index adapters into the wells of an 8-well strip tube, with a different adapter in each well.
  - Add 2.5 µl of the appropriate/desired adapter index from the well of the 8-well strip tube to each well of the plate containing a sample, using a multichannel pipette.

Preparing 12–24 Samples

When preparing 12–24 samples, follow these reagent setup and distribution guidelines as you perform each procedure described in Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) 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 or cut the plate to separate the columns if desired. Use the appropriate plate for the protocol being performed:

- LT protocol - 0.3 ml PCR plate
- HT protocol - 96-well MIDI plate and 96-well HSP plate

Reagents in Reservoirs

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

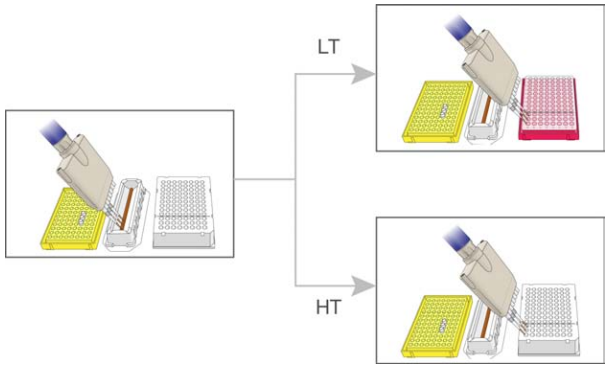
- 80% Ethanol
- AMPure XP Beads
- Resuspension Buffer

1. Determine the volume needed using the equation (# of samples x volume per sample) + 600 µl dead volume. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol 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 multichannel pipette with three tips, transfer the reagent in the reservoir to the samples in the plate as follows, holding the pipette vertically. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol for the required reagent volume per sample.
   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.

Figure 3 Transfer Reagent from Reservoir to Sample Plate with 12–24 Samples



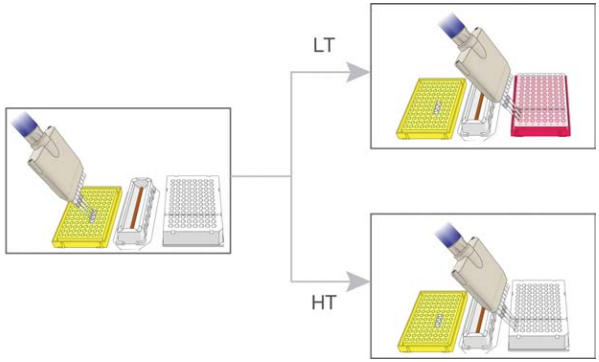
Reagents in Strip Tubes

When the remaining reagents not mentioned above, except the adapters, are required in the protocol, distribute each evenly across the three wells of an 8-well strip tube. Add an allowance of 5 µl for dead volume per well.

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

1 Using an multichannel pipette with three tips, transfer the reagent in the strip tube to the samples in the plate as follows, holding the pipette vertically. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol for the required reagent volume per sample.
   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.

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



Index Adapter Usage

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 reagent setup and distribution guidelines as you perform each procedure described in Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol.

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

Temperature Considerations

Temperature is an important consideration for making gDNA libraries:

- Keep libraries at temperatures ≤37°C.
- Avoid elevated temperatures, particularly in the steps preceding the adapter ligation.
- 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.

DNA Input Recommendations

It is important to quantitate the input DNA and assess the DNA quality prior to performingTruSeq DNA Sample Preparation v2.

Input DNA Quantitation

Follow these gDNA input recommendations:

- Correct quantification of genomic DNA is essential.
- Illumina recommends 1 µg input DNA.
- The ultimate success or failure of a library preparation strongly depends on using an accurately quantified amount of input DNA.
- Illumina recommends using fluorometric based methods for quantification including Qubit or PicoGreen to provide accurate quantification for 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.
- 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.
  - Ensure 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.
  - Genomic DNA samples should be carefully collected to ensure 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.
  - Impurities, such as detergents or proteins, can be revealed by smearing of DNA bands.
  - 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 may indicate nicking or other damage to DNA.
  - Where possible, or necessary, a gel should be run to assess the condition of the DNA sample.

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, 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 (Table 4). 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 4 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
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.

Tracking Tools

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

- **Experienced User Cards** to guide you through the protocol, but with less detail than provided in this user guide.
- **Lab Tracking Forms** to record lab equipment start and stop times and record the barcode of each reagent and plate used in the protocol.
- **Sample Sheet Template** to record information about your samples for later use in data analysis.

NOTE
All of these documents can be downloaded via http://www.illumina.com/support/documentation.ilmn.

Lab Tracking Form

Create a copy of the Lab Tracking Form for each run. Use it to track information about your sample preparation such as operator information, start and stop times, reagent lot numbers, and barcodes. This form can be filled out and saved online or printed and filled in by hand.

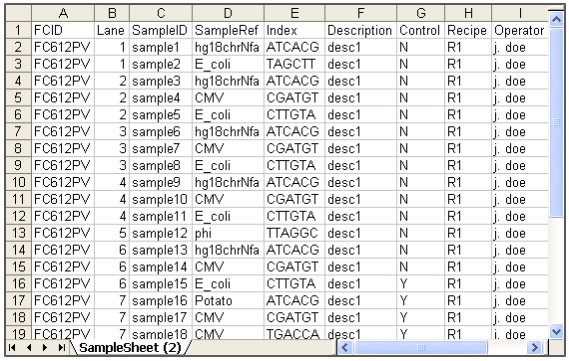
Sample Sheet

The sample sheet is a file that describes the samples in each lane, including the indexes used, and is required for demultiplexing following sequencing. For instructions on using the sample sheet to direct demultiplexing, see the analysis pipeline documentation.

The sample sheet is a comma-separated values (*.csv) file that contains the sample name and related information, as shown below. Create the sample sheet using Excel or another text editing tool that supports .csv files. Fill in your sample sheet according to the guidelines provided in this section.

Include lanes with multiplexed samples in the sample sheet, listing the information below. Lanes with a single sample can be left out of the sample sheet. These single sample lanes can then be aligned to a reference genome, as specified in the CASAVA config.template.txt file.

Figure 5 Example: Sample Sheet



	A	B	C	D	E	F	G	H	I
1	FCID	Lane	SampleID	SampleRef	Index	Description	Control	Recipe	Operator
2	FC612PV	1	sample1	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
3	FC612PV	1	sample2	E_coli	TAGCTT	desc1	N	R1	j. doe
4	FC612PV	2	sample3	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
5	FC612PV	2	sample4	CMV	CGATGT	desc1	N	R1	j. doe
6	FC612PV	2	sample5	E_coli	CTTGTA	desc1	N	R1	j. doe
7	FC612PV	3	sample6	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
8	FC612PV	3	sample7	CMV	CGATGT	desc1	N	R1	j. doe
9	FC612PV	3	sample8	E_coli	CTTGTA	desc1	N	R1	j. doe
10	FC612PV	4	sample9	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
11	FC612PV	4	sample10	CMV	CGATGT	desc1	N	R1	j. doe
12	FC612PV	4	sample11	E_coli	CTTGTA	desc1	N	R1	j. doe
13	FC612PV	5	sample12	phi	TTAGGC	desc1	N	R1	j. doe
14	FC612PV	6	sample13	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
15	FC612PV	6	sample14	CMV	CGATGT	desc1	N	R1	j. doe
16	FC612PV	6	sample15	E_coli	CTTGTA	desc1	Y	R1	j. doe
17	FC612PV	7	sample16	Potato	ATCACG	desc1	Y	R1	j. doe
18	FC612PV	7	sample17	CMV	CGATGT	desc1	Y	R1	j. doe
19	FC612PV	7	sample18	CMV	TGACCA	desc1	Y	R1	j. doe

SampleSheet (2)

The sample sheet has the following fields:

Table 5 Sample Sheet Fields

Column Header	Description
FCID	The flow cell ID
Lane	A positive integer indicating the lane number (1–8)
Sample ID	The sample ID. This can be used to specify samples in the CASAVA config.template.txt file.
Sample Ref	The reference sequence for the sample. This can be used to specify a reference genome in the CASAVA config.template.txt.
Index	The index sequence
Description	The sample description
Control	Y indicates the lane is a control lane N indicates a sample
Recipe	The recipe used during sequencing
Operator	The name or ID of the operator

NOTE

To avoid misidentifying samples, ensure that the sample IDs entered in the sample sheet correctly correspond to the DNA samples used.

Adapter Index Sequences

The following table lists the TruSeq DNA Sample Prep v2 adapter index sequences.

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) may not be A, and this will be seen in the seventh cycle of the index read.

Table 6 TruSeq DNA Sample Prep v2 Adapter Index Sequences

Indexed Adapter	Sequence	Indexed Adapter	Sequence
AD001	ATCACG(A)	AD013	AGTCAA(C)
AD002	CGATGT(A)	AD014	AGTTCC(G)
AD003	TTAGGC(A)	AD015	ATGTCA(G)
AD004	TGACCA(A)	AD016	CCGTCC(C)
AD005	ACAGTG(A)	AD018	GTCCGC(A)
AD006	GCCAAT(A)	AD019	GTGAAA(C)
AD007	CAGATC(A)	AD020	GTGGCC(T)
AD008	ACTTGA(A)	AD021	GTTTCG(G)
AD009	GATCAG(A)	AD022	CGTACG(T)
AD010	TAGCTT(A)	AD023	GAGTGG(A)
AD011	GGCTAC(A)	AD025	ACTGAT(A)
AD012	CTTGTA(A)	AD027	ATTCCT(T)

Kit Contents

Check to ensure that you have all of the reagents identified in this section before proceeding. Each TruSeq DNA Sample Prep Kit v2 can be used to process up to 48 samples. Set A and B differ in the index pooling level.

- TruSeq DNA Sample Prep Kit v2 - Set A (48rxn with PCR), catalog # FC-122-2001
- TruSeq DNA Sample Prep Kit v2 - Set B (48rxn with PCR), catalog # FC-122-2002

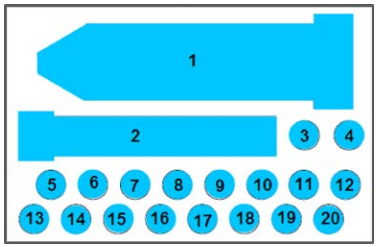
Kit Contents, Boxes A and B

You will receive either box A or B with you 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.

Figure 6 TruSeq DNA Sample Prep Kit v2, Box A



1 Resuspension Buffer (RSB)

2 End Repair Mix (ERP)

3 A-Tailing Mix (ATL)

4 Ligation Mix (LIG)

5 End Repair Control (CTE)

6 A-Tailing Control (CTA),

7 Ligation Control (CTL)

8 Stop Ligation Buffer (STL)

9 DNA Adapter Index 2 (AD002)

10 DNA Adapter Index 4 (AD004)

11 DNA Adapter Index 5 (AD005)

12 DNA Adapter Index 6 (AD006)

13 DNA Adapter Index 7 (AD007)

14 DNA Adapter Index 12 (AD012)

15 DNA Adapter Index 13 (AD013)

16 DNA Adapter Index 14 (AD014)

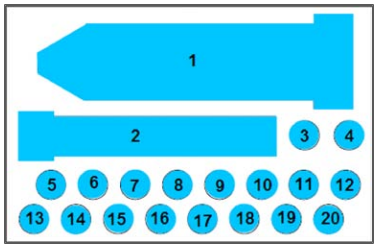
17 DNA Adapter Index 15 (AD015)

18 DNA Adapter Index 16 (AD016)

19 DNA Adapter Index 18 (AD018)

20 DNA Adapter Index 19 (AD019)

Figure 7 TruSeq DNA Sample Prep Kit v2, Box B



1 Resuspension Buffer (RSB)

2 End Repair Mix (ERP)

3 A-Tailing Mix (ATL)

4 Ligation Mix (LIG)

5 End Repair Control (CTE)

6 A-Tailing Control (CTA)

7 Ligation Control (CTL)

8 Stop Ligation Buffer (STL)

9 DNA Adapter Index 1 (AD001)

10 DNA Adapter Index 3 (AD003)

11 DNA Adapter Index 8 (AD008)

12 DNA Adapter Index 9 (AD009)

13 DNA Adapter Index 10 (AD010)

14 DNA Adapter Index 11 (AD011)

15 DNA Adapter Index 20 (AD020)

16 DNA Adapter Index 21 (AD021)

17 DNA Adapter Index 22 (AD022)

18 DNA Adapter Index 23 (AD023)

19 DNA Adapter Index 25 (AD025)

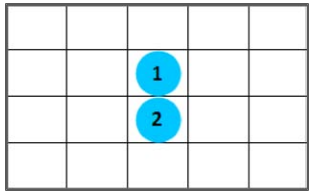
20 DNA Adapter Index 27 (AD027)

Kit Contents, PCR Prep 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 Sample Prep Kit v2, PCR Prep Box



1 PCR Master Mix (PMM)

2 PCR Primer Cocktail (PPC)

Consumables and Equipment

Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation. These consumables and equipment are Illumina recommended for the TruSeq DNA Sample Preparation v2 protocols. The requirement of some supplies are dependent upon the protocol performed (LT or HT) and these items are specified in separate tables below.

Table 7 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
2 µl barrier pipette tips	General lab supplier
2 µl multichannel pipettes	General lab supplier
2 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
96-well storage plates, round well, 0.8 ml (“MIDI” plate)	Fisher Scientific, part # AB-0859

Consumable	Supplier
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, catalog # PE-102-1001 (10 samples), or Illumina, catalog # PE-102-1001 (40 samples)
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 8 User-Supplied Consumables - Additional Items for LT 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 9 User-Supplied Consumables - Additional Items for HT Processing

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

Table 10 User-Supplied Equipment

Equipment	Supplier
96-well thermal cycler (with heated lid)	General lab supplier
Covaris S2 System, or Covaris E210 System	Covaris, part # S2, or Covaris, part # E210
Dark reader transilluminator	Clare Chemical Research, part # D195M
Electrophoresis power supply	General lab supplier
Magnetic stand-96	Ambion, part # 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 11 User-Supplied Equipment - Additional Items for HT 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)

Chapter 3

Low-Throughput (LT) Protocol

Introduction

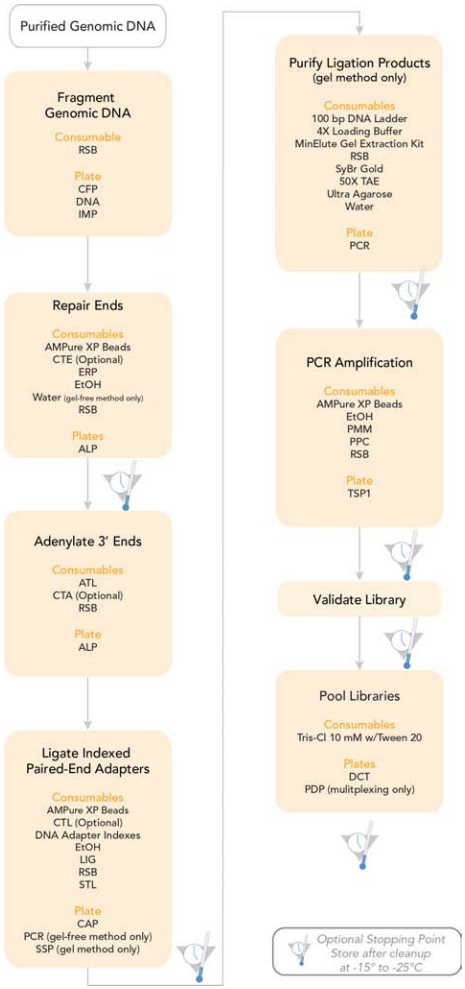
This chapter describes the TruSeq DNA Sample Preparation v2 low-throughput (LT) protocol. Illumina recommends this protocol for processing 48 or fewer samples. Follow the protocol in the order shown. For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample preparation.

When processing more than 48 samples, Illumina recommends following the protocol described in Chapter 4 High-Throughput (HT) Protocol.

Sample Prep Workflow

The following figure illustrates the processes of the LT TruSeq DNA Sample Preparation v2 protocol to prepare templates using 24 indexed adapters.

Figure 9 TruSeq DNA Sample Preparation v2 LT 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 12 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 20.
- Remove one tube of Resuspension Buffer from -15° to -25°C storage and thaw it at room temperature.
- 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 may 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 may not be preventable during the process run.

2 Fragment the DNA using the following settings:

	Whole-genome Resequencing	TruSeq Enrichment
Duty cycle	10%	10%
Intensity	5.0	5.0

	Whole-genome Resequencing	TruSeq Enrichment
Cycles per burst	200	200
Duration	40 seconds	2 x 60 seconds (120 seconds total)
Mode	Frequency sweeping	Frequency sweeping
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, 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.

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 or Resuspension Buffer
  - 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 *AMPure XP Handling* on page 10.
- 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.
- 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:
     - Briefly 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. Change the tip after each sample.
   - 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. Change the tip after each sample.
2. Add 40 µl of End Repair Mix to each well of the IMP plate containing the fragmented DNA. Change the tip after each sample.
3. Adjust the pipette to 100 µl, then gently pipette the entire volume of each pooled library up and down 10 times to mix thoroughly. Change the tip after each sample.
4. Seal the IMP plate with a Microseal 'B' adhesive seal.

Incubate 1 IMP

1. Pre-heat the thermal cycler lid to 100°C.
2. Incubate the IMP plate on the pre-heated thermal cycler, with the lid closed, for 30 minutes at 30°C.
3. Remove the IMP plate from the thermal cycler.

Clean Up IMP

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 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 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.
   - 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 Adjust the pipette to 200 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

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 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. Change the tip after each sample.

8 Repeat step 7 once.

NOTE

Leave the IMP plate on the magnetic stand while performing the following 80% EtOH wash steps (9–11).

9 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.

10 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. Change the tip after each sample.

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

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

13 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.

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

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

16 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. Change the tip after each sample.

SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3' Ends* on page 47 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)

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 Control or Resuspension Buffer
  - A-Tailing Mix

  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 44 and let stand to thaw at room temperature.
  - Briefly 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.

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.
3. Adjust the pipette to 30 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.
4. Seal the ALP plate with a Microseal 'B' adhesive seal.

Incubate 1 ALP

1. Pre-heat the thermal cycler lid to 100°C.
2. Incubate the ALP plate on the pre-heated thermal cycler, with the lid closed, for 30 minutes at 37°C.
3. Immediately remove the ALP plate from the thermal cycler, then proceed immediately to *Ligate Adapters* on page 49.

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)
- DNA Adapter Indexes (AD001–AD016, AD018–AD023, AD025, AD027) (1 tube per column of 8 reactions, depending on the DNA Adapter Indexes being used)
- (Optional) Ligation Control (CTL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB)
- Stop Ligation Buffer (STL) (1 tube per 48 reactions)
- CAP (Clean Up ALP Plate) barcode label
- 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 Index tubes, depending on the DNA Adapter Indexes being used
  - Stop Ligation Buffer
  - Ligation Control or Resuspension Buffer

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

- Review *AMPure XP Handling* on page 10.
- 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.
- 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, 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.

Add LIG

1. Briefly centrifuge the appropriate/desired thawed DNA Adapter Index tubes, Ligation Control (if using Ligation Control), and Stop Ligation Buffer tubes to 600 xg for 5 seconds.
2. Immediately before use, remove the Ligation Mix tube from -15° to -25°C storage.
3. Remove the adhesive seal from the ALP plate.
4. 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.
5. Add 2.5 µl of Ligation Mix to each well of the ALP plate.
6. Return the Ligation Mix tube back to -15° to -25°C storage immediately after use.

7 Add 2.5 µl of the appropriate/desired thawed DNA Adapter Index to each well of the ALP plate.

8 Adjust the pipette to 37.5 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

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

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.

3 Adjust the pipette to 42.5 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

Clean Up ALP

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 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.

2 Adjust the pipette to 85 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

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 and discard 80 µl of the supernatant from each well of the ALP plate. Change the tip after each sample.

NOTE
Leave the ALP plate on the magnetic stand while performing the following 80% EtOH wash steps (6–8).

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

7 Incubate the ALP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well. Change the tip after each sample.

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

9 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.

10 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. Change the tip after each sample.

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

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

13 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. Change the tip after each sample.

14 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.

15 Adjust the pipette to 100 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

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

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

18 Remove and discard 95 µl of the supernatant from each well of the CAP plate. Change the tip after each sample.

NOTE
Leave the CAP plate on the magnetic stand while performing the following 80% EtOH wash steps (19–21)

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

20 Incubate the CAP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well. Change the tip after each sample.

21 Repeat steps 19 and 20 once for a total of two 80% EtOH washes.

22 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.

23 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. Change the tip after each sample.

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

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

26 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. Change the tip after each sample.
  - Proceed to *Enrich DNA Fragments* on page 58.
- 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. Change the tip after each sample.
  - Proceed to *Purify Ligation Products (gel method only)* on page 54.

SAFE STOPPING POINT
If you do not plan to proceed to *Enrich DNA Fragments* on page 58 or *Purify Ligation Products (gel method only)* on page 54 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 for enrichment, proceed to *Enrich DNA Fragments* on page 58.

This process purifies the products of the ligation reaction on a gel and removes unligated adapters, as well as any adapters that may 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 may be desired and the cut size adjusted accordingly.

Table 13 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 may 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 51 and let stand to thaw at room temperature.
  - Briefly 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.

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 may 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 may 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 Exome 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 58 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)
- 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 one tube each of PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature. Once thawed, keep the tubes on ice.
- Briefly centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.

- Review *AMPure XP Handling* on page 10.
- 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 51 or *Size Separate Gel* on page 57 and let stand to thaw at room temperature.
  - Briefly 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 as follows:
  - 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 may also be performed.

- Pre-heat the thermal cycler lid to 100°C.
- 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. Change the tip after each sample.
2. Add 25 µl of thawed PCR Master Mix to each well of the PCR plate. Change the tip after each sample.
3. Adjust the pipette to 40 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.
4. Seal the PCR plate with a Microseal 'B' adhesive seal.

Amp PCR

1. Pre-heat the thermal cycler lid to 100°C.
2. Amplify the PCR plate in the pre-programmed thermal cycler, with the lid closed, as follows:
   a. 98°C for 30 seconds
   b. 10 cycles of:
      98°C for 10 seconds
      60°C for 30 seconds
      72°C for 30 seconds
   c. 72°C for 5 minutes
   d. Hold at 10°C

Clean Up PCR

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 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 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. Change the tip after each sample.
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. Change the tip after each sample.

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. Change the tip after each sample.

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. Change the tip after each sample.

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. Change the tip after each sample.

14 Do one of the following:

- If performing whole-genome resequencing, proceed to *Validate Library* on page 62.
- 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 62 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.

- 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. Running samples on a Bioanalyzer should be used for qualitative purposes only.

Figure 10 Example of DNA Library Size Distribution for Whole-Genome Resequencing

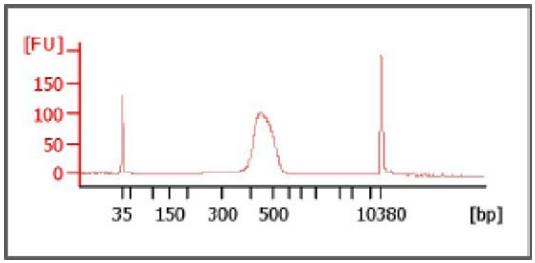
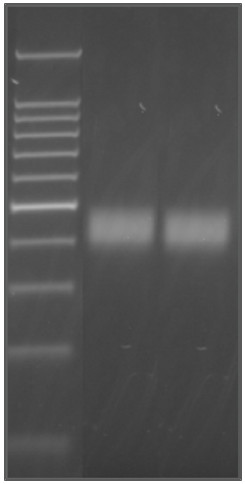


Figure 11 DNA PCR Product



NOTE

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 54.

Normalize and Pool Libraries

This process describes how to prepare DNA templates that will be applied to cluster generation. Multiplexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. Non-multiplexed DNA libraries 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 multiplexing only)

User-Supplied Consumables

- 96-well 0.3 ml PCR plate (for multiplexing 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 multiplexing only).
- Remove the TSP1 plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 60, and let stand to thaw at room temperature.
  - Briefly 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. Change the tip after each sample.
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 may 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-multiplexed paired-end libraries, the protocol stops here. Do one of the following:
  - Proceed to cluster generation. 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 multiplexed paired-end libraries, proceed to Make PDP.

Make PDP (for multiplexing 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.

2 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 10–120 µl (1–12 libraries).

Table 14 Pooled Sample Volumes

Number of pooled samples	Volume (µl)
1	10
2	20
3	30
4	40

Number of pooled samples	Volume (µl)
5	50
6	60
7	70
8	80
9	90
10	100
11	110
12	120

NOTE
Keep track of which sample goes into which well, to avoid pooling two samples with the same index.

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

4 Do one of the following:
- Proceed to cluster generation. See the *Illumina Cluster Generation User Guide*.
- Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.

Chapter 4

High-Throughput (HT) Protocol

Introduction

This chapter describes the TruSeq DNA Sample Preparation v2 high-throughput (HT) protocol. Illumina recommends this protocol when processing more than 48 samples. Follow the protocols in the order shown. For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample preparation.

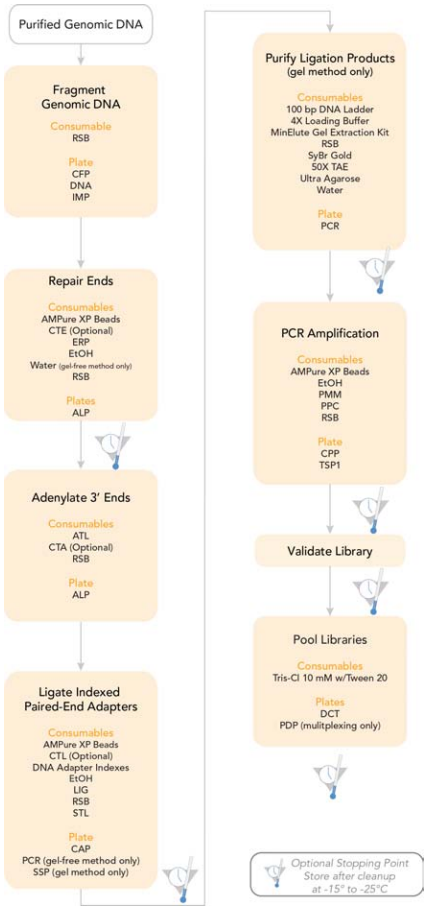
When processing 48 or fewer samples, Illumina recommends following the Chapter 3 Low-Throughput (LT) Protocol.

The HT protocol requires shaking and heating equipment to mix reagents and for incubation (see *Consumables and Equipment* on page 31).

Sample Prep Workflow

The following figure illustrates the processes of the HT TruSeq DNA Sample Preparation v2 protocol to prepare templates using 24 indexed adapters.

Figure 12 TruSeq DNA Sample Preparation v2 HT 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 15 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 20.
- Remove one tube of Resuspension Buffer from -15° to -25°C storage and thaw it at room temperature.
- 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 may 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 may not be preventable during the process run.

2 Fragment the DNA using the following settings:

	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)

	Whole-genome Resequencing	TruSeq Enrichment
Mode	Frequency sweeping	Frequency sweeping
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, 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.

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 or Resuspension Buffer
  - 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 *AMPure XP Handling* on page 10.
- 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:
     - Briefly 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. Change the tip after each sample.
   - 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. Change the tip after each sample.
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. Incubate the IMP plate on the pre-heated microheating system, with the lid closed, for 30 minutes at 30°C.
2. Remove the IMP plate from the microheating system.

Clean Up IMP

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 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 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. Change the tip after each sample.

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. Change the tip after each sample.

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. Change the tip after each sample.

SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3' Ends* on page 77 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)

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 Control or Resuspension Buffer
  - A-Tailing Mix

  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 74 and let stand to thaw at room temperature.
  - Briefly 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 Incubate the ALP plate on the pre-heated microheating system, with the lid closed, for 30 minutes at 37°C.

2 Immediately remove the ALP plate from the microheating system, then proceed immediately to *Ligate Adapters* on page 79.

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)
- DNA Adapter Indexes (AD001–AD016, AD018–AD023, AD025, AD027) (1 tube per column of 8 reactions, depending on the DNA Adapter Indexes being used)
- (Optional) Ligation Control (CTL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB)
- Stop Ligation Buffer (STL) (1 tube per 48 reactions)
- CAP (Clean Up ALP Plate) barcode label
- 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 Index tubes, depending on the DNA Adapter Indexes being used
  - Stop Ligation Buffer
  - Ligation Control or Resuspension Buffer

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

- Review *AMPure XP Handling* on page 10.
- 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.
- 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, 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.

Add LIG

1. Briefly centrifuge the appropriate/desired thawed DNA Adapter Index tubes, Ligation Control (if using Ligation Control), and Stop Ligation Buffer tubes to 600 xg for 5 seconds.
2. Immediately before use, remove the Ligation Mix tube from -15° to -25°C storage.
3. Remove the adhesive seal from the ALP plate.
4. 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.
5. Add 2.5 µl of Ligation Mix to each well of the ALP plate.
6. Return the Ligation Mix tube back to -15° to -25°C storage immediately after use.

7 Add 2.5 µl of the appropriate/desired thawed DNA Adapter Index 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 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 *AMPure XP Handling* on page 10 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. Change the tip after each sample.

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. Change the tip after each sample.

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. Change the tip after each sample.

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. Take care not to disturb the beads. Change the tip after each sample.

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. Take care not to disturb the beads. Change the tip after each sample.

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. Change the tip after each sample.
     — Proceed to *Enrich DNA Fragments* on page 89.
   - 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. Change the tip after each sample.
- Proceed to *Purify Ligation Products (gel method only)* on page 85.

SAFE STOPPING POINT

If you do not plan to proceed to *Enrich DNA Fragments* on page 89 or *Purify Ligation Products (gel method only)* on page 85 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 for enrichment, proceed to *Enrich DNA Fragments* on page 89.

This process purifies the products of the ligation reaction on a gel and removes unligated adapters, as well as any adapters that may 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 may be desired and the cut size adjusted accordingly.

Table 16 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 may 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 81 and let stand to thaw at room temperature.
  - Briefly 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.

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 may 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 may 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 Exome 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 89 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)
- 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 one tube each of PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature. Once thawed, keep the tubes on

ice.

- Briefly centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- Review *AMPure XP Handling* on page 10.
- 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 81 or *Size Separate Gel* on page 88 and let stand to thaw at room temperature.
  - Briefly 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 as follows:
  - 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 may also be performed.

- Pre-heat the thermal cycler lid to 100°C.
- 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. Change the tip after each sample.

2 Add 25 µl of thawed PCR Master Mix to each well of the PCR plate. Change the tip after each sample. Mix thoroughly as follows:
   a Seal the PCR plate with a Microseal 'A' film.
   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.

WARNING
Read and 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).

Amp PCR

1 Pre-heat the thermal cycler lid to 100°C.

2 Amplify the PCR plate in the pre-programmed thermal cycler, with the lid closed, as follows:
   a 98°C for 30 seconds
   b 10 cycles of:
      98°C for 10 seconds
      60°C for 30 seconds
      72°C for 30 seconds
   c 72°C for 5 minutes
   d Hold at 10°C

Clean Up PCR

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 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 add 50 µl of the mixed AMPure XP Beads to each well of the new MIDI plate labeled with the CPP barcode. Change the tip after each sample.

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. Take care not to disturb the beads. Change the tip after each sample.

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. Take care not to disturb the beads. Change the tip after each sample.

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. Change the tip after each sample.

17 Do one of the following:

- If performing whole-genome resequencing, proceed to *Validate Library* on page 94.
- 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 94 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.

- 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. Running samples on a Bioanalyzer should be used for qualitative purposes only.

Figure 13 Example of DNA Library Size Distribution for Whole-Genome Resequencing

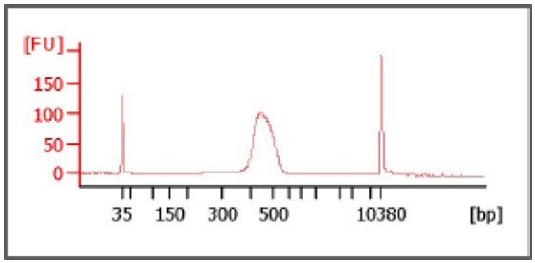
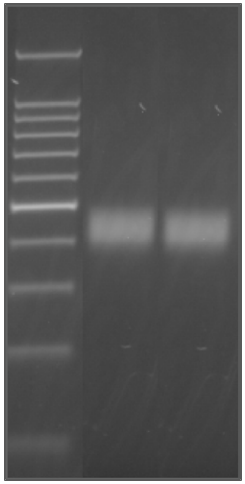


Figure 14 DNA PCR Product



NOTE

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 85.

Normalize and Pool Libraries

This process describes how to prepare DNA templates that will be applied to cluster generation. Multiplexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. Non-multiplexed DNA libraries 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 multiplexing only)

User-Supplied Consumables

- 96-well HSP plate (for multiplexing 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 multiplexing only).
- Remove the TSP1 plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 91, and let stand to thaw at room temperature.
  - Briefly 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. Change the tip after each sample.
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 may 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-multiplexed paired-end libraries, the protocol stops here. Do one of the following:
     — Proceed to cluster generation. 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 multiplexed paired-end libraries, proceed to Make PDP.

Make PDP (for multiplexing 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.

2 Transfer 10 µl of each normalized sample library to be pooled from the DCT plate to one well of the new HSP 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 10–120 µl (1–12 libraries).

Table 17 Pooled Sample Volumes

Number of pooled samples	Volume (µl)
1	10
2	20

Number of pooled samples	Volume (µl)
3	30
4	40
5	50
6	60
7	70
8	80
9	90
10	100
11	110
12	120

NOTE
Keep track of which sample goes into which well, to avoid pooling two samples with the same index.

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. 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 v2 to the procedures described in *Fragment DNA* on page 40 for the LT protocol or *Fragment DNA* on page 70 for the HT 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 LT protocol), or
- 96-well MIDI plate (for HT 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

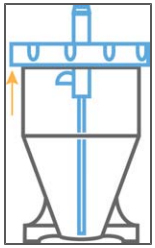
Procedure

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.

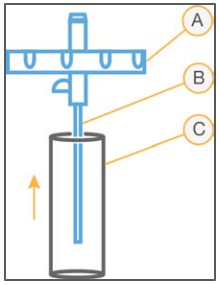
1 Remove a nebulizer from the plastic packaging and unscrew the blue lid.

Figure 15 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 16 Assemble the Nebulizer



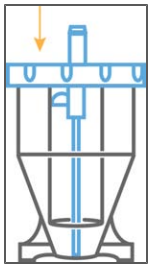
A Blue Lid
B Atomizer
C Vinyl Tubing

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).

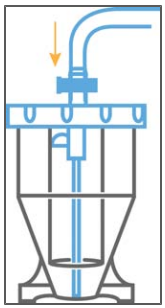
Figure 17 Replace the Nebulizer Lid



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 18 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 ensure the air is delivered at 32-35 psi.

10 Nebulize for 6 minutes. You may notice vapor rising from the nebulizer; this is normal. Also, the Nebulization Buffer may 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 LT processing, proceed to *Perform End Repair* on page 43.
- For HT processing, proceed to *Perform End Repair* on page 73.

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 18 Illumina General Contact Information

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

Table 19 Illumina Customer Support Telephone Numbers

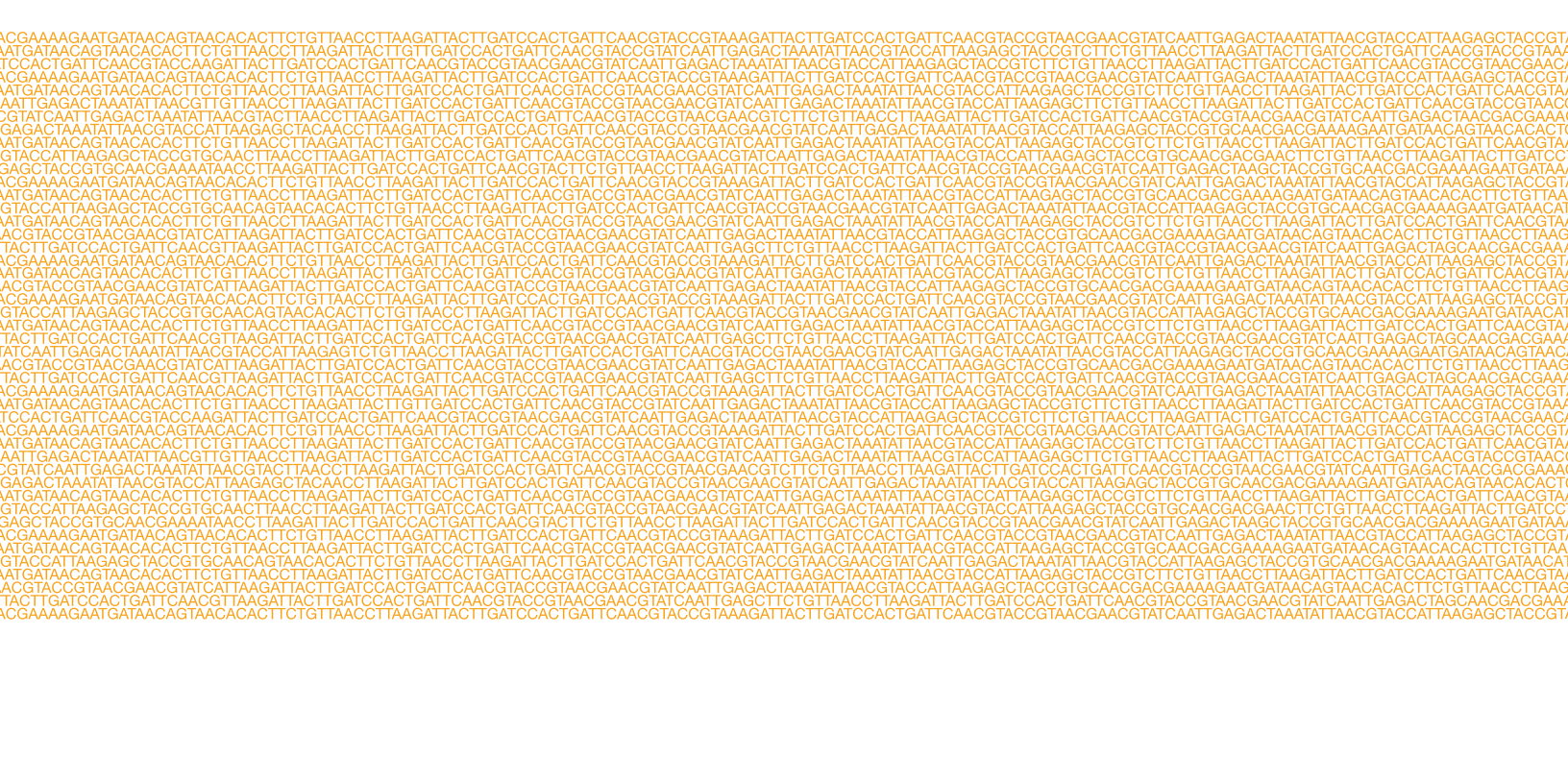
Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	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

If you require additional product documentation, you can obtain PDFs from the Illumina website if PDFs are available. Go to http://www.illumina.com/support/documentation.ilmn. When you click on a link, you will be asked to log in to iCom. After you log in, you can view or save the PDF. To register for an iCom account, please visit https://icom.illumina.com/Account/Register.



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