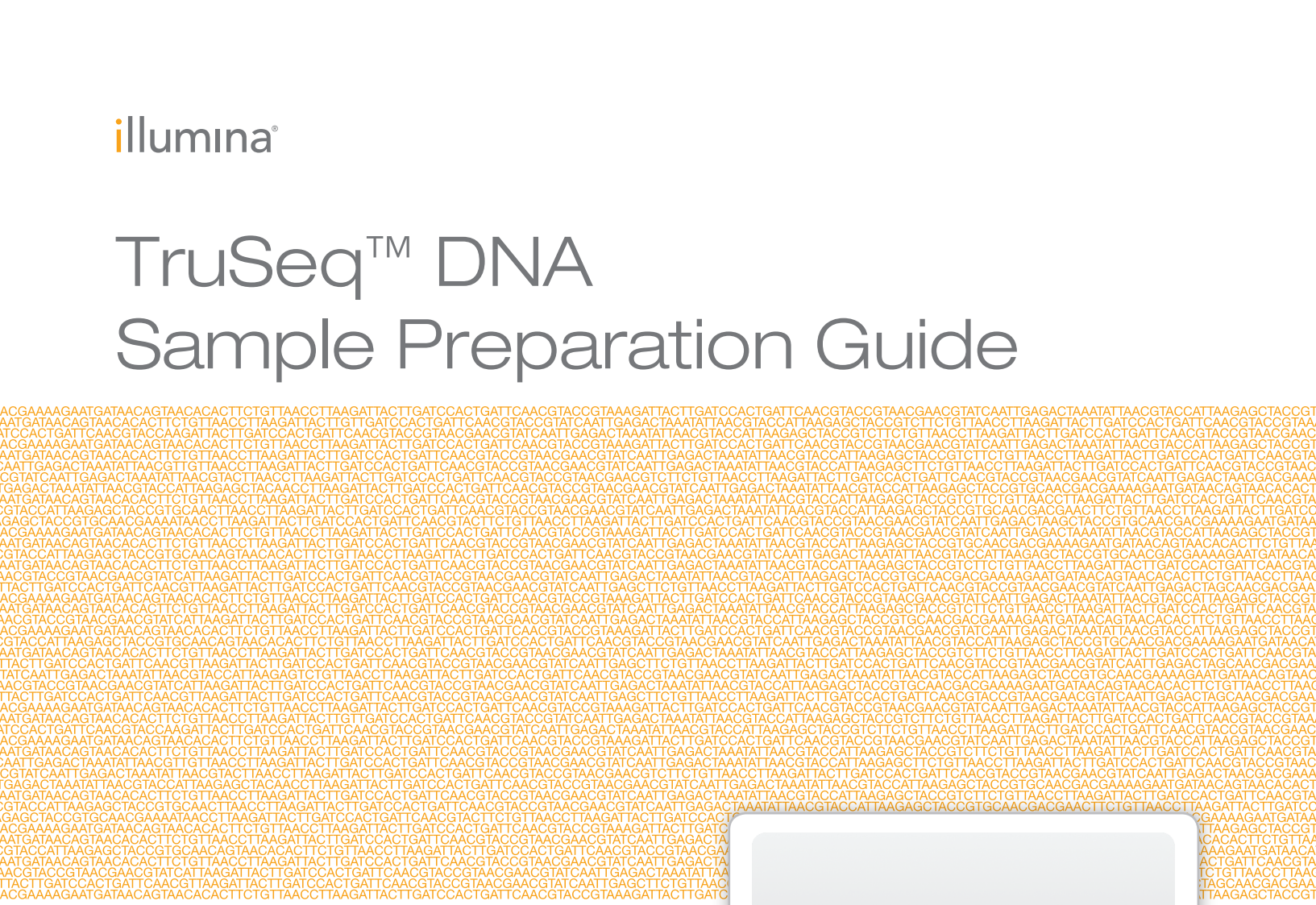
illumina®

TruSeq™ DNA Sample Preparation Guide



FOR RESEARCH USE ONLY

ILLUMINA PROPRIETARY
Catalog # PE-940-2001
Part # 15005180 Rev. A
November 2010

Notice

This document and its contents are proprietary to Illumina, Inc. and its affiliates ("Illumina"), and

are intended solely for the contractual use of its customer in connection with the use of the product(s) described herein and for no other purpose. This document and its contents shall not be used or distributed for any other purpose and/or otherwise communicated, disclosed, or reproduced in any way whatsoever without the prior written consent of Illumina. Illumina does not convey any license under its patent, trademark, copyright, or common-law rights nor similar rights of any third parties by this document.

The instructions in this document must be strictly and explicitly followed by qualified and properly trained personnel in order to ensure the proper and safe use of the product(s) described herein. All of the contents of this document must be fully read and understood prior to using such product(s).

FAILURE TO COMPLETELY READ AND EXPLICITLY FOLLOW ALL OF THE INSTRUCTIONS CONTAINED HEREIN MAY RESULT IN DAMAGE TO THE PRODUCT(S), INJURY TO PERSONS, INCLUDING TO USERS OR OTHERS, AND DAMAGE TO OTHER PROPERTY.

ILLUMINA DOES NOT ASSUME ANY LIABILITY ARISING OUT OF THE IMPROPER USE OF THE PRODUCT(S) DESCRIBED HEREIN (INCLUDING PARTS THEREOF OR SOFTWARE) OR ANY USE OF SUCH PRODUCT(S) OUTSIDE THE SCOPE OF THE EXPRESS WRITTEN LICENSES OR PERMISSIONS GRANTED BY ILLUMINA IN CONNECTION WITH CUSTOMER'S ACQUISITION OF SUCH PRODUCT(S).

FOR RESEARCH USE ONLY

Revision History

Part #	Revision	Date	Description of Change
15005180	A	November 2010	Initial Release

Revision History

Table of Contents

List of Tables

List of Tables

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 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, pipetting and hands-on time
- Universal adapter for preparation of SR, PE, and Multiplexing

Higher Throughput

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

Improved Troubleshooting

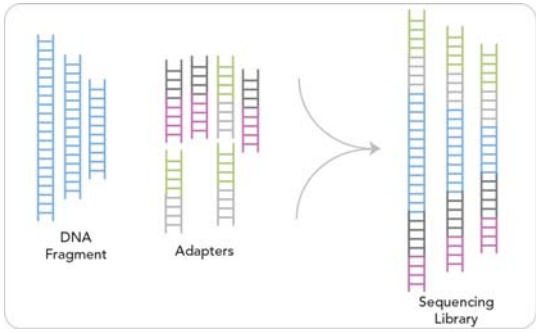
- Process control checks built-in for QC

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.

Figure 1 Sequencing Library After TruSeq DNA Sample Preparation



Audience and Purpose

This guide documents the sample preparation protocol using the Illumina TruSeq DNA Sample Preparation Kit.

- Chapter 3, explains how to perform the TruSeq DNA Sample Preparation using the *TruSeq DNA Sample Preparation Low Throughput (LT) Protocol*
- Chapter 4, explains how to perform the TruSeq DNA Sample Preparation using the *TruSeq DNA Sample Preparation 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 TCY 96-well MIDI
Incubation Equipment	96-well thermal cycler	Microheating system
Mixing Method	Pipetting	Micro plate shaker

Standard Operating Procedures

Chapter 2

Introduction

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

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

Acronyms

Table 2 TruSeq DNA Sample Preparation Acronyms

Acronym	Definition
ALP	Adapter Ligation Plate
ATL	A-Tailing Mix
CAP	Clean Up ALP Plate
CFP	Covaris Fragmentation Plate
CPP	Clean Up PCR Plate
CTA	A-Tailing Control
CTE	End Repair Control
CTL	Ligase Control
DCT	Diluted Cluster Template
dsDNA	double-stranded DNA
ERP	End Repair Mix
EUC	Experienced User Card
gDNA	genomic DNA
HT	High Throughput
IMP	Insert Modification Plate
ISP	Intermediate Source Plate
LIG	DNA Ligation Mix
LT	Low Throughput
LTF	Lab Tracking Form

Table 2 TruSeq DNA Sample Preparation Acronyms (Continued)

Acronym	Definition
PCR	Polymerase Chain Reaction
PDP	Pooled Dilution Plate
PMM	PCR Master Mix
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
SSP	Size Separate Plate
STL	Stop Ligase Mix
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, because solutions of high molecular weight double-stranded (ds)DNA can be viscous and not evenly dispersed, resulting in aliquot measurements that are not representative of the true concentration of the solution.

AMPure XP Handling

The following indicates the appropriate handling methods when working with Agencourt AMPure XP Beads:

- 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 thoroughly by pipetting up and down 10 times.
- **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.
- Change the tips for each sample or when using a multichannel pipette, change the tips after each column.

- 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.
- For the wash steps, prepare fresh 80% ethanol. Eighty-percent 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.
- Remove the reaction plate from the magnetic stand and let it air-dry at room temperature. Allow for the complete evaporation of residual ethanol, as it impacts 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 for DNA elution.
  - **When performing the LT protocol**, gently pipette 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**, seal the plate and shake the plate on a microplate shaker at 1,800 rpm for 2 minutes,

Avoid Cross-Contamination

- 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

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.

Temperature Considerations

Temperature is another important consideration for making gDNA libraries. Elevated temperatures should be particularly avoided 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. As a general rule, libraries should be kept at temperatures $\leq$37°C. Temperature is less of an issue after the adapters have been ligated onto the ends of the DNA, although care should be taken not to denature the library prior to the agarose gel electrophoresis process, because single-stranded DNA has a different migration rate.

DNA Input Recommendations

Input DNA Quantitation

This protocol is optimized for 1 µg input DNA. The ultimate success or failure of a library preparation strongly depends on using an accurately quantified amount of input DNA. Therefore, the correct quantitation of gDNA is essential.

Illumina strongly recommends quantifying the starting genomic material by a fluorescence-based quantification, rather than a UV-spectrometer-based method. This is because fluorescence-based methods, which employ a double-stranded (ds) DNA specific dye, will specifically and accurately quantitate dsDNA even in the presence of many common contaminants. UV spectrometer methods based on 260 OD readings are prone to overestimating the DNA concentration due to the presence of RNA and other contaminants commonly found in gDNA preparations.

Assessing DNA Quantity and Quality

Absorbance measurements at 260 nm are commonly used to quantify DNA. 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. However, both measurements can be compromised by the presence of RNA or small nucleic acid fragments such as nucleotides. Thus, gDNA samples should be carefully collected to ensure that they are free of contaminants, and the most accurate spectrophotometric method available should be used to quantify the input gDNA.

DNA quantitation methods that rely on intercalating fluorescent dyes measure only dsDNA and are less subject to excess nucleic acids. However, 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.

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 Ligase Control reagents contain DNA fragments used as controls for the enzymatic activities of the End Repair Mix, A-Tailing Mix, and DNA Ligase 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 a control's sequence 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 3). 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 3 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
DNA Ligase Mix	Ligation: Join adapters to inserts	Ligase 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 1.8 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

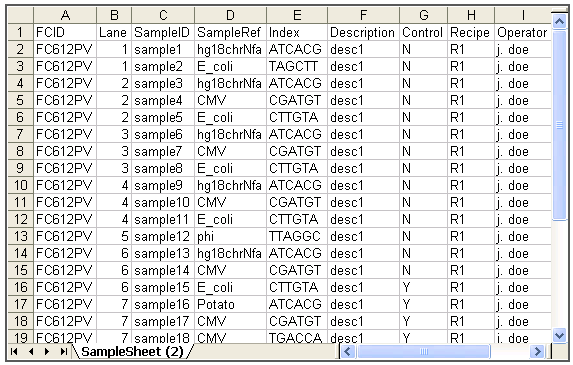
Create a copy of the lab tracking form for each run. Use it to track information about your sample preparation such as operator information, start and stop times, reagent lot numbers, and barcodes. This form can be filled out and saved online or printed and filled in by hand.

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. 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.

Figure 2 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 4 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
Sample Ref	The reference sequence for the sample
Index	The index sequence or 0 for no indexing
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

CAUTION

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

Kit Contents

Check to ensure that you have all of the reagents identified in this section before proceeding. Each TruSeq DNA Sample Preparation Kit can be used to process up to 48 samples. The kit cartons also contains plate barcode labels.

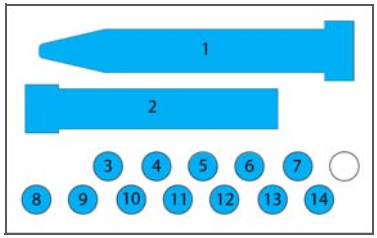
Kit Contents, Boxes A and B

You will choose to receive either box A or B with the kit depending on the index pooling level you require.

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 3 TruSeq DNA Sample Preparation Kit, Box A, part # 15012999



1 Resuspension Buffer (RSB), part # 15012547

2 End Repair Mix (ERP), part # 15012494

3 A-Tailing Mix (ATL), part # 15012495

4 Ligation Mix (LIG), part # 15012496

5 End Repair Control (CTE), part # 15012360

6 A-Tailing Control (CTA), part # 15012361

7 Ligase Control (CTL), part # 15012362

8 Stop Ligase Mix (STL), part # 15012546

9 DNA Adapter Index 2 (AD002), part # 15012348

10 DNA Adapter Index 4 (AD004), part # 15012350

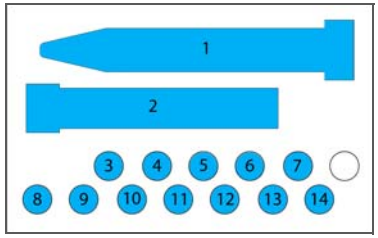
11 DNA Adapter Index 5 (AD005), part # 15012351

12 DNA Adapter Index 6 (AD006), part # 15012352

13 DNA Adapter Index 7 (AD007), part # 15012353

14 DNA Adapter Index 12 (AD012), part # 15012358

Figure 4 TruSeq DNA Sample Preparation Kit, Box B, part # 15013001



1 Resuspension Buffer (RSB), part # 15012547

2 End Repair Mix (ERP), part # 15012494

3 A-Tailing Mix (ATL), part # 15012495

4 Ligation Mix (LIG), part # 15012496

5 End Repair Control (CTE), part # 15012360

6 A-Tailing Control (CTA), part # 15012361

7 Ligase Control (CTL), part # 15012362

8 Stop Ligase Mix (STL), part # 15012546

9 DNA Adapter Index 1 (AD001), part # 15012347

10 DNA Adapter Index 3 (AD003), part # 15012349

11 DNA Adapter Index 8 (AD008), part # 15012354

12 DNA Adapter Index 9 (AD009), part # 15012355

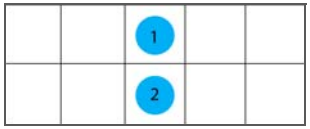
13 DNA Adapter Index 10 (AD010), part # 15012356

14 DNA Adapter Index 11 (AD011), part # 15012357

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 5 TruSeq DNA Sample Preparation Kit, PCR Prep Box, part # 15012995



1 PCR Master Mix (PMM), part # 15013681

2 PCR Primer Cocktail (PPC), part # 15013682

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 protocols. The requirement of some supplies are dependent upon the exact protocol followed (LT or HT) and these items are specified in separate tables below.

Table 5 User-Supplied Consumables

Consumable	Supplier
2 µl multichannel pipettes	General lab supplier
2 µl barrier pipette tips	General lab supplier
2 µl single channel pipettes	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl barrier pipette tips	General lab supplier
10 µl single channel pipettes	General lab supplier
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
BenchTop 100 bp DNA ladder	Promega, part # G829B
200 µl multichannel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl single channel pipettes	General lab supplier

Table 5 User-Supplied Consumables (Continued)

Consumable	Supplier
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
Certified low-range ultra agarose	BIO-RAD, part # 161-3107
Clean scalpels	General lab supplier
Covaris 100 µl (6 x 16 mm) round bottom tubes with AFA fiber	KBiosciences, catalog # 520045
Distilled water	General lab supplier
DNase/RNase zapper (to decontaminate surfaces)	General lab supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma Aldrich, part # E7023
4X Loading buffer (50 mM Tris pH 8.0, 40 mM EDTA, 40% (w/v) sucrose, 0.3% Bromophenol blue)	General lab supplier
Microseal 'B' adhesive seals	BioRad, part # MSB1001
MinElute Gel Extraction Kit	QIAGEN, part# 28604
RNase/DNase-free Multichannel reagent reservoirs, disposable	General lab supplier
RNase/DNase-free Strip tubes and caps	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

Table 6 User-Supplied Consumables - Additional Items for LT Processing

Consumable	Supplier
96-well 0.3 ml skirtless PCR plates	E&K Scientific, part # 480096

Table 7 User-Supplied Consumables - Additional Items for HT Processing

Consumable	Supplier
Microseal 96-well PCR plates (“TCY” plate)	BIO-RAD, part # HSP-9601

Table 8 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
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 9 User-Supplied Equipment - Additional Items for HT Processing

Consumable	Supplier
High Speed Micro Plate Shaker	Illumina, part # 175732
MIDI plate insert for heating system	Illumina, part # 211191
Stroboscope (to calibrate the micro plate shaker)	General lab supplier
Tru Temp Microheating System	Illumina, part # 11191471

Low-Throughput (LT) Protocol

Chapter 3

Introduction

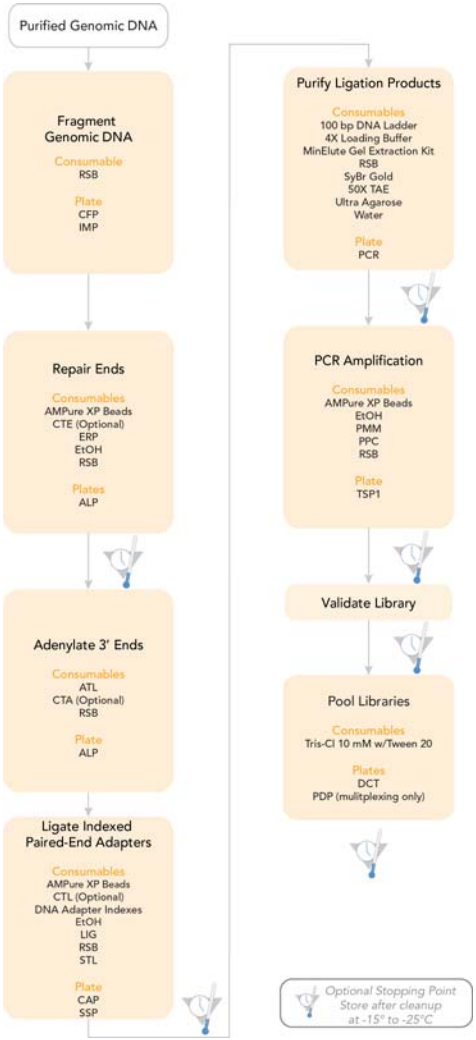
This chapter describes the TruSeq DNA Sample Preparation low-throughput (LT) protocol. Illumina recommends this protocol for processing 48 or fewer samples. Follow the protocol in the order shown.

When processing more than 48 samples, Illumina recommends following the *High-Throughput (HT) Protocol*.

Sample Prep Workflow

The following illustrates the processes of the LT TruSeq DNA Sample Preparation protocol to prepare a template composed of 12 pooled indexed libraries.

Figure 6 TruSeq DNA Sample Preparation LT Workflow



Fragment DNA

This process describes how to optimally fragment the gDNA to obtain a final library of 300–400 bp average insert size. Covaris shearing generates dsDNA fragments with 3' or 5' overhangs. The fragmentation process described below was optimized to obtain both final libraries of 200–300 bp average insert size, recommended for TruSeq Exome Enrichment, and 300–400 bp average insert size for general genomic sequencing.

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
- IMP (Insert Modification Plate) barcode label

User-Supplied Consumables

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

Preparation

- 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 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 normalize the gDNA samples to 55 µl at 20 ng/µl into each well of the new 0.3 ml PCR plate labeled with the CFP barcode.

Fragment DNA

1 Shear 1 µg of gDNA sample by transferring 52.5 µl of each DNA from the CFP plate to each Covaris tube.

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

2 Fragment the DNA using the following settings:

NOTE
These settings are optimized for creating library inserts of 200–400 bp and may need to be modified for other insert sizes.

- Duty cycle—10%
- Intensity—5.0
- Bursts per second—200
- Duration—120 seconds
- Mode—Frequency sweeping
- Power—23W
- Temperature—5.5° to 6°C

3 Seal the Covaris tube and briefly centrifuge to 600 xg for 5 seconds.

4 Transfer 50 µl of fragmented DNA from the Covaris tube to each well of the new 0.3 ml PCR plate labeled with the IMP barcode using a single channel pipette.

NOTE
For better handling of the 0.3 ml PCR plate, it is recommended to put it in the 96-well PCR tube rack.

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
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)

Preparation

- Remove one tube of End Repair Mix and, if using the End Repair Control, one tube of End Repair Control per 48 reactions from -15° to -25°C storage and thaw them at room temperature.

NOTE

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

NOTE

If you do not intend to consume the End Repair Control and End Repair Mix reagents in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- Review *AMPure XP Handling* on page 19.

- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Apply a ALP barcode label to a new 96-well 0.3 ml PCR plate.

NOTE

When using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Make IMP

1. If using the in-line End Repair Control, briefly centrifuge the thawed End Repair Control tube to 600 xg for 5 seconds.
2. Add 10 µl of thawed End Repair Control (or 10 µl of RSB if not using End Repair Control) to each well of the IMP plate that contains 50 µl of fragmented DNA using a single channel or multichannel pipette. Change the tip after each sample.
3. Add 40 µl of End Repair Mix to each well of the IMP plate containing the fragmented DNA. Change the tips after each sample.
4. Adjust the single channel or multichannel pipette to 100 µl. Gently pipette the entire volume of each pooled library up and down 10 times to mix thoroughly. Change the tips after each sample.
5. Seal the IMP plate with a Microseal 'B' adhesive seal.

Incubate 1 IMP

1. Incubate the IMP plate on the thermal cycler, with the lid closed, for 30 minutes at 30°C
2. Remove the IMP plate from the thermal cycler.

Clean Up IMP

NOTE

Before performing clean up, review *AMPure XP Handling* on page 19 when working with AMPure XP Beads.

1 Remove the adhesive seal from the IMP plate.

1 Vortex the AMPure XP Beads until they are well dispersed, then add 160 µl of well-mixed AMPure XP Beads to each well of the IMP plate containing 100 µl of End Repair Mix using a single channel or multichannel pipette.

2 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.

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

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

5 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. Some liquid may remain in each well. Take care not to disturb the beads. Change the tip after each sample.

6 Repeat step 5 once. Some liquid may remain in each well.

NOTE

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

7 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.

8 Incubate the IMP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well using a single channel or multichannel pipette. Take care not to disturb the beads. Change the tip after each sample.

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

10 Remove the IMP plate from the magnetic stand and let the plate stand at room temperature for 15 minutes to dry.

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

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

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

14 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 using a single channel or multichannel pipette. Change the tip after each sample.

SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3' Ends* immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store it 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

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

User-Supplied Consumables

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

Preparation

- Remove one tube of A-Tailing Mix and, if using the A-Tailing Control, one tube of A-Tailing Control per 48 reactions from -15° to -25°C storage and thaw them at room temperature.

NOTE

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

NOTE

If you do not intend to consume the A-Tailing Mix and A-Tailing Control reagents in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Perform End Repair* and let stand to thaw at room temperature.

- Briefly centrifuge the thawed ALP plate to 280 xg for 1 minute, then remove the adhesive seal from the plate.

NOTE

When using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Add ATL

1. Add 2.5 µl of A-Tailing Control (or 2.5 µl of Resuspension Buffer, if not using A-Tailing Control) to the bottom of each well of the ALP plate.
2. Add 12.5 µl of A-Tailing Mix to the bottom of each well of the ALP plate.
3. Adjust the single channel or multichannel pipette to 30 µl and 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. Incubate the ALP plate on the thermal cycler, with the lid closed, for 30 minutes at 37°C.
2. Immediately remove the ALP plate from the thermal cycler, then proceed immediately to *Ligate Adapters*.

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

- DNA Ligase Mix (LIG) (1 tube per 48 reactions)
- DNA Adapter Indexes 1–12 (AD001–AD012)
  (1 tube per 8 reactions, depending on the DNA Adapter Indexes being used)
- (Optional) Ligase Control (CTL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)
- Stop Ligase Mix (STL)
- CAP (Clean Up ALP Plate) barcode label
- SSP (Size Separate Plate) barcode label

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 appropriate DNA Adapter Index tubes (AD001–AD012, depending on the DNA Adapter Indexes being used), and one tube each of Ligase Control and Stop Ligase Mix per 48 reactions from -15° to -25°C storage and thaw them at room temperature.

NOTE

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

NOTE

If you do not intend to consume the Ligase Control in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- Review *AMPure XP Handling* on page 19.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Apply a CAP barcode label to a new 96-well 0.3 ml PCR plate.
- 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.

NOTE

When using a multichannel pipette:

- Review best practices to *Avoid Cross-Contamination* on page 20.
- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Add LIG

1. Briefly centrifuge the thawed DNA Adapter Index tubes (AD001–AD012 depending on the DNA Adapter Indexes being used), Ligase Control, and Stop Ligase Mix tubes to 600 xg for 5 seconds.
2. Immediately before use, remove the DNA Ligase Mix tube from -15° to -25°C storage.

NOTE

If you do not intend to consume the DNA Ligase Mix in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

3 Remove the adhesive seal from the ALP plate.

4 Add 2.5 µl of Ligase Control (or 2.5 µl of Resuspension Buffer, if not using Ligase Control) to each well of the ALP plate.

5 Add 2.5 µl of DNA Ligase Mix to each well of the ALP plate.

6 Return the DNA Ligase Mix tube back to -15° to -25°C storage immediately after use.

7 Add 2.5 µl of each thawed DNA Adapter Index (AD001–AD012 depending on the DNA Adapter Indexes being used) to each well of the ALP plate.

8 Adjust the single channel or multichannel pipette to 37.5 µl and 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 thermal cycler, with the lid closed, for 10 minutes at 30°C.

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 Ligase Mix to each well of the ALP plate to inactivate the ligation.

3 Adjust the single channel or multichannel pipette to 42.5 µl and 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 19 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 using a single channel or multichannel pipette.
2. Adjust the single channel or multichannel pipette to 85 µl and 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 2 minutes or until the liquid appears clear.
5. Remove and discard 80 µl of the supernatant from each well of the ALP plate using a single channel or multichannel pipette. Some liquid may remain in each well. Take care not to disturb the beads. 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 at least 30 seconds, then remove and discard all of the supernatant from each well using a single channel or multichannel pipette. Take care not to disturb the beads. Change the tip after each sample.
8. Repeat steps 6 and 7 once for a total of two 80% EtOH washes.
9. Let the ALP plate stand at room temperature for 15 minutes to dry 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 2 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 using a single channel or multichannel pipette. 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 using a single channel or multichannel pipette.

15 Adjust the single channel or multichannel pipette to 100 µl and 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 2 minutes or until the liquid appears clear.

18 Remove and discard 95 µl of the supernatant from each well of the CAP plate using a single channel or multichannel pipette. Some liquid may remain in each well. 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 (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 at least 30 seconds, then remove and discard all of the supernatant from each well using a single channel or multichannel pipette. Take care not to disturb the beads. Change the tip after each sample.

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

22 Let the CAP plate stand at room temperature for 15 minutes to dry 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 2 minutes or until the liquid appears clear.

26 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 using a single channel or multichannel pipette. Change the tip after each sample. The samples are ready for loading onto the gel.

SAFE STOPPING POINT

If you do not plan to proceed to *Purify Ligation Products* immediately, the protocol can be safely stopped here. If you are stopping, seal the SSP plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C for up to seven days.

Purify Ligation Products

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.

For genomic sequencing, Illumina suggests a 300–400 bp insert size target (+/- 1 standard deviation of 20 bp, i.e, a < 20% variance) for read lengths of 2 × 75 bp or shorter. This translates to a 3 mm gel slice at 400–500 bp to account for the length of the adapter sequences flanking the inserts.

For exome enrichment, Illumina suggests a 200–300 bp insert size target. This translates to a 3 mm gel slice at 300–400 bp to account for the length of the adapter sequences flanking the inserts. For other applications, other size ranges may be desired and the cut size can be adjusted accordingly.

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
- 4X Loading Buffer
- MinElute Gel Extraction Kit
- SyBr Gold Nucleic Acid Gel Stain

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 *Ligate Adapters* and let stand to thaw at room temperature. Briefly centrifuge the thawed SSP plate to 280 xg for 1 minute.

- 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 7 µl of 4X Loading Buffer to each well of the SSP plate.

4 Add 17 µl Resuspension Buffer and 7 µl of 4X Loading Buffer 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 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.

NOTE

If proceeding with the TruSeq Exome Enrichment protocol, excise a band ranging in size from 300–400 bp. 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* immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store it 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)
- PCR Primer Cocktail (PPC) (1 tube)
- Resuspension Buffer (RSB) (1 tube)
- TSP1 (Target Sample Plate) barcode label

User-Supplied Consumables

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

Preparation

- Remove one tube each of PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature.
- Briefly centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- Review *AMPure XP Handling* on page 19.
- 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 *Purify Ligation Products* 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 4°C
- Apply a TSP1 barcode label to a new 96-well 0.3 ml PCR plate.

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.

NOTE

When using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Make PCR

The following procedure assumes 1 µg of input DNA to library preparation and is designed to get higher library yields.

1. Add 5 µl of thawed PCR Primer Cocktail to each well of the PCR plate using a single channel or multichannel pipette. Change the tip after each sample.
2. Add 25 µl of thawed PCR Master Mix to each well of the PCR plate using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

Amp PCR

1. Amplify the PCR plate in the pre-programed 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 4°C

Clean Up PCR

NOTE

Before performing clean up, review *AMPure XP Handling* on page 19 when working with AMPure XP Beads.

1. 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 using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each column.
2. Incubate the PCR plate at room temperature for 15 minutes.
3. Place the PCR plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.

4 Remove and discard 95 µl of the supernatant from each well of the PCR plate using a single channel or multichannel pipette. Some liquid may remain in each well. Take care not to disturb the beads. Change the tip after each sample.

NOTE

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

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

6 Incubate the PCR plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well using a single channel or multichannel pipette. Take care not to disturb the beads. Change the tip after each sample.

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

8 Remove the PCR plate from the magnetic stand and let the plate stand at room temperature for 15 minutes to dry.

9 Resuspend the dried pellet in each well with 32.5 µl Resuspension Buffer using a single channel or multichannel pipette. Gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

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

11 Place the PCR plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.

12 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 using a single channel or multichannel pipette. Change the tip after each sample.

SAFE STOPPING POINT

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

Validate Library

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

Quantify Libraries

1 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 *qPCR Quantification Protocol Guide*.

2 If performing exome enrichment, proceed to the *TruSeq Exome Enrichment Guide*. For all other sequencing applications, proceed to *Pool Libraries*.

Quality Control

1 [Optional] To verify the size of your PCR enriched fragments, check the template size distribution by running an aliquot of the enriched library on a gel or on a Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip.

a 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.

b If using the Agilent Bioanalyzer with a high sensitivity DNA chip, make a 1:100 dilution of the library using water and load 1 µl of the diluted library on the Agilent High Sensitivity DNA chip.

Figure 7 Example of DNA Library Size Distribution

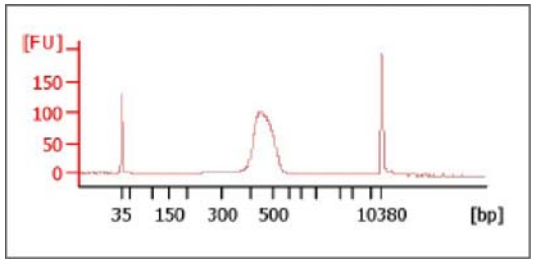
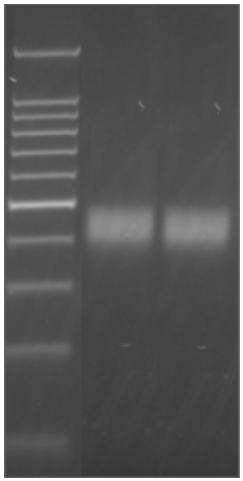


Figure 8 DNA PCR Product



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* on page 51.

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 (Diluted Cluster Template) plate and then pooled in equal volumes in the PDP (Pooled DCT 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 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 using a single channel or multichannel pipette. 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:

a For non-multiplexed 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 it at -15° to -25°C.

b For multiplexed libraries, proceed to *Make PDP*.

Make PDP

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 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 10 Pooled Sample Volumes

Number of pooled samples	Volume (µl)
1	10
2	20
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 Gently pipette the entire volume up and down 10 times to mix thoroughly.

4 Do one of the following:

a Proceed to cluster generation. See the *Illumina Cluster Generation User Guide*.

b Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.

High-Throughput (HT) Protocol

Chapter 4

Introduction

This chapter describes the TruSeq DNA Sample Preparation high-throughput (HT) protocol. Illumina recommends this protocol when processing more than 48 samples. Follow the protocols in the order shown.

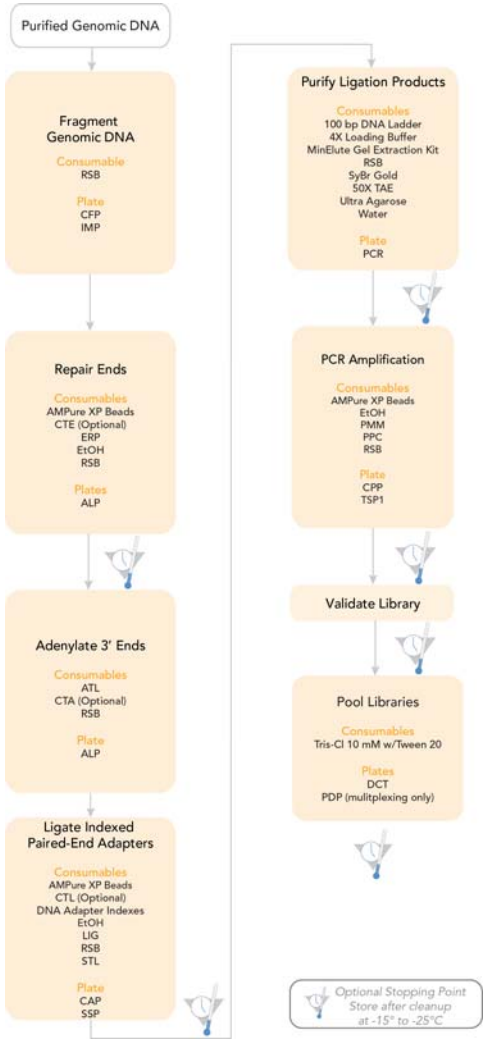
When processing 48 or fewer samples, Illumina recommends following the *Low-Throughput (LT) Protocol*.

The HT protocol requires shaking and heating equipment to mix reagents and for incubation (see *User-Supplied Consumables - Additional Items for HT Processing* and *User-Supplied Equipment - Additional Items for HT Processing*).

Sample Prep Workflow

The following illustrates the processes of the HT TruSeq DNA Sample Preparation protocol to prepare a template composed of 12 pooled indexed libraries.

Figure 9 TruSeq DNA Sample Preparation HT Workflow



Fragment DNA

This process describes how to optimally fragment the gDNA to obtain a final library of 300–400 bp average insert size. Covaris shearing generates dsDNA fragments with 3' or 5' overhangs. The fragmentation process described below was optimized to obtain both final libraries of 200–300 bp average insert size, recommended for TruSeq Exome Enrichment, and 300–400 bp average insert size for general genomic sequencing.

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
- IMP (Insert Modification Plate) barcode label

User-Supplied Consumables

- 96-well MIDI plate
- 96-well TCY plate
- Covaris Tubes
- DNA

Preparation

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

Make CFP

1 Illumina recommends to normalize the gDNA samples to 55 µl at 20 ng/µl into each well of the new TCY plate labeled with the CFP barcode.

Fragment DNA

1 Shear 1 µg of gDNA sample by transferring 52.5 µl of each DNA from the CFP plate to each Covaris tube.

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

2 Fragment the DNA using the following settings:

NOTE
These settings are optimized for creating library inserts of 200–400 bp and may need to be modified for other insert sizes.

- Duty cycle—10%
- Intensity—5.0
- Bursts per second—200
- Duration—120 seconds
- Mode—Frequency sweeping
- Power—23W
- Temperature—5.5° to 6°C

3 Seal the Covaris tube and briefly centrifuge to 600 xg for 5 seconds.

4 Transfer 50 µl of fragmented DNA from the Covaris tube to each well of the new MIDI plate labeled with the IMP barcode using a single channel pipette.

NOTE
For better handling of the MIDI plate, it is recommended to put it in the 96-well PCR tube rack.

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
- RNase/DNase-free Reagent Reservoirs
- RNase/DNase-free Strip Tubes and Caps

Preparation

- Remove one tube of End Repair Mix and, if using the End Repair Control, one tube of End Repair Control per 48 reactions from -15° to -25°C storage and thaw them at room temperature.

NOTE

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

NOTE

If you do not intend to consume the End Repair Control and End Repair Mix reagents in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- Review *AMPure XP Handling* on page 19.

- 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.

NOTE

When using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Make IMP

1. If using the in-line End Repair Control, briefly centrifuge the thawed End Repair Control tube to 600 xg for 5 seconds.
2. Add 10 µl of thawed End Repair Control (or 10 µl of RSB if not using End Repair Control) to each well of the IMP plate that contains 50 µl of fragmented DNA using a single channel or multichannel pipette. Change the tip after each sample.
3. Add 40 µl of End Repair Mix to each well of the IMP plate containing the fragmented DNA using a single channel or multichannel pipette. 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 19 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, then add 160 µl of well-mixed AMPure XP Beads from the stock bottle to each well of the IMP plate containing 100 µl of End Repair Mix. 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.
3. Incubate the IMP plate at room temperature for 15 minutes.
4. Place the IMP plate on the magnetic stand at room temperature for 15 minutes or until the liquid appears clear.
5. Remove the adhesive seal from the IMP plate.
6. Using a 200 µl single channel or multichannel pipette set to 127.5 µl, remove and discard 127.5 µl of the supernatant from each well of the IMP plate. Some liquid may remain in each well. Take care not to disturb the beads. Change the tip after each sample.
7. Repeat step 6 once. Some liquid may remain in each well.

NOTE

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

8. With the IMP plate on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well with a sample without disturbing the beads.
9. Incubate the IMP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well using a single channel or multichannel pipette. 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. Remove the IMP plate from the magnetic stand and let the plate stand at room temperature for 15 minutes to dry.

12 Resuspend the dried pellet in 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.

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

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

15 Remove the adhesive seal from the IMP plate.

16 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 using a single channel or multichannel pipette. Change the tip after each sample.

SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3' Ends* immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store it 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

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

User-Supplied Consumables

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

Preparation

- Remove one tube of A-Tailing Mix and, if using the A-Tailing Control, one tube of A-Tailing Control per 48 reactions from -15° to -25°C storage and thaw them at room temperature.

NOTE

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

NOTE

If you do not intend to consume the A-Tailing Mix and A-Tailing Control reagents in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Perform End Repair* and let stand to thaw at room temperature.

- Briefly centrifuge the thawed ALP plate to 280 xg for 1 minute, then remove the adhesive seal from the plate.
- Pre-heat the microheating system to 37°C.

NOTE

When using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Add ATL

1 Add 2.5 µl of A-Tailing Control (or 2.5 µl of Resuspension Buffer, if not using A-Tailing Control) to the bottom of each well of the ALP plate.

1 Add 12.5 µl of A-Tailing Mix to the bottom of 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*.

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

- DNA Ligase Mix (LIG) (1 tube per 48 reactions)
- DNA Adapter Indexes 1–12 (AD001–AD012)
  (1 tube per 8 reactions, depending on the DNA Adapter Indexes being used)
- (Optional) Ligase Control (CTL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)
- Stop Ligase Mix (STL)
- CAP (Clean Up ALP Plate) barcode label
- SSP (Size Separate Plate) barcode label

User-Supplied Consumables

- 96-well MIDI plate
- 96-well TCY 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 appropriate DNA Adapter Index tubes (AD001–AD012, depending on the DNA Adapter Indexes being used), and one tube each of Ligase Control and Stop Ligase Mix per 48 reactions from -15° to -25°C storage and thaw them at room temperature.

NOTE

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

NOTE

If you do not intend to consume the Ligase Control in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- Review *AMPure XP Handling* on page 19.
- 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.
- Apply a SSP barcode label to a new 96-well TCY 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.

NOTE

When using a multichannel pipette:

- Review best practices to *Avoid Cross-Contamination* on page 20.
- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Add LIG

1. Briefly centrifuge the thawed DNA Adapter Index tubes (AD001–AD012 depending on the DNA Adapter Indexes being used), Ligase Control, and Stop Ligase Mix tubes to 600 xg for 5 seconds.
2. Immediately before use, remove the DNA Ligase Mix tube from -15° to -25°C storage.

NOTE

If you do not intend to consume the DNA Ligase Mix in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

3 Remove the adhesive seal from the ALP plate.

4 Add 2.5 µl of Ligase Control (or 2.5 µl of Resuspension Buffer, if not using Ligase Control) to each well of the ALP plate.

5 Add 2.5 µl of DNA Ligase Mix to each well of the ALP plate.

6 Return the DNA Ligase Mix tube back to -15° to -25°C storage immediately after use.

7 Add 2.5 µl of each thawed DNA Adapter Index (AD001–AD012 depending on the DNA Adapter Indexes being used) 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, for 10 minutes at 30°C.

2 Remove the ALP plate from the heat block.

Add STL

1 Remove the adhesive seal from the ALP plate.

2 Add 5 µl of Stop Ligase Mix 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 19 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 2 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 using a single channel or multichannel pipette. Some liquid may remain in each well. Take care not to disturb the beads. 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 at least 30 seconds, then remove and discard all of the supernatant from each well using a single channel or multichannel pipette. Take care not to disturb the beads. Change the tip after each sample.

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

10 Let the ALP plate stand at room temperature for 15 minutes to dry and then remove the plate from the magnetic stand.

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 Place the ALP plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.

13 Remove the adhesive seal from the ALP plate.

14 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 using a single channel or multichannel pipette. Change the tip after each sample.

15 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. 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.

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

17 Place the CAP plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.

18 Remove the adhesive seal from the CAP plate.

19 Remove and discard 95 µl of the supernatant from each well of the CAP plate using a single channel or multichannel pipette. Some liquid may remain in each well. 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 (20–22).

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

21 Incubate the CAP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well using a single channel or multichannel pipette. Take care not to disturb the beads. Change the tip after each sample.

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

23 Let the CAP plate stand at room temperature for 15 minutes to dry and then remove the plate from the magnetic stand.

24 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.

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

26 Place the CAP plate on the magnetic stand at room temperature for 2 minutes or until the liquid appears clear.

27 Remove the adhesive seal from the CAP plate.

28 Transfer 20 µl of the clear supernatant from each well of the CAP plate to the corresponding well of the new TCY plate labeled with the SSP barcode using a single channel or multichannel pipette. Change the tip after each sample. The samples are ready for loading onto the gel.

SAFE STOPPING POINT

If you do not plan to proceed to *Purify Ligation Products* immediately, the protocol can be safely stopped here. If you are stopping, seal the SSP plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C for up to seven days.

Purify Ligation Products

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.

For genomic sequencing, Illumina suggests a 300–400 bp insert size target (+/- 1 standard deviation of 20 bp, i.e, a < 20% variance) for read lengths of 2 × 75 bp or shorter. This translates to a 3 mm gel slice at 400–500 bp to account for the length of the adapter sequences flanking the inserts.

For exome enrichment, Illumina suggests a 200–300 bp insert size target. This translates to a 3 mm gel slice at 300–400 bp to account for the length of the adapter sequences flanking the inserts. For other applications, other size ranges may be desired and the cut size can be adjusted accordingly.

Illumina-Supplied Consumables

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

User-Supplied Consumables

- 50 X TAE Buffer
- 96-well TCY plate
- BenchTop 100 bp DNA Ladder
- Clean Scalpels
- Certified Low-range Ultra Agarose
- Distilled Water
- 4X Loading Buffer
- MinElute Gel Extraction Kit
- SyBr Gold Nucleic Acid Gel Stain

Preparation

- Prepare 1X TAE buffer (> 1 L)
- Apply a PCR barcode label to a new 96-well TCY plate.
- Remove the SSP plate from -15° to -25°C storage, if it was stored at the conclusion of *Ligate Adapters* and let stand to thaw at room temperature. Briefly centrifuge the thawed SSP plate to 280 xg for 1 minute.

- 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 7 µl of 4X Loading Buffer to each well of the SSP plate.
4. Add 17 µl Resuspension Buffer and 7 µl of 4X Loading Buffer 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 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.

NOTE

If proceeding with the TruSeq Exome Enrichment protocol, excise a band ranging in size from 300–400 bp. 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 TCY 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* immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store it 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)
- PCR Primer Cocktail (PPC) (1 tube)
- Resuspension Buffer (RSB) (1 tube)
- CPP (Clean Up PCR Plate) barcode label
- TSP1 (Target Sample Plate) barcode label

User-Supplied Consumables

- 96-well MIDI plate
- 96-well TCY 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 one tube each of PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature.
- Briefly centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- Review *AMPure XP Handling* on page 19.
- 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 *Purify Ligation Products* 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 4°C
- Apply a CPP barcode label to a new 96-well MIDI plate.
- Apply a TSP1 barcode label to a new 96-well TCY plate.

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.

NOTE

When using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Make PCR

The following procedure assumes 1 µg of input DNA to library preparation and is designed to get higher library yields.

1 Add 5 µl of thawed PCR Primer Cocktail to each well of the PCR plate using a single channel or multichannel pipette.

2 Add 25 µl of thawed PCR Master Mix to each well of the PCR plate using a multichannel pipette. Change the tip after each sample. Mix thoroughly as follows:

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

   b Shake the PCR plate on a microplate shaker at 1,600 rpm for 20 seconds.

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

Amp PCR

1 Amplify the PCR plate in the pre-programed 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 4°C

Clean Up PCR

NOTE

Before performing clean up, review *AMPure XP Handling* on page 19 when working with AMPure XP Beads.

1 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.

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

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 2 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 using a single channel or multichannel pipette. Some liquid may remain in each well. 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 at least 30 seconds, then remove and discard all of the supernatant from each well using a single channel or multichannel pipette. 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 Remove the CPP plate from the magnetic stand and let the plate stand at room temperature for 15 minutes to dry.

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 2 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 TCY plate labeled with the TSP1 barcode using a single channel or multichannel pipette. Change the tip after each sample.

SAFE STOPPING POINT

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

Validate Library

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

Quantify Libraries

1 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 *qPCR Quantification Protocol Guide*.

2 If performing exome enrichment, proceed to the *TruSeq Exome Enrichment Guide*. For all other sequencing applications, proceed to *Pool Libraries*.

Quality Control

1 [Optional] To verify the size of your PCR enriched fragments, check the template size distribution by running an aliquot of the enriched library on a gel or on a Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip.

a 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.

b If using the Agilent Bioanalyzer with a high sensitivity DNA chip, make a 1:100 dilution of the library using water and load 1 µl of the diluted library on the Agilent High Sensitivity DNA chip.

Figure 10 Example of DNA Library Size Distribution

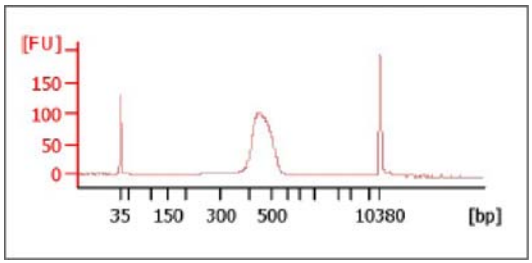
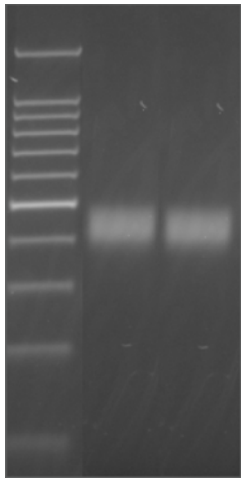


Figure 11 DNA PCR Product



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* on page 83.

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 (Diluted Cluster Template) plate and then pooled in equal volumes in the PDP (Pooled DCT 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 TCY 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 TCY plate (for multiplexing only).
- Remove the TSP1 plate from -15° to -25°C storage 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 using a single channel or multichannel pipette. 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:
   a For non-multiplexed 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 it at -15° to -25°C.
   b For multiplexed libraries, proceed to *Make PDP*.

Make PDP

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 TCY plate labeled with 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 11 Pooled Sample Volumes

Number of pooled samples	Volume (µl)
1	10
2	20
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:
   a Proceed to cluster generation. See the *Illumina Cluster Generation User Guide*.
   b Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.

Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 12 Illumina General Contact Information

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

Table 13 Illumina Customer Support Telephone Numbers

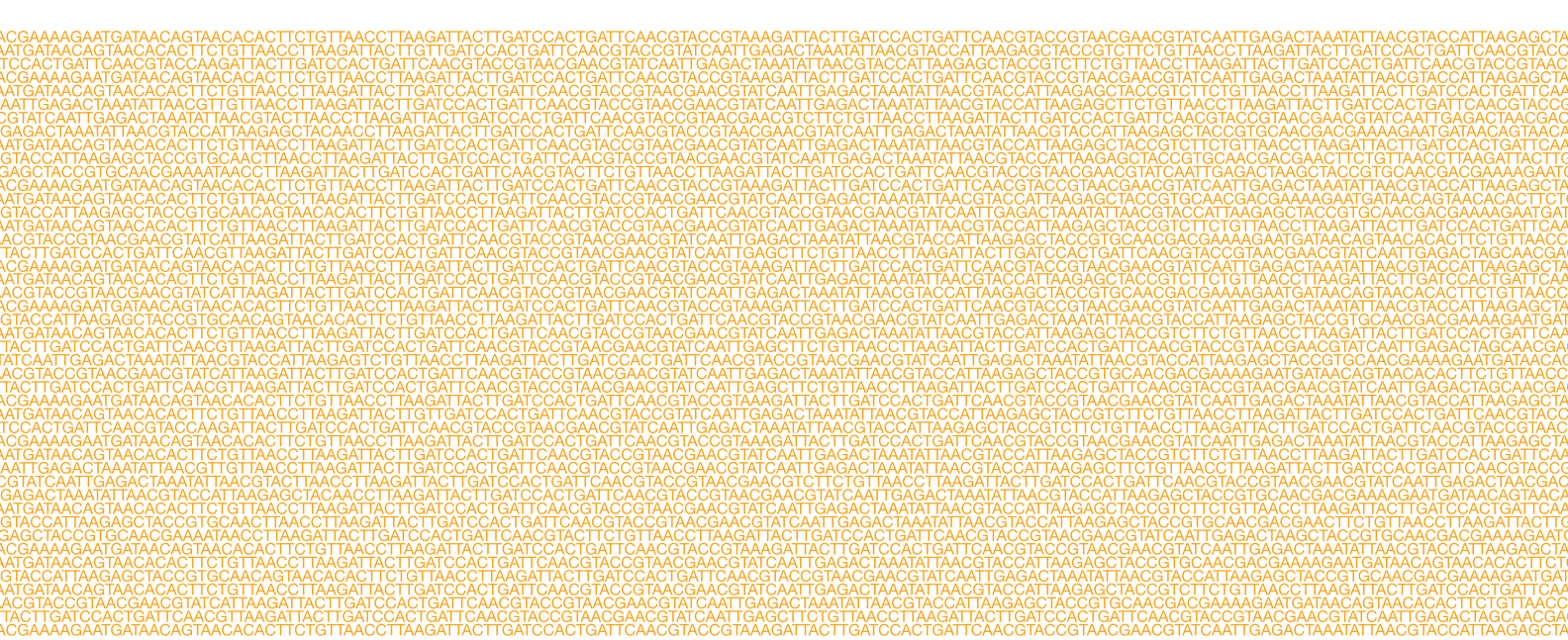
Region	Contact Number
North America toll-free	1.800.809.ILMN (1.800.809.4566)
United Kingdom toll-free	0800.917.0041
Germany toll-free	0800.180.8994
Netherlands toll-free	0800.0223859
France toll-free	0800.911850
Other European time zones	+44.1799.534000
Other regions and locations	1.858.202.ILMN (1.858.202.4566)

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. 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.



Illumina, Inc.
9885 Towne Centre Drive
San Diego, CA 92121-1975
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com