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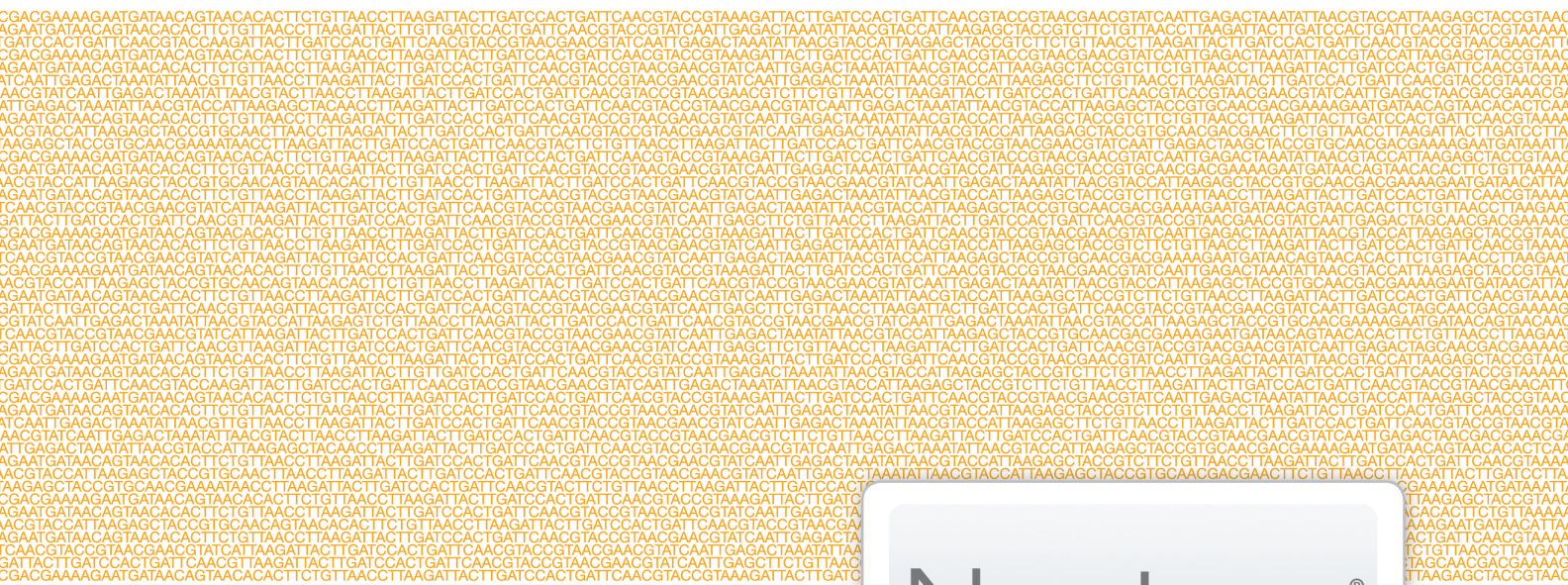
Nextera® Mate Pair Sample Preparation Guide



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Overview

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Introduction

This protocol explains how to generate mate pair libraries from genomic DNA, for paired-end sequencing, using the Illumina Nextera Mate Pair Sample Preparation Kit.

The Nextera Mate Pair Sample Preparation Protocol Offers:

- Transposome mediated fragmentation and adapter tagging of genomic DNA
- An identifiable mate pair junction sequence
- TruSeq DNA Sample Prep master-mixed reagents
- TruSeq DNA Sample Prep adapter indexing compatibility – 12 indexes included
- Gel-Free protocol – A low input, simple, and robust protocol generating mate pair libraries with a broad distribution fragment size
- Optional Gel-Plus protocol with size-selection - For more challenging mate pair applications requiring larger fragment sizes or tighter fragment size distribution
- On-bead reactions for ease of automation, reduced sample loss and simple purification steps

Gel-Free & Gel-Plus Versions of Nextera Mate Pair Sample Preparation Protocol

Two different versions of the protocol are described in this guide that differ from one another in the use or avoidance of gel electrophoresis to select fragments of a particular size. Each has its merits and challenges: for example, the Gel-Free protocol is shorter, more robust, yields a higher diversity of fragments, and requires less input DNA than the Gel-Plus protocol, however produces a much broader range of fragment sizes. In contrast, the Gel-Plus protocol is longer and more challenging to do, but allows the user the freedom to select a narrower range of fragment sizes. Which protocol to use will depend on preference of workflows and the final application of the mate pair dataset.

Upon sequencing, the Gel-Free protocol will typically yield a library with a mate pair fragment distribution ranging from 2 to 15 kb, and with a median fragment size between 2.5 and 4 kb. The Gel-Plus protocol will yield libraries with mate pair fragment size distributions determined by the gel based size-selection procedure.

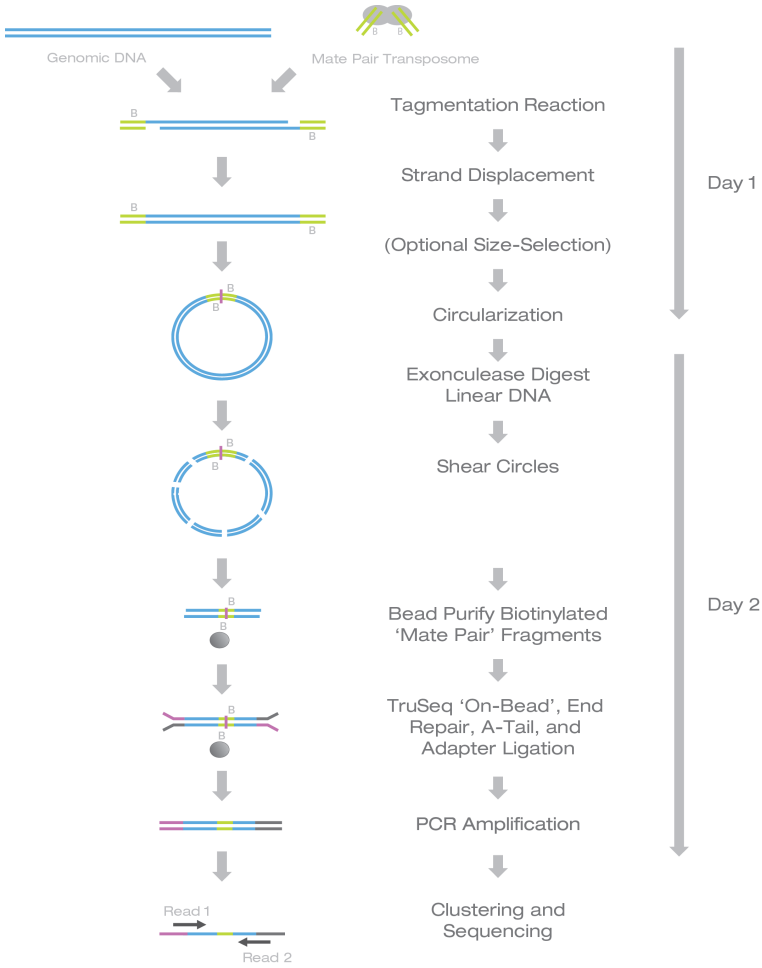
When following the Gel-Free protocol, the recommended input is 1 µg of genomic DNA and the kit is formulated to generate 48 libraries from 48 independent samples. When following the Gel-Plus protocol, the recommended input is 4 µg of genomic DNA and this

kit is formulated to either prepare 12 libraries with single size-selections per sample, or 48 libraries if making multiple size-selections from up to 12 samples.

Table 1 Summary Table

Protocol	DNA Input	Number of Samples	Number of Size-Selections per Sample	Number of Libraries
Nextera Mate Pair Gel-Free Protocol	1 µg	48	n/a	48
Nextera Mate Pair Gel-Plus Protocol:				
Pippin Prep size Selection	4 µg	12	1	12
Agarose Gel size-selection	4 µg	12	up to 4	up to 48

Figure 1 Nextera Mate Pair Sample Preparation Procedure



Nextera Mate Pair Sample Preparation Process Overview

The Nextera Mate Pair Sample Preparation process can be summarized into the following steps:

Tagmentation Reaction

During this step, by use of a specially formulated mate pair transposome, the genomic DNA sample is simultaneously fragmented and tagged with a biotinylated mate pair junction adapter. In later steps this biotin adapter serves to facilitate purification of mate pair fragments. During post sequencing analysis, the sequence of this junction adapter is used to identify the location where the ends of the larger fragments have joined to form a mate pair fragment.

Strand Displacement Reaction

The previous tagmentation step leaves a short single stranded sequence gap in the tagmented DNA. The strand displacement reaction uses a polymerase to fill this gap and ensure that all fragments are flush and ready for circularization.

AMPure Purification of Strand Displacement Reaction

AMPure Beads are employed to purify the DNA from the strand displacement reaction mix. They also are used to remove smaller DNA fragments (< 1500 bp). When the AMPure beads are added to the reaction volume, the larger desired DNA fragments are bound to the beads, while the smaller unwanted DNA fragments remain in solution and are removed.

Size-Selection (Gel-Plus Protocol Only)

This step offers a more stringent size-selection process than the AMPure purification step alone. It allows the generation of libraries with larger mate pair fragment sizes and tighter distributions. Size-selection can be carried out by Sage Pippin Prep or by standard agarose gel electrophoresis. Both protocols are described in this guide.

Circularization

The AMPure purified and size-selected fragments are circularized in a blunt ended intramolecular ligation, with an overnight incubation optimized to maximize the number

of fragments that will form circular molecules.

Exonuclease Digestion

During this step, any linear molecules still remaining in the circularization reaction are removed by DNA exonuclease treatment. This treatment leaves the desired circular molecules intact.

Fragmentation of Circularized Fragments

During this step the large circularized DNA fragments are physically sheared to smaller sized fragments (approximately 300–1000 bp). Shearing can be carried out by either of two alternative methods, Covaris sonication or nebulization. Both protocols are described in this guide.

Purification of Biotinylated Mate Pair Fragments

During this step the sheared DNA fragments that contain the biotinylated junction adapter (mate pair fragments) are purified by means of binding to streptavidin magnetic beads, and the unwanted, unbiotinylated molecules are removed through a series of washes.

End Repair

This process converts the overhangs resulting from the DNA shearing step 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. The end repair reaction is carried out on-bead, and the DNA remains bound to the beads throughout this reaction and subsequent bead wash steps.

A-Tailing

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. The A-tailing reaction is carried out on-bead, and the DNA remains bound to the beads throughout this step.

Adapter Ligation

This process ligates TruSeq indexing adapters to the ends of the DNA fragments, preparing them for PCR amplification and subsequent hybridization onto a flow cell. The adapter ligation reaction is carried out on-bead, and the DNA remains bound to the beads throughout this reaction and subsequent bead wash steps.

PCR

PCR amplification is used to enrich for the mate pair fragments that have TruSeq DNA adapters on both ends. The template material is bound to the streptavidin beads, however the resulting PCR amplified copies are not biotinylated and are not bound to the beads.

PCR Clean-Up

The product of the PCR amplification is a DNA smear of different fragment sizes. An AMPure bead purification step is used to clean up the PCR reaction and remove the smallest fragments (<300 bp) from the final library.

Documentation

Additional documentation is available for download from the Illumina website. Refer to the inside back cover of this guide for more information.

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Nextera Mate Pair Sample Preparation Kit (FC-132-1001)

Check to make sure that you have all of the reagents identified in this section before proceeding. The Nextera Mate Pair Sample Preparation Kit contains four component boxes.

- Nextera Mate Pair Sample Preparation Kit - Box 1
- Nextera Mate Pair Sample Preparation Kit - Box 2 Wash Solutions
- TruSeq DNA LT Sample Prep Kit - Set A
- TruSeq DNA Sample Prep Kit - PCR Box

The Nextera Mate Pair Sample Preparation Kit Box 1 and Box 2 contain all the Illumina supplied reagents required for the first half of the protocol up until the *End Repair* step. The TruSeq Sample Prep Kit boxes in combination with the Nextera Mate Pair Sample Preparation Kit - Wash Solutions Box, provide the remaining Illumina supplied reagents required for the *End Repair* step onwards.

Nextera Mate Pair Sample Preparation Kit - Box 1

This box is shipped on dry ice. As soon as you receive your kit, store the kit components at the specified temperature.

Quantity	Reagent	Part #	Storage Temperature	Description
1	MTP	15034805	-15° to -25°C	Mate Pair Tagment Enzyme
1	NPT	15034806	-15° to -25°C	dNTPs
1	SDP	15034807	-15° to -25°C	Strand Displacement Polymerase
1	PS1	15034808	-15° to -25°C	Exonuclease
1	TB1	15034809	-15° to -25°C	Tagment Buffer Mate Pair
1	NT	15038589	Room temperature	Neutralize Tagment Buffer
1	CB	15038590	-15° to -25°C	Circularization Buffer 10X
1	SDB	15034810	-15° to -25°C	10X Strand Disp Buffer
1	CL	15038591	-15° to -25°C	Circularization Ligase
1	STL*	15012546	-15° to -25°C	Stop Ligation Buffer*

NOTE

*Two Tubes of Stop Ligation Buffer are provided in the Nextera Mate Pair Sample Preparation Kit: one tube in the Nextera Mate Pair Sample Preparation Kit – Box 1, and another tube in the TruSeq DNA LT Sample Prep – Set A box.

Nextera Mate Pair Sample Preparation Kit – Box 2 Wash Solutions

This box is shipped at room temperature. As soon as you receive your kit, store the kit components at the specified temperature.

Quantity	Reagent	Part #	Storage Temperature	Description
1	RSB	15038592	Room temperature	Resuspension Buffer*
1	BBB	15034811	Room temperature	Bead Bind Buffer
1	BWB	15034813	Room temperature	Bead Wash Buffer

NOTE

*Two aliquots of Resuspension Buffer are provided in the Nextera Mate Pair Sample Preparation Kit: One larger volume bottle in the Nextera Mate Pair Sample Preparation Kit – Box 2, and a second smaller volume tube in the TruSeq DNA LT Sample Prep – Set A box.

TruSeq DNA LT Sample Prep Kit - Set A Box

This box is shipped on dry ice. As soon as you receive your kit, store the kit components at the specified temperature.

Quantity	Reagent	Part #	Storage Temperature	Description
1	RSB	15026770	-15° to -25°C	Resuspension Buffer
1	ERP	15012494	-15° to -25°C	End Repair Mix
1	ATL	15012495	-15° to -25°C	A-Tailing Mix
1	LIG	15026773	-15° to -25°C	Ligation Mix
1	CTE	15026774	-15° to -25°C	End Repair Control
1	CTA	15026775	-15° to -25°C	A-Tailing Control
1	CTL	15026776	-15° to -25°C	Ligation Control
1	STL	15012546	-15° to -25°C	Stop Ligation Buffer
1	AD002	15026621	-15° to -25°C	DNA Adapter Index 2
1	AD004	15026623	-15° to -25°C	DNA Adapter Index 4
1	AD005	15026624	-15° to -25°C	DNA Adapter Index 5

Quantity	Reagent	Part #	Storage Temperature	Description
1	AD006	15026625	-15° to -25°C	DNA Adapter Index 6
1	AD007	15026627	-15° to -25°C	DNA Adapter Index 7
1	AD012	15026632	-15° to -25°C	DNA Adapter Index 12
1	AD013	15024641	-15° to -25°C	DNA Adapter Index 13
1	AD014	15024642	-15° to -25°C	DNA Adapter Index 14
1	AD015	15024643	-15° to -25°C	DNA Adapter Index 15
1	AD016	15024644	-15° to -25°C	DNA Adapter Index 16
1	AD018	15024646	-15° to -25°C	DNA Adapter Index 18
1	AD019	15024647	-15° to -25°C	DNA Adapter Index 19

NOTE

The control reagents supplied in the TruSeq LT Sample Prep Kit cannot be used when preparing Nextera Mate Pair Sample Preparation Libraries. However, these controls can still be used with the TruSeq DNA LT Sample Preps kit if performing Standard TruSeq DNA Sample Preparation.

TruSeq DNA LT Sample Prep Kit - PCR Box

This box is shipped on dry ice. As soon as you receive your kit, store the kit components at the specified temperature.

Quantity	Reagent	Part #	Storage Temperature	Description
1	PMM	15026785	-15° to -25°C	PCR Master Mix
1	PPC	15031748	-15° to -25°C	PCR Primer Cocktail

Indexed Adapter Sequences

The Nextera Mate Pair Sample Preparation Kit is supplied with a TruSeq DNA LT Sample Prep Kit containing DNA Adapter Index tubes (Set A) that can be used to perform pooled sequencing.

- Each tube contains a unique single 6 base index adapter on the P7 strand and contains enough reagent for 20 reactions.
- Samples prepared with these adapters can be sequenced on any Illumina sequencing platform using the TruSeq Single Index Recipe.

For more information on pooling guidelines when using adapter index tubes, see *Pooling Guidelines* on page 14.

TruSeq DNA LT Sample Prep Kit Indexed Adapter Sequences

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

NOTE

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

Table 2 TruSeq DNA LT Sample Prep Kit Indexed Adapter Sequences Set A

Index Adapter	Sequence
AD002	CGATGT(A)
AD004	TGACCA(A)
AD005	ACAGTG(A)
AD006	GCCAAT(A)
AD007	CAGATC(A)

Index Adapter	Sequence
AD012	CTTGTA(A)
AD013	AGTCAA(C)
AD014	AGTTCC(G)
AD015	ATGTCA(G)
AD016	CCGTCC(C)
AD018	GTCCGC(A)
AD019	GTGAAA(C)

Pooling Guidelines

Illumina uses a green laser to sequence G/T and a red laser to sequence A/C. At each cycle at least one of two nucleotides for each color channel needs to be read to ensure proper image registration. It is important to maintain color balance for each base of the index read being sequenced, otherwise index read sequencing could fail due to registration failure.

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

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

Table 3 Single-Indexed Pooling Strategies for 2–4 Samples

Plexity	Option	Set A Only
2	1	AD006 and AD012
	2	AD005 and AD019

Plexity	Option	Set A Only
3	1	AD002 and AD007 and AD019
	2	AD005 and AD006 and AD015
	3	2-plex options with any other adapter
4	1	AD005 and AD006 and AD012 and AD019
	2	AD002 and AD004 and AD007 and AD027
	3	3-plex options with any other adapter

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

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 Nextera Mate Pair Sample Preparation protocols.

Table 4 User-Supplied Consumables

Consumable	Supplier
1.7 ml Axygen MAXYMum Recovery Microcentrifuge tubes	Axygen Scientific, part # MCT-175-L-C
0.2 ml thin wall PCR tubes	Axygen Scientific, part # PCR-02-C or equivalent
Qubit® dsDNA BR Assay Kit (recommended)	Invitrogen, Cat # Q32850
Agilent High Sensitivity DNA Kit (recommended)	Agilent Technologies, Cat # 5067-4626
Agilent DNA 12000 Kit (recommended)	Agilent Technologies, Cat #5067-1508
Zymo Genomic DNA Clean & Concentrator™	Zymo Research, Cat # D4010 (25 preps) or D4011 (100 preps)
AMPure XP Beads	Beckman Coulter, Cat # A63880
Dynabeads M-280 streptavidin magnetic beads	Invitrogen, part # 112-05D

If performing the Covaris Shearing protocol, the following consumables are required:

Table 5 User-Supplied Consumables for the Covaris Shearing Protccol

Consumable	Supplier
Covaris T6 (6 x 32 mm) glass tubes	Covaris, part # 520031
Covaris Snap-Cap - Teflon Silicone Septa 8mm	Covaris, part # 520042

If performing the Nebulization protocol, the following consumables are required:

Table 6 User-Supplied Consumables for the Nebulization Protocol

Consumable	Supplier
Glycerol	Sigma, part # G5516
PVC tubing or equivalent	Intersurgical, part # 1174-003
Nebulizers and nebulization buffer	Illumina, catalog # FC-301-1001
Zymo DNA Clean & Concentrator™-5	Zymo Research, Cat # D4013

Table 7 User-Supplied Equipment

Equipment	Supplier
Heat blocks (20°C - 70°C)	General lab supplier
Magnetic rack for 1.7 ml microcentrifuge tubes	Invitrogen, part # CS15000
Micro-Centrifuge for 1 minute spins > 16,000 g	General lab supplier (e.g. Eppendorf Cat # 5424 000.410)
Thermal cycler or PCR machine	General lab supplier
Mini-centrifuge for quick ~2000 g spins (recommended)	General lab supplier (e.g. Fisher, Cat # 05-090-100)
2100 Bioanalyzer (recommended)	Agilent
Qubit Fluorometer or equivalent (recommended)	Invitrogen, Cat # Q32866

If following the Covaris Shearing protocol, the following equipment is required:

Table 8 Covaris Shearing Protocol Equipment

Equipment	Supplier
Covaris AFA™ Ultrasonicator	Covaris, S2 or S220 Ultrasonicator device

If performing the Nebulization protocol, the following equipment is required:

Table 9 Nebulization Protocol Equipment

Equipment	Supplier
Compressed Nitrogen or Air source of at least 32 psi	General lab supplier
Benchtop centrifuge with swing-out rotor - Capable of holding nebulizer units.	General lab supplier

If following the Gel-Plus protocol and using a Sage Pippin Prep, the following equipment and consumables are required:

Table 10 Sage Pippin Prep Equipment and Consumables

Equipment & Consumables	Supplier
Sage Pippin Prep DNA size selection system	Sage Science
Pippin Prep 0.75 % Agarose Cassettes and Marker	Sage Science, Cat # CSD7510

If following the Gel-Plus protocol and using an agarose gel for size-selection, the following equipment and consumables are required:

Table 11 Agarose Gel Size Selection Equipment and Consumables

Equipment & Consumables	Supplier
Gel tray and electrophoresis unit (Dimensions—12 cm width x 14 cm length)	Fisher, part # FB58325 (or similar)
Gel comb with wide wells (Well dimensions—9 mm width x 1 mm length)	Fisher, part # FB58325 (or similar)
Dark reader transilluminator	Clare Chemical Research, part # D195M
Megabase Agarose	Bio-Rad, Cat # 161-3108
50 X TAE Buffer	Bio-Rad, Cat # 161-0743

Equipment & Consumables	Supplier
1 kb plus DNA ladder	Invitrogen, Cat # 10787-018
6X Gel Loading Dye	BioLabs, Cat # B7021S
Sybr Safe	Invitrogen, Cat # S33102
3.5 ml screw cap tubes (or equivalent)	Sarstedt, Cat # 62.613
DNA Gel Extraction kit - Zymoclean™ Large Fragment DNA Recovery Kit	Zymo Research, Cat # D4045

Best Practices

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

General Advice on Sample Handling

- To minimize sample loss throughout the Nextera Mate Pair Sample Preparation we highly recommend using Axygen Maxymum Recovery 1.7 ml tubes (Cat # MCT-175-L-C), for their low retention and heat resistance properties.
- When adding and mixing reagents or bead wash solutions, try to avoid mixing reactions by pipetting up and down, as small amounts of sample may remain in the tip and be lost. Instead add the reagents or wash buffers and mix the solution by flicking the sample tube. Then briefly spin the sample in a mini-centrifuge to collect sample to the bottom of tube.
- When handling DNA samples with fragment sizes above 2 kb (all steps prior to Shear Circularized DNA), do not vortex samples to mix, as this may further shear the DNA.

Handling AMPure Magnetic Beads

Follow appropriate handling methods when working with AMPure XP Beads and the Nextera Mate Pair Sample Preparation Protocol:

NOTE
AMPure purification procedures in this user guide have only been validated using the Axygen Maxymum Recovery 1.7 ml microcentrifuge tubes and the magnetic stand specified in the Consumables and Equipment list. Comparable performance is not guaranteed when using other microcentrifuge tubes or tube formats, or other magnets.

- Prior to use, allow the beads to come to room temperature.
- Do not reuse beads. Always add fresh beads when performing these procedures.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous.
- When carrying out the AMPure purification of the Strand Displacement Reaction, use of the specified volumes and accurate pipetting is critical to the success of this step to remove the unwanted smaller DNA fragments (<1500 bp).
- Take care to minimize bead loss which can impact final yields.

- Change the tips for each sample.
- When aspirating the cleared solution, it is important to keep the tubes firmly 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 tube and into the pipette tips.
- Prepare fresh 70% ethanol. Ethanol tends to absorb water from the air, therefore fresh 70% ethanol should be prepared for optimal results.
- Be sure to remove all of the ethanol from the bottom of the wells, as it can contain residual contaminants.
- Remaining ethanol can be removed with a 20 µl pipette.
- Keep the microcentrifuge tube on the magnetic stand and let it air-dry at room temperature to prevent potential bead loss due to electrostatic forces. Allow for the complete evaporation of residual ethanol, as the presence of ethanol will impact the performance of the subsequent reactions.
- Avoid over drying the beads, which can impact final yields.
- Use the Resuspension Buffer (RSB) for DNA elution.
- To maximize sample recovery during elution, incubate the sample/bead mix for 5 minutes at room temperature before placing the samples onto the magnet.

Handling the Streptavidin Magnetic Beads and Performing the On-Bead Reactions

- The M280 Dynabeads beads do not need to come to room temperature before use.
- Do not reuse beads. Always add fresh beads when performing these procedures.
- Immediately prior to use, mix the beads well by shaking, until they are well dispersed. The color of the liquid should appear homogeneous.
- Once the DNA has been bound to the Streptavidin beads, it remains bound to the beads until it is amplified off the beads in the PCR amplification reaction.
- The following steps are all carried out on-bead: End Repair, A-Tailing, Adapter Ligation, and PCR Amplification.
- To avoid unnecessary sample loss due to pipette tip carryover, do not resuspend the beads using a pipette, instead resuspend them by flicking and then spin in a mini-centrifuge to collect the sample to the bottom of the tube.
- When washing the beads during the on-bead reaction steps, please note that the Resuspension Buffer is used as a wash buffer and does not elute the DNA from the beads.
- When washing the beads after the on-bead reactions, resuspend the beads in the wash buffer by flicking tube multiple times, and then spin in a mini-centrifuge. By

centrifuging the bead/wash solution for a longer period of time (up to 10 seconds), the beads can be collected to the bottom of the tube, thus aiding the subsequent collection of the beads to the magnet.

- Before adding the on-bead reaction mixtures, remove as much of the final Resuspension Buffer wash solution as possible with a 200 µl pipette tip, spin the tube again briefly, and place back on the magnet. Then remove any remaining Resuspension Buffer with a 10 µl pipette.
- After adding the on-bead reaction mixtures, resuspend the beads in the reaction mixture by flicking the tube multiple times, and then spin very briefly in a mini-centrifuge. Centrifuge the reactions only long enough to collect the solution to the bottom of the tube, but not so long as to begin to pellet the beads (approximately 1–2 seconds at low speed).
- During the on-bead reaction incubations the beads will settle to the bottom of the tube, this is normal and will not affect library preparation.

Handling Reagents

- Minimize freeze-thaw cycles. If you do not intend to consume the reagents in one use, dispense the reagent into aliquots after the initial thaw and refreeze the aliquots in order to avoid excessive freeze-thaw cycles. However, if you aliquot, you might not have enough reagents for the full number of reactions over multiple uses.
- Add reagents in the order indicated.
- Due to their viscosity, take extra care while adding the following reagents; Mate Pair Tagment Enzyme, Strand Displacement Polymerase, Circularization Ligase, End Repair Mix, A-Tailing Mix, and Ligation Mix.

DNA Input Recommendations

The Nextera Mate Pair Sample Preparation Kit protocol is optimized for 1 µg of genomic DNA if performing the Gel-Free protocol or 4 µg of DNA if performing the Gel-Plus protocol. Illumina strongly recommends quantifying the starting genomic material.

Input DNA Quantitation

Nextera Mate Pair Sample Preparation kit uses an enzymatic DNA fragmentation step and is more sensitive to DNA input than mechanical fragmentation methods. The ultimate success of library preparation strongly depends on using an accurate amount of input genomic DNA. Therefore, the correct quantitation of the input DNA sample is essential. To obtain an accurate quantification of the genomic DNA, it is recommended to quantify it using a fluorometric based method specific for duplex DNA such as the Qubit dsDNA BR Assay system. Illumina recommends using 2 µl of each DNA sample with 198 µl of the Qubit working solution for sample quantification. Methods that measure total nucleic acid content (e.g. nanodrop or other UV absorbance methods) should be avoided because common contaminants such as ssDNA, RNA, and oligos are not substrates for the Nextera Mate Pair Sample Preparation protocol.

Assessing DNA Quality

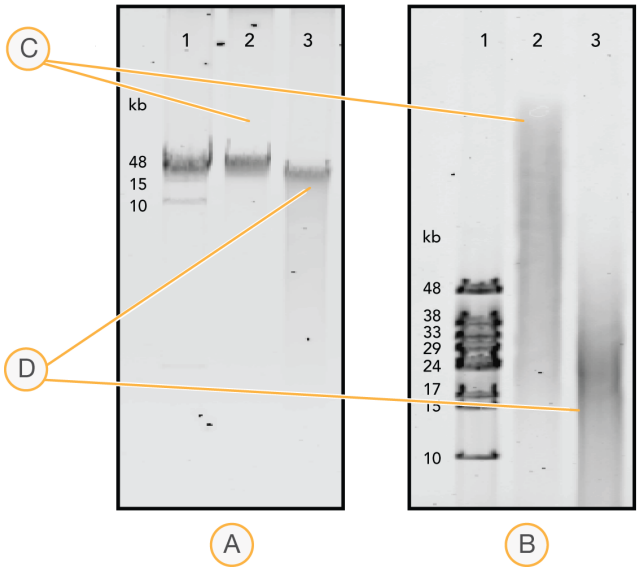
Use of high-quality, high molecular weight genomic starting material is also important for successful library generation. If degraded genomic DNA is used, the tagmentation step of the protocol is likely to fragment the DNA to a size below the desired size range. Furthermore, damaged and degraded DNA samples will PCR amplify less efficiently, leading to diminished library yields and diversity.

A simple way to assess the quality of the starting material is to run a small amount on a low-percentage agarose gel. High-quality DNA should run as a high molecular weight band with the majority of DNA greater than 50 kb in size and with minimal lower molecular weight smearing. If the majority of the DNA is below 50 kb or smearing is visible, this suggests that the DNA is degraded. If the sample is partially degraded, the Nextera Mate Pair Sample Preparation Protocol can still be used, but the amount of Transposome used in the tagmentation step may have to be reduced to prevent over-tagmentation and generate DNA fragments in the desired size range.

Figure 2 shows agarose gel analysis of two genomic DNA samples. Approximately 200 ng of sample was loaded per lane. Figure A is a 0.6% standard agarose gel stained with

ethidium bromide. Figure B is a higher resolution Pulse Field Gel, which more clearly shows the differences in quality and integrity.

Figure 2 Analysis of Genomic DNA Sample Integrity



A 0.6% Standard Agarose Gel Stained with Ethidium Bromide
B Higher Resolution Pulse Field Gel
C Intact, High-Quality DNA has Large Fragments (> 50 kb)
D Partially Degraded DNA has Small Fragments (< 29 kb)

Prevent PCR Product Contamination

The PCR process is commonly used in the laboratory to amplify specific DNA sequences. Unless proper laboratory hygiene is used, PCR products can contaminate reagents, instrumentation, and genomic DNA samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations.

Make sure that the lab is set up appropriately to reduce the risk of PCR product contamination:

- Physically separate laboratory space where pre-PCR processes are performed from the laboratory space where PCR products are made and processed during the post-PCR processes.
- Dedicate separate full sets of equipment and supplies (pipettes, centrifuges, oven, heat block, etc.) to pre-PCR and post-PCR lab processes.
- Illumina recommends that you practice daily and weekly cleaning of lab areas using 0.5% Sodium Hypochlorite (10% Bleach). To prevent sample or reagent degradation, make sure that all vapors from the cleaning solution have fully dissipated before you begin any processes.

Sequencing and Data Analysis

Mate pair libraries are generated using unique molecular biology protocols that share some common characteristics with standard Illumina library generation workflows. Although the initial fragmentation of the DNA is performed with Nextera based transposomes, the final library contains TruSeq adapter sequences. Thus, for sequencing purposes, these libraries should be sequenced using TruSeq DNA workflows and sequencing chemistry, and can be sequenced on any Illumina platform. There is no specified read length limit when sequencing, however, increasing the read length will inherently increase the chances of sequencing into the mate pair junction adaptor (see below). For more information on sequencing samples prepared using the Nextera Mate Pair Sample Preparation Kit, see your sequencing platform guide.

When analyzing and interpreting sequence data from a Nextera Mate Pair library, the following unique features that differentiate these libraries from other standard Illumina libraries should be considered:

- The presence of a junction adapter sequence within the templates. This junction adapter can occur at a random position within the template; therefore, its recognition during sequencing depends on its location within a template, the length of a cluster template, and the length of the reads.
- Sequenced read pairs align in an outward-facing (or 'reverseforward', RF) orientation to one another rather than inward facing (or 'forward-reverse', FR). This is a consequence of circularization, whereby the fragment ends are inverted and linked together.

For details of mate pair adapter sequences, and information on how best to utilize mate pair data, please refer to Illumina documentation *Data processing of Nextera Mate Pair reads on Illumina sequencing platforms* available on my.illumina.com.

Nextera Mate Pair Protocol

Chapter 3

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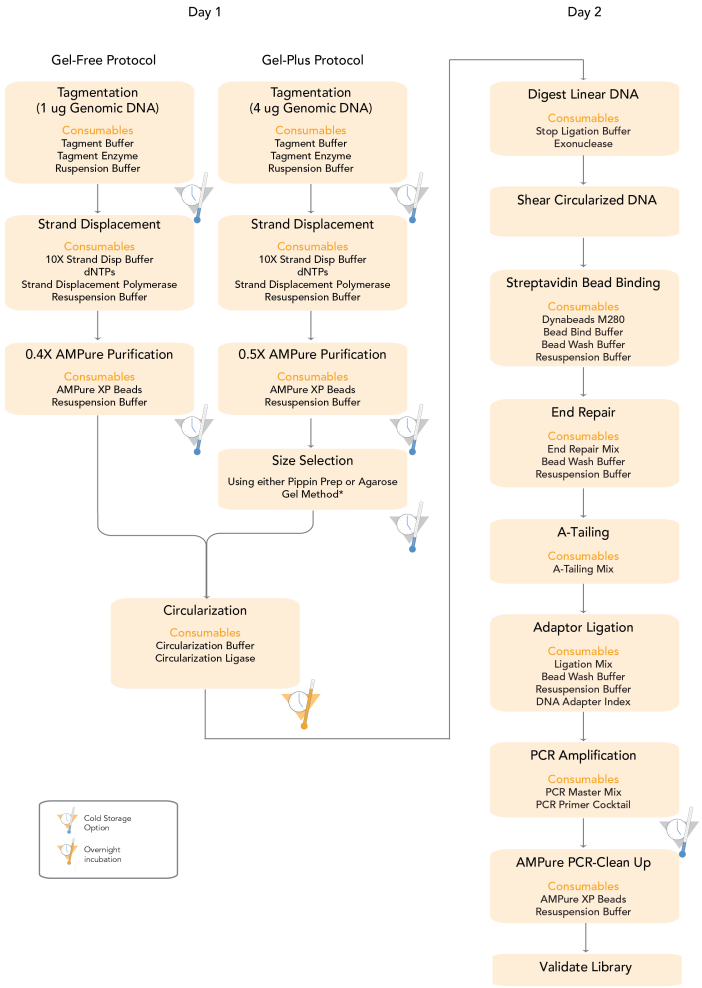
Nextera Mate Pair Sample Preparation Workflow

The following diagrams illustrate the Nextera Mate Pair Sample Preparation Kit. Safe stopping points are marked between steps.

Figure 3 Nextera Mate Pair Sample Preparation Workflow



Tagmentation

During this step, by use of a specially formulated mate pair transposome, the genomic DNA sample is simultaneously fragmented and tagged with a biotinylated mate pair junction adaptor. In later steps this biotin adapter serves to facilitate purification of mate pair fragments. During post sequencing analysis, the sequence of this junction adapter is used to identify the location where the ends of the larger fragments have joined to form a mate pair fragment.

Estimated Time

- Hands-on: 15 minutes
- Total duration: 45 minutes

Illumina-Supplied Consumables

- Mate Pair Tagment Enzyme
- Tagment Buffer Mate Pair
- Resuspension Buffer

User-Supplied Consumables

- Low retention 1.7 ml microcentrifuge tubes
- Genomic DNA:
  - Gel-Free Protocol - 1 µg
  - Gel-Plus Protocol - 4 µg
- Water
- Zymo Genomic DNA Clean & Concentrator kit

Preparation

NOTE

See *[Optional Alternative Procedure] AMPure Purification of Tagmentation Reaction* on page 68 for an alternative Tagmentation purification procedure using AMPure XP beads rather than Zymo columns. When carrying out the Gel-Free protocol this offers a more automation friendly alternative.

NOTE

To minimize sample loss throughout sample preparation we highly recommend using Axygen Maxymum Recovery 1.7 ml tubes for their low retention and heat resistance properties.

- Remove the following from -15° to -25°C storage and place on ice:
  - Tagment Buffer Mate Pair
  - Mate Pair Tagment Enzyme
  - Genomic DNA Sample
- Pre-heat the heat block to 55°C.
- Ensure DNA sample concentration has been accurately quantified.

Procedure

1 In a clean 1.7 ml microcentrifuge tube, set up the tagmentation reaction, adding each component in the order shown below:

	Gel-Free Volume	Gel-Plus Volume
Genomic DNA	x µl (1 µg)	x µl (4 µg)
Water	76–x µl	308–x µl
Tagment Buffer Mate Pair	20 µl	80 µl
Mate Pair Tagment Enzyme	4 µl	12 µl
Total	100 µl	400 µl

2 Mix the reaction well by firmly flicking the tube 5 times, then spin briefly (1–2 seconds) in a mini-centrifuge to collect reaction to the bottom of the tube.

3 To ensure tagmentation reaction is well mixed, repeat step 2.

4 Incubate at 55°C for 30 minutes.

5 Purify the tagmentation reaction using a Zymo Genomic DNA Clean & Concentrator™, as descried below:

NOTE
All centrifugation steps should be performed between 10,000–16,000 xg.

a Add 2 volumes of Zymo ChIP DNA Binding Buffer to the tagmentation reaction and mix thoroughly.

b Transfer up to 800 µl of mixture to a Zymo-Spin™ IC-XL column in a collection tube.
c Centrifuge for 30 seconds. Discard the flow-through.
d If applicable, transfer the remaining tagmentation mixture to the same Zymo-Spin™ IC-XL column.
e Centrifuge for 30 seconds. Discard the flow-through.
f Add 200 µl Zymo DNA Wash Buffer to the column. Centrifuge for 1 minute. Discard the flow-through.
g Repeat the wash step once.
h Centrifuge the empty column for 1 minute with lid open to ensure any residual ethanol wash is removed. Discard any flow-through and collection tube.
i Transfer the column to a clean 1.7 ml microcentrifuge tube and add 30 µl of Resuspension Buffer. Incubate at room temperature for 1 minute.
j Centrifuge for 1 minute to elute the DNA.

6 [Optional QC Step] Remove 1 µl of sample to test tagmentation size on a 12000 Bioanalyzer LabChip. Before running on Bioanalyzer chip, dilute the 1 µl sample with the appropriate volume of water:

	Gel-Free Volume	Gel-Plus Volume
Water	1 µl	7 µl

7 Proceed to *Strand Displacement on page 32*.

Strand Displacement

The previous tagmentation step leaves a short single stranded sequence gap in the tagmented DNA. The strand displacement reaction uses a polymerase to fill this gap and ensure that all fragments are flush and ready for circularization.

Estimated Time

- Hands-on: 5 minutes
- Total duration: 35 minutes

Illumina-Supplied Consumables

- 10X Strand Disp Buffer
- dNTPs
- Strand Displacement Polymerase
- Resuspension Buffer

User-Supplied Consumables

- Tagmented DNA Sample (from previous step)
- Water

Preparation

- Remove the following from -15° to -25°C storage, thaw at room temperature, then place on ice:
  - 10X Strand Disp Buffer
  - dNTPs
- Remove the following from -15° to -25°C storage, then place on ice:
  - Strand Displacement Polymerase
- Pre-heat the heat block to 20°C.

Procedure

1 Using the 1.7 ml microcentrifuge tube already containing the Tagmented DNA, set up the strand displacement reaction, adding each additional component in the order shown below:

	Gel-Free Volume	Gel-Plus Volume
Tagmented DNA Sample	30 µl	30 µl
Water	10.5 µl	132 µl
10x Strand Disp Buffer	5 µl	20 µl
dNTPs	2 µl	8 µl
Strand Displacement Polymerase	2.5 µl	10 µl
Total	50 µl	200 µl

2 Mix the reaction by flicking the tube 5 times, then spin briefly (1–2 seconds) in a mini-centrifuge to collect reaction to the bottom of the tube.

3 Incubate at 20°C for 30 minutes.

4 Proceed to *AMPure Purification of Strand Displacement Reaction on page 34*.

AMPure Purification of Strand Displacement Reaction

During this step AMPure Beads are employed to purify the DNA from the Strand Displacement Reaction mix. The AMPure Beads are also used to remove smaller DNA fragments (< 1500 bp). When the AMPure beads are added to the reaction volume, the larger desired DNA fragments are bound to the beads, while the smaller unwanted DNA fragments remain in solution and are removed.

Estimated Time

- Hands-on: 10 minutes
- Total duration: 30–40 minutes

Illumina-Supplied Consumables

- Resuspension Buffer

User-Supplied Consumables

- Strand Displacement DNA Sample (from previous step)
- Water
- AMPure XP Beads
- 70% Ethanol, freshly prepared
- Low retention 1.7 ml microcentrifuge tubes

Preparation

- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Prepare Fresh 70% Ethanol. 800 µl per sample.
- Review *Best Practices* on page 20 and the *Handling AMPure Magnetic Beads* section.

Procedure

1 Bring the AMPure XP beads to room temperature for 30 minutes. Vortex well to resuspend all the beads.

2 Set up the following incubation mix, ensuring that the volumes are measured and pipetted accurately. Using the 1.7 ml microcentrifuge tube already containing the Strand Displaced DNA sample, add each additional component in the order shown below:

	Gel-Free Volume	Gel-Plus Volume
Strand Displaced DNA	50 µl	200 µl
Water	50 µl*	0 µl**
AMPure XP Beads	40 µl	100 µl
Total	140 µl	300 µl

NOTE
Ensuring that the correct volumes are used is critical to the success of this purification step. *For the Gel-Free Protocol the sum of the Strand Displaced DNA and water should be 100 µl. **For the Gel-Plus Protocol the sum of the Strand Displaced DNA and water should be 200 µl. If necessary, the volume of water added to this mixture should be adjusted to achieve these specified volumes.

3 Mix the reaction by flicking the tube 5 times, then spin briefly (1–2 seconds) in a mini-centrifuge to collect reaction to the bottom of the tube.

4 Incubate DNA-bead mixture for 15 minutes at room temperature, resuspend the beads every 2 minutes by gently flicking tubes.

5 Briefly spin (1–2 seconds) in a mini-centrifuge to collect contents to the bottom of the tube.

6 Place the tube on magnet for 5 minutes, and then carefully remove all liquid from tube.

7 Leaving the tube on magnet, add 400 µl of fresh 70% Ethanol, and incubate for 30 seconds. Carefully remove and discard the ethanol without disturbing beads.

8 Repeat step 7 once.

9 Air dry beads for 10–15 minutes.

10 Remove the tube from magnet and add 30 µl of Resuspension Buffer to elute DNA from beads, mix by flicking tube.

11 Briefly spin (1–2 seconds) in a mini-centrifuge to collect contents to the bottom of the tube, and incubate for 5 minutes at room temperature.

12 Place the tube on magnet for 5 minutes and then carefully transfer supernatant containing DNA to a clean 1.7 ml microcentrifuge tube.

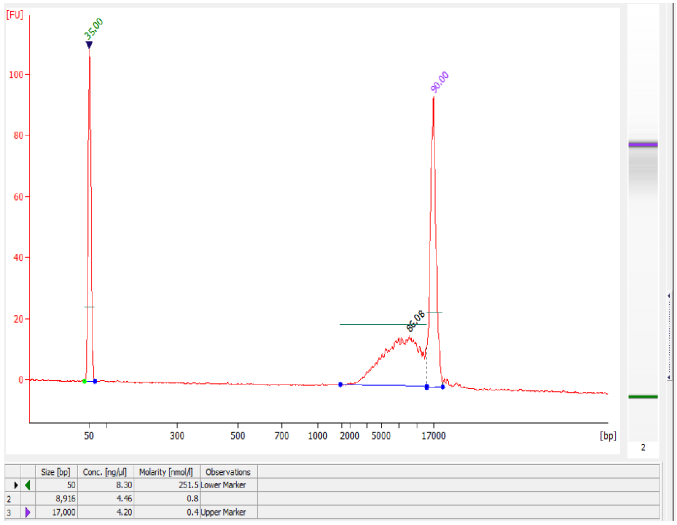
13 [Optional QC Step] Remove 1 µl of sample to run on a Bioanalyzer 12000 chip or to quantify using Qubit High Sensitivity kit or similar. Before running Bioanalyzer chip, dilute the sample with the appropriate volume of water:

	Gel-Free Volume	Gel-Plus Volume
Water	1 µl	7 µl

14 If performing the Gel-Free Protocol proceed directly to *Circularization* on page 38. If performing the Gel-Plus Protocol proceed to *Size Selection (Gel-Plus Protocol Only)* on page 44.

Figure 4 shows an example of quantification and sizing of Tagmented/Strand Displaced DNA, when following the Gel-Free Protocol. 1 ul of diluted AMPure Purified Strand Displaced DNA was run on an Agilent BioAnazlyzer 12000 LabChip. The size of distribution of the fragments is approximately 2–20 kb, with a peak fragment size of 8916 bp. There is approximately 267 ng of DNA (4.46 ng/µl x 8 x 30) remaining for circularization.

Figure 4 Example Quantification and Sizing of Tagmented / Strand Displaced DNA



Circularization

The AMPure purified and size-selected fragments are circularized in a blunt ended intramolecular ligation, with an overnight incubation optimized to maximize the number of fragments that will form circular molecules.

Prior to starting the circularization reaction, calculate how much DNA is remaining for each sample using the Agilent Bioanalyzer or Qubit HS quantification. An expected yield at this stage is 250–700 ng from the Gel-Free protocol, and 150–400 ng from the Gel-Plus protocol.

Using greater amounts of DNA in the circularization will increase library yields and increase library diversity, but will also have the unwanted consequence of increasing chimeric read pairs. Conversely, adding less DNA to the circularization reaction will reduce the number of chimeric read pairs, but will reduce library yield and diversity. To balance these constraints Illumina recommends using up to 600 ng of DNA in a total circularization volume of 300 µl.

NOTE
From the Circularization step onwards, the workflow and reagents volumes used by the Gel-Free and Gel-Plus protocols are identical and only one protocol is described.

Estimated Time

- Hands-on: 5 minutes
- Incubation time: 12–16 hours

Illumina-Supplied Consumables

- Circularization Buffer 10X
- Circularization Ligase

User-Supplied Consumables

- AMPure purified OR size-selected DNA sample (from previous step)
- Water
- Low retention 1.7 ml microcentrifuge tubes

Preparation

- Remove the following from -15° to -25°C storage, thaw at room temperature, then place on ice:
  - Circularization Buffer 10x
- Remove the following from -15° to -25°C storage, then place on ice:
  - Circularization Ligase
- Pre-heat the heat block to 30°C.
- Quantify DNA samples.

Procedure

1 In a clean 1.7 ml microcentrifuge tube, set up the circularization reaction, adding up to 600 ng of DNA. Add the components in the order shown below:

	Volume
AMPure Purified or Size Selected DNA	x µl (up to 600 ng)
Water	263–x µl
Circularization Buffer 10x	30 µl
Circularization Ligase	7 µl
Total	300 µl

2 Mix the reaction by flicking and inverting the tube several times, then spin briefly (1–2 seconds) in a mini-centrifuge to collect reaction to the bottom of the tube.

3 Incubate at 30°C overnight (12–16 hours).

4 After the overnight incubation proceed to *Digest Linear DNA* on page 40.

Digest Linear DNA

During this step, any linear molecules still remaining in the circularization reaction are removed by DNA exonuclease treatment. This treatment leaves the desired circular molecules intact. The exonuclease and ligase enzymes are then both inactivated by heat treatment and the addition of Stop Ligation Buffer.

Estimated Time

- Hands-on: 5 minutes
- Total duration: 65 minutes

Illumina-Supplied Consumables

- Exonuclease
- Stop Ligation Buffer

User-Supplied Consumables

- Circularized DNA sample (from previous step)

Preparation

- Remove the following from -15° to -25°C storage, thaw at room temperature, then place on ice:
  - Stop Ligation Buffer
- Remove the following from -15° to -25°C storage, then place on ice:
  - Circularization Ligase
- Pre-heat the heat blocks to 37°C and 70°C.

Procedure

1. Add 9 µl Exonuclease directly to the overnight circularization reaction. Mix by flicking the tube, then spin briefly (1–2 seconds) in a mini-centrifuge to collect sample to the bottom of the tube.
2. Incubate at 37°C for 30 minutes.
3. Inactivate the Exonuclease by incubating tube at 70°C for 30 minutes.

4 Add 12 µl of Stop Ligation Buffer, mix by flicking the tube, then spin briefly (1–2 seconds) in a mini-centrifuge to collect sample to the bottom of the tube.

5 Proceed to *Shear Circularized DNA* on page 42.

Shear Circularized DNA

During this step the large circularized DNA fragments are physically sheared to smaller sized fragments (approximately 300–1000 bp). The shearing procedure generates dsDNA fragments with 3' or 5' overhangs.

Illumina recommends the use of a Covaris S2 or S220 device to shear the DNA. If using a Covaris device other than the S2 or S220, please refer to the Covaris User Manual for equivalent settings and parameters.

In the *[Optional Alternative Procedure] Shear Circularized DNA - Nebulizer Procedure* on page 70 at the end of this guide we provide an alternative procedure to using a Covaris, describing how to shear the DNA using a nebulizer device.

Estimated Time

- Hands-on: 5 minutes
- Total duration: 6 minutes

User-Supplied Consumables

- Exonuclease treated circularized DNA (from previous step)
- Low retention 1.7 ml microcentrifuge tubes
- Covaris T3 Tubes and Snap Caps
- Water

NOTE
To provide a good fit to the Covaris T6 tubes, ensure you are using the recommended Covaris Snap-Caps # 520042 and not the looser fitting Covaris Snap-Caps # 520030.

Preparation

- Turn on the Covaris instrument at least 30 minutes before starting.
- Following the manufacturer's instructions, de-gas and pre-chill the water to a temperature of 3° to 6°C. You can start the fragmentation procedure at 6°C.

Procedure

1 Transfer the entire sample to a Covaris T6 glass tube (approximately 320 µl).

2 Ensure tube is full to the very top by adding any extra water if needed. Cap the tube. Check that there is no air bubble present in the tube, as this may disrupt shearing.

3 Shear DNA using Covaris S2 or S220 device and the parameters shown below:

Settings	S2	S220
Peak Power Intensity	n/a	240
Intensity	8	n/a
Duty Cycle/Duty Factor	20%	20%
Cycles Per Burst	200	200
Time	40 s	40 s
Temperature	6°C	6°C

4 After shearing, transfer the ~320 µl DNA sample to a fresh 1.7 ml microcentrifuge tube.

5 Proceed to *Streptavidin Bead Binding* on page 50.

Size Selection (Gel-Plus Protocol Only)

This step offers a more stringent size-selection process than the AMPure purification step alone. It allows the generation of libraries with larger mate pair fragment sizes and tighter distributions than the Gel-Free Protocol.

The size of the fragments selected in this step determines the distance between the paired reads upon sequencing the mate pair library. The precise size of the size-selected fragments is user defined and will depend on experimental design, the application of the mate pair data set, and on the fragment size distribution generated by the tagmentation process.

When carrying out mate pair sample preparation, the size-selection procedures present some of the biggest challenges to successful library construction. The DNA selection step itself and the inefficiencies of the purification step can lead to significant sample loss and to 'sample to sample' variability in the final libraries. The key consideration is the amount of DNA passing through the protocol at each step. This is dependent on a number of factors, including those that are chosen by the user: the amount of starting DNA used in the prep; the correct quantification of starting DNA, and the selection of a portion of the total fragments by size-selection on a gel.

Crucially, selecting a narrow range of fragment sizes inherently diminishes the amount of DNA going forward in the protocol and increases the risk of a low yield at the end of the prep. This can be avoided by broadening the range of fragment sizes (see below) or increasing the number of PCR cycles at the end of the procedure (see *PCR Cycle Number Guidelines* on page 63). When performing the size-selection step, the aim should be to recover between 150 and 500 ng of DNA per size-selection. The selection of a broader range of fragment sizes will help increase the likelihood of recovering DNA within this ng range.

We have validated two different size-selection procedures for use in the Nextera Mate Pair Gel-Plus Protocol:

- Sage Science Pippin Prep with 0.75% Cassette
- Agarose gel electrophoresis and DNA extraction with Zymo Purification kit

NOTE

The size-selection procedures described in this document have not been exhaustively optimized. There may be other electrophoresis conditions and DNA extraction methods that would yield comparable or even superior results. Users are encouraged to apply their current optimized gel sizing protocol if it consistently results in desired size and final yield.

Pippin Prep

This procedure requires the use of a Sage Science Pippin Prep size selection system. When using the Pippin Prep only a single size-selection is possible per sample.

Illumina recommends eluting fragments with a broad range of sizes, of 3 to 6 kb in width, increasing in width with increasing fragment length (e.g. 2–5 kb, 4–8 kb or 6–12 kb). The selection of a broad fragment size range will increase the yield of DNA recovered and increases the chances of generating a high diversity mate pair library. Selection of tighter distributions is possible, but this will result in the recovery of less DNA, diminished library yields, and lower diversity libraries.

Illumina recommends checking the size of the AMPure purified DNA sample prior to selecting the elution range. The size can be verified by running a diluted aliquot of sample on an Bioanalyzer DNA 12000 LabChip (see step 13 of the Optional QC step at the end of *AMPure Purification of Strand Displacement Reaction* on page 34). Maximum recoveries of DNA can be achieved by using the peak fragment size from Bioanalyzer electrophoreogram as a guide for selecting the Pippin Prep elution range.

User-Supplied Consumables

- AMPure Purified DNA (from previous step)
- Pippin Prep 0.75% agarose cassette and solutions (Cat # CSD7510)
- Low retention 1.7 ml microcentrifuge tubes

Preparation

- Familiarize yourself with the Sage Science Pippin Prep size selection system and User Manual.

Procedure

1. Follow the Sage Pippin Prep user guide to load the 30 µl of AMPure purified DNA on single lane of a Pippin Prep 0.75% agarose cassette.
2. Program the Pippin Prep Protocol to run for 2 hours and use the range mode to define the start and end of the desired sample elution size.

NOTE

Be sure to seal the elution wells with the adhesive tape, supplied with the 0.75% Cassette, to ensure sample is not lost to overflow from the elution well.

3 Once run is complete remove all the DNA from elution well, and transfer to a clean 1.7 ml microcentrifuge tube.

4 [Optional QC Step] 1 µl of the elution can be run undiluted on a Bioanalyzer 12000 LabChip to quantify how much DNA was recovered and confirm the size of the eluted fraction.

5 Proceed to *Circularization on page 38* step. The elution from the Pippin Prep can be used directly in the circularization reaction.

Agarose Size Selection

In our experience running a standard agarose gel does not provide as robust and reproducible results as the Sage Pippin Prep; it however does allow greater user flexibility over fragment sizes selected, and allows the easy selection of multiple size cuts from a single sample.

When using the agarose gel protocol Illumina recommends selecting a fragment range of several kb in width (e.g. 4–6 kb, 7–10 kb or 9–12 kb). See *Example agarose size-selection gel* on page 49 for an example gel. The selection of a broader fragment size range will increase the yield of DNA recovered and increases the chances of generating a high diversity mate pair library.

The Agarose Size-selection procedure detailed below has been optimized for the following equipment:

- Gel tray and electrophoresis unit (Gel dimensions—12 cm width x 14 cm length, Fisher, part # FB57161).
- Gel comb with wide wells (Well dimensions—9 mm width x 1 mm length Fisher, part # FB58325).

If you use alternative electrophoresis equipment, ensure that the agarose gel and well dimensions are as similar as possible to those recommended. When using alternative equipment, optimize the voltage and run times before processing a sample.

Illumina-Supplied Consumables

- Resuspension Buffer

User-Supplied Consumables

- AMPure Purified DNA (from previous step)
- 0.6 % Megabase Agarose
- 50 X TAE Buffer

- 1 kb plus DNA ladder
- 6X Gel Loading Dye
- Agarose Gel Electrophoresis Equipment
- SYBR safe DNA Gel Stain
- Clean Scalpels
- Zymoclean™ Large Fragment DNA Recovery Kit
- 3.5 ml screw cap tubes
- Low retention 1.7 ml microcentrifuge tubes

Preparation

- Prepare 1X TAE Buffer (> 1 liter).
- Clean the tray, comb, and the gel tank with ethanol and rinse thoroughly with deionized water to avoid cross contamination.
- Dilute 1 kb plus DNA ladder 1:10 in a 1X solution of Gel Loading Dye.
- Set an incubator oven (or heat block that holds 3.5 ml tubes) to 50°C.

Procedure

1. Prepare a 100 ml, 0.6% megabase agarose gel using 100 ml 1X TAE buffer.
   a. Add 0.6 g of agarose powder to 100 ml of 1X TAE buffer.
   b. Microwave the gel buffer until agarose powder is completely dissolved.
   c. Cool the gel buffer on the bench for 5 minutes. Add 10 µl of SYBR Safe DNA gel stain. Swirl to Mix.
   d. Pour the entire gel solution into the gel tray, and allow to cool.
2. 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.
3. Add 6 µl of 6X Loading Dye to 30 µl of AMPure purified DNA sample.
4. Load the entire sample over two consecutive lanes of the gel. Pipette 18 µl per well.
5. Load 20 µl of diluted pre-prepared 1 kb plus ladder into the lanes either side of the sample lanes.
6. Run the gel for 120 minutes at 100 V (constant voltage).
7. View the gel on a Dark Reader transilluminator to visualize DNA.
8. Using a clean scalpel blade, and the 1 kb plus DNA ladder as a size guide, carefully excise DNA fraction(s) from the gel containing the desired fragment sizes. Transfer

agarose gel fraction to a 3.5 ml screw cap tube. Use a clean scalpel blade for each new size excision.

NOTE
Multiple fractions can be taken when using the agarose gel method, see *Example agarose size-selection gel* on page 49 for an example of an agarose gel image with multiple gel fractions excised.

9 Purify the DNA from the agarose fractions using a Zymoclean™ Large Fragment DNA Recovery Kit, as described below:

NOTE
All centrifugation steps should be performed between 10,000–16,000 xg.

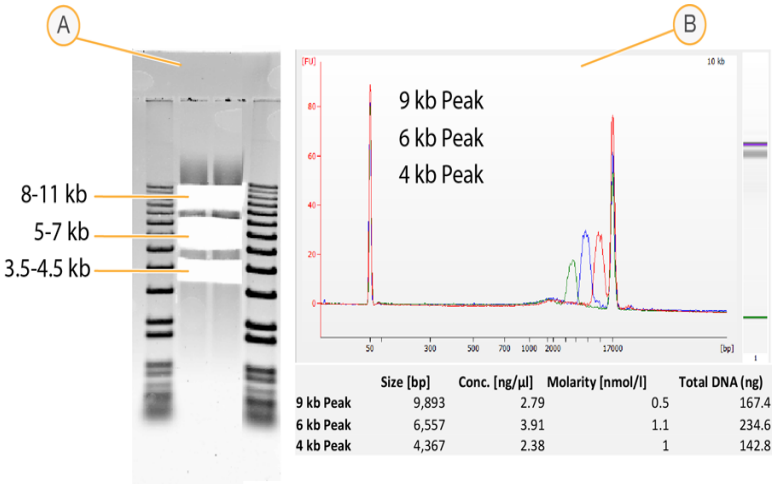
a Add 3 volumes of Zymo ADB to each volume of agarose excised from the gel (e.g. for a 600 mg agarose gel add 1800 µl of ADB).
b Incubate at 50°C, inverting the tubes to mix every 2 minutes until the gel is completely dissolved, approximately 10–15 minutes.
c Transfer the melted agarose solution to two Zymo-Spin™ IC-XL Columns per gel fraction. Loading up to 800 µl per column, and distributing the solution evenly across both columns.
d Centrifuge for 1 minute. Discard the flow-through.
e If needed repeat steps c and d, until all the melted agarose has been loaded on the columns.
f Add 200 µl Zymo DNA Wash Buffer to each column. Centrifuge for 1 minute. Discard the flow-through.
g Repeat the wash step f once.
h Centrifuge the empty columns for 1 minute with lid open to ensure any residual ethanol wash is removed. Discard any flow-through and the collection tube.
i Transfer the columns to clean 1.7 ml microcentrifuge tubes, add 30 µl of Resuspension Buffer to each column, incubate for 1 minute and then centrifuge for 1 minute to elute the DNA.
j Combine elutions from the two matching columns, to give a total 60 µl of sample per size-selection.

10 1 µl of the elution should be run undiluted on a Bioanalyzer 12000 LabChip to confirm the size of the extracted fraction and quantify how much DNA was recovered.

11 Proceed to *Circularization on page 38*.

Figure 5 shows an example of agarose gel size-selection. Image A shows an agarose gel with three gel fractions removed, 3.5-4.5 kb, 5-7 kb and 8-11 kb. Image B shows a Bioanalyzer 12000 LabChip trace showing sizing and quantification the same three fractions. For each fraction 1 µl of the 60 µl elution volume was run on a 12000 LabChip. The total amount of recovered size-selected DNA for the 4 kb, 6 kb, and 9 kb fragments are 167 ng, 234 ng, and 167 ng respectively.

Figure 5 Example agarose size-selection gel



	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Total DNA (ng)
9 kb Peak	9,893	2.79	0.5	167.4
6 kb Peak	6,557	3.91	1.1	234.6
4 kb Peak	4,367	2.38	1	142.8

Streptavidin Bead Binding

During this step the sheared DNA fragments that contain the biotinylated junction adapter (mate pair fragments) are purified by means of binding to streptavidin magnetic beads, and the unwanted, unbiotinylated molecules are removed through a series of washes.

NOTE
After binding the biotinylated DNA to the streptavidin beads, all sample processing can be carried out on-bead, using in a single 1.7 ml microcentrifuge tube per sample. It is not necessary to transfer the sample to a clean tube until the PCR step, which requires a thin-walled 0.2 ml PCR tube. Avoiding unnecessary tube transfers reduces sample loss.

Estimated Time

- Hands-on: 15 minutes
- Total duration: 30 minutes

Illumina-Supplied Consumables

- Bead Bind Buffer
- Bead Wash Buffer
- Resuspension Buffer

User-Supplied Consumables

- Sheared DNA (from previous step)
- Dynabeads M-280 Streptavidin Magnetic Beads
- 1.7 ml microcentrifuge tubes

Preparation

- Pre-heat the heat block to 20°C.
- Review *Best Practices* on page 20 and the *Handling the Streptavidin Magnetic Beads and Carrying out the On-bead Reactions* section.

NOTE
To reduce sample loss throughout the on-bead reaction set up and bead wash steps, do not mix reactions or resuspended beads by pipetting. Instead, mix the reactions and resuspend the beads by closing the lids and flicking the tubes. After flicking, the tubes should be spun briefly in a mini-centrifuge to collect the contents to the bottom.

Procedure

Bead Preparation

NOTE
The bead preparation step below describes how to prepare sufficient beads for 1 library prep. You can prepare beads for several samples at once by multiplying the volumes used by the number of samples you are preparing. If using a 1.7 ml tube to prepare the beads, there is only adequate volume in the tube to prepare sufficient beads for 5 library preparations. If processing more than 5 samples you may need to use a larger volume tube to prepare the beads.

1. Resuspend the magnetic streptavidin M280 Dynabeads by shaking the bottle well.
2. Transfer 20 µl of the M280 Dynabeads into a clean 1.7 ml tube.
3. Place the tube on a magnet for 1 minute, remove the supernatant while the tube remains on the magnet.
4. Wash the beads by resuspending them in 50 µl of Bead Bind Buffer.
5. Place the tube on a magnet for 1 minute, remove the supernatant while the tube remains on the magnet.
6. Repeat wash once (steps 4 and 5).
7. Remove tube from magnet and resuspend the beads in 300 µl of Bead Bind Buffer.
8. Proceed to *Bead Binding*.

Bead Binding

1. Add 300 µl of resuspended beads prepared in the previous *Bead Preparation* section (step 7) to the 300 µl of sheared DNA sample from the *Shear Circularized DNA* step.
2. Incubate at 20°C for 15 minutes. Resuspend the beads every 2 minutes.
3. Briefly spin samples (5–10 seconds) in a mini-centrifuge and place on a magnet for 1 minute.
4. Remove and discard the supernatant, then add 200 µl of Bead Wash Buffer.
5. Remove tube from magnet, resuspend beads by flicking, briefly spin tube (5–10 seconds) and place on magnet for 30 seconds.

6 Repeat steps 4 and 5, three times. Washing beads four times total with Bead Wash Buffer.

7 Remove and discard the supernatant, then add 200 µl of Resuspension Buffer.

8 Remove tube from magnet, resuspend beads by flicking, briefly spin tube (5–10 seconds) and place on magnet for 30 seconds.

9 Repeat steps 7 and 8, once. Washing beads twice in total with Resuspension Buffer.

10 Leave beads in final wash solution on the magnet until you are ready to add enzyme reaction mix in the next section.

11 Proceed to *End Repair* on page 53.

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. The DNA remains bound to the beads throughout this reaction and subsequent bead wash steps.

Estimated Time

- Hands-on: 15 minutes
- Total duration: 45 minutes

Illumina-Supplied Consumables

- End Repair Mix
- Bead Wash Buffer
- Resuspension Buffer

User-Supplied Consumables

- Bead bound DNA (from previous step)
- 1.7 ml microcentrifuge tubes

Preparation

- Remove the following from -15° to -25°C storage, thaw at room temperature, then place on ice:
  - End Repair Mix
- Pre-heat the heat block to 30°C.

Procedure

1 In a clean tube, set up the End Repair Reaction as shown below. For processing multiple samples, master mixing is allowed.

	Volume
End Repair Mix	40 µl
Water	60 µl
Total	100 µl

2 Remove the final bead wash solution from the DNA sample. Spin the tube briefly to collect any left over solution to the bottom of the tube, place tube back on the magnet and then carefully remove any remaining liquid with a P10 pipette.

3 Add 100 µl of End Repair Reaction directly to the beads.

4 Remove from magnet and resuspend beads by flicking tube, spin briefly (1–2 seconds), sufficient to collect sample to bottom of tube, but not long enough to pellet any of the beads.

5 Incubate at 30°C for 30 minutes.

6 Proceed to *Bead Wash*.

Bead Wash

1 Briefly spin samples (5–10 seconds) in a mini-centrifuge and place on a magnet for 1 minute.

2 Remove and discard the supernatant, then add 200 µl of Bead Wash Buffer.

3 Remove tube from magnet, resuspend beads by flicking, briefly spin tube (5–10 seconds) and place on magnet for 30 seconds.

4 Repeat steps 2 and 3, three times. Wash beads four times total with Bead Wash Buffer.

5 Remove and discard the supernatant, then add 200 µl of Resuspension Buffer.

6 Remove tube from magnet, resuspend beads by flicking, briefly spin tube (5–10 seconds) and place on magnet for 30 seconds.

7 Repeat steps 5 and 6, once. Wash beads two times total with Resuspension Buffer.

8 Leave beads in final wash solution on the magnet until you are ready to add enzyme reaction mix in the next section.

9 Proceed to *A-Tailing* on page 56.

A-Tailing

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. The A-tailing reaction is carried out on-bead, and the DNA remains bound to the beads throughout this step.

Estimated Time

- Hands-on: 5 minutes
- Total duration: 35 minutes

Illumina-Supplied Consumables

- A-Tailing Mix

User-Supplied Consumables

- Water
- 1.7 ml microcentrifuge tubes

Preparation

- Remove the following from -15° to -25°C storage, thaw at room temperature, then place on ice:
  - A-Tailing Mix
- Pre-heat the heat block to 37°C.

Procedure

1 In a clean tube, set up the A-Tailing Reaction as shown below. For processing multiple samples, master mixing is allowed:

	Volume
A-Tailing Mix	12.5 µl
Water	17.5 µl
Total	30 µl

2 Remove the final bead wash solution from the DNA sample. Spin the tube briefly to collect any left over solution to the bottom of the tube, place tube back on the magnet and then carefully remove any remaining liquid with a P10 pipette.

3 Add 30 µl of A-Tailing Reaction Mix directly to the beads

4 Remove from magnet and resuspend beads by flicking tube, spin briefly (1–2 seconds), sufficient to collect sample to bottom of tube, but not long enough to pellet any of the beads.

5 Incubate at 37°C for 30 minutes.

6 Proceed directly to *Adaptor Ligation* on page 58. Do not wash beads at this stage.

Adaptor Ligation

This process ligates indexing adapters to the ends of the DNA fragments, preparing them for PCR amplification and subsequent hybridization onto a flow cell. The adapter ligation reaction is carried out on-bead, and the DNA remains bound to the beads throughout this reaction and subsequent bead wash steps.

Estimated Time

- Hands-on: 15 minutes
- Total duration: 35 minutes

Illumina-Supplied Consumables

- Ligation Mix
- DNA Adapter Index
- Bead Wash Buffer
- Resuspension Buffer

User-Supplied Consumables

- Water

NOTE
When used in low-plex combinations, not all indexes combinations are compatible. Please refer to the *Pooling Guidelines* on page 14 for more information.

Preparation

- Remove the following from -15° to -25°C storage, thaw at room temperature, then place on ice:
  - DNA Adapter Index(es)
- Remove the following from -15° to -25°C storage and place immediately on ice:
  - Ligation Mix
- Pre-heat the heat block to 30°C.

Procedure

1 Set up the Adapter Ligation Reaction as shown below, adding reagents in the order shown directly to the A-Tailing Reaction. Master mixing is not possible in this step:

	Volume
A-Tailing Reaction/Bead Mix	30 µl
Ligation Mix	2.5 µl
Water	4 µl
DNA Adapter Index	1 µl
Total	37.5 µl

2 Resuspend beads and mix reaction by flicking tube, spin briefly (1–2 seconds), sufficient to collect sample to bottom of tube, but not long enough to pellet any of the beads.

3 Incubate at 30°C for 10 minutes .

4 After the incubation, add 5 µl of Ligation Stop Buffer.

5 Proceed to *Bead Wash*.

Bead Wash

1 Briefly spin samples (5–10 seconds) in a mini-centrifuge and place on a magnet for 1 minute.

2 Remove and discard the supernatant, then add 200 µl of Bead Wash Buffer.

3 Remove tube from magnet, resuspend beads by flicking, briefly spin tube (5–10 seconds) and place on magnet for 30 seconds.

4 Repeat steps 2 and 3, three times. Wash beads four times total with Bead Wash Buffer.

5 Remove and discard the supernatant, then add 200 µl of Resuspension Buffer.

6 Remove tube from magnet, resuspend beads by flicking, briefly spin tube (5–10 seconds) and place on magnet for 30 seconds.

7 Repeat steps 5 and 6, once. Wash beads two times total with Resuspension Buffer.

8 Leave beads in final wash solution on the magnet until you are ready to add enzyme reaction mix in the next section.

9 Proceed to *PCR Amplification* on page 61.

PCR Amplification

PCR amplification is used to enrich for the mate pair fragments that have TruSeq DNA adapters on both ends. The template material is bound to the streptavidin beads, however the resulting PCR amplified copies are not biotinylated and are not bound to the beads.

Estimated Time

- Hands-on: 5 minutes
- Total duration: 40 minutes

Illumina-Supplied Consumables

- PCR Master Mix
- PCR Primer Cocktail

User-Supplied Consumables

- Water
- 0.2 ml thin wall PCR tubes
- 1.7 ml microcentrifuge tubes

Preparation

- Remove the following from -15° to -25°C storage, thaw at room temperature, then place on ice:
  - PCR Master Mix
  - PCR Primer Cocktail

Procedure

1 In a clean 1.7 ml microcentrifuge tube, set up the PCR Reaction as shown below. For processing multiple samples, master mixing is allowed:

	Volume
PCR Master Mix	25 µl
PCR Primer Cocktail	5 µl
Water	20 µl
Total	50 µl

2 Remove the final bead wash solution from the DNA sample. Spin the tube briefly to collect any left over solution to the bottom of the tube, place tube back on the magnet and then carefully remove any remaining liquid with a P10 pipette.

3 Add 50 µl of PCR Reaction Mix directly to the beads

4 Resuspend beads in PCR Reaction Mix by gently pippetting up and down, then transfer mix to PCR tubes.

5 Place tubes in PCR block and perform PCR using the following program on a thermal cycler:
- 98°C for 30 seconds
- 10 or 15 cycles of PCR (see *PCR Cycle Number Guidelines* on page 63):
  - 98°C for 10 seconds
  - 60°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°C

6 Proceed to *PCR Clean-Up* on page 64.

PCR Cycle Number Guidelines

To achieve sufficient library yields to meet sequencing needs, 10 Cycles of PCR may not be sufficient for all library preps. Increasing PCR cycles from 10 to 15 cycles may be necessary, especially when following the Gel-Plus protocol to generate libraries from fragments greater than 5 kb or when the amount of DNA used in the circularization reaction was less than 200 ng.

	Circularized DNA	PCR cycles
Gel-Free	200 – 600 ng	10
Gel-Plus	> 200 ng and < 5 kb	10
Gel-Plus	< 200 ng or > 5 kb	15

PCR Clean-Up

The product of the PCR amplification is a DNA smear of different fragment sizes. An AMPure bead purification step is used to clean up the PCR reaction and remove the smallest fragments (<300 bp) from the final library.

Estimated Time

- Hands-on: 10 minutes
- Total duration: 30 minutes

Illumina-Supplied Consumables

- Resuspension Buffer

User-Supplied Consumables

- AMPure XP beads
- 70% Ethanol, freshly-prepared
- 1.7 ml microcentrifuge tube

Preparation

- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Prepare Fresh 70% Ethanol. 400 µl per sample.
- Review *Best Practices* on page 20 and the *Handling AMPure Magnetic Beads* section.

Procedure

1. Place PCR reactions on a magnet (transfer to clean 1.7 ml tubes if necessary), leave on magnet for 1 minute.
2. Carefully transfer 45 µl of supernatant to a clean tube.
3. Add 30 µl of AMPure XP beads to the PCR reaction and mix by flicking tube and spin briefly to collect sample.
4. Incubate for 5 minutes at room temperature.
5. Place tube on magnet for 5 minutes, then carefully remove all liquid from tube.

6 Leaving tube attached to the magnet, add 200 µl of fresh 70% ethanol to wash beads, incubate for 30 seconds before carefully removing ethanol.

7 Repeat step 6 once.

8 Air dry beads for approximately 10–15 minutes.

9 Remove tube from magnet and add 20 µl of Resuspension Buffer to elute DNA from beads. Resuspend beads in Resuspension Buffer by flicking tube and incubate for 5 minutes at room temperature.

10 Place tube back on magnet for 5 minutes, then carefully transfer supernatant containing final DNA library to a clean tube.

11 Proceed to *Validate Library* on page 66.

Validate Library

Illumina recommends performing the following procedures for quality control analysis and quantification of your Nextera Mate Pair Library.

Quality Control

To verify the size of your PCR enriched fragments, check the template size distribution by running an aliquot of the DNA library on a gel or on an Agilent Technologies 2100 Bioanalyzer.

- For libraries generated with the Gel-Free protocol:
  - Load 1 µl of sample on a 7,500 or 12,000 DNA LabChip. The expected library size range is 300 bp to 1500 bp, with a concentration of 5 nM to 50 nM.
- For libraries generated with the Gel-Plus protocol:
  - Load 1 µl of sample on a High Sensitivity DNA LabChip. The expected library size range is 300 bp to 1500 bp, with a concentration of 1.5 nM to 20 nM.
- 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 DNA smear of 300 to 1000 bp is expected.

Figure 6 Example of a Gel-Plus mate pair Library Bioanalyzer Profile

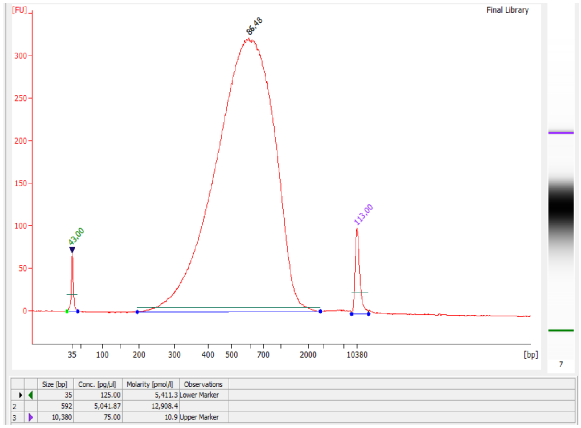


Figure 6 shows an Agilent 2100, High Sensitivity Bioanalyzer LabChip profile of a typical mate pair library. The DNA fragments range in size from 300 bp up to 1200 bp. The concentration of the final library is 12.9 nM.

Library Quantification

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 the flow cell. This requires accurate quantitation of DNA library templates. It is possible to use the Bioanalyzer quantification as a rough quantitation of mate pair libraries, however for more accurate results, Illumina recommends quantifying your library using qPCR according to the *Illumina Sequencing Library qPCR Quantification Guide*.

1 Calculate concentration of library by mean of qPCR or Bioanalyzer analysis.

2 Normalize the libraries to 2 nM by diluting with Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20.

3 Do one of the following:
- Proceed to *Pooling Indexed Library* on page 67.
- Proceed to cluster generation. For more information, see the appropriate user guide for the sequencing platform being used.
- Store library at -15° to -25°C.

Pooling Indexed Library

NOTE
Important – Please read *Indexed Adapter Sequences* on page 13 to ensure indexed libraries are pooled with compatible indexing.

This section describes how to pool indexed libraries for cluster generation and sequencing.

1 Ensure all libraries have been accurately quantified and normalized to 2 nM, using either a Bioanalyzer or preferably a QPCR quantification method.

2 Combine 10 µl each library to be pooled into a 1.7 ml tube.

3 Vortex briefly to mix and spin to collect solution at bottom of tube.

4 Do one of the following:
- Proceed to cluster generation. For more information, see the appropriate user guide for the sequencing platform being used.
- Store pooled libraries at -15° to -25°C.

[Optional Alternative Procedure] AMPure Purification of Tagmentation Reaction

This step provides an alternative method to the Zymo column to purify the tagmentation reaction. This step uses AMPure beads and is more automation friendly. If carrying out this step manually, the AMPure purification is more time consuming than using the Zymo column.

Due to volume constraints it is only practical to perform this procedure with the Gel-Free Protocol.

Estimated Time

- Hands-on: 15 minutes
- Total duration: 45 minutes

Illumina-Supplied Consumables

- Neutralize Tagment Buffer
- Resuspension Buffer

User-Supplied Consumables

- AMPure XP beads
- 70% Ethanol, freshly-prepared
- 1.7 ml microcentrifuge tube

Preparation

- Prepare Fresh 70% Ethanol. 400 µl per sample.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Review *Best Practices* on page 20 and the *Handling AMPure Magnetic Beads* section.

Procedure

After incubating the Tagmentation reaction at 55°C for 30 minutes:

1. Add 25 µl of Neutralize Tagment Buffer to the tagmentation reaction, and mix well by pipetting up and down 10 times. Incubate for 5 minutes at room temperature.
2. Add 125 µl of AMPure XP beads, mix and incubate for 15 minutes at room temperature, resuspend the beads every 2 minutes by gently shaking or flicking tubes.
3. Place the tube on the magnetic stand for 5 minutes, then carefully remove all liquid from tube.
4. Leaving the tube on the magnet stand, add 400 µl of fresh 70% Ethanol, incubate for 30 seconds and then carefully remove ethanol wash without disturbing the beads.
5. Repeat step 4 once.
6. Air dry beads for 10–15 minutes.
7. Remove the tube from the magnetic stand and add 30 µl of Resuspension Buffer to elute DNA from beads, mix gently and incubate for 5 minutes.
8. Place tube back on magnetic stand for 5 minutes and then carefully transfer supernatant containing DNA to a clean 1.7 ml tube.
9. [Optional QC step] Remove 1 µl of sample to test tagmentation size on a 12000 Bioanalyzer Chip. Dilute 1 µl aliquot of sample with 1 µl of water before running the Bioanalyzer chip.

[Optional Alternative Procedure] Shear Circularized DNA - Nebulizer Procedure

The following steps describe the use of nebulization as an alternative procedure to Covaris sonication for the shearing of the circularized DNA.

Estimated Time

- Hands-on: 30 minutes
- Total duration: 30 minutes

Illumina-Supplied Consumables

- Resuspension Buffer

User-Supplied Consumables

- Exonuclease treated circularized DNA (from previous step)
- Nebulizers and nebulization buffer
- PVC tubing or equivalent
- Compressed Nitrogen or Air source of at least 32 PSI
- Zymo™ Genomic DNA Clean & Concentrator™-5
- 1.7 ml microcentrifuge tube

Procedure

1. Remove a nebulizer from the plastic packaging and unscrew the blue lid.
2. Using gloves, remove a piece of vinyl tubing from the packaging and slip it over the central atomizer tube, pushing it all the way to the inner surface of the blue lid.
3. Transfer all the exonuclease-treated DNA to the nebulizer.
4. Add 400 µl of the nebulization buffer and approximately 150 µl of 100% glycerol to the DNA and mix well.
5. Screw the lid back on (finger tight).
6. Chill the nebulizer containing the DNA solution on ice while performing the next step.

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

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

9 Use the regulator on the compressed air source to ensure the air is delivered at 32 psi.

10 Nebulize for 6 minutes. Vapor may rise from the nebulizer.

11 Centrifuge the nebulizer to 450 xg for 2 minutes in a benchtop centrifuge to collect the droplets from the side of the nebulizer.

12 Measure the recovered volume. Typically, you should recover approximately 400 µl.

13 Purify DNA using a Zymo 5 Clean and Concentrator Kit, eluting DNA in 300 µl of Resuspension Buffer. Due to the large volume of the nebulized sample, it is necessary to load the Zymo column multiple times with DNA-Binding buffer solution.

NOTE
All centrifugation steps should be performed between 10,000–16,000 xg.

a Add 5 volumes (~2000 ml) of Zymo DNA Binding Buffer to the nebulized sample Mix thoroughly by pipetting.
b Transfer 750 µl mixture to a Zymo-Spin column in a collection tube.
c Centrifuge for 30 seconds. Discard the flow-through.
d Repeat steps b and c until all of the nebulized sample has been loaded onto the Zymo-Spin.
e Add 200 µl Zymo DNA Wash Buffer to the column. Centrifuge for 1 minute. Repeat the wash step.
f Add 50 µl of Resuspension Buffer directly to the column matrix and incubate at room temperature for 1 minute. Transfer the column to a 1.7 ml microcentrifuge tube and centrifuge at for 30 seconds to elute the DNA.
g Add another 250 µl of Resuspension Buffer to the samples to bring the final volume up to 300 µl.

14 Proceed to *Streptavidin Bead Binding* on page 50.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 12 Illumina General Contact Information

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

Table 13 Illumina Customer Support Telephone Numbers

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

MSDSs

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

Product Documentation

Additional product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support and select a product. A MyIllumina login is required. To register for a MyIllumina account, please visit my.illumina.com/Account/Register.