

Mate Pair Library v2

Sample Preparation Guide

For 2–5 kb Libraries

For Research Use Only
Not for use in diagnostic procedures

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Introduction

This protocol explains how to prepare 2–5 kb mate pair genomic DNA libraries for cluster generation and analysis using the Illumina Mate Pair Library Preparation Kit v2. The v2 kit is formulated to generate 10 mate pair libraries with a gap size ranging from 2–5 kb and includes several modifications to the first version of the Illumina Mate Pair Library Preparation Kit. The modifications, which increase protocol robustness, improve usability, and generate higher quality and more diverse libraries, include:

- ▶ Combined end repair and biotinylation reaction
- ▶ Using QIAEX II suspension instead of QIAquick spin columns, because of its superior performance in isolating large DNA fragments
- ▶ Improved size selection and gel running conditions
- ▶ Increased circularization volume and amount of size-selected DNA used in circularization reaction
- ▶ Recommendations for using the Covaris S2 shearing device
- ▶ Changes to the streptavidin bead wash procedure

Procedure

Using the Mate Pair Library Preparation Kit v2, purified genomic DNA is first fragmented to reduce the high molecular weight DNA into smaller fragments of a desired size range. The DNA fragments are then end-repaired and biotin-labeled, placing biotinylated nucleotides at the ends of these fragments.

Next, DNA fragments of a particular size range are selected from an agarose gel. The length and range of the size-selected material determine the gap size, and its variance, of the paired reads of the final library.

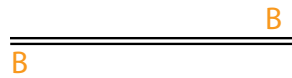


Figure 1 Biotin Labeled and Size-Selected Fragment

The size-selected fragments are circularized by an intramolecular ligation. Any remaining linear molecules are removed by DNA exonuclease treatment.

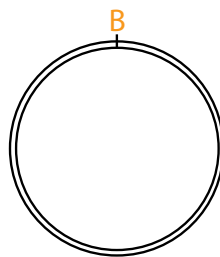


Figure 2 Circularized Fragment

The circular DNA fragments are sheared again either by Covaris shearing or nebulization to a fragment length with an average size of 450 bp.

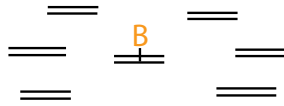


Figure 3 Fragmented Circle

The fragments that contain the biotinylated ends of the original size-selected fragment are purified using streptavidin-coated magnetic beads. It is important to follow the protocol carefully to ensure that all non-biotinylated fragments are washed away.



Figure 4 Purified Fragments, Bound to Streptavidin Beads

The fragments still bound to the streptavidin beads are end-repaired and A-tailed and then the illumina paired-end oligo adapters are attached by ligation.

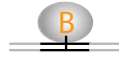


Figure 5 A-Tailed Fragments with Ligated Adapters

After the adapter ligation, PCR is carried out to simultaneously enrich and amplify those DNA fragments that have adapter molecules on both ends.



Figure 6 Amplified Fragments with Cluster and Sequencing Sites Attached

The product of the PCR amplification is a DNA smear of different fragment sizes. Fragments in the range of 350–650 bp are excised from the gel. This size is optimal in order to attain high-quality mate pair sequence reads.

Sample Prep Workflow

Figure 7 shows a typical workflow to prepare a 2–5 kb mate pair library. However, the time you take to complete the protocol may be longer or shorter as needed.

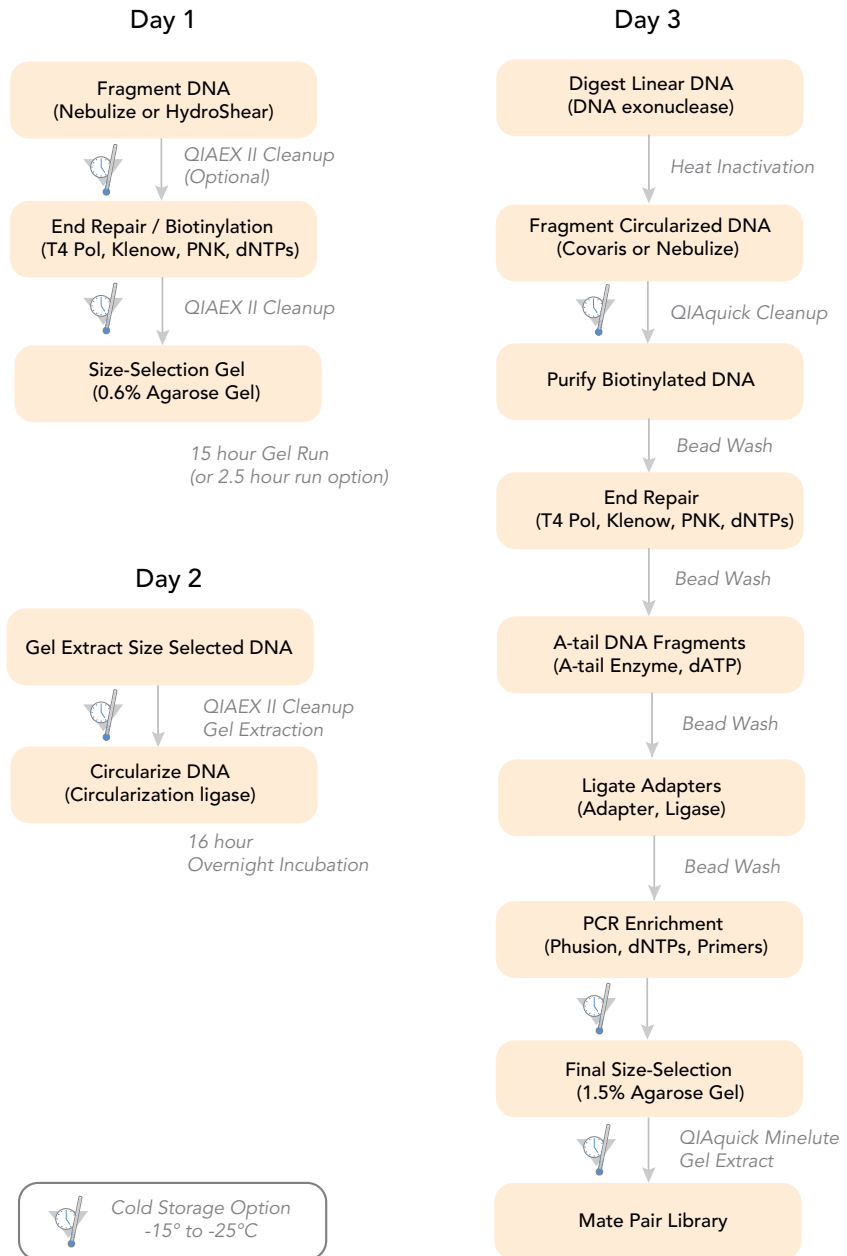


Figure 7 Mate Pair 2–5 kb Library Preparation Workflow

Best Practices

Before you start, read this section to learn important information about generating mate pair libraries.

Quantitate the Starting Material

This mate pair 2–5 kb protocol requires 10 µg of starting genomic material per library. It is very important to accurately quantitate the starting genomic material. If you use less than the recommended amount of starting material, it significantly reduces the amount of size-selected DNA recovered and lowers the diversity of the final library.

Use fluorescence-based quantification such as Invitrogen's Qubit Quantitation Platform, rather than a UV spectrometer based method. This is because fluorescence-based methods, which employ a double-stranded (ds) DNA-specific dye, specifically and accurately quantitate dsDNA even in the presence of many common contaminants. UV spectrometer methods based on 260 OD readings tend to overestimate DNA concentrations due to the presence of RNA and other contaminants.

Assess Quality of Starting Material

High-quality starting material is extremely important for successful library generation. Low quality, damaged, or degraded DNA diminishes library yield and diversity and can lead to an increase in 'inward-facing' reads (refer to *Inward and Outward-Facing Reads* on page 9). Damaged DNA samples PCR-amplify less efficiently, provide less DNA of the desired size range, and may disrupt the specificity of the biotinylation reaction.

The negative effect of damaged DNA on the resulting mate pair libraries increases proportionally with the gap size, because longer DNA fragments are more likely to contain damaged nucleotides.

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.

Figure 8 shows agarose gel analysis of two genomic DNA samples. Approximately 200 ng of sample was loaded per lane. Figure 8A is a 0.6% standard agarose gel stained with ethidium bromide. Figure 8B is a higher-resolution Pulse Field Gel, which more clearly shows the differences in quality and integrity.

- ▶ Lane 1—Lambda Mono Cut DNA marker (NEB, N3019S)
- ▶ Lane 2—Intact high quality genomic DNA sample
- ▶ Lane 3—Partially degraded genomic sample

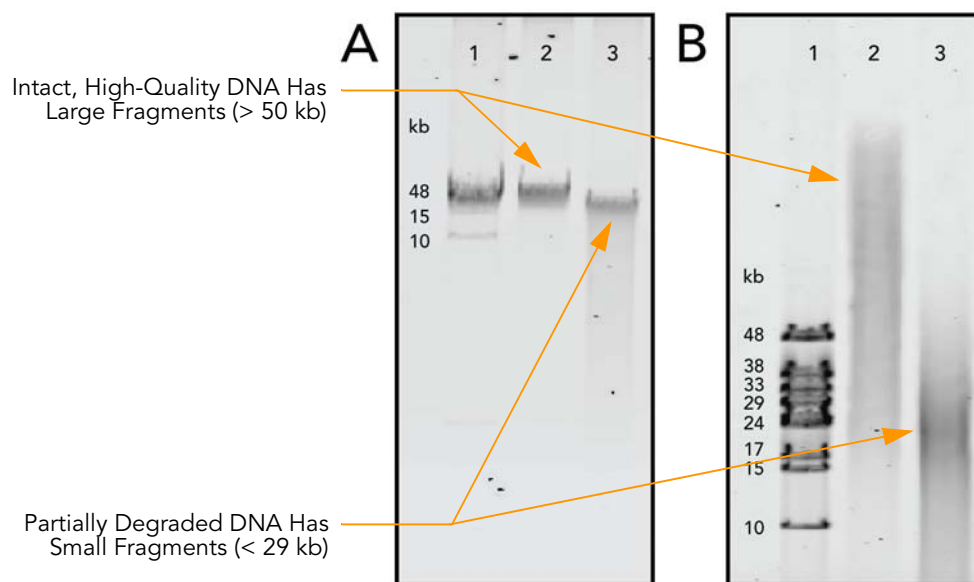


Figure 8 Analysis of Genomic DNA Sample Integrity

Use HydroShear to Fragment DNA

Illumina recommends using a HydroShear device to shear DNA for the initial shearing event, but the mate pair protocol has also been successfully validated using a Nebulization protocol.

The Nebulizer and HydroShear protocols offer comparable results in the 2–4 kb gap size range. However, when the target gap size increases, the HydroShear protocol can provide more sheared DNA in a desired size range. This is because it has a more controllable shearing mechanism and provides tighter distribution of sheared DNA fragments. The tighter the initial shearing event, the greater the fraction of the sample will be in the desired size range, thus increasing the yield of DNA recovered during the size-selection step.

Recovered Size-Selected Material

The final diversity achieved for a given library is strongly influenced by the quantity of size-selected material recovered from the first size selection gel. This mate pair 2–5 kb protocol is optimized for up to 600 ng of size-selected material in the circularization ligation.

In this protocol, you use agarose gel electrophoresis to select DNA fragments of a precise and defined size range. The labelled DNA appears as a smear on the gel, typically with a size range spanning several kilobases, depending on the shearing method used. The actual length of the fragments selected is user-defined and depends on the experimental design and ultimate use of the mate pair library.

The gel running conditions described in this protocol have been carefully optimized and experimentally verified. Follow the conditions closely to achieve optimal and reproducible results.

Library Size Range and Sequencing Length

When sequencing a mate pair library, Illumina recommends a read length no longer than 36 bases. A longer read length elevates error rates, because longer reads are more likely to cross over the junction of the two joined ends of a size-selected fragment. The Illumina analysis pipeline discards these junction reads, since they do not align to the reference sequence.

To minimize junction reads, the mate pair library uses a template size range of 350–650 bp. This is larger than a typical paired-end library template of 300–400 bp. Increasing the size range of the library in the mate pair protocol minimizes the number of sequence reads that pass through a junction.

Data Analysis and Interpretation

Library Diversity

The greater the diversity of a library, the greater the depth of data it yields. Illumina defines library diversity as the total number of unique fragments in a DNA library. The final diversity achieved for a given library is influenced by a number of factors, including:

- The quantity and quality of the starting material
- The efficiency of the fragmentation method
- The quantity of size-selected material used in the circularization reaction
- The size and variance of the size-selected fragments
- The genome size of the organism being sequenced

Additional details on measuring library diversity can be found on the Illumina website (<http://www.illumina.com>) under **Downloads | Software**.

Inward and Outward-Facing Reads

After circularization, the DNA is fragmented, and two populations of fragments are generated:

- ▶ **Biotinylated fragments** originating from the junction of the two ligated ends of the large size-selected molecules
- ▶ **Unbiotinylated fragments** of contiguous sequence originating from the internal sequence of size-selected molecules

Only the fragments originating from the ends of the size-selected fragments are biotin-labelled. These fragments are enriched during the streptavidin bead purification steps, and the majority of unbiotinylated fragments are washed away.

Sequencing these biotinylated fragments generates the desired 'outward-facing' paired reads, meaning they read towards the ends of the original size-selected fragment and align to a reference sequence outward-facing from each other. These outward-facing reads align to the reference sequence with a gap size of approximately the original size-selected fragment (e.g., 3 kb).

A smaller proportion of inward-facing paired reads may also be found in mate pair libraries. These reads can be easily identified as they align to the reference sequence in an inward-facing orientation and with a smaller gap size of around 200–400 bp. The undesired inward-facing reads are caused by contiguous internal sequences that were not successfully washed away.

Figure 9 depicts the origin of the large gap sized outward-facing reads and the smaller gap sized inward-facing reads. In an successful Illumina Mate Pair Library, the vast majority of reads will be outward-facing paired reads.

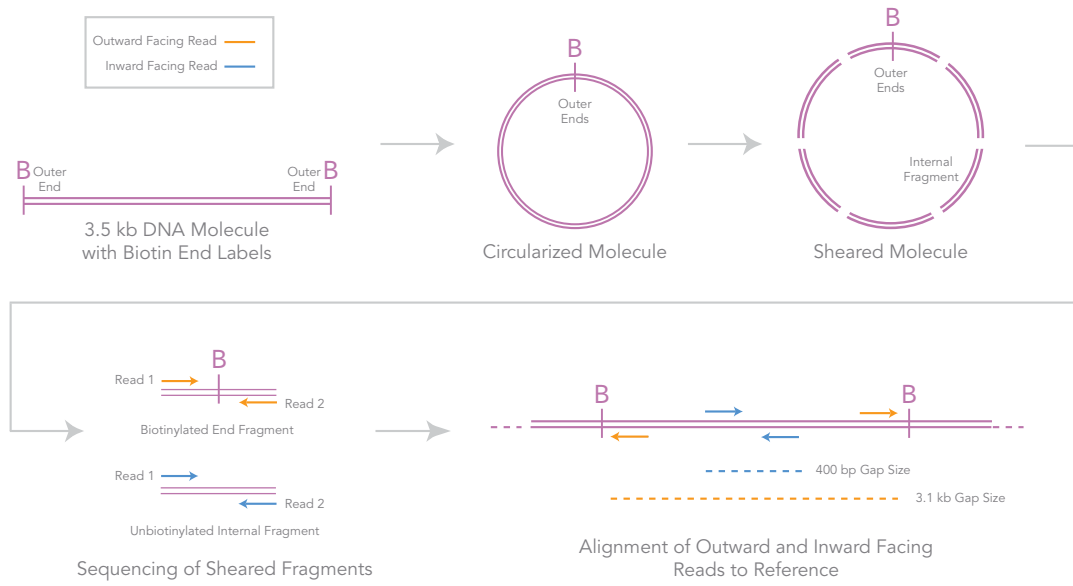


Figure 9 Origin and Alignment of Inward and Outward-Facing Reads

Potential Errors

Chimeras Normally, the circularization step joins the two ends of a single size-selected fragment together, with the junction marked by the biotin label. Chimeras, or false mate pairs, occur when two separate fragments are accidentally ligated together. In this event, the sequences on either side of the biotin label bear no relation to each other.

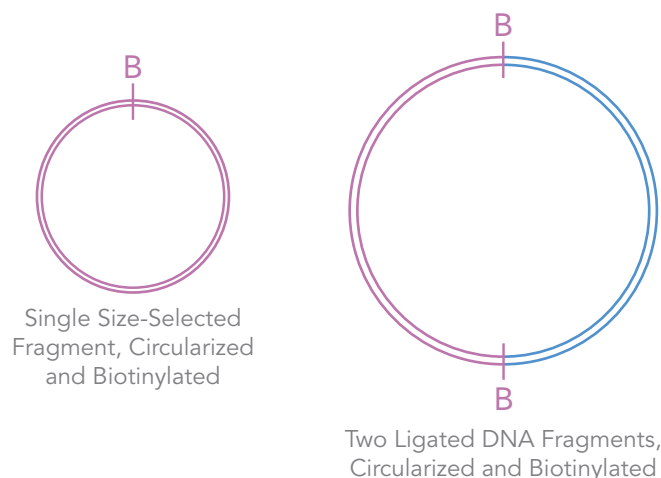


Figure 10 Normal DNA Fragment vs. Chimera

While it is important to avoid chimeras, a trade-off is involved. Greater amounts of DNA increase the number of chimeras, but also provide a more diverse library. Diluting the DNA solves the problem by minimizing the likelihood that DNA fragments encounter each other, but utilizing larger volumes also has practical disadvantages. The protocol balances these constraints by using up to 600 ng of DNA in a reaction volume of 300 μ l. In addition, the protocol calls for visualizing and quantifying the size-selected DNA prior to circularization as an additional quality-control step.

Low Quality DNA and Inward-Facing Reads

Illumina recommends using only high quality, high molecular weight DNA for the generation mate pair libraries (refer to *Assess Quality of Starting Material* on page 6). If the initial starting material is of low quality and partially degraded, this can lead to the generation of size selected fragments that are internally biotinylated and consequently can lead to elevated numbers inward-facing reads. Internal biotinylation becomes more likely the more degraded the DNA is and the larger the target gap sized.

Perform an Extra Wash on Lower-Quality DNA

If you run the protocol using lower-quality DNA, you may want to perform an extra, more stringent streptavidin bead wash after adding the adapters (refer to *Ligate Adapters to DNA Fragments* on page 38). A disadvantage of using this wash buffer is that it may also remove some of the desired biotinylated end fragments, leading to a reduction in the overall diversity of the library. Make sure to ligate the adapters before using streptavidin bead wash buffer 2.

Mate Pair Library Kit v2 Contents

This kit is used to generate mate pair libraries with gap sizes of 2–5 kb. Check to ensure that you have all of the reagents identified in this section before proceeding to library preparation.

The reagent contents may have settled on the inner walls of the tubes. Illumina recommends centrifuging the tubes briefly before use.

Kit Contents, Box 1 **Store at -15° to -25°C**

This box is shipped at -80°C. As soon as you receive it, store the following components at -15° to -25°C.

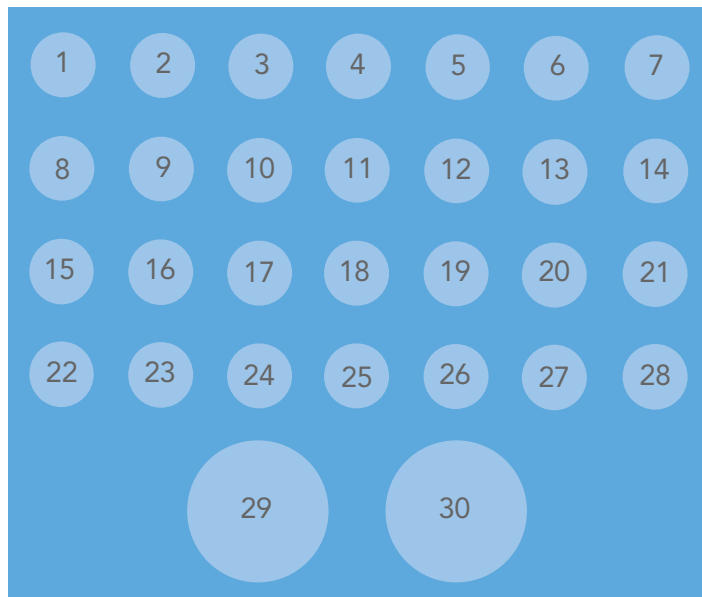


Figure 11 Mate Pair Library Prep Kit v2, Box 1

1. Ultra Pure Water, part # 1001913
2. End Repair Buffer 10X, part # 1006578
3. Natural dNTP Mix, part # 15007985
4. T4 DNA Polymerase, part # 15008323
5. T4 Polynucleotide Kinase, part # 15008327
6. Klenow DNA Polymerase, part # 15008325
7. Biotin dNTP Mix, part # 15008011
8. Circularization Buffer 10X, part # 1006640
9. Circularization Ligase, part # 15008331
10. DNA Exonuclease, part # 15008329
11. A-Tailing Buffer 10X, part # 1006579
12. 1 mM dATP, part # 11318081
13. A-Tailing Enzyme, part # 1006679

14. Adapter Ligation Buffer 2X, part # 1006574
15. PE Adapter Oligo Mix 15 μ M, part # 1006582
16. Adapter Ligase, part # 1006573
17. Phusion™ DNA Polymerase (Finnzymes Oy), part # 1000524
18. PCR Primer PE 1.0, part # 1001783
19. PCR Primer PE 2.0, part # 1001784
20. Positions 20–30 are empty

Kit Contents, Box 2 Store at Room Temperature

This box is shipped at room temperature. Store the following components at room temperature.

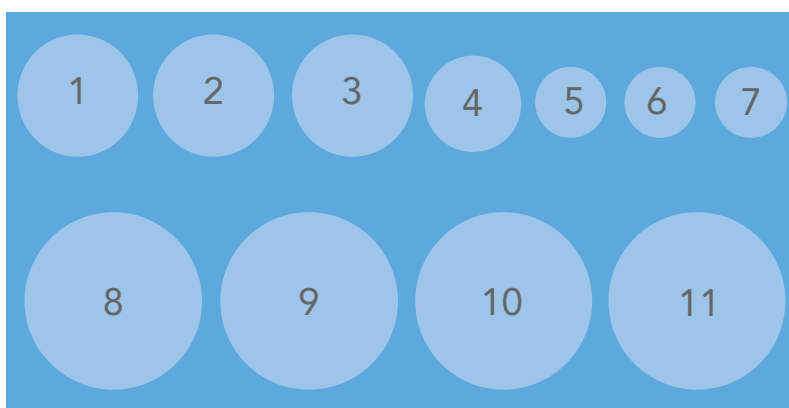


Figure 12 Mate Pair Library Prep Kit v2, Box 2

1. Streptavidin Bead Binding Buffer, part # 1006643
2. Streptavidin Bead Wash Buffer 2, part # 1006645
3. Positions 3–6 are empty
7. EDTA (0.5 M), part # 15008333
8. Streptavidin Bead Wash Buffer 1, part # 1006644
9. Positions 9–11 are empty

User-Supplied Consumables and Equipment

Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation. The requirement of some supplies are dependant upon the exact protocol followed and the items for specific protocols are specified where necessary.

Table 1 *User-Supplied Consumables*

Consumable	Supplier
0.2 ml thin wall PCR tubes	Axygen, part # PCR-02-C or equivalent
1 kb Plus DNA ladder	Invitrogen, part # 10787-026
1.5 ml microcentrifuge tubes	Axygen, part # MTC-175 or equivalent
2.0 ml microcentrifuge tubes	Axygen, part # MCT-200 or equivalent
50 ml Greiner tubes	Sigma, part # T2318 or equivalent
50X TAE buffer	BIO-RAD, part # 161-0743
Covaris T6 (6 x 32 mm) round bottom glass tubes (required for Covaris Shearing protocol only)	Covaris, part # 520031
Covaris 8 mm Snap Caps (required for Covaris Shearing protocol only)	Covaris, part # 520042
Disposable scalpels	General lab supplier
Distilled water	General lab supplier
Dynabeads M-280 streptavidin magnetic beads	Invitrogen, part # 112-05D
Ethidium bromide	Sigma, part # E1510
Glycerol (required for nebulization protocol only)	Sigma, part # G5516
HydroShear wash solutions (required for HydroShear protocol only) 0.2 M HCl 0.2 M NaOH TE pH 8.0	General lab supplier
Loading buffer 10X (0.5% bromophenol blue, 60% glycerol, 100 mM Tris pH 8.0, 100 mM EDTA)	General lab supplier
Megabase agarose	BIO-RAD, part # 161-3108
MinElute Gel Extraction Kit	QIAGEN, part # 28604
Molecular biology agarose	BIO-RAD, part # 161-3101
Nebulizers and nebulization buffer (required for nebulization protocol only)	Illumina, catalog # FC-301-1001
PVC tubing (required for nebulization protocol only)	Intersurgical, part # 1174-003

Table 1 *User-Supplied Consumables (Continued)*

Consumable	Supplier
QIAGEN EB Buffer	QIAGEN, part # 19086
QIAEX II Gel Extraction Kit	QIAGEN, part # 20021
QIAquick PCR Purification Kit	QIAGEN, part # 28104
TWEEN 20	Sigma, part # P7949

Table 2 *Equipment Checklist*

Equipment	Supplier
Benchtop microcentrifuge	General lab supplier
Benchtop centrifuge with swing-out rotor (for 50 ml Greiner tubes)	General lab supplier
2100 Bioanalyzer (recommended)	Agilent
Compressed air source of at least 32 psi	General lab supplier
Covaris S2 System	Covaris, part # S2
Dark reader transilluminator	Clare Chemical Research, part # D195M
Gel comb with wide wells (Well dimensions—9 mm width x 1 mm length x 0.7 height (65 µl well size))	Fisher, part # FB58325
Gel tray and electrophoresis unit (Gel dimensions—12 cm width x 14 cm length x 0.75 cm height (125 ml))	Fisher, part # FB57161
Heat block	General lab supplier
HydroShear Device (required for HydroShear protocol only)	Genomic Solutions, part # 4387834
Invitrogen Qubit Fluorometer or equivalent (recommended)	Invitrogen, part # Q32860
Magnetic rack for microcentrifuge tubes	General lab supplier (e.g. Invitrogen, part # CS15000)
Mini-centrifuge (recommended)	General lab supplier (e.g. Fisher, part # 05-090-100)
Shaking incubator or hybridization oven	General lab supplier
Thermal cycler or PCR machine	General lab supplier

Fragment Genomic DNA

The first step of the protocol shears the genomic DNA. The DNA needs to be fragmented to sizes approximately equal to the desired gap size. DNA can be fragmented in numerous ways. Two examples, HydroShear and nebulization, are described. HydroShear is recommended because it has a more controllable shearing mechanism and provides tighter distribution of sheared DNA fragments.



NOTE

To reduce the risk of unwanted shearing and sample loss, when handling DNA fragments of larger sizes (> 2 kb) do not vortex sample.

HydroShear Method

User-Supplied Consumables

- ▶ Genomic DNA 10 µg
- ▶ QIAGEN EB Buffer
- ▶ Wash Solutions (0.2 micron filtered)
 - Solution 1: 0.2 M HCl
 - Solution 2: 0.2 M NaOH
 - Solution 3: TE pH 8.0
- ▶ QIAEX II Gel Extraction Kit



NOTE

DNA should be as intact as possible, uncontaminated, and preferably quantitated by a fluorescence-based method. Illumina recommends using the Qubit quantification Platform and Quant-iT BR assay (Invitrogen). Illumina also suggests running a small quantity of genomic DNA on an agarose gel to assess DNA integrity.

Sample Preparation

Ensure that you are familiar with the HydroShear equipment before performing this protocol.

1. Dilute 10 µg of genomic DNA to approximately 150 ng/µl in QIAGEN EB, for a final volume of approximately 70 µl per sample preparation.
2. To ensure the genomic DNA is dissolved, incubate at room temperature for 30 minutes, occasionally mixing the solution.
3. Immediately before shearing, centrifuge to > 14,000 xg in a microcentrifuge for 20–30 minutes. Centrifugation removes any particulate matter in the solution that may block the HydroShear device.
4. Transfer the DNA solution to a clean tube, leaving behind any pellet that may have formed during centrifugation. The sample is now ready for shearing.

Shearing

The volume and speed code used depends upon user preferences, total sample volume, and desired size range of fragmentation.

1. To generate 2–5 kb fragments, set the shearing parameters described below:
 - Volume—70 μ l
 - Cycle Number—20
 - Shearing Assembly Size—Standard
 - Speed Code—8–14 (user optimized condition)
2. Perform 12 wash cycles (4 cycles per wash solution) as per the manufacturer's recommendations.
3. Load and shear the sample following the manufacturer's directions and eject the sheared sample into a clean tube.
4. The sample can now be used in *Perform End Repair and Biotinylation Reaction* on page 21. However, if the genomic sample was in a solution containing high levels of EDTA, it is advisable to purify the sample first.
5. [Optional] Purify the sample solution using the QIAEX II Gel Extraction Kit and protocol for 'Desalting and Concentrating DNA Solutions'. Follow the modified QIAEX II protocol described below:
 - a. Add 3 volumes of QIAGEN QX1 buffer and 2 volumes of water to the DNA sample.
 - b. Resuspend the QIAEX II by vortexing for 30 seconds and add 20 μ l of the QIAEX II suspension, gently mixing by flicking the tube.
 - c. Incubate the sample for 10 minutes at room temperature, gently mixing the tube every 2 minutes to keep the QIAEX II in suspension.
 - d. Centrifuge the sample for 60 seconds and remove the supernatant.
 - e. Resuspend the pellet in 500 μ l of QIAGEN wash buffer PE by flicking the tube, centrifuge for 60 seconds, and then remove the supernatant.
 - f. Repeat step e once.
 - g. Air-dry the pellet until a bit of it starts to look powdery and bright white. Most of it will still be pearlescent. This takes approximately 15–30 minutes. After 10 minutes, monitor the pellet so that it does not overdry.



CAUTION

Do not wait until the pellet has become completely white. This may over-dry the pellet and reduce DNA recovery.

- h. To elute DNA, add 35 μ l of QIAGEN EB buffer, mix by gently flicking the tube and incubate at 50°C for 5 minutes.
- i. Centrifuge for 60 seconds and carefully transfer the supernatant into a clean tube.
- j. Repeat elution steps h and i and combine the eluates. There should be a final volume of 70 μ l.



If you do not plan to proceed to *Perform End Repair and Biotinylation Reaction* immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer.

Nebulization Method

User-Supplied Consumables and Equipment

- ▶ Genomic DNA 10 μg
- ▶ Nebulizers and nebulization buffer
- ▶ PVC Tubing
- ▶ Compressed Air of at least 7.5 psi
- ▶ QIAEX II Gel Extraction Kit

The following instructions are guidelines for using the nebulizer. You can alter the distribution of fragment sizes by slight alterations in air pressure, varying from 2–10 psi. Illumina recommends that you optimize this nebulization process prior to processing samples, using an air pressure regulator with good resolution in the 0–15 psi range.

Familiarize yourself with the QIAEX II directions before performing this protocol.

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

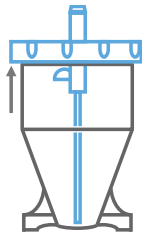


Figure 13 Remove the Nebulizer Lid

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.

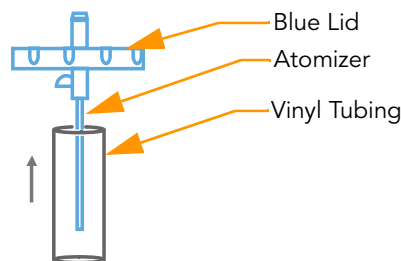


Figure 14 Assemble the Nebulizer

3. Add 10 μg of purified DNA for a total volume of up to 100 μl to the nebulizer.
4. Add 700 μl nebulization buffer to the DNA and mix well.

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

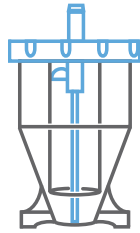


Figure 15 Replace the Nebulizer Lid

- Chill the nebulizer containing the DNA solution on ice while performing the next step.
- Connect the compressed air source to the inlet port on the top of the nebulizer with the PVC tubing, ensuring a tight fit.

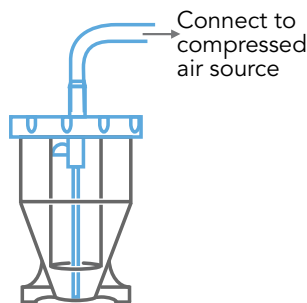


Figure 16 Connect Compressed Air

- Bury the nebulizer in an ice bucket and place it in a fume hood.
- Use the regulator on the compressed air source to ensure the air is delivered at 7.5 psi (or user optimized pressure).
- Pump the air for 30 seconds at a low pressure to nebulize the sample and generate fragment sizes in the 2–4 kb range.
- Centrifuge the nebulizer to 450 xg for 2 minutes in a benchtop centrifuge to collect the droplets from the side of the nebulizer. If necessary, use an old nebulizer as a counter-balance.
If a benchtop centrifuge is not available, then use the binding buffer from the QIAEX II Gel Extraction Kit to rinse the sides of the nebulizer and collect the DNA solution at the base of the nebulizer.
- Measure the recovered volume. Typically, you should recover approximately 800 μ l. Transfer to a 50 ml Greiner tube or equivalent.



NOTE

A 50 ml Greiner tube is used to accommodate the large liquid volume required for this clean up procedure.

- 13.** Purify the sample solution using the QIAEX II Gel Extraction Kit and protocol for 'Desalting and Concentrating DNA Solutions'. Follow the modified QIAEX II protocol described below:
- a. Add 3 volumes of QIAGEN QX1 buffer and 2 volumes of water to the DNA sample.
 - b. Resuspend the QIAEX II by vortexing for 30 seconds and add 20 μ l of QIAEX II suspension, gently mixing by flicking the tube.
 - c. Incubate the sample for 10 minutes at room temperature, gently mixing the tube every 2 minutes to keep the QIAEX II in suspension.
 - d. Centrifuge the 50 ml Greiner tube to 2500 xg for 5 minutes and carefully pour off the supernatant.
 - e. Resuspend the pellet in 500 μ l of QIAGEN wash buffer PE by shaking the tube, and transfer to a 1.5 ml microcentrifuge tube.
 - f. Centrifuge for 60 seconds at high speed in a microcentrifuge and remove the supernatant.
 - g. Resuspend the pellet for a second time in 500 μ l of QIAGEN wash buffer PE, centrifuge for 60 seconds and carefully remove the supernatant.
 - h. Air-dry the pellet until a bit of it starts to look powdery and bright white. Most of it will still be pearlescent. This takes approximately 15–30 minutes. After 10 minutes, monitor the pellet so that it does not overdry.

**CAUTION**

Do not wait until the pellet has become completely white. This may over-dry the pellet and reduce DNA recovery.

- i. To elute DNA, add 35 μ l of QIAGEN EB buffer, mix by gently flicking the tube and then incubate at 50°C for 5 minutes.
- j. Centrifuge for 60 seconds and carefully transfer the supernatant into a clean tube.
- k. Repeat elution steps 'i' and 'j' and combine the eluates. There should be a final volume of 70 μ l.



If you do not plan to proceed to *Perform End Repair and Biotinylation Reaction* immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer.

Perform End Repair and Biotinylation Reaction

Shearing the genomic DNA generates double-stranded DNA fragments containing 3' or 5' overhangs. This process first converts the overhangs into blunt ends using T4 DNA polymerase and Klenow DNA polymerase in the presence of natural nucleotides. The same enzymes are then used to incorporate biotinylated nucleotides to the repaired ends of the DNA fragments with the addition of biotin nucleotides to the reaction after 15 minutes.

Consumables

Illustrina-Supplied

- ▶ End Repair Buffer 10X
- ▶ Ultra Pure Water
- ▶ Natural dNTP Mix
- ▶ Biotin dNTP Mix
- ▶ T4 DNA Polymerase
- ▶ T4 Polynucleotide Kinase
- ▶ Klenow DNA Polymerase

User-Supplied

- ▶ Fragmented DNA
- ▶ QIAEX II Gel Extraction Kit

Procedure

1. Vortex the end repair buffer until any precipitate is dissolved.
2. Prepare the following reaction mix:

Reagent	Volume (μl)
Fragmented DNA	70
End Repair Buffer	10
Ultra Pure Water	7.5
Natural dNTP mix	1.5
T4 DNA Polymerase	5
T4 Polynucleotide Kinase	5
Klenow DNA Polymerase	1
Total Volume	100

3. Incubate for 15 minutes at 20°C in a heating block, then place immediately on ice.
4. Add 2.5 µl of the biotin dNTP mix to the reaction and mix by flicking the tube.
5. Incubate for an additional 15 minutes at 20°C, then place immediately on ice.
6. Purify the sample solution using the QIAEX II Gel Extraction Kit and protocol for 'Desalting and Concentrating DNA Solutions'. Follow the modified QIAEX II protocol described below:
 - a. Add 3 volumes of the QIAGEN QX1 buffer and 2 volumes of water to the DNA sample.
 - b. Resuspend the QIAEX II by vortexing for 30 seconds and add 20 µl of QIAEX II suspension, gently mixing by flicking the tube.
 - c. Incubate the sample for 10 minutes at room temperature, gently mixing the tube every 2 minutes to keep the QIAEX II in suspension.
 - d. Centrifuge for 60 seconds at high speed in a microcentrifuge and remove the supernatant.
 - e. Resuspend the pellet in 500 µl of QIAGEN wash buffer PE by flicking the tube, centrifuge for 60 seconds, and then remove the supernatant.
 - f. Repeat step 'e' once.
 - g. Air-dry the pellet until a bit of it starts to look powdery and bright white. Most of it will still be pearlescent. This takes approximately 15–30 minutes. After 10 minutes, monitor the pellet so that it does not overdry.

**CAUTION**

Do not wait until the pellet has become completely white. This may over-dry the pellet and reduce DNA recovery.

- h. To elute DNA, add 35 µl of QIAGEN EB buffer, mix by gently flicking the tube and incubate at 50°C for 5 minutes.
- i. Centrifuge for 60 seconds and carefully transfer the supernatant into a clean tube.
- j. Repeat elution steps 'h' and 'i' and combine the eluates. There should be a final volume of 70 µl.



If you do not plan to proceed to *Size-Select DNA* immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer.

Size-Select DNA

This process purifies DNA fragments of a particular size range from a gel. The size of the fragments purified determines the gap size of the paired reads. The length of the size-selected fragments is user-defined and depends on the experimental design and ultimate use of the Mate Pair Library.

Consumables and Equipment

User-Supplied

- ▶ Biotin-labeled DNA
- ▶ Megabase Agarose
- ▶ 50X TAE Buffer
- ▶ Distilled Water
- ▶ Ethidium Bromide
- ▶ 10X Loading Buffer
- ▶ 1 kb Plus DNA Ladder diluted 1:10 in 1X loading buffer
- ▶ 50 ml Greiner Tube
- ▶ Disposable Scalpels
- ▶ QIAEX II Gel Extraction Kit
- ▶ Gel Tray and Electrophoresis Unit
- ▶ Gel Comb with wide wells



CAUTION

It is extremely important to follow the gel preparation and run conditions given below to ensure reproducibility of the procedure.



NOTE

Using the Illumina recommended electrophoresis equipment and gel comb, the sample should be loaded over 4 wells, loading only 20 μ l per well. This has not been rigorously tested, but using more wells appears to decrease the chance that short fragments become entangled with longer fragments in the gel.

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.

Procedure

1. Prepare a 0.6% megabase agarose gel in a final volume of 125 ml 1X TAE buffer. Use a gel comb with wide wells (recommended well size: 1 mm (length) x 9 mm (width) x 10 mm (height)). Do not add ethidium bromide to the gel at this stage.
2. Place the gel in the electrophoresis tank, and fill with 1X TAE to the fill line. For overnight runs this is essential to ensure that there is sufficient buffer present to prevent buffer exhaustion and gel distortions.
3. Prepare the DNA sample for loading. Add 10 μ l of 10X loading buffer to the biotin-labeled DNA sample to give a final volume of 80 μ l.

4. Load the sample over the four middle lanes of the cast gel, loading approximately 20 μ l per well. The sample should sink to the bottom of the well and fill only 1/3 of the possible well volume.
5. Load 20 μ l of diluted 1 kb ladder in the wells either side of the sample wells.
6. Run the gel long enough to give sufficient separation at the size range for purification. The run time depends on the gel apparatus used, user preferences, and desired fragment size. Using the recommended reagents and apparatus as a guide, run the gel at 20 V (1 V/cm) for 15 hours (overnight).

**NOTE**

It is also possible to run the gel for a shorter time period at a higher voltage (100 V for 2.5 hours), and complete the gel extraction during day 1. However the overnight run is an easier workflow and allows time to quantitate the size-selection material before proceeding to the circularization reaction. It may also help to generate better fragment resolution.

7. Stain the gel in buffer with 0.5 μ g/ml ethidium bromide solution in 1X TAE buffer for 60 minutes with gentle rocking.
8. View the gel on a Dark Reader transilluminator.
9. Excise a single slice of gel with a clean scalpel, and place in a 50 ml Greiner tube. The size of the fragments purified determines the gap size of the paired reads and the width of the gel slice determines the spread of the gap size distribution.

Target Gap Size	Approximate Fragment Size Range Purified From Gel
2 kb	2–2.6 kb
3 kb	3–3.7 kb
4 kb	4–4.8 kb
5 kb	5–5.9 kb

Figure 17 shows a 0.6% agarose gel containing nebulized and biotinylated genomic DNA in the 2–5 kb size range, with a gel slice excised at approximately 3 kb.



Figure 17 Excised 3 kb Gel Slice

10. Purify the sample using the QIAEX II kit and protocol for 'Agarose Gel Extraction'. Follow the modified QIAEX II protocol described below:
 - a. Weigh the gel slices in the 50 ml Greiner tubes. The slices should be in the range of 1000–2000 mg depending on the size of the gel cut.
 - b. Add 3 volumes of QIAGEN QX1 buffer and 2 volumes of water to 1 volume of gel.
 - c. Resuspend QIAEX II by vortexing for 30 seconds and add 30 μ l of QIAEX II suspension, gently mixing by flicking the tube.
 - d. Incubate the sample at 50°C with constant mixing in a shaking incubator at approximately 150 rpm or rotating hybridization oven, until all the agarose has solubilized.



NOTE

Solubilization of the agarose using the protocol described may take 60 minutes or longer, which is longer than the time suggested in the QIAEX II protocol. Before proceeding to the next step ensure that all the agarose has been solubilized.

- e. Once all the agarose has solubilized, continue to incubate the sample for 10 minutes at room temperature, gently mixing the tube every 2 minutes to keep the QIAEX II in suspension.
- f. Centrifuge the 50 ml Greiner tube to 2500 xg for 5 minutes and carefully pour off the supernatant.
- g. Resuspend the pellet in 500 μ l of QIAGEN QX1 buffer and transfer to a 1.5 ml microcentrifuge tube.
- h. Centrifuge for 60 seconds at high speed in a microcentrifuge and remove the supernatant.
- i. Resuspend the pellet in 500 μ l of QIAGEN wash buffer PE by flicking the tube, centrifuge for 60 seconds, and then carefully remove the supernatant.
- j. Repeat step 'i' once.

- k. Air-dry the pellet until a bit of it starts to look powdery and bright white. Most of it will still be pearlescent. This takes approximately 15–30 minutes. After 10 minutes, monitor the pellet so that it does not overdry.

**CAUTION**

Do not wait until the pellet has become completely white. This may over-dry the pellet and reduce DNA recovery.

- l. To elute DNA, add 75 μ l of QIAGEN EB buffer, mix by gently flicking the tube and incubate at 50°C for 5 minutes.
 - m. Centrifuge for 60 seconds and carefully transfer the supernatant into a clean tube.
 - n. Repeat elution steps 'l' and 'm' and combine the eluates. There should be a final volume of 150 μ l.
11. Check the size distribution and quantify the purified DNA using an Agilent Bioanalyzer as shown in Figure 18.

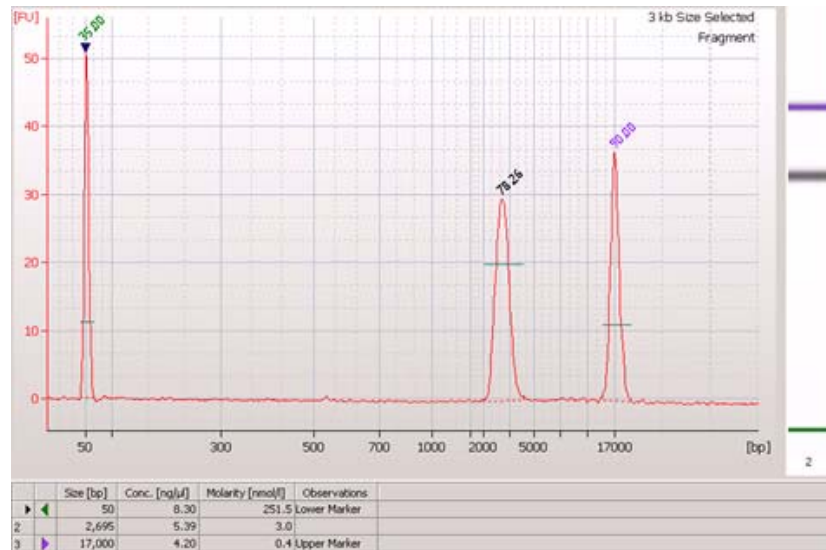


Figure 18 Agilent Profile of Purified 2.5 kb Size-Selected Sample

Figure 18 shows an Agilent 2100 12000 LabChip profile of a purified size-selected sample. 1 μ l of 150 μ l the sample was loaded on the LabChip. The calculated concentration of this sample is 5.39 ng/ μ l, giving a total of 808 ng of size-selected DNA.



If you do not plan to proceed to *Size-Select DNA* immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer.

Circularize DNA

This protocol circularizes the size-selected DNA fragments by intramolecular ligation of their blunt ends. The original ends of the linear DNA fragment are identified in the circle by the location of the biotin label.

Consumables Illumina-Supplied

- ▶ Circularization Buffer 10X
- ▶ Ultra Pure Water
- ▶ Circularization Ligase

User-Supplied

- ▶ Size-selected DNA (up to 600 ng as quantified on an Agilent Bioanalyzer)



NOTE

The circularization buffer contains ATP, which can degrade with repeated freeze thaw cycles. To avoid reduced performance of the circularization buffer, it is recommended that upon thawing for the first time the buffer is dispensed into single use aliquots.

Procedure

1. Vortex the circularization buffer until any precipitate is dissolved.
2. Prepare the following reaction mix:

Reagent	Volume (μ l)
Size-selected DNA	x (up to 600 ng)
Circularization Buffer 10X	30
Ultra Pure Water	256.6 - x
Circularization Ligase	13.4
Total Volume	300

3. Incubate overnight for 16 hours at 30°C in a heating block or water bath.

Digest Linear DNA

This protocol uses a DNA exonuclease to remove any remaining linear DNA fragments from the circularization reaction.

Consumables **Illumina-Supplied**

- ▶ DNA Exonuclease
- ▶ EDTA 0.5 M

User-Supplied

- ▶ Circularized DNA

- ### Procedure
1. Add 3 μ l of DNA exonuclease to 300 μ l of the circularization reaction. Mix by gently flicking the tube and briefly centrifuge.
 2. Incubate at 37°C for 20 minutes followed by 70°C for 30 minutes in a heating block or water bath.
 3. Add 12 μ l of EDTA to the exonuclease-treated sample. Mix well by flicking the tube.
 4. Proceed to *Fragment Circularized DNA* on page 29.

Fragment Circularized DNA

This protocol randomly fragments the circularized DNA to lengths of approximately 400 bp in size. Illumina recommends using a Covaris S2 device, because its closed system reduces the risk of contamination and sample loss. Nebulization can also be used, but is prone to sample loss. Both methods are described.

Covaris S2 Fragmentation

User-Supplied Consumables and Equipment

- ▶ Exonuclease-treated DNA
- ▶ Covaris S2
- ▶ Covaris T6 Round Bottom Glass Tubes
- ▶ Covaris Snap Caps
- ▶ QIAquick PCR Purification Kit

The following instructions are guidelines for using the Covaris S2 for preparing a Mate Pair library. Familiarize yourself with this device before attempting to use it in a Mate Pair library preparation.

Procedure

1. Turn on Covaris S2 device. Degas and pre-chill water in water bath to 6° to 8°C, as per the manufacturer's instructions.
2. Transfer the exonuclease-treated DNA into a T6 glass tube. To ensure consistent shearing, the liquid should reach the very top of the tube. If necessary, add additional volume by adding ultrapure water to remove air gaps. Place the snap cap firmly on the tube.



CAUTION

Air gaps present in the shearing tube can impair the fragmentation process. This can result in longer fragments than desired. Foaming or bubbling in the tube after shearing indicate the presence of an air gap

3. Shear the DNA sample using the following parameters:
 - Use frequency sweeping
 - Intensity—8
 - Duty Cycle—20 %
 - Bursts per second—200
 - Time—50 seconds
4. Transfer the sample to a 2 ml microcentrifuge tube and follow the instructions in the QIAquick PCR Purification Kit to purify the sample solution, eluting in 50 µl of QIAGEN EB buffer.



If you do not plan to proceed to *Purify Biotinylated DNA* immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer.

Nebulization User-Supplied Consumables and Equipment

- ▶ Exonuclease-treated DNA
- ▶ Glycerol
- ▶ Nebulizers and nebulization buffer
- ▶ PVC Tubing
- ▶ Compressed Air source of at least 32 psi
- ▶ QIAquick PCR Purification Kit

Procedure

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

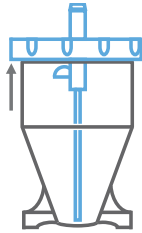


Figure 19 Remove the Nebulizer Lid

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.



NOTE

Calibrate the air pressure required for nebulization, to optimize shearing of DNA to the desired size range.

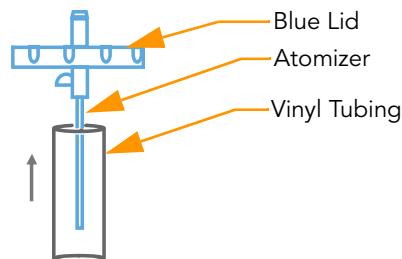


Figure 20 Assemble the Nebulizer

3. Transfer all the exonuclease-treated DNA to the nebulizer.
4. Add 400 μ l of the nebulization buffer and approximately 150 μ l of 100% glycerol to the DNA and mix well.

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

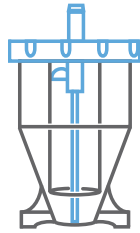


Figure 21 Replace the Nebulizer Lid

- Chill the nebulizer containing the DNA solution on ice while performing the next step.
- Connect the compressed air source to the inlet port on the top of the nebulizer with the PVC tubing, ensuring a tight fit.

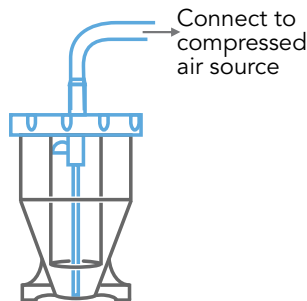


Figure 22 Connect Compressed Air

- Bury the nebulizer in an ice bucket and place in a fume hood.
- Use the regulator on the compressed air source to ensure the air is delivered at 32 psi.
- Nebulize for 6 minutes. Vapor may rise from the nebulizer, which is normal.
- Centrifuge the nebulizer to 450 xg for 2 minutes in a benchtop centrifuge to collect the droplets from the side of the nebulizer. If necessary, use an old nebulizer as a counterbalance.
If a benchtop centrifuge is not available, the binding buffer from the QIAquick PCR Purification Kit can be used to rinse the side of the nebulizer and to collect the DNA solution at the base of the nebulizer.
- Measure the recovered volume. Typically, you should recover approximately 400 μ l.
- Follow the instructions in the QIAquick PCR Purification Kit to purify the sample solution and concentrate it on one QIAquick column, eluting in 50 μ l of QIAGEN EB buffer.



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

Purify Biotinylated DNA

This protocol uses Dynal magnetic M-280 streptavidin beads to purify the biotinylated DNA fragments. The biotin label marks the site of circularization, and so the biotinylated DNA contains the two ends of the original size-selected DNA fragment.

Consumables **Illumina-Supplied**

- ▶ Streptavidin Bead Binding Buffer
- ▶ Streptavidin Bead Wash Buffer 1

User-Supplied

- ▶ Fragmented DNA
- ▶ Dynabeads M-280 Streptavidin Magnetic Beads
- ▶ QIAGEN EB Buffer
- ▶ 1.5 ml Microcentrifuge Tube



After purifying the biotinylated DNA, all sample processing can be carried out in a single 1.5 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.

Procedure

1. Ensure that each of the bead buffers is well-mixed by inverting the tube a few times before use.
2. Resuspend the streptavidin magnetic beads by shaking the bottle.
3. Transfer 20 μ l of resuspended beads into a 1.5 ml microcentrifuge tube.
4. Place the tube in the magnetic rack for about 1 minute, until the beads are separated from the solution.
5. Remove the supernatant with a pipette, while the tube remains in the magnetic rack, and then discard the supernatant.
6. Wash the beads in 50 μ l of streptavidin bead binding buffer as follows:
 - a. Remove the tube from the magnetic rack.
 - b. Add the buffer solution and resuspend the beads by gently flicking the tube.
 - c. Centrifuge the tube for 1–2 seconds.
 - d. Place the tube in the magnetic rack for 1 minute, and then remove and discard the supernatant.
7. Repeat step 6 once.
8. Remove the tube from the magnetic rack and resuspend the beads in 50 μ l of fresh streptavidin bead binding buffer.
9. Add 50 μ l of the bead solution to the 50 μ l of fragmented DNA sample and incubate for 15 minutes at 20°C. Resuspend the beads every 2 minutes by gentle mixing.

10. Place the tube in the magnetic rack for 1 minute. Remove and discard the supernatant.
11. Wash the beads in 200 μ l of streptavidin bead wash buffer 1 as follows:
 - a. Remove the tube from the magnetic rack.
 - b. Add the buffer solution and resuspend the beads by gently flicking the tube.
 - c. Centrifuge the tube for 1–2 seconds.
 - d. Place the tube in the magnetic rack for 1 minute, then remove and discard the supernatant.

**NOTE**

During this initial wash, a few beads may adhere to the sides of the tube. Take extra care when aspirating to avoid collecting the beads.

12. Repeat the same wash three times, discarding each wash supernatant.
13. Wash the beads in 200 μ l of QIAGEN EB buffer as follows:
 - a. Remove the tube from the magnetic rack.
 - b. Add the buffer solution and resuspend the beads by gently flicking the tube.
 - c. Centrifuge the tube for 1–2 seconds.
 - d. Place the tube in the magnetic rack for 1 minute, then remove and discard the supernatant.
14. Wash the beads for a second time in 200 μ l of QIAGEN EB buffer. Place the sample on ice without removing the final wash solution.

**CAUTION**

The final wash solution is removed during the next step of the protocol, just before the addition of the enzymatic reaction mix. It is important to remove this final wash solution when instructed to do so.

15. Proceed immediately to *Perform End Repair* on page 34.

Perform End Repair

Shearing the circularized DNA generates double-stranded DNA fragments containing 3' or 5' overhangs. This process converts the overhangs resulting from fragmentation into blunt ends, using T4 DNA polymerase and Klenow DNA polymerase. The end-repair reaction is carried out on the biotinylated DNA immobilized to the streptavidin beads.

Consumables

Illumina-Supplied

- ▶ End Repair Buffer 10X
- ▶ Ultra Pure Water
- ▶ Natural dNTP Mix
- ▶ T4 DNA Polymerase
- ▶ T4 Polynucleotide Kinase
- ▶ Klenow DNA Polymerase
- ▶ Streptavidin Bead Wash Buffer 1

User-Supplied

- ▶ Washed beads with bound, sheared DNA
- ▶ QIAGEN EB Buffer

Procedure

1. Vortex the end repair buffer until any precipitate is dissolved.
2. Prepare the following end-repair reaction mix on ice:

Reagent	Volume (µl)
End Repair Buffer	10
Ultra Pure Water	75
Natural dNTP Mix	4
T4 DNA Polymerase	5
T4 Polynucleotide Kinase	5
Klenow DNA Polymerase	1
Total Volume	100

3. Place the washed beads back on the magnet for 1 minute and remove and discard the supernatant.
4. Resuspend the beads immediately in 100 µl of the end-repair reaction mix.
5. Incubate for 30 minutes at 20°C in a heat block or water bath.
6. Place the tube in the magnetic rack for 1 minute to separate the beads from the solution.

7. Remove the supernatant with a pipette, while the tube remains in the magnetic rack. Discard the supernatant.
8. Wash the beads in 200 μ l of streptavidin bead wash buffer 1 as follows:
 - a. Remove the tube from the magnetic rack.
 - b. Add the buffer solution and resuspend the beads by gently flicking the tube.
 - c. Centrifuge the tube for 1–2 seconds.
 - d. Place the tube in the magnetic rack for 1 minute, then remove and discard the supernatant.
9. Repeat the same wash three times, discarding each wash supernatant.
10. Wash the beads in 200 μ l of QIAGEN EB buffer as follows:
 - a. Remove the tube from the magnetic rack.
 - b. Add the buffer solution and resuspend the beads by gently flicking the tube.
 - c. Centrifuge the tube for 1–2 seconds.
 - d. Place the tube in the magnetic rack for 1 minute, then remove and discard the supernatant.
11. Wash the beads for a second time in 200 μ l of QIAGEN EB buffer. Place the sample on ice without removing the final wash solution.



The final wash solution is removed during the next step of the protocol, just before the addition of the enzymatic reaction mix. It is important to remove this final wash solution when instructed to do so.

12. Proceed immediately to *A-Tail DNA Fragments* on page 36.

A-Tail DNA Fragments

This protocol adds an 'A' base to the 3' ends of the blunt phosphorylated DNA fragments. The A-Tailing prevents intermolecular ligation of mate pair DNA fragments and prepares the DNA fragments to be ligated to the adapters, which have a single 'T' base overhang at their 3' end. The A-Tailing reaction is carried out on the biotinylated DNA immobilized to the streptavidin beads.

Consumables

Illumina-Supplied

- ▶ A-Tailing Buffer 10X
- ▶ 1 mM dATP
- ▶ A-Tailing Enzyme
- ▶ Ultra Pure Water
- ▶ Streptavidin Bead Wash Buffer 1

User-Supplied

- ▶ Washed beads from previous *Perform End Repair* process
- ▶ QIAGEN EB Buffer

Procedure

1. Prepare the following reaction mix:

Reagent	Volume (µl)
A-Tailing Buffer	5
Ultra Pure Water	32
1 mM dATP	10
A-Tailing Enzyme	3
Total Volume	50

2. Place the washed beads back on the magnet for 1 minute and remove and discard the supernatant.
3. Resuspend the beads immediately in 50 µl of A-Tailing reaction mix.
4. Incubate for 30 minutes at 37°C in a heat block or water bath.
5. Place the tube in the magnetic rack for 1 minute to separate the beads from the solution.
6. Remove the supernatant with a pipette, while the tube remains in the magnetic rack. Discard the supernatant.
7. Wash the beads in 200 µl of streptavidin bead wash buffer 1 as follows:
 - a. Remove the tube from the magnetic rack.
 - b. Add the buffer solution and resuspend the beads by gently flicking the tube.

- c. Centrifuge the tube for 1–2 seconds.
 - d. Place the tube in the magnetic rack for 1 minute, then remove and discard the supernatant.
8. Repeat the same wash three times, discarding each wash supernatant.
9. Wash the beads in 200 μ l of QIAGEN EB buffer as follows:
 - a. Remove the tube from the magnetic rack.
 - b. Add the buffer solution and resuspend the beads by gently flicking the tube.
 - c. Centrifuge the tube for 1–2 seconds.
 - d. Place the tube in the magnetic rack for 1 minute, then remove and discard the supernatant.
10. Wash the beads for a second time in 200 μ l of QIAGEN EB buffer. Place the sample on ice without removing the final wash solution.



The final wash solution is removed during the next step of the protocol, just before the addition of the enzymatic reaction mix. It is important to remove this final wash solution when instructed to do so.

11. Proceed immediately to *Ligate Adapters to DNA Fragments* on page 38.

Ligate Adapters to DNA Fragments

This protocol ligates adapters to the ends of the DNA fragments, preparing them to be hybridized to a flow cell and sequenced. A large excess of adapters is used to maximize the number of mate pair library fragments that will have adapters ligated at both ends. The adapters have a single 'T' base overhang at their 3' end to prevent the formation of adapter dimers. The ligation reaction is carried out on the biotinylated DNA immobilized to the streptavidin beads.

Consumables

ILLUMINA-SUPPLIED

- ▶ Adapter Ligation Buffer 2X
- ▶ PE Adapter Oligo Mix 15 uM
- ▶ Ultra Pure Water
- ▶ Adapter Ligase
- ▶ Streptavidin Bead Wash Buffer 1
- ▶ [Optional] Streptavidin Bead Wash Buffer 2

USER-SUPPLIED

- ▶ QIAGEN EB Buffer
- ▶ Washed beads from *A-Tail DNA Fragments* process

Procedure

1. Vortex the adapter ligation buffer until any precipitate is dissolved.
2. Prepare the following reaction mix:

Reagent	Volume (µl)
Adapter Ligation Buffer 2X	25
Ultra Pure Water	19
PE Adapter Oligo Mix	1
Total Volume	45

3. Place the washed beads back on the magnet for 1 minute and remove and discard the supernatant.
4. Resuspend the beads immediately in 45 µl of the ligation reaction mix.
5. Add 5 µl of adapter ligase to the reaction and mix by pipetting gently up and down several times.
6. Incubate for 15 minutes at 20°C in a heat block or water bath.
7. Place the tube in the magnetic rack for 1 minute to separate the beads from the solution.
8. Remove the supernatant with a pipette, while the tube remains in the magnetic rack. Discard the supernatant.

9. Wash the beads in 200 μ l of streptavidin bead wash buffer 1 as follows:
 - a. Remove the tube from the magnetic rack.
 - b. Add the buffer solution and resuspend the beads by gently flicking the tube.
 - c. Centrifuge the tube for 1–2 seconds.
 - d. Place the tube in the magnetic rack for 1 minute, then remove and discard the supernatant.
10. Repeat the same wash three times, discarding each wash supernatant.
11. [Optional] Wash the beads in 200 μ l of streptavidin bead wash buffer 2 as follows:

**NOTE**

This optional stringent wash step should only be used if you suspect the starting genomic material to be of low quality and wish to reduce levels of inward-facing reads present in the final library (refer to 'Low Quality DNA and Inward-Facing Reads' on page 11 for more information). The streptavidin bead wash buffer 2 should only be used for the stringent wash step and only **after** the ligation of the PE oligo adapters. Care should be taken not to use it for any other wash step.

- a. Remove the tube from the magnetic rack.
 - b. Add the buffer solution and resuspend the beads by gently flicking the tube.
 - c. Centrifuge the tube for 1–2 seconds.
 - d. Place the tube in the magnetic rack for 1 minute, then remove and discard the supernatant.
 - e. Repeat wash once, discarding wash supernatant.
12. Wash the beads in 200 μ l of QIAGEN EB buffer as follows:
 - a. Remove the tube from the magnetic rack.
 - b. Add the buffer solution and resuspend the beads by gently flicking the tube.
 - c. Centrifuge the tube for 1–2 seconds.
 - d. Place the tube in the magnetic rack for 1 minute, then remove and discard the supernatant.
 13. Wash the beads for a second time in 200 μ l of QIAGEN EB buffer and place the sample on ice without removing the final wash solution.

**CAUTION**

The final wash solution is removed during the next step of the protocol, just before the addition of the enzymatic reaction mix. It is important to remove this final wash solution when instructed to do so.

14. Proceed immediately to *Enrich Adapter-Modified DNA Fragments by PCR* on page 40.

Enrich Adapter-Modified DNA Fragments by PCR

This protocol 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 two primers that anneal to the ends of the adapters. The number of PCR cycles is minimized to avoid skewing the representation of the library. The biotinylated DNA immobilized on the streptavidin beads is amplified, and the amplified DNA is harvested from the bead supernatant.

Consumables **Illumina-Supplied**

- ▶ Phusion DNA Polymerase (Finnzymes Oy)
- ▶ Ultra Pure Water
- ▶ PCR Primer PE 1.0
- ▶ PCR Primer PE 2.0

User-Supplied

- ▶ Washed beads from *Ligate Adapters to DNA Fragments* process

Procedure

1. Prepare the following PCR mix

Reagent	Volume (µl)
Phusion DNA Polymerase	25
Ultra Pure Water	23
PCR Primer 1.0	1
PCR Primer 2.0	1
Total Volume	50

2. Place the washed beads back on the magnet for 1 minute and remove and discard the supernatant.
3. Resuspend the beads in 50 µl of the PCR mix and transfer to a 0.2 ml PCR tube.
4. Amplify in a thermal cycler using the following PCR protocol:
 - a. 30 seconds at 98°C
 - b. 18 cycles of:
 - 10 seconds at 98°C
 - 30 seconds at 65°C
 - 30 seconds at 72°C
 - c. 5 minutes at 72°C
 - d. Hold at 4°C
5. Remove and retain the PCR supernatant from the beads using a magnetic rack.

6. Discard the beads.



If you do not plan to proceed to *Size-Select Library* immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer.

Size-Select Library

This protocol purifies the mate pair library fragments from a gel, selecting an optimal size range of 350–650 bp for cluster formation and sequencing.

Consumables and Equipment

User-Supplied

- ▶ Amplified DNA
- ▶ Molecular Biology Agarose
- ▶ 50X TAE Buffer
- ▶ Distilled Water
- ▶ Ethidium Bromide
- ▶ Loading Buffer 10X
- ▶ 1 kb Plus DNA Ladder diluted to 1:10 in 1 X Loading Buffer
- ▶ Disposable Scalpels
- ▶ MinElute Gel Extraction Kit
- ▶ TWEEN 20 diluted to 1% in QIAGEN EB Buffer
- ▶ Gel Tray and Electrophoresis Unit
- ▶ Gel Comb with wide wells



CAUTION

It is extremely important to follow the gel preparation and run conditions given below to ensure reproducibility of the procedure.



NOTE

If you use an alternative to the Illumina recommended electrophoresis equipment and gel comb, 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.

Procedure

1. Prepare a 1.5% agarose gel in a final volume of 150 ml 1X TAE buffer.
2. Use a comb with wide wells to accommodate 66 μ l of sample per well. Recommended well size: 1 mm (length) x 9 mm (width) x 10 mm (height).
3. Add the ethidium bromide to the gel during preparation at a concentration of 400 ng/ml.
4. Load 20 μ l of the ladder solution in 1 lane of the gel.
5. Add 5 μ l of the 10X loading buffer to the amplified DNA.
6. Load the entire sample in 1 wide lane of the gel, leaving at least a gap of 1 empty lane between ladder and sample.
7. Run the gel at 100 V (5 V/cm) for 45 minutes using the Illumina-recommended apparatus. The running conditions depend on the gel apparatus used.

8. View the gel on a Dark Reader transilluminator.
9. Excise a piece of gel, purifying DNA 350–650 bp in size with a clean scalpel, as shown in Figure 23.

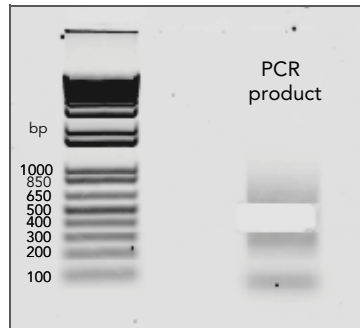


Figure 23 Gel Containing Amplified Mate Pair Sample After Removal of Size-Selected Final Library

10. Follow the instructions in the MinElute Gel Extraction Kit to purify the sample solution and concentrate on 1 MinElute column, eluting in 15 μ l of QIAGEN EB buffer.
11. Add 1.5 μ l of 1% TWEEN 20 to the library and store at -15° to -25°C .

Validate the Library

Illumina recommends performing the following validation steps on your DNA library.

1. Perform library validation by running a sample on either a gel or an Agilent Bioanalyzer chip. An example of a Mate Pair library prepared using this protocol is shown in Figure 24.

Figure 24 shows an Agilent 2100 Bioanalyzer profile of a purified Mate Pair library using a 1000 LabChip. The typical concentration of a final library eluted in 15 μ l of QIAGEN EB buffer is between 10 and 40 nM.

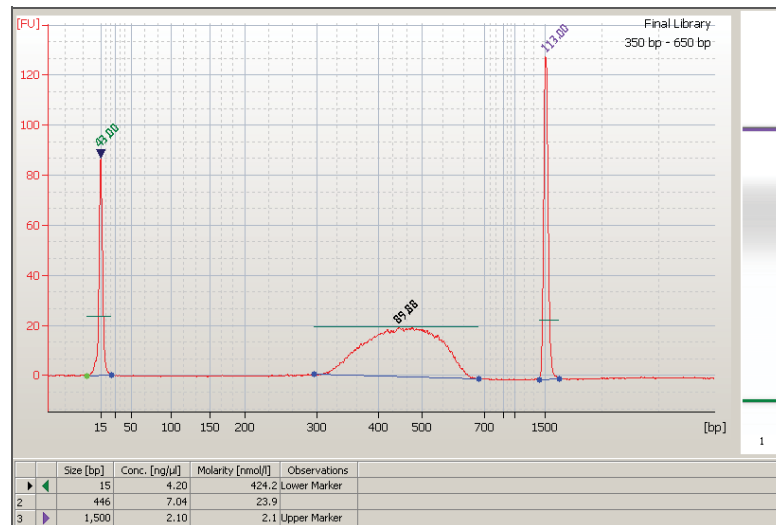


Figure 24 Agilent Profile of Purified Mate Pair Library

2. Determine the concentration of the library for cluster formation. Illumina recommends using qPCR, although the concentration may also be determined using an Agilent Bioanalyzer.

DNA Template Storage

Illumina recommends storing prepared DNA template at a concentration of 10 nM. For paired-end libraries, 10 nM is ~2.0 ng/μl for 310 bp fragments including adapter length.

Adjust the concentration for your prepared DNA samples to 10 nM using Tris-Cl 10 mM, pH 8.5. For long-term storage of DNA samples at a concentration of 10 nM, add Tween 20 to the sample to a final concentration of 0.1% Tween. This helps to prevent adsorption of the template to plastic tubes upon repeated freeze-thaw cycles, which would decrease the cluster numbers from a sample over time.



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