Preparing 2–5kb Samples for Mate Pair Library Sequencing

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Topics

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Introduction

This protocol explains how to prepare mate pair genomic DNA libraries for analysis on the Illumina Cluster Station and Genome Analyzer. The Mate Pair Library Preparation Kit fills the need for attaining paired reads with a gap size of 2–5kb in order to facilitate contig assembly and detection of structural variation.

Mate Pair Library Preparation Overview

In the Mate Pair Library Preparation Kit, the purified genomic DNA is 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 a gel. The length and range of the size-selected material will determine the gap size and its variance of the paired reads of the final library.

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

The circular DNA fragments are then sheared again by nebulization to a fragment length of around 400 bp. The fragments containing the biotinylated ends of the original size-selected fragment are then purified by the use of Streptavidin-coated magnetic beads.

These fragments are then end-repaired and A-tailed in preparation for the ligation of the Illumina paired-end oligo adapters. After the adapter ligation, PCR is carried out to both amplify and enrich those DNA fragments that have adapter molecules on both ends.

The product of the PCR amplification is a DNA smear. Gel size selection is carried out to select for fragments in the range of 400–600 bp, which are optimal for attaining high-quality mate pair sequence reads.

Figure 1 shows the schematic preparation of the Mate Pair Library.

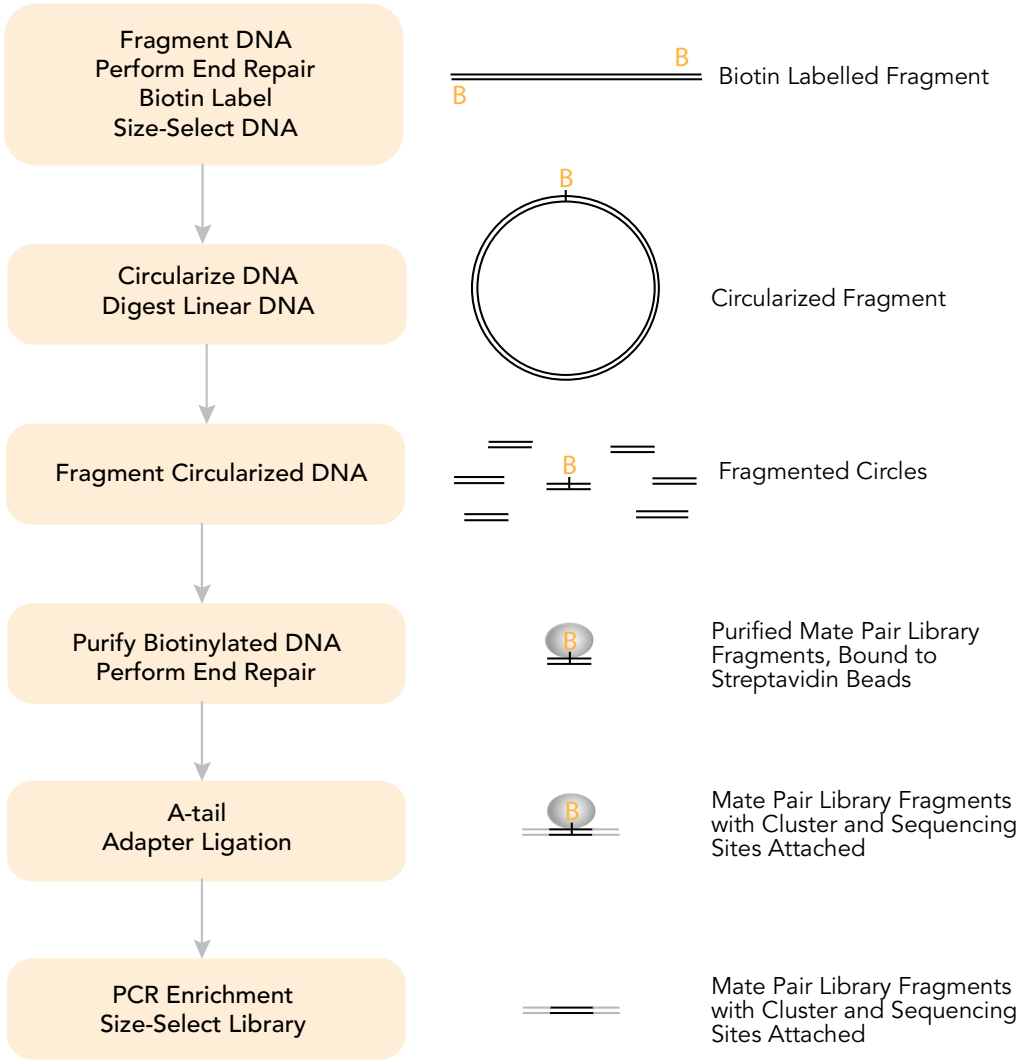


Figure 1 Mate Pair Library Preparation Overview

Workflow

Figure 2 shows an example of the workflow for a 2–5kb gap size mate pair library. You can pause this protocol at several stopping points, which are marked by an asterisk (*) in Figure 2. At a safe stopping point, you can store the sample at -15° to -25°C overnight or for longer periods of time.

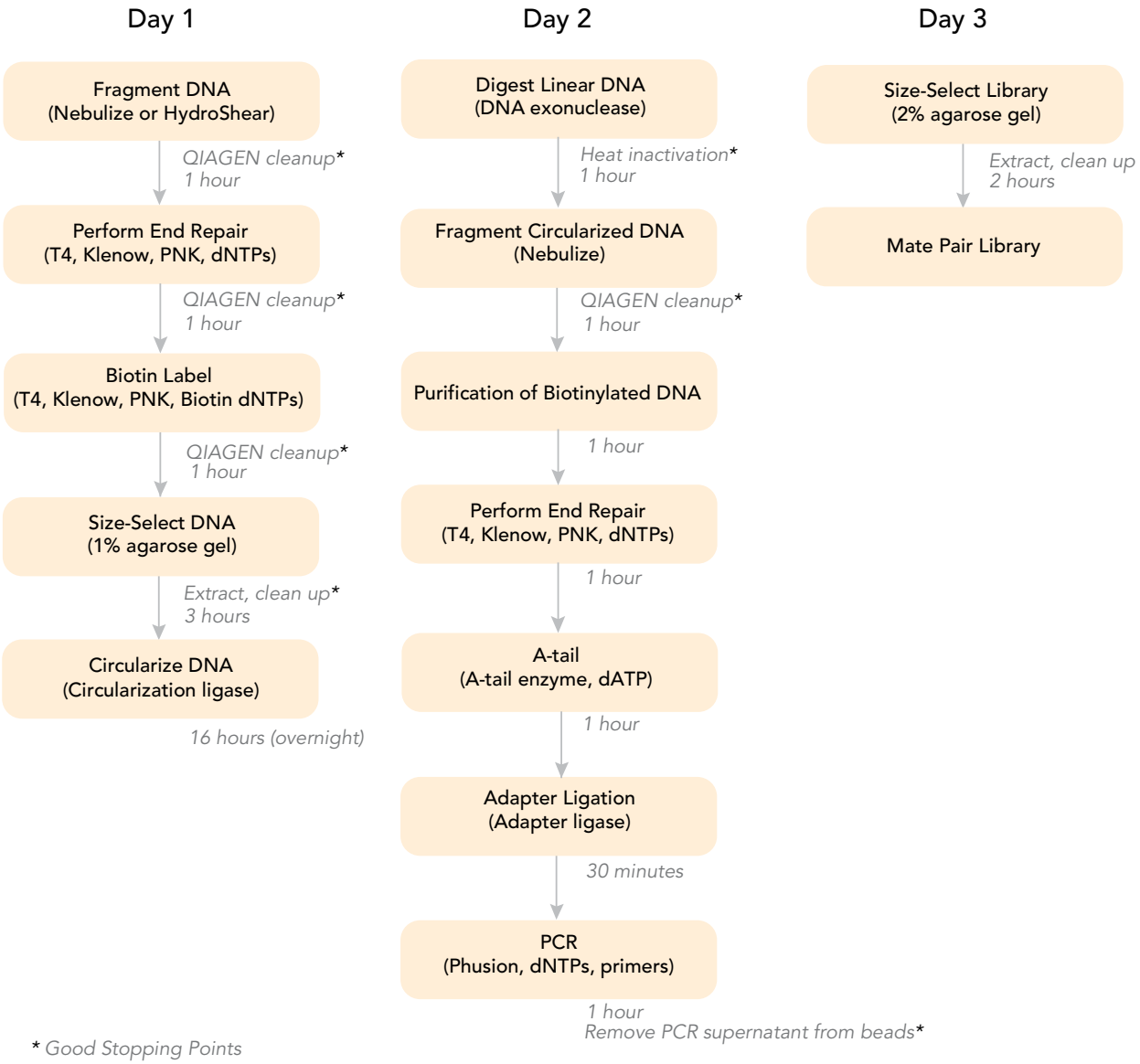


Figure 2 *Mate Pair Library Preparation Workflow for 2–5kb Gap Size*

Important Mate Pair Library Information

Before you start, read this section to learn important information about generating Mate Pair libraries.

Library Preparation

The Mate Pair Library Preparation Kit provides a protocol and reagents for generating mate pair libraries with a gap size of 2–5kb. The protocol recommends using 10 µg of starting genomic material, and this kit provides sufficient reagents to generate ten 2–5kb libraries.

Library Diversity

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 recovered size-selected material
- The size and variance of the size-selected fragments
- The genome size of the organism being sequenced

Further details on measuring library diversity, including a download for the GA Mate Pair Evaluation Script, can be found on the iCom website at icom.illumina.com under **Downloads | Software**.

Starting Material

High-quality starting material is extremely important for successful library generation. The DNA sample should be highly pure, having an OD 260/280 ratio of between 1.8 and 2, and should be as intact as possible. It is important to quantitate the input material carefully, preferably using a fluorescence-based quantitation method. This is because UV spectrometer methods based on 260 OD readings are prone to overestimating genomic DNA concentrations due to the presence of RNA and other contaminants commonly found in genomic DNA preparations.

Poor-quality or lower quantities of starting DNA can significantly impact the amount of size-selected DNA recovered and lower the diversity achieved in the final library.

DNA Fragmentation

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 2–3kb 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 yields 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 Library Preparation Kit is optimized around obtaining 200 ng of size-selected material for input into the circularization ligation.

This protocol uses agarose gel electrophoresis to allow selection of DNA fragments of a precise and defined size range. The labelled DNA will appear 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 will depend on the experimental design and ultimate use of the mate pair library.

Chimeras

The circularization step joins the two ends of the size-selected DNA molecules together via a blunt intramolecular ligation, with the junction of the two joined ends of the molecules identified in the circle by the biotin label. The quantity of the size-selected material used in the ligation will affect library diversity and the levels of chimeras (i.e., false mate pairs). Chimeras form from intermolecular ligation events where two or more DNA molecules are joined together.

Prior to the circularization step, Illumina advocates visualizing and quantifying the size-selected DNA using an Agilent Bioanalyzer. Our protocol recommends using up to 200 ng of DNA in a circularization reaction volume of 100 µl. Using greater DNA amounts leads to elevated levels of chimeric reads, although it also advantageously generates a more diverse library. Conversely, using lower amounts of DNA leads to fewer chimeric reads, but also a less diverse library.

Inward and Outward Facing Paired Reads

A consequence of the circularization of the size-selected fragments and the subsequent random shearing of ligated circles is the generation of two populations of fragments:

- 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 internal sequence fragments are washed away.

However, a small fraction of these unbiotinylated internal fragments can persist within the library preparation, leading to two distinct and identifiable populations of paired reads upon sequencing.

The sequence reads from fragments originating from the ends of the size-selected fragment will align back to a reference sequence as outward facing reads with a gap size of approximately the original size-selected fragment (e.g. 3kb). Fragments originating from the internal sequence of the size-selected fragments will align back to the reference as inward facing reads, with a smaller gap size of around 300–500 bp.

Figure 3 shows a schematic explanation of the origin of inward and outward facing reads.

Inward Facing Paired Read Reduction

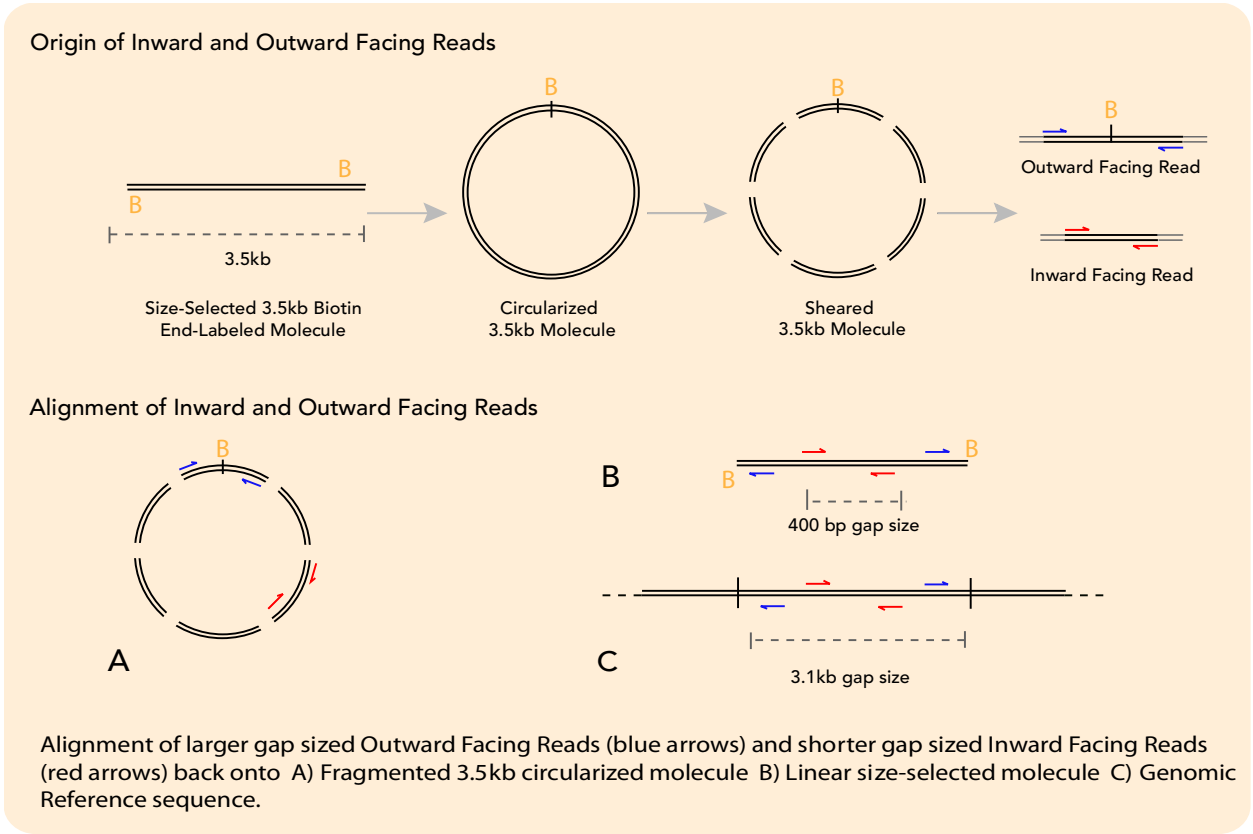
When generating mate pair libraries with larger fragments (e.g., 5kb), there is a tendency for the relative number of inward facing reads to increase in the library. This kit supplies an optional wash buffer (Streptavidin Bead Wash Buffer 2), which provides a more stringent wash step for removal of the unbiotinylated fragments that lead to inward facing reads. A disadvantage of using this additional wash buffer is the removal of some of the desired biotinylated end fragments too, leading to a reduction in the overall diversity of the library. The user can choose to implement this step or not, depending on whether they wish to minimize inward facing reads or maximize the diversity of the library.

Sequencing Length and Junction Reads

When sequencing a mate pair library, Illumina recommends a read length no longer than 36 bases. Increasing read length will lead to elevated error rates. This is due to the increased possibility that any particular sequence read will pass through the junction of the two joined ends of a size-selected fragment. When using the standard Illumina analysis pipeline, such junction reads are discarded, since they would not align to the reference sequence.

Due to these potential junction reads, a library of a size range of 400–600 bp is excised from the gel during the final library size selection. This is larger than a typical paired-end library of 200–300 bp. Increasing the size range of the library in the mate pair protocol minimizes the number of sequence reads that will pass through a junction.

Figure 3 illustrates the origin of the inward and outward facing reads.



Alignment of larger gap sized Outward Facing Reads (blue arrows) and shorter gap sized Inward Facing Reads (red arrows) back onto A) Fragmented 3.5kb circularized molecule B) Linear size-selected molecule C) Genomic Reference sequence.

Figure 3 *Origin and Alignment of Inward and Outward Facing Reads*

Kit Contents

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 that you centrifuge the tubes briefly before use.

Mate Pair Library Prep Kit, 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.

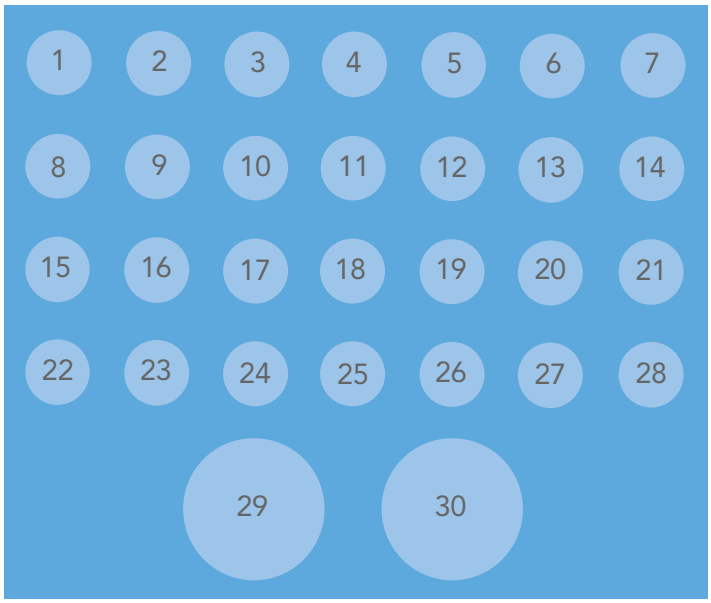


Figure 4 Mate Pair Library Prep Kit, Box 1

1. Ultra Pure Water, part # 1001913
2. End Repair Buffer 10X, part # 1006578
3. dNTP Mix 25 mM, part # 11318102
4. T4 DNA Polymerase, part # 1006575
5. T4 Polynucleotide Kinase, part # 1006577
6. Klenow DNA Polymerase, part # 1006576
7. Biotin dNTP Mix 1 mM, part # 1006584
8. Circularization Buffer 10X, part # 1006640
9. Circularization Ligase, part # 1006583
10. DNA Exonuclease, part # 1006580
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 uM, part # 1006582

16. Adapter Ligase, part # 1006573

17. Phusion DNA Polymerase, part # 1000524

18. PCR Primer PE 1.0, part # 1001783

19. PCR Primer PE 2.0, part # 1001784

20. -30. Empty

Mate Pair Library Prep Kit, Box 2

Store at Room Temperature

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

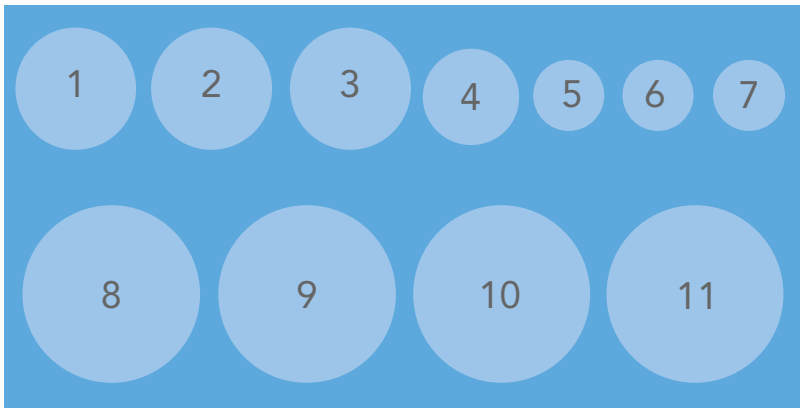


Figure 5 Mate Pair Library Prep Kit, Box 2

1. Streptavidin Bead Binding Buffer, part # 1006643

2. Streptavidin Bead Wash Buffer 2, part # 1006645

3. Empty

4. Empty

5. Empty

6. Empty

7. EDTA (0.5 M), part # 1006585

8. Streptavidin Bead Wash Buffer 1, part # 1006644

9. Empty

10. Empty

11. Empty

User-Supplied Consumables and Equipment

Check that you have all of the following user-supplied consumables and equipment before beginning library preparation.

Consumables

- QIAquick PCR Purification Kit (QIAGEN, part # 28104)
- QIAquick Gel Extraction Kit (QIAGEN, part # 28704)
- MinElute PCR Purification Kit (QIAGEN, part # 28004)
- Nebulizers (Illumina, catalog # FC-301-1001)
- PVC tubing (Intersurgical, part # 1174-003)
- Compressed air source of at least 32 psi
- HydroShear wash solutions
  - 0.2 M HCl
  - 0.2 M NaOH
  - TE pH 8.0
- Molecular biology agarose (BIO-RAD, part # 161-3101)
- Low range ultra agarose (BIO-RAD, part # 161-3107)
- 50X TAE Buffer (BIO-RAD, part # 161-0743)
- Distilled water
- Ethidium bromide (Sigma, part # E1510)
- Loading Buffer 4X (50 mM Tris pH 8.0, 40 mM EDTA, 40% (w/v) sucrose, 0.25% (w/v) bromophenol blue)
- 1 kb Plus DNA ladder (Invitrogen, part # 10787-026)
- Dynabeads M-280 Streptavidin Magnetic Beads (Invitrogen, part # 112-05D)
- Disposable scalpels
- 0.2 ml thin wall PCR tubes (Axygen, part # PCR-02-C)
- TWEEN 20 (Sigma, part # P7949)

Equipment

- Benchtop microcentrifuge
- Benchtop centrifuge with swing-out rotor (e.g., Sorvall Legend RT)
- Thermal cycler or PCR machine
- Heat block
- HydroShear (GeneMachines)
- Gel tray and electrophoresis unit (Fisher, part # FB57161)
- Gel comb with wide wells (Fisher, part # FB58325)
- Dark reader transilluminator (Clare Chemical Research, part # D195M)
- Magnetic rack for microfuge tubes (Invitrogen, part # CS15000)
- Bioanalyzer (Agilent) or UV-Spectrophotometer

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 gap size required for the paired sequencing reads. This protocol is recommended for gap sizes of 2–5kb.

DNA can be fragmented by a number of techniques. Two examples, HydroShear and nebulization, are outlined in this protocol.

HydroShear Method

User-Supplied Consumables

- Genomic DNA
  - 10 µg for 2–5kb gap size libraries

  DNA should be as intact as possible, with no contamination and preferably quantitated by a fluorescence-based method.
- QIAGEN EB (from PCR Purification Kit, QIAGEN part # 28104) or TE Buffer
- Wash Solutions (0.2 micron filtered)
  - Buffer 1: 0.2 M HCl
  - Buffer 2: 0.2 M NaOH
  - Buffer 3: TE pH 8.0
- QIAquick PCR Purification Kit (QIAGEN, part # 28104)

The following instructions are guidelines for using the HydroShear. Illumina recommends that you familiarize yourself with the manufacturer's instructions and calibration of the individual HydroShear assemblies to optimize shearing of DNA to your desired size range.

Sample Preparation

1. Dilute 10 µg of genomic DNA to approximately 150 ng/µl in QIAGEN EB or TE Buffer, for a final volume of approximately 70 µl per sample preparation.
2. Incubate at 37°C for 30 minutes in a hot block, occasionally mixing the solution.
3. Immediately before shearing, centrifuge at 18,000 g on a bench-top centrifuge for 15–30 minutes.
4. Transfer the DNA solution to a clean tube, leaving behind any pellet that may have formed during centrifugation.

   The sample is ready for shearing.

Shearing

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

1. Set the shearing conditions as follows:

   For 2–5kb fragments:

   - Volume: 70 µl
   - Cycle Number: 20
   - Assembly / Speed Code: Standard / 6–14

2. Perform 12 wash cycles (4 cycles per buffer) as per manufacturer's recommendations.
3. Load and shear the sample following manufacturer's directions and eject the sheared sample into a clean tube.
4. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample solution on one QIAquick column, eluting in 50 µl of QIAGEN EB.

   *This is a possible stopping point. Store the sample at -15° to -25°C.

Nebulization Method

User-Supplied Consumables

- 10 µg genomic DNA for 2–5kb gap size libraries

  DNA should be as intact as possible, with no contamination and preferably quantitated by a fluorescence-based method.
- Nebulizers (Illumina, catalog # FC-301-1001)
- PVC tubing (Intersurgical, part # 1174-003)
- Compressed air of at least 7.5 psi
- QIAquick PCR Purification Kit (QIAGEN, part # 28104)

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

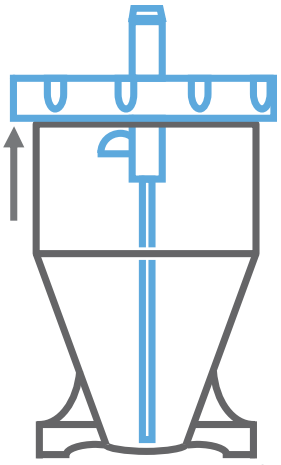


Figure 6 Remove the Nebulizer Lid

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

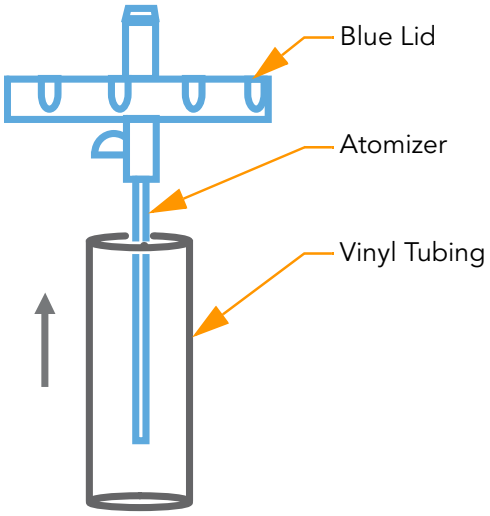


Figure 7 Assemble the Nebulizer

3. Add 10 µg of purified DNA in a total volume of 50 µl of TE Buffer to the nebulizer. Illumina recommends 10 µg as a standard amount of DNA to start.
4. Add 700 µl Nebulization Buffer to the DNA and mix well.
5. Screw the lid back on (finger-tight).

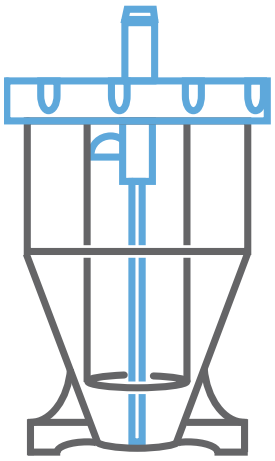


Figure 8 Replace the Nebulizer Lid

6. Chill the nebulizer containing the DNA solution on ice.
7. Connect the compressed air source to the inlet port on the top of the nebulizer with the PVC tubing, ensuring a tight fit.

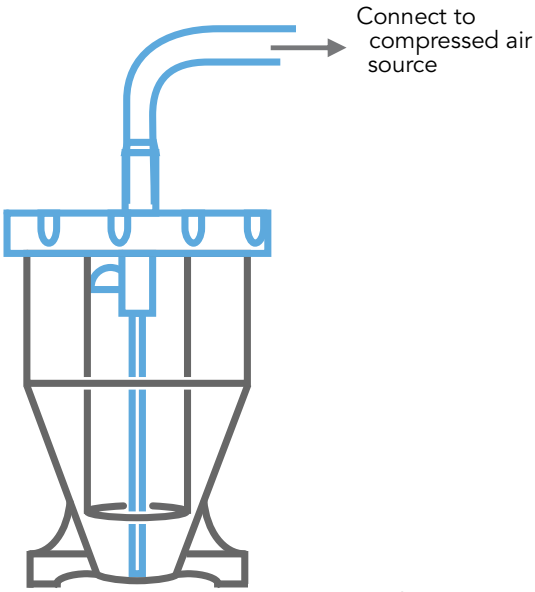


Figure 9 *Connect Compressed Air*

8. Bury the nebulizer in an ice bucket and place it in a fume hood.
9. Use the regulator on the compressed air source to ensure the air is delivered at 7.5 psi.
10. Nebulize for 30 seconds. You may notice vapor rising from the nebulizer; this is normal. These conditions generate fragment sizes in the 2–5kb range.
11. Centrifuge the nebulizer at 450 xg in a benchtop centrifuge for 2 minutes 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 QIAquick PCR Purification Kit to rinse the sides of the nebulizer and collect the DNA solution at the base of the nebulizer.
12. Measure the recovered volume. Typically, you should recover approximately 750 µl.
13. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample solution and concentrate it on one QIAquick column, eluting in 50 µl of QIAGEN EB.

*This is a possible stopping point. Store the sample at -15° to -25°C.

Perform End Repair

Shearing the genomic DNA generates double-stranded DNA fragments containing 3' or 5' overhangs. This protocol converts the overhangs resulting from fragmentation into blunt ends, using T4 DNA polymerase and Klenow DNA polymerase. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs, and the polymerase activity fills in the 5' overhangs. T4 Polynucleotide Kinase phosphorylates the 5' ends of the DNA.

Consumables

Illumina-Supplied

- End Repair Buffer 10X
- Ultra Pure Water
- dNTP Mix 25 mM
- T4 DNA Polymerase
- T4 Polynucleotide Kinase
- Klenow DNA Polymerase

User-Supplied

- Fragmented DNA
- QIAquick PCR Purification Kit (QIAGEN, part # 28104)

Procedure

1. Vortex the End Repair Buffer until all of the precipitate is dissolved.
2. Prepare the following reaction mix:

Table 1 *Reaction Mix Measurements*

Reagent	2–5kb
Fragmented DNA	50 µl
End Repair Buffer	10 µl
Ultra Pure Water	27.4 µl
dNTP mix	1.6 µl
T4 DNA Polymerase	5 µl
T4 Polynucleotide Kinase	5 µl
Klenow DNA Polymerase	1 µl
Total Volume	100 µl

3. Incubate for 30 minutes at 20°C in a thermal cycler.
4. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample solution on one QIAquick column, eluting in 50 µl of QIAGEN EB.

*This is a possible stopping point. Store the sample at -15° to -25°C.

Biotin Label

This protocol uses T4 and Klenow DNA polymerase to incorporate biotin dNTPs near the 3' ends of the DNA fragments. The exonuclease and polymerase activity of these enzymes allows an exchange reaction at the 3’ termini of the DNA, removing natural dNTPs and replacing them with biotin dNTPs. The ends of the DNA fragments remain blunt but are labeled by the biotin. T4 Polynucleotide Kinase phosphorylates the 5' ends of the DNA.

Consumables

Illumina-Supplied

- End Repair Buffer 10X
- Ultra Pure Water
- Biotin dNTP Mix 1 mM
- T4 DNA Polymerase
- T4 Polynucleotide Kinase
- Klenow DNA Polymerase

User-Supplied

- End-Repaired DNA
- QIAquick PCR Purification Kit (QIAGEN, part # 28104)

Procedure

1. Vortex the End Repair Buffer until all of the precipitate is dissolved.
2. Prepare the following reaction mix:

Table 2 *Reaction Mix Measurements*

Reagent	2–5kb
End-Repaired DNA	50 µl
End Repair Buffer	10 µl
Ultra Pure Water	19 µl
Biotin dNTP Mix	10 µl
T4 DNA Polymerase	5 µl
T4 Polynucleotide Kinase	5 µl
Klenow DNA Polymerase	1 µl
Total Volume	100 µl

3. Incubate for 30 minutes at 20°C in a thermal cycler.
4. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample solution on one QIAquick column, eluting in 30 µl of QIAGEN EB.

*This is a possible stopping point. Store the sample at -15° to -25°C.

Size-Select DNA

This protocol 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.

This Mate Pair Library Preparation Kit is optimized around obtaining 200 ng of size-selected material for input into the subsequent circularization step.

Consumables

User-Supplied

- Biotin-labeled DNA
- Molecular Biology Agarose (BIO-RAD, part # 161-3101)
- 50X TAE Buffer (BIO-RAD, part # 161-0743)
- Distilled Water
- Ethidium Bromide (Sigma, part # E1510)
- Loading Buffer 4X (50 mM Tris pH 8.0, 40 mM EDTA, 40% (w/v) sucrose, 0.25% (w/v) bromophenol blue)
- 1kb Plus DNA Ladder (Invitrogen, part # 10787-026)
- Disposable scalpels
- QIAquick Gel Extraction Kit (QIAGEN, part # 28704)

It is important to follow the gel preparation and run conditions given below to ensure reproducibility of the procedure. Illumina does not recommend purifying multiple samples on a single gel due to the risk of cross-contamination of libraries.

Procedure

1. Prepare a 1% agarose gel in a final volume of 150 ml 1X TAE Buffer (dilute stock solution with distilled water). Use a gel comb with wide wells. Recommended well size is 1 mm (d) x 9 mm (w) x 10 mm (h).
2. Add the ethidium bromide to the gel during preparation at a concentration of 400 ng/ml.
3. Add 1 µl of Loading Buffer to 3 µl of the ladder.
4. Load all of the ladder solution in one lane of the gel.
5. Add 10 µl of Loading Buffer to the 30 µl biotin-labeled DNA.
6. Load the entire sample in one lane of the gel, leaving at least one empty lane between ladder and sample.
7. Run the gel long enough to give sufficient separation at the size range for purification.

   The running conditions will depend on the type of agarose and the gel apparatus used. Using the recommended reagents and apparatus, the following can be used as a guide.

   - Run the gel at 120 V for 2 hours.
8. View the gel on a Dark Reader transilluminator.

9. Excise a single slice of gel with a clean scalpel.

   The size of the fragments purified determines the gap size of the paired reads. The size of the gel piece will determine the spread of the gap size distribution.

Table 3 Gap Sizes and Purified Fragment Sizes

Gap Size Required	Fragment Size Range Purified From Gel
2kb	2–2.5kb
3kb	3–3.5kb
4kb	4–4.5kb
5kb	5–5.5kb

Figure 10 shows a 1% agarose gel containing sheared and biotinylated genomic DNA, with a gel slice excised at approximately 2.5kb.

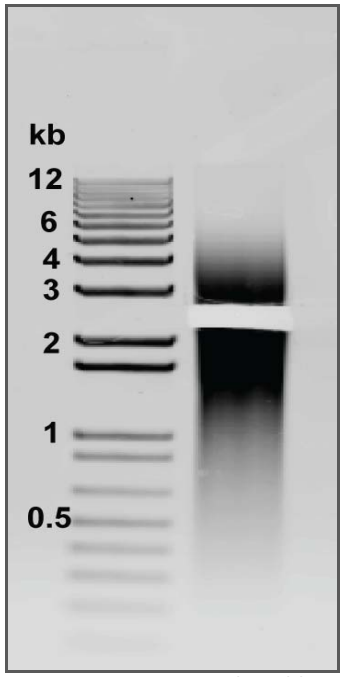


Figure 10 Excised 2.5kb Gel Slice

Use a QIAquick Gel Extraction kit to purify the gel-excised fragment. Follow the instructions to purify the DNA from the agarose slices, eluting in 50 µl of QIAGEN EB.

10. Check the size distribution and quantify the purified DNA using an Agilent Bioanalyzer, as shown in Figure 11.

*This is a possible stopping point. Store the sample at -15° to -25°C.

Figure 11 shows an Agilent 2100 profile of a purified size-selected sample using a 7500 LabChip. The concentration of this sample is

5.73 ng/µl. 35 µl (200 ng) of this purified sample is used directly in the subsequent circularization reaction.

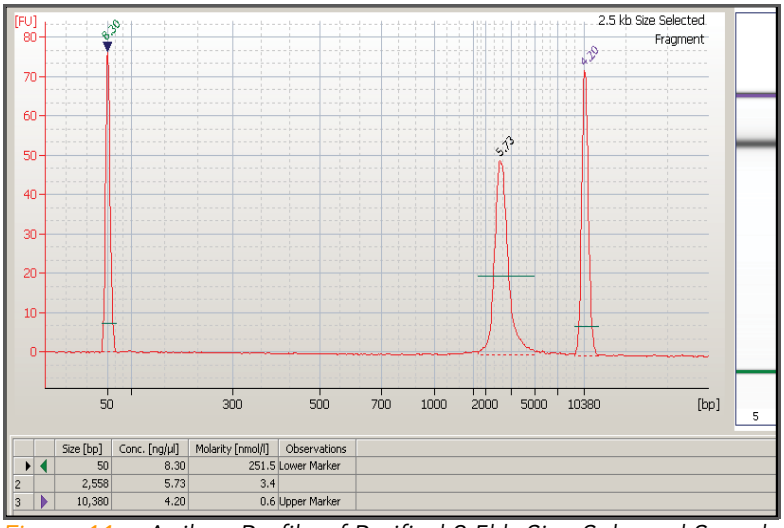


Figure 11 Agilent Profile of Purified 2.5kb Size-Selected Sample

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 biotin label.

Consumables

Illumina-Supplied

- Circularization Buffer
- Ultra Pure Water
- Circularization Ligase

User-Supplied

- Size-selected DNA (up to 200 ng as quantified on an Agilent Bioanalyzer)

Procedure

1. Vortex the Circularization Buffer until all of the precipitate is dissolved.
2. Prepare the following reaction mix in a 0.2 ml PCR tube:
   - Size-selected DNA (up to 200 ng, quantified on Bioanalyzer)
   - Circularization Buffer (10 µl)
   - Ultra Pure Water (add for a final reaction volume of 100 µl)
   - Circularization Ligase (6.7 µl)

   The total volume should be 100 µl.
3. Incubate overnight for 16 hours at 16°C.

Digest Linear DNA

This protocol uses a DNA exonuclease to remove any linear DNA fragments from the circularization reaction. Linear fragments result from DNA that did not ligate and intermolecular ligation products (linear concatemers).

Consumables

Illumina-Supplied

- DNA Exonuclease
- EDTA 0.5 M

User-Supplied

- Circularized DNA

Procedure

1. Add 1 µl of DNA Exonuclease to 100 µl of circularized DNA.
2. Incubate at 37°C for 20 minutes followed by 70°C for 30 minutes.
3. Add 4 µl of EDTA to the exonuclease-treated sample.

*This is a possible stopping point. Store the sample at -15° to -25°C.

Fragment Circularized DNA

This protocol randomly fragments the circularized DNA to lengths of approximately 400 bp in size. Nebulization is outlined in this protocol, which fragments DNA to less than 800 bp in minutes using a disposable device. A Covaris shearing device could also be used.

Consumables

User-Supplied

- Exonuclease-treated DNA
- Nebulizers (Illumina, catalog # FC-301-1001)
- PVC tubing (Intersurgical, part # 1174-003)
- Compressed air source of at least 32 psi
- QIAquick PCR Purification Kit (QIAGEN, part # 28104)

NOTE

The following instructions are guidelines for using the nebulizer. Illumina recommends calibrating the air pressure required for nebulization, to optimize shearing of DNA to your desired size range.

Procedure

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

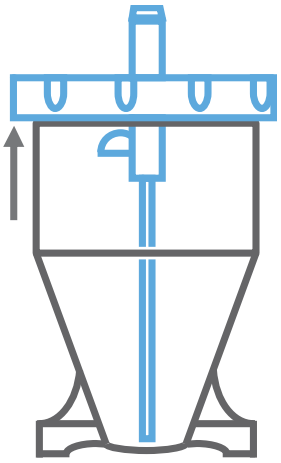


Figure 12 Remove the Nebulizer Lid

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.

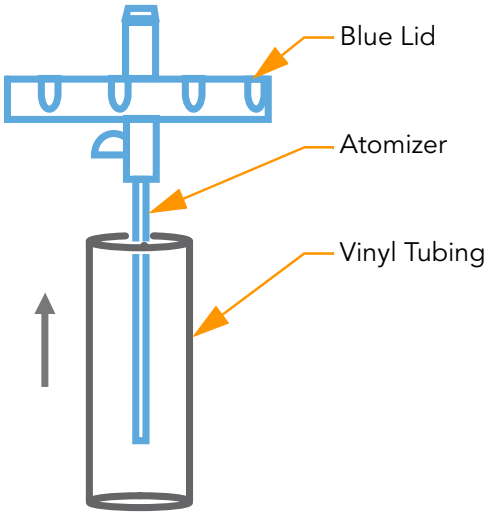


Figure 13 Assemble the Nebulizer

3. Add exonuclease-treated DNA to the nebulizer.
4. Add 700 µl of Nebulization Buffer to the DNA and mix well.
5. Screw the lid back on finger-tight.

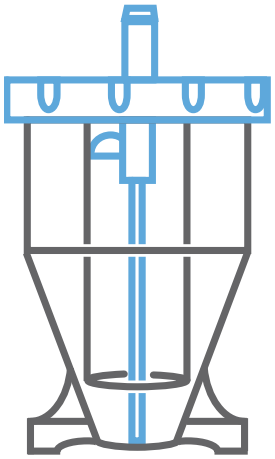


Figure 14 Replace the Nebulizer Lid

6. Chill the nebulizer containing the DNA solution on ice.
7. Connect the compressed air source to the inlet port on the top of the nebulizer with the PVC tubing, ensuring a tight fit.

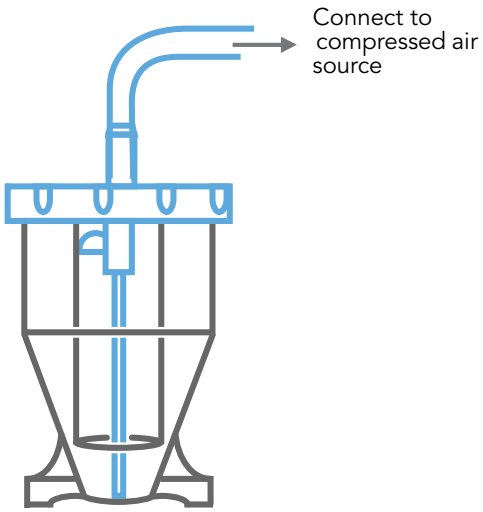


Figure 15 Connect Compressed Air

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. You may notice vapor rising from the nebulizer; this is normal.
11. Centrifuge the nebulizer to 450 xg for 2 minutes in a benchtop centrifuge (e.g., Sorvall Legend RT) 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.
12. Measure the recovered volume. Typically, recovery is approximately 400 µl.
13. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample solution and concentrate it on one QIAquick column, eluting in 50 µl of QIAGEN EB.

*This is a possible stopping point. Store the sample at -15° to -25°C.

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 (Invitrogen, part # 112-05D)
- QIAGEN EB Buffer (from PCR Purification Kit, QIAGEN, part # 28104)

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 pipetting up and down.
3. Transfer 20 µl of resuspended beads into a 1.5 ml microfuge tube.
4. Place the tube in the magnetic rack for 1 minute to separate the beads from the solution.
5. Remove the supernatant with a pipette, while the tube remains in the magnetic rack, and then discard the supernatant.
6. Remove the tube from the magnetic rack and wash the beads in 50 µl of Streptavidin Bead Binding Buffer by carefully pipetting up and down.
7. Place the tube in the magnetic rack for one minute, and then remove and discard the supernatant.
8. Repeat the wash once and discard the wash supernatant.
9. Remove the tube from the magnetic rack and resuspend the beads in 50 µl of fresh Streptavidin Bead Binding Buffer.
10. Add 50 µl of fragmented DNA and incubate for 15 minutes at 20° C. Resuspend the beads every two minutes by gentle mixing.
11. Place the tube in the magnetic rack for 1 minute. Remove and discard the supernatant.
12. Wash the beads in 200 µl of Streptavidin Bead Wash Buffer 1. Ensure that Wash Buffer 1 is used at this step of the protocol and not Wash Buffer 2. Remove the tube from the magnetic rack and resuspend the beads in the wash solution by pipetting up and down five times.
13. Remove the supernatant from the beads using the magnet and discard. Repeat the same wash three times, discarding each wash supernatant.

14. Wash the beads in 200 µl of QIAGEN EB. Remove the tube from the magnetic rack and resuspend the beads in the wash solution by pipetting up and down five times.
15. Remove the supernatant from the beads using the magnet and discard. Repeat the same wash once.
16. Remove the final wash solution from the beads using the magnet and discard. Remove the tube from the magnetic rack and resuspend the beads in 50 µl of QIAGEN EB.

Perform End Repair

Shearing the circularized DNA generates double-stranded DNA fragments containing 3' or 5' overhangs. This protocol converts the overhangs resulting from fragmentation into blunt ends, using T4 DNA polymerase and Klenow DNA polymerase. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs. T4 Polynucleotide Kinase phosphorylates the 5' ends of the DNA. 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
- dNTP Mix 25 mM
- T4 DNA Polymerase
- T4 Polynucleotide Kinase
- Klenow DNA Polymerase
- Streptavidin Bead Wash Buffer 1

User-Supplied

- Washed beads from previous section
- QIAGEN EB (from PCR Purification Kit, QIAGEN, part # 28104)

Procedure

1. Vortex the End Repair Buffer until all of the precipitate is dissolved.
2. Prepare the following reaction mix in a 0.2 ml PCR tube:
   - Beads from previous section (50 µl)
   - End Repair Buffer (10 µl)
   - Ultra Pure Water (27.4 µl)
   - dNTP Mix (1.6 µl)
   - T4 DNA Polymerase (5 µl)
   - T4 Polynucleotide Kinase (5 µl)
   - Klenow DNA Polymerase (1 µl)

   The total volume should be 100 µl.
3. Ensure that the beads are resuspended in the end repair mix by pipetting up and down gently.
4. Incubate for 30 minutes at 20°C in a thermal cycler.
5. Transfer the bead mix to a 1.5 ml microfuge tube.
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 µl of Streptavidin Bead Wash Buffer 1. Ensure that Wash Buffer 1 is used at this step of the protocol and not Wash Buffer 2. Remove the tube from the magnetic rack and resuspend the beads in the wash solution by pipetting up and down five times.
9. Remove the supernatant from the beads using the magnet and discard. Repeat the same wash three times, discarding each wash supernatant.
10. Wash the beads in 200 µl of QIAGEN EB. Remove the tube from the magnetic rack and resuspend the beads in the wash solution by pipetting up and down five times.
11. Remove the supernatant from the beads using the magnet and discard. Repeat the same wash once.
12. Remove the final wash solution from the beads using the magnet and discard. Remove the tube from the magnetic rack and resuspend the beads in 32 µl of QIAGEN EB.

Add ‘A’ Bases to the 3' Ends of 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
- Streptavidin Bead Wash Buffer 1

User-Supplied

- Washed beads from previous section
- QIAGEN EB (from PCR Purification Kit, QIAGEN, part # 28104)

Procedure

1. Prepare the following reaction mix in a 0.2 ml PCR tube:
   - Beads from previous section (32 µl)
   - A-Tailing Buffer (5 µl)
   - 1 mM dATP (10 µl)
   - A-Tailing Enzyme (3 µl)

   The total volume should be 50 µl.
2. Ensure the beads are resuspended in the A-Tailing mix by pipetting up and down gently.
3. Incubate for 30 minutes at 37°C in a thermal cycler.
4. Transfer the bead mix to a 1.5 ml microfuge tube.
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. Ensure that Wash Buffer 1 is used at this step of the protocol and not Wash Buffer 2. Remove the tube from the magnetic rack and resuspend the beads in the wash solution by pipetting up and down five times.
8. Remove the supernatant from the beads using the magnet and discard. Repeat the same wash three times, discarding each wash supernatant.
9. Wash the beads in 200 µl of QIAGEN EB. Remove the tube from the magnetic rack and resuspend the beads in the wash solution by pipetting up and down five times.

10. Remove the supernatant from the beads using the magnet and discard. Repeat the same wash once.
11. Remove the final wash solution from the beads using the magnet and discard. Remove the tube from the magnetic rack and resuspend the beads in 19 µl of QIAGEN EB.

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
- Adapter Ligase
- Streptavidin Bead Wash Buffer 1
- Streptavidin Bead Wash Buffer 2 (optional)

User-Supplied

- Washed beads from previous section
- QIAGEN EB (from PCR Purification Kit, QIAGEN, part # 28104)

Procedure

1. Vortex the Adapter Ligation Buffer until all of the precipitate is dissolved.
2. Prepare the following reaction mix in a 0.2 ml PCR tube:
   - Beads from previous section (19 µl)
   - Adapter Ligation Buffer (25 µl)
   - PE Adapter Oligo Mix (1 µl)
   - Adapter Ligase (5 µl)

   The total volume should be 50 µl.
3. Ensure the beads are resuspended in the ligation mix by pipetting up and down gently.
4. Incubate for 15 minutes at 20°C in a thermal cycler.

   During the incubation, you can prepare the PCR mix as described on page 36.
5. Transfer the bead mix into a 1.5 ml microfuge tube.
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 µl of Streptavidin Bead Wash Buffer 1. Ensure that Wash Buffer 1 is used at this step of the protocol and not Wash Buffer 2. Remove the tube from the magnetic rack and resuspend the beads in the wash solution by pipetting up and down five times.

9. Remove the supernatant from the beads using the magnet and discard. Repeat the same wash three times, discarding each wash supernatant.
10. (Optional) For libraries with gap sizes of 5kb, Illumina recommends an extra wash step to remove more of the non-biotinylated DNA fragments, as follows:
    a. Wash the beads in 200 µl of Streptavidin Bead Wash Buffer 2.
    b. Remove the tube from the magnetic rack and resuspend the beads in the wash solution by pipetting up and down five times.
    c. Remove the supernatant from the beads using the magnet and discard.
    d. Repeat the same wash once, discarding the wash supernatant.
11. Wash the beads in 200 µl of QIAGEN EB. Remove the tube from the magnetic rack and resuspend the beads in the wash solution by pipetting up and down five times.
12. Remove the supernatant from the beads using the magnet and discard. Repeat the same wash once.
13. Remove the final wash solution from the beads using the magnet and discard. Remove the tube from the magnetic rack and resuspend the beads immediately in 50 µl of PCR mix.

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
- Ultra Pure Water
- PCR Primer PE 1.0
- PCR Primer PE 2.0

User-Supplied

- Washed beads from previous section

Procedure

1. Prepare the following PCR mix in a 0.2 ml PCR tube:
   - Phusion DNA Polymerase (25 µl)
   - Ultra Pure Water (23 µl)
   - PCR Primer PE 1.0 (1 µl)
   - PCR Primer PE 2.0 (1 µl)

   The total volume should be 50 µl.
2. Resuspend the beads in 50 µl of the PCR mix.
3. 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
4. Remove and retain the PCR supernatant from the beads using a magnetic rack.
5. Discard the beads.

*This is a possible stopping point. Store the sample at -15° to -25°C.

Size-Select Library

This protocol purifies the mate pair library fragments from a gel, selecting a particular size range (400–600 bp optimally) for cluster formation and sequencing.

Consumables

User-Supplied

- Amplified DNA
- Low-Range Ultra Agarose (BIO-RAD, part # 161-3107)
- 50X TAE Buffer (BIO-RAD, part # 161-0743)
- Distilled water
- Ethidium bromide (Sigma, part # E1510)
- Loading Buffer 4X (50 mM Tris pH 8.0, 40 mM EDTA, 40% (w/v) sucrose, 0.25% (w/v) bromophenol blue)
- 1kb Plus DNA ladder (Invitrogen, part # 10787-026)
- Disposable scalpels
- MinElute PCR Purification Kit (QIAGEN, part # 28004)
- TWEEN 20 (Sigma, part # P7949)

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

Procedure

1. Prepare a 2% agarose gel in a final volume of 150 ml 1X TAE buffer (dilute stock solution with distilled water).
2. Use a comb with wide wells (to accommodate 66 µl of sample per well).
   Recommended well size: 1 mm (d) x 9 mm (w) x 10 mm (h).
3. Add the ethidium bromide to the gel during preparation at a concentration of 400 ng/ml.
4. Add 1 µl of Loading Buffer to 3 µl of the ladder.
5. Load all of the ladder solution in one lane of the gel.
6. Add 16 µl of Loading Buffer to the amplified DNA.
7. Load the entire sample in one wide lane of the gel leaving at least a gap of one empty lane between ladder and sample.
8. Run the gel at 120 V for 60 minutes using the Illumina-recommended apparatus.
   The running conditions will depend on the gel apparatus used.
9. View the gel on a Dark Reader transilluminator.
10. Excise a piece of gel purifying DNA 400–600 bp in size with a clean scalpel, as shown in Figure 16.

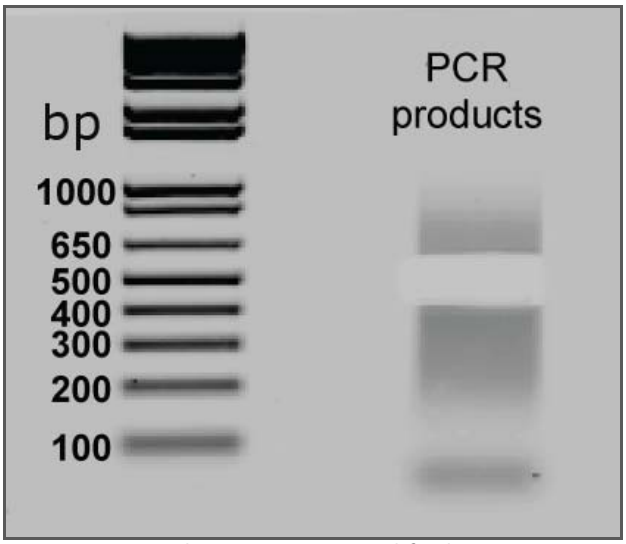


Figure 16 *Gel Containing Amplified Mate Pair Sample After Removal of Size-Selected Final Library*

11. Follow the instructions in the MinElute PCR Purification Kit to purify the sample solution and concentrate on one MinElute column, eluting in 15 µl of QIAGEN EB.

12. Add 1.5 µl 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-paired library prepared using this protocol is shown in Figure 17.

   Figure 17 shows an Agilent 2100 profile of a purified mate pair library using a 1000 LabChip. The typical concentration of a final library eluted in 15 µl of QIAGEN EB is between 5 and 25 nM.

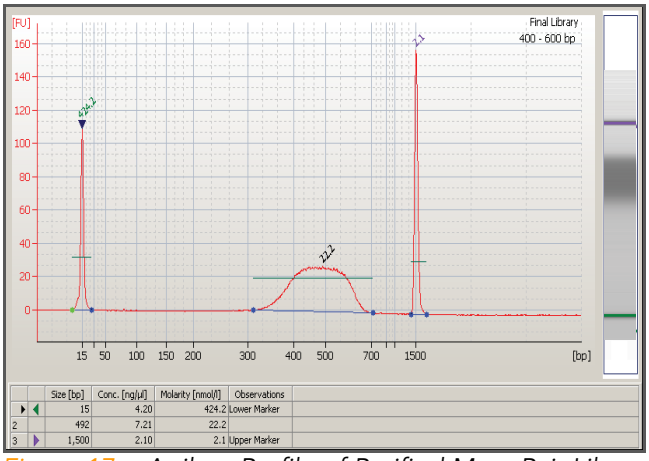


Figure 17 Agilent Profile of Purified Mate Pair Library

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 or a Nanodrop.

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

illumina®