

Preparing Samples for Paired-End Sequencing

FOR RESEARCH ONLY

Topics

- 3 Introduction
- 5 Sample Preparation Kit Contents
- 7 Fragment the Genomic DNA
- 11 Perform End Repair
- 12 Add 'A' Bases to the 3' End of the DNA Fragments
- 13 Ligate Adapters to DNA Fragments
- 14 Purify Ligation Products
- 16 Enrich the Adapter-Modified DNA Fragments by PCR
- 17 Validate the Library

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Introduction

This protocol explains how to prepare libraries of genomic DNA for paired-end analysis on the Illumina Cluster Station and Genome Analyzer. You will add adapter sequences onto the ends of DNA fragments to generate the following template format:

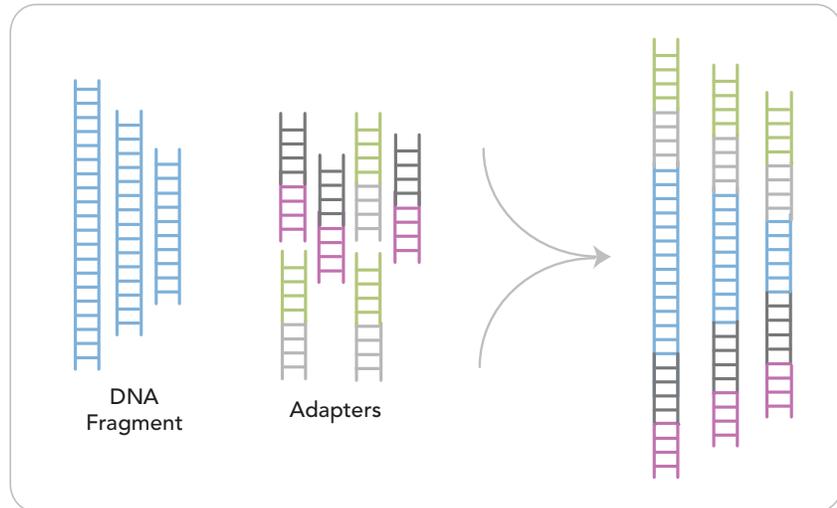


Figure 1 Fragments after Sample Preparation

The adapters contain sequences that correspond to the two surface-bound amplification primers on the flow cells used in the Cluster Station.

Sample Prep Workflow

The following figure illustrates the steps required to prepare samples for paired-end sequencing.

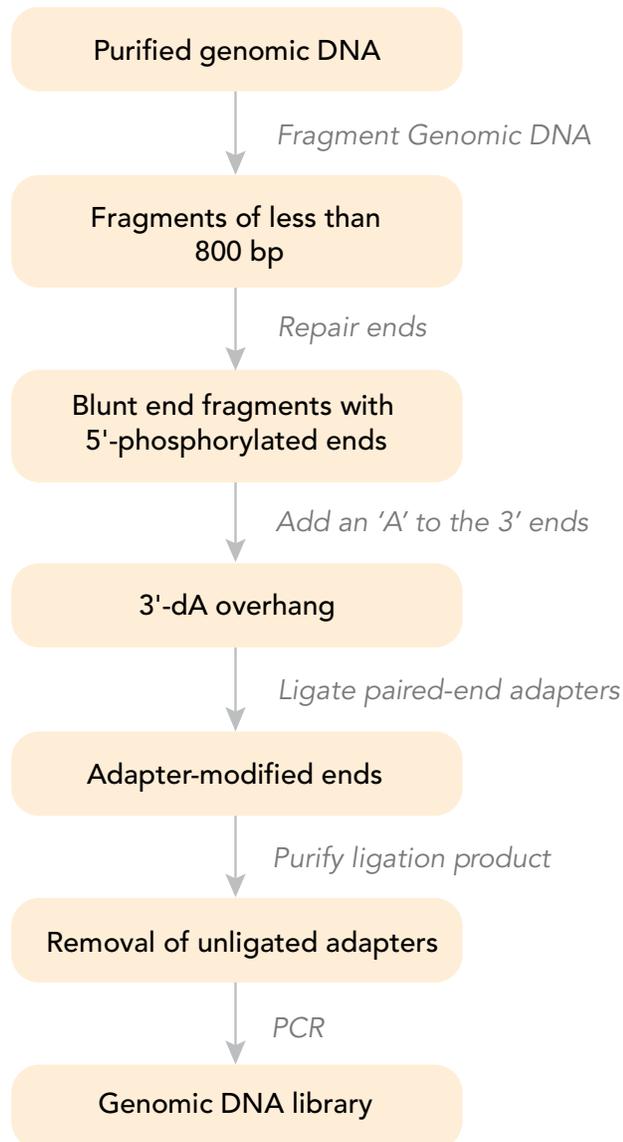


Figure 2 Sample Preparation Workflow

Sample Preparation Kit Contents

Check to ensure that you have all of the reagents identified in this section before proceeding to sample preparation.

Paired-End Sample Prep Kit, Box 1

Store at -20°C

This box is shipped at -80°C. As soon as you receive it, store the components at -20°C.

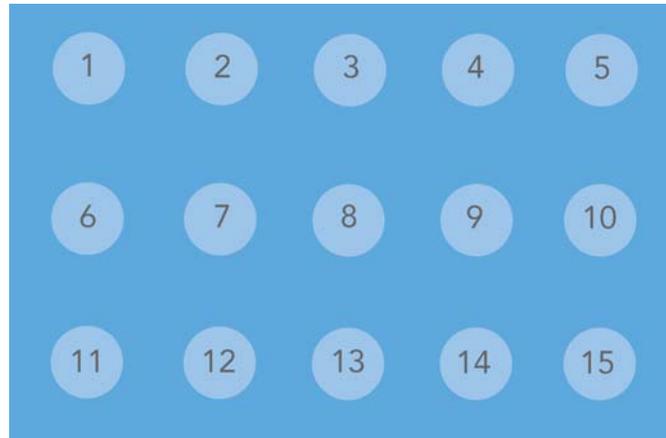


Figure 3 Paired-End Sample Prep Kit, Box 1

1. T4 DNA Ligase Buffer with 10 mM ATP, part # 1000534
2. Klenow Enzyme, part # 1000515
3. Klenow Buffer, part # 1000535
4. DNA Ligase Buffer 2X, part # 1000523
5. Phusion DNA Polymerase (Finnzymes Oy), part # 1000524
6. 10 mM dNTP Mix, part # 1001932
7. T4 PNK, part # 1000519
8. 1 mM dATP, part # 1000520
9. PE Adapter Oligo Mix, part # 1001782
10. PCR Primer PE 1.0, part # 1001783
11. T4 DNA Polymerase, part # 1000514
12. Empty
13. Klenow Fragment (3' to 5' exo minus), part # 1000536
14. DNA Ligase, part # 1000522
15. PCR Primer PE 2.0, part # 1001784

Paired-End Sample
Prep Kit, Box 2

Store at Room Temperature

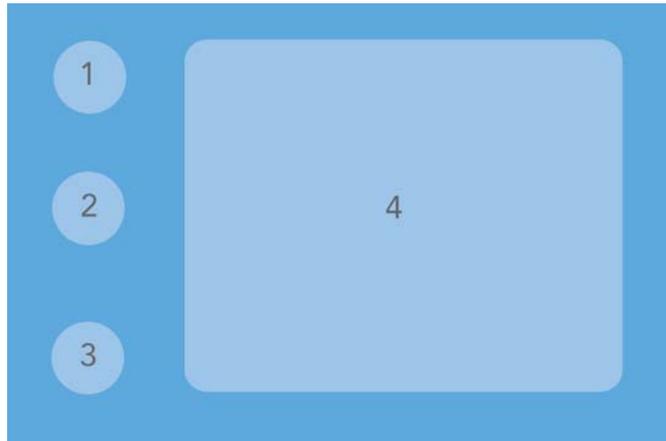


Figure 4 Paired-End Sample Prep Kit, Box 2

1. Nebulization Buffer, part # 1000466
2. TE Buffer, part # 1000465
3. Ultra Pure Water, part # 1000467
4. Nebulizer Kit (10 each), part # 1000541

Fragment the Genomic DNA

This protocol fragments the genomic DNA using a nebulization technique, which fragments DNA to less than 800 bp in minutes using a disposable device. Nebulization generates double-stranded DNA fragments comprised of 3' or 5' overhangs.

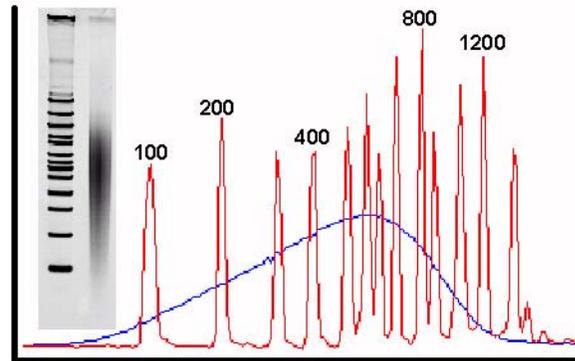


Figure 5 Fragment Genomic DNA

Consumables Illumina-Supplied

- ▶ Nebulizers (box of 10 nebulizers and vinyl accessory tubes)
- ▶ Nebulization buffer (7 ml)
- ▶ TE Buffer

User-Supplied

- ▶ QIAquick PCR Purification Kit (QIAGEN, part # 28104)
- ▶ Purified DNA (1–5 μ g, 5 μ g recommended)
DNA should be as intact as possible, with an OD₂₆₀/OD₂₈₀ ratio of 1.8–2
- ▶ Compressed air of at least 32 psi
- ▶ Clamp (1 per nebulizer)
- ▶ PVC tubing
 - Fisher Scientific, catalog # 14-176-102
 - Nalgene Labware, catalog # 8007-0060

Table 1 PVC Tubing Dimensions

ID	OD	Wall	Length
1/4 in.	3/8 in.	1/16 in.	1 meter



CAUTION

If you intend to nebulize DNA that could possibly contain any pathogenic sequences such as pathogenic viral DNA, perform the nebulization process under containment conditions (e.g., a biosafety cabinet) to prevent exposure to aerosols.

Procedure

The DNA sample to be processed should be highly pure, having an OD₂₆₀/280 ratio of between 1.8 and 2, and should be as intact as possible.



NOTE

If you are not familiar with this shearing method, Illumina recommends that you test this procedure on test samples before proceeding with your sample DNA.

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

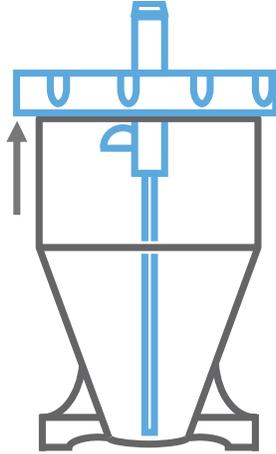


Figure 6 Remove the Nebulizer Lid

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

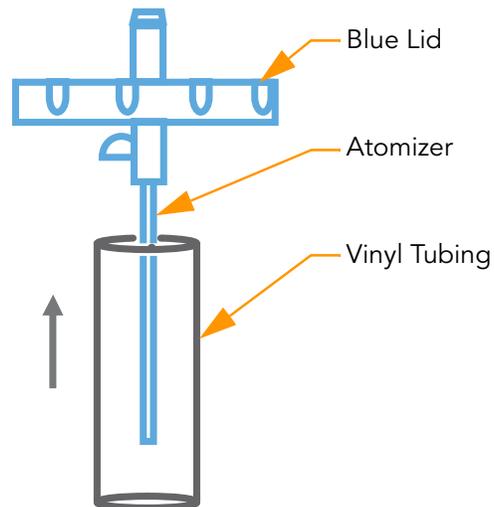


Figure 7 Assemble the Nebulizer

3. Add 1–5 μg of purified DNA in a total volume of 50 μl of TE buffer to the nebulizer.
4. Add 700 μl nebulization buffer to the DNA and mix well.
5. Screw the lid back on (finger-tight).

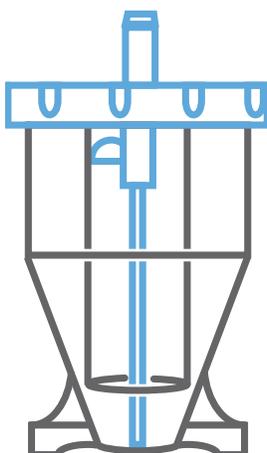


Figure 8 Replace the Nebulizer Lid

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. Secure with the small clamp.

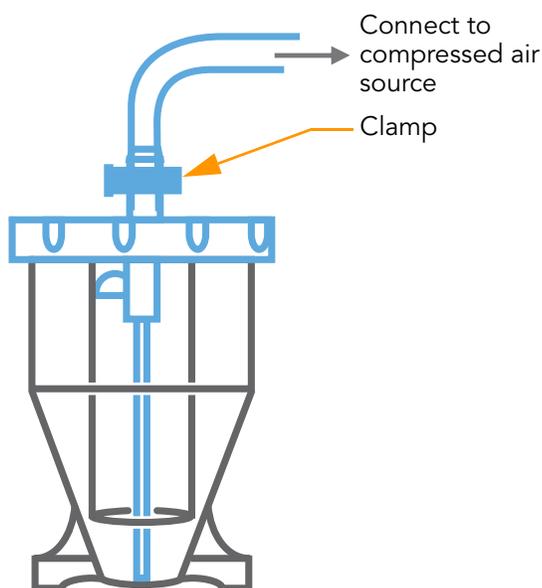


Figure 9 Connect Compressed Air

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 32–35 psi.
10. Nebulize for 6 minutes. You may notice vapor rising from the nebulizer; this is normal.
11. Centrifuge the nebulizer at 450 xg for 2 minutes to collect the droplets from the side of the nebulizer. If necessary, use an old nebulizer as a counter-balance.

12. If a centrifuge is not available, then use 2 ml of the binding buffer (PB or PBI buffer) from the QIAquick PCR Purification Kit to rinse the sides of the nebulizer and collect the DNA solution at the base of the nebulizer.
13. Measure the recovered volume. Typically, you should recover 400–600 μ l.
14. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample solution and concentrate it on one QIAquick column, eluting in 30 μ l of EB.

Perform End Repair

This protocol converts the overhangs resulting from fragmentation into blunt ends, using T4 DNA polymerase and Klenow enzyme. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs.

Consumables **Illumina-Supplied**

- ▶ T4 DNA ligase buffer with 10mM ATP
- ▶ 10 mM dNTP mix
- ▶ T4 DNA polymerase
- ▶ Klenow enzyme
- ▶ T4 PNK
- ▶ Water

User-Supplied

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

Procedure

1. Prepare the following reaction mix:
 - DNA sample (30 μ l)
 - Water (45 μ l)
 - T4 DNA ligase buffer with 10mM ATP (10 μ l)
 - 10 mM dNTP mix (4 μ l)
 - T4 DNA polymerase (5 μ l)
 - Klenow enzyme (1 μ l)
 - T4 PNK (5 μ l)The total volume should be 100 μ l.
2. Incubate in the thermal cycler for 30 minutes at 20°C.
3. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick column, eluting in 32 μ l of EB.

Add 'A' Bases to the 3' End of the DNA Fragments

This protocol adds an 'A' base to the 3' end of the blunt phosphorylated DNA fragments, using the polymerase activity of Klenow fragment (3' to 5' exo minus). This prepares the DNA fragments to be ligated to the adapters, which have a single 'T' base overhang at their 3' end.

Consumables **Illumina-Supplied**

- ▶ Klenow buffer
- ▶ 1 mM dATP
- ▶ Klenow exo (3' to 5' exo minus)

User-Supplied

- ▶ MinElute PCR Purification Kit (QIAGEN, part # 28004)



NOTE

This protocol requires a QIAquick MinElute column rather than a normal QIAquick column.

Procedure

1. Prepare the following reaction mix:
 - DNA sample (32 μ l)
 - Klenow buffer (5 μ l)
 - 1 mM dATP (10 μ l)
 - Klenow exo (3' to 5' exo minus) (3 μ l)The total volume should be 50 μ l.
2. Incubate for 30 minutes at 37°C.
3. Follow the instructions in the MinElute PCR Purification Kit to purify on one QIAquick MinElute column, eluting in 10 μ l of 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.

Consumables **Illumina-Supplied**

- ▶ DNA ligase buffer, 2X
- ▶ PE adapter oligo mix
- ▶ DNA ligase

User-Supplied

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

Procedure

This procedure uses a 10:1 molar ratio of adapter to genomic DNA insert, based on a starting quantity of 5 µg of DNA before fragmentation. If you started with less than 5 µg, titrate the volume of adapter reagent accordingly to maintain the 10:1 ratio of DNA.

1. Prepare the following reaction mix:

- DNA sample (10 µl)
- DNA ligase buffer, 2X (25 µl)
- PE adapter oligo mix (10 µl)
- DNA ligase (5 µl)

The total volume should be 50 µl.

2. Incubate in a thermal cycler for 15 minutes at 20°C.

3. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick column, eluting in 30 µl of EB.

Purify Ligation Products

This protocol purifies the products of the ligation reaction on a gel to remove all unligated adapters, remove any adapters that may have ligated to one another, and select a size-range of templates to go on the cluster generation platform.

Consumables User-Supplied

- ▶ Certified low-range Ultra Agarose (BIO-RAD, part # 161-3106)
- ▶ 50x TAE buffer
- ▶ Distilled water
- ▶ Ethidium bromide
- ▶ Loading buffer (50 mM Tris pH 8.0, 40 mM EDTA, 40% (w/v) sucrose)
- ▶ Low molecular weight DNA ladder (NEB, part # N3233L)
- ▶ QIAquick PCR Purification Kit (QIAGEN, part # 28104)

Procedure



CAUTION

Illumina does not recommend purifying multiple samples on a single gel due to the risk of cross-contamination between libraries.



NOTE

It is important to perform this procedure exactly as described, to ensure reproducibility.



NOTE

Illumina recommends that a Dark Reader is used to visualize DNA on agarose gels.



NOTE

It is important to excise as narrow a band as possible from the gel during gel purification. Paired-end libraries should consist of templates of the same size or nearly the same size.

1. Prepare a 150 ml, 2% agarose gel with distilled water and TAE. Final concentration of TAE should be 1X at 150 ml.
2. Add ethidium bromide (EtBr) after the TAE-agarose has cooled. Final concentration of EtBr should be 400 ng/ml (i.e., add 60 µg EtBr to 150 ml of 1X TAE).
3. Cast the gel in a tray that is approximately 14 cm in length. No ethidium bromide is required in the running buffer.

4. Add 3 μ l of loading buffer to 8 μ l of the ladder.
5. Add 10 μ l of loading buffer to 30 μ l of the DNA from the purified ligation reaction.
6. Load all of the ladder solution to one lane of the gel.
7. Load the entire sample in another lane of the gel, leaving a gap of at least one empty lane between ladder and sample.
8. Run gel at 120 V for 120 minutes.
9. View the gel on a Dark Reader transilluminator or a UV transilluminator.
10. Place a clean scalpel vertically above the sample in the gel at the desired size of the template.
11. Excise a 2 mm slice at approximately 300 bp using the markers as a guide.
12. Follow the instructions in the QIAquick Gel Extraction Kit to purify on one QIAquick column, eluting in 30 μ l of EB.
13. Discard the scalpel.

Enrich the 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.

Consumables Illumina-Supplied

- ▶ Phusion DNA polymerase
- ▶ PCR primer PE 1.0
- ▶ PCR primer PE 2.0
- ▶ Ultra pure water

User-Supplied

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

Procedure

This protocol assumes 5 μg of DNA input into library prep. If you use 0.5 μg , adjust the protocol as described in the following table.

Input of DNA to Library Prep	Volume of Purified Library into PCR	Volume of Water	Number of PCR Cycles
5 μg	1 μl	22 μl	10
0.5 μg	10 μl	13 μl	12

1. Prepare the following PCR reaction mix:

- DNA (1 μl)
- Phusion DNA polymerase (25 μl)
- PCR primer PE 1.0 (1 μl)
- PCR primer PE 2.0 (1 μl)
- Ultra Pure Water (22 μl)

The total volume should be 50 μl .

2. Amplify using the following PCR protocol:

- a. 30 seconds at 98°C
- b. 10 or 12 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

3. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick column, eluting in 50 μl of EB.

Validate the Library

Illumina recommends performing the following quality control steps on your DNA library.

1. Determine the concentration of the library by measuring the absorbance at 260 nm. The yield from the protocol should be between 500 and 1000 ng of DNA.
2. Measure the 260/280 ratio. It should be approximately 1.8.
3. Load 10% of the volume of the library on a gel and check that the size range is as expected: a narrow smear similar in size to the DNA excised from the gel after the ligation.

If the DNA is not a narrow smear but instead comprises a long smear of several hundred base pairs, then another gel purification step is recommended. Repeat the procedure as described in *Purify Ligation Products* on page 25.

4. To determine the molar concentration of the library, examine the gel image and estimate the median size of the library smear.
 - a. Multiply this size by 650 (the molecular mass of a base-pair) to get the molecular weight of the library.
 - b. Use this number to calculate the molar concentration of the library.
5. Clone 4% of the volume of the library into a sequencing vector.
 - a. Sequence individual clones by conventional Sanger sequencing.
 - b. Verify that the insert sequences are from the genomic source DNA.



Figure 10 Library Validation Gel

This example shows a library run on a 4–20% TBE polyacrylamide gel, stained with Vistra Green (GE Healthcare # RPN5786) and visualized on a fluorescence scanner. The left lane shows a marker ladder. The center lane and right lane show paired-end libraries with insert sizes of approximately 250 bp to 550 bp, respectively. The two bands around 50 bp in size are primers from the enrichment PCR step and have no effect on the subsequent formation of clusters.

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

