

Preparing Samples for Multiplexed Paired-End Sequencing

Topics

- 3 Introduction
- 4 Sample Preparation Workflow
- 5 Kit Contents and User-Supplied Consumables
- 8 Fragment the DNA
- 12 Perform End Repair
- 13 Add 'A' Bases to the 3' End of the DNA Fragments
- 14 Ligate Adapters to DNA Fragments
- 15 Purify Ligation Products
- 17 Enrich the Adapter-Modified DNA Fragments by PCR
- 19 Validate the Library

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Introduction

This guide explains how to prepare libraries of DNA fragments for multiplexed paired-end analysis on the Illumina Cluster Station and Genome Analyzer. However, libraries produced with this protocol may also be used for single-read analysis. You will add adapter sequences onto the ends of DNA fragments to generate the following template format:

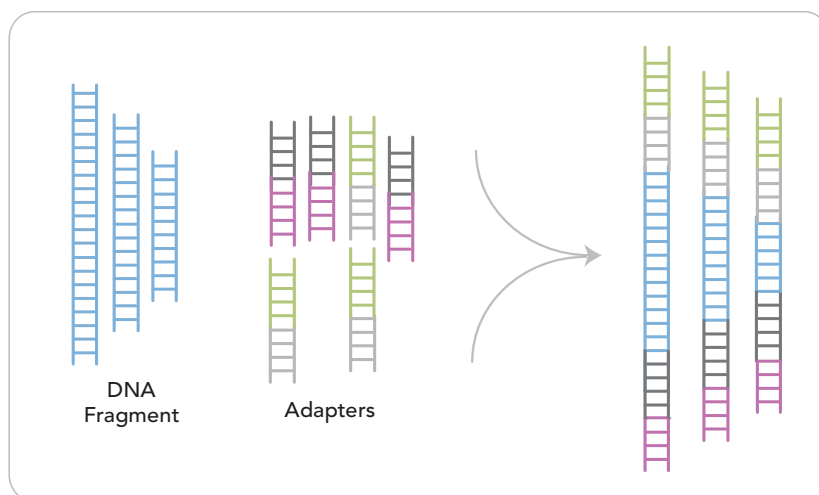


Figure 1 Fragments After Multiplexed Sample Preparation

This sample preparation protocol uses a novel set of oligonucleotides but is otherwise the same as the standard paired-end sample preparation procedure. A six-base sequence index is introduced into the adapter at the PCR stage of sample preparation.

The Multiplexing Sample Preparation Oligonucleotide Kit contains 12 index primers. These have been designed so that there are no more than three positions of agreement between any two indexes. Therefore, if there is one error in the index, the correct index can be recovered.

The adapters contain sequences that correspond to the two surface-bound amplification primers on the flow cells used in the Cluster Station. One of the adapters also contains the index sequence.

The Multiplexing Sample Preparation Oligonucleotide Kit contains all the oligonucleotides required for preparation of up to 96 indexed samples. Several of the user-supplied consumables required for indexed sample preparation are available in other Illumina sample preparation kits. See *Kit Contents and User-Supplied Consumables* on page 5 for a list of required kits and additional user-supplied consumables.

Sample Preparation Workflow

The following figure illustrates the steps required to prepare samples for multiplexed sequencing.

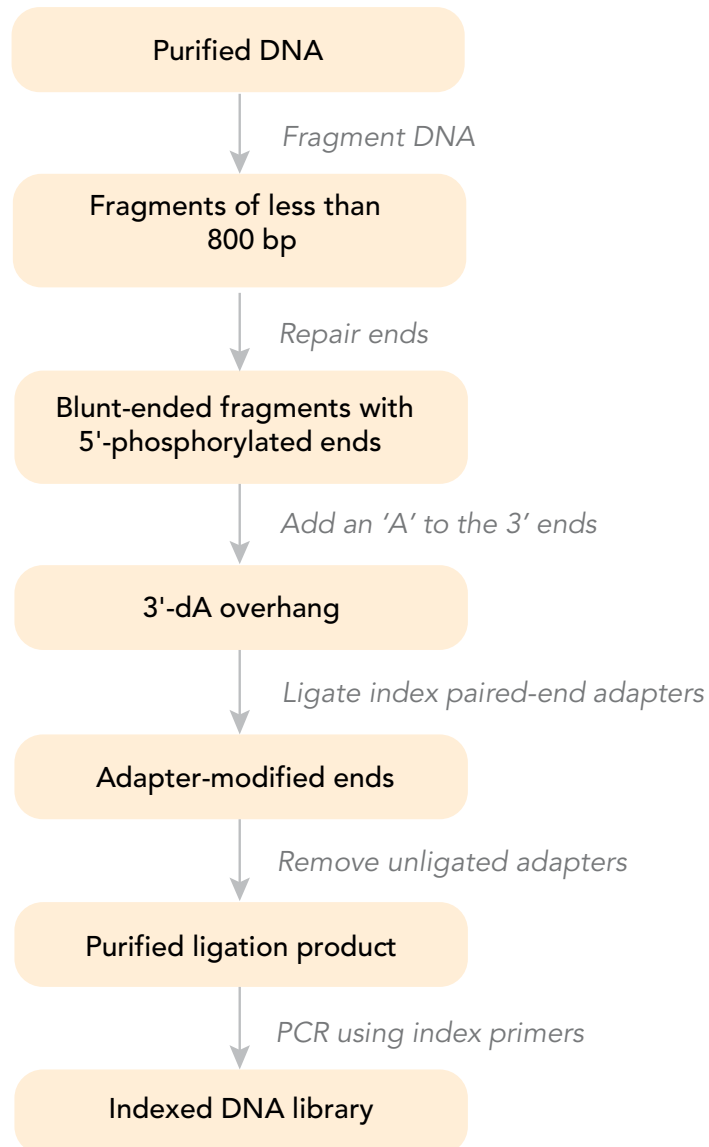


Figure 2 Sample Preparation Workflow

Kit Contents and User-Supplied Consumables

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

This guide describes the protocol for preparing samples for paired-end multiplex sequencing. However, it should be noted that these samples are also compatible with single-read sequencing. Please refer to the following tables for a list of required Illumina kits for multiplexing sample preparation.

Table 1 Sample Prep Kits for Paired-End Multiplexing

Catalog Number	Quantity	Product Name
PE-400-1001	1	Multiplexing Sample Preparation Oligonucleotide Kit
PE-102-1001	1	Paired End DNA Sample Prep Kit

For every 10 samples to be prepared, a Paired-End DNA Sample Preparation Kit is required.

Multiplexing Sample Preparation Oligonucleotide Kit

Store at -15° to -25°C

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

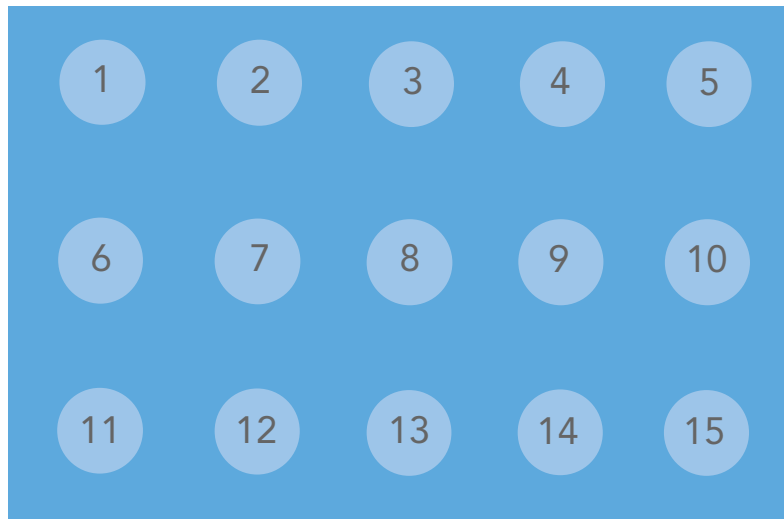


Figure 1 Multiplexing Sample Preparation Oligonucleotide Kit

1. Index PE Adapter Oligo Mix, part # 1005711
2. PCR Primer InPE 1.0, part # 1005712
3. PCR Primer InPE 2.0, part # 1005713
4. PCR Primer Index 1, part # 1005714
5. PCR Primer Index 2, part # 1005715
6. PCR Primer Index 3, part # 1005716

7. PCR Primer Index 4, part # 1005717
8. PCR Primer Index 5, part # 1005718
9. PCR Primer Index 6, part # 1005719
10. PCR Primer Index 7, part # 1005720
11. PCR Primer Index 8, part # 1005721
12. PCR Primer Index 9, part # 1005722
13. PCR Primer Index 10, part # 1005723
14. PCR Primer Index 11, part # 1005724
15. PCR Primer Index 12, part # 1005725

Paired-End Sample Preparation Kit

The following consumables are available in Illumina sample preparation kits:

- ▶ Klenow Enzyme
- ▶ Klenow Buffer
- ▶ Klenow Fragment (3' to 5' exo minus)
- ▶ T4 DNA Ligase Buffer with 10 mM ATP
- ▶ T4 DNA Polymerase
- ▶ DNA Ligase
- ▶ DNA Ligase Buffer 2X
- ▶ Phusion DNA Polymerase (Finnzymes Oy)
- ▶ TE Buffer
- ▶ 10 mM dNTP Mix
- ▶ 1 mM dATP, diluted from dATP vial
- ▶ T4 PNK
- ▶ Nebulization Buffer
- ▶ Nebulizers
- ▶ Ultra Pure Water

User-Supplied Consumables

Check to ensure that you have all of the following user-supplied consumables:

- ▶ Purified DNA (1–5 µg, 5 µg recommended)
 - DNA should be as intact as possible, with an OD₂₆₀/280 ratio of 1.8–2.0
- ▶ Compressed air of at least 32 psi
- ▶ Clamp (1 per nebulizer)
- ▶ PVC tubing
 - Fisher Scientific, catalog # 14-176-102
 - Nalgene Labware, catalog # 8007-0060
- ▶ QIAquick Gel Extraction Kit (QIAGEN, part # 28704)
- ▶ MinElute PCR Purification Kit (QIAGEN, part # 28004)
- ▶ QIAquick PCR Purification Kit (QIAGEN, part # 28104)
- ▶ Certified low range ultra agarose (BIO-RAD, part # 161-3106)

- ▶ Low molecular weight DNA ladder (NEB, part # N3233L)
- ▶ DNase/RNase-Free distilled water
- ▶ 50X TAE buffer
- ▶ Ethidium bromide
- ▶ Loading buffer
- ▶ Disposable scalpels

Fragment the DNA

This protocol fragments the 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 containing 3' or 5' overhangs.

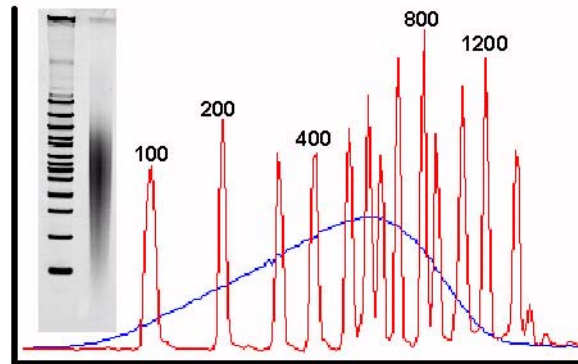


Figure 1 Fragment DNA

Consumables Illumina-Supplied

The following consumables are provided in the Paired-End Sample Preparation Kit:

- ▶ 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 µg, 5 µg recommended)
DNA should be as intact as possible, with an OD₂₆₀/OD₂₈₀ ratio of 1.8–2.0
- ▶ 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 2.0, 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.

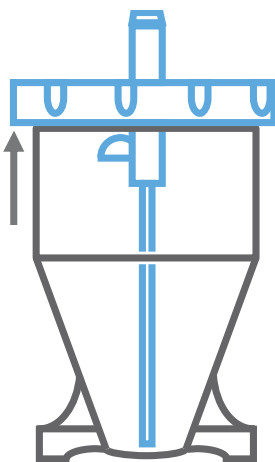


Figure 2 Remove the Nebulizer Lid

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.

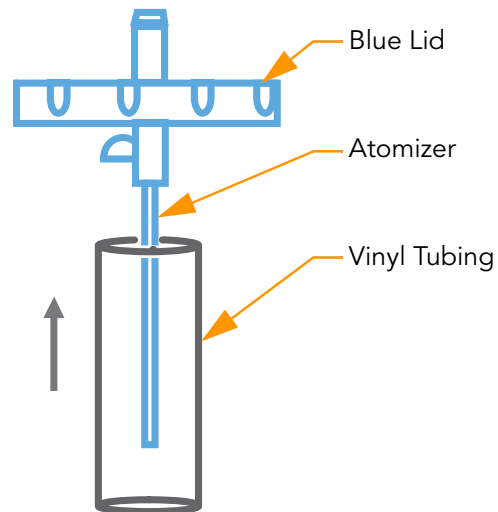


Figure 3 Assemble the Nebulizer

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).

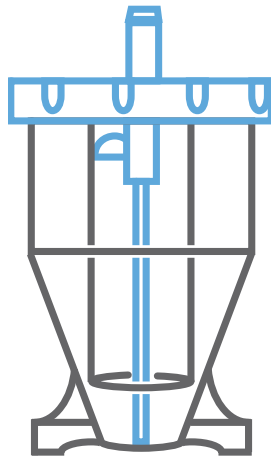


Figure 4 Replace the Nebulizer Lid

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.

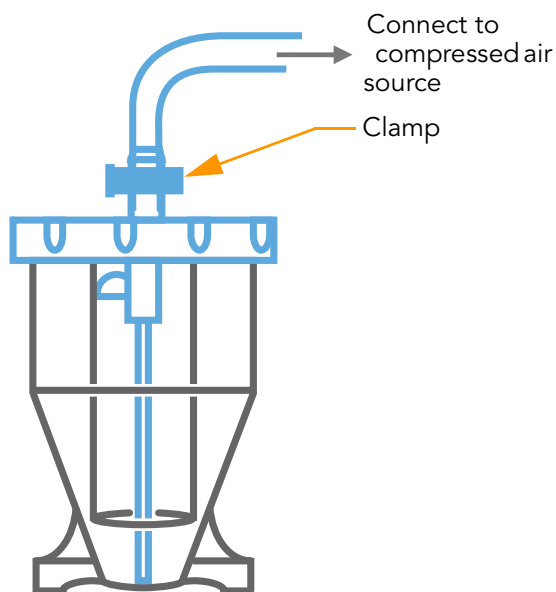


Figure 5 Connect Compressed Air

- 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 QIAGEN Buffer 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 5' overhangs.

Consumables **Illumina-Supplied**

The following consumables are provided in the Paired-End Sample Preparation Kit:

- ▶ T4 DNA Ligase Buffer with 10 mM ATP
- ▶ 10 mM dNTP Mix
- ▶ Klenow Enzyme
- ▶ T4 PNK
- ▶ T4 DNA Polymerase
- ▶ 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 the sample solution and concentrate it on one QIAquick column, eluting in 32 μ l of QIAGEN Buffer 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**

The following consumables are provided in the Paired-End Sample Preparation Kit:

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

User-Supplied

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



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 Fragment (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 the sample solution and concentrate it on one QIAquick MinElute column, eluting in 10 μ l of QIAGEN Buffer 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**

The following consumable is provided in the Paired-End Sample Preparation Kit:

- ▶ DNA Ligase Buffer 2X
- ▶ DNA Ligase

The following consumable is provided in the Multiplexing Sample Preparation Oligonucleotide Kit:

- ▶ Index PE Adapter Oligo Mix

User-Supplied

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

Procedure

This procedure uses a 10:1 molar ratio of adapter to DNA insert, based on a starting quantity of 5 µg of DNA before fragmentation. If you started with less than 5 µg, the volume of adapter can be reduced (e.g., to 5 µl for 1 µg of DNA).

1. Prepare the following reaction mix:
 - DNA sample (10 µl)
 - DNA Ligase Buffer 2X (25 µl)
 - Index 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 the sample solution and concentrate it on one QIAquick column, eluting in 30 µl of QIAGEN Buffer 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 Gel Extraction Kit (QIAGEN, part # 28704)

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

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

Illumina recommends using a Dark Reader to visualize DNA on agarose gels.

1. Prepare a 150 ml, 2% agarose gel with distilled water and 50X TAE. Final concentration of TAE should be 1X at 150 ml.
2. Add ethidium bromide (EtBr) after the TAE-agarose has cooled to approximately 50°C. 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 µl of loading buffer to 8 µl of the ladder.
5. Add 10 µl of loading buffer to 30 µl 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.



Prolonged personnel exposure to UV light can damage your DNA.

10. Place a clean scalpel vertically above the sample in the gel at the desired size of the template.



The adapters flanking the insert add approximately 70 bp to the length of the insert.

11. Excise a 2 mm slice of the sample lane at approximately 300 bp using the markers as a guide.



In order to avoid contamination with adapters that have ligated to each other, Illumina recommend that the excised fraction should be not less than 300 bp.

12. Follow the instructions in the QIAquick Gel Extraction Kit to purify the sample solution and concentrate it on one QIAquick column, eluting in 30 μ l of QIAGEN Buffer 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 six-base index is introduced into the adapter at the PCR stage. The PCR is performed with three primers that anneal to the ends of the adapters. Two of these primers are used in all sample preparations, while the third contains the index tag that will be used to discriminate between samples after the sequencing is completed.

Consumables **Illumina-Supplied**

The following consumables are provided in the Paired-End Sample Preparation Kit:

- ▶ Phusion DNA Polymerase
- ▶ Ultra Pure Water

The following consumables are provided in the Multiplexing Sample Preparation Oligonucleotide Kit:

- ▶ PCR Primer InPE 1.0
- ▶ PCR Primer InPE 2.0
- ▶ PCR Primer Index 1
- ▶ PCR Primer Index 2
- ▶ PCR Primer Index 3
- ▶ PCR Primer Index 4
- ▶ PCR Primer Index 5
- ▶ PCR Primer Index 6
- ▶ PCR Primer Index 7
- ▶ PCR Primer Index 8
- ▶ PCR Primer Index 9
- ▶ PCR Primer Index 10
- ▶ PCR Primer Index 11
- ▶ PCR Primer Index 12

User-Supplied

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

Procedure

This protocol assumes 5 µg of DNA input into library prep. If you use 1 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	21 µl	18
1 µg	5 µl	17 µl	18

1. Prepare the following PCR reaction mix using the appropriate PCR Index Primer for each separate sample:
 - DNA (1 µl)
 - Phusion DNA Polymerase (25 µl)
 - PCR Primer InPE 1.0 (1 µl)
 - PCR Primer InPE 2.0 (1 µl)
 - Ultra Pure Water (21 µl)
 - PCR Primer Index (1 µl)

The total volume should be 50 µl.
2. Amplify 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
3. 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 Buffer EB.

Validate the Library

Illumina recommends performing the following quality control steps on your DNA libraries:

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



NOTE

If the DNA is not a narrow smear but instead comprises a long smear of several hundred base pairs, and/or if there is an adapter-dimer band present at ~110 bp, then Illumina recommends you complete another gel purification step. Repeat the procedure as described in *Purify Ligation Products* on page 15. Illumina does not recommend that you re-amplify the library following this second gel.

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 fragments in the library.
 - b. Use this number to calculate the molar concentration of the library.
 At this stage, indexed libraries can be mixed together in known concentrations.
5. Clone 4% of the volume of the mixed indexing libraries into a sequencing vector.
 - a. Sequence individual clones by conventional Sanger sequencing.



NOTE

The 5' ends of the library molecules are not phosphorylated and therefore require a phosphorylated vector for cloning.

- b. Verify that the insert sequences are from the source DNA and that the index sequences associate with the appropriate sample.

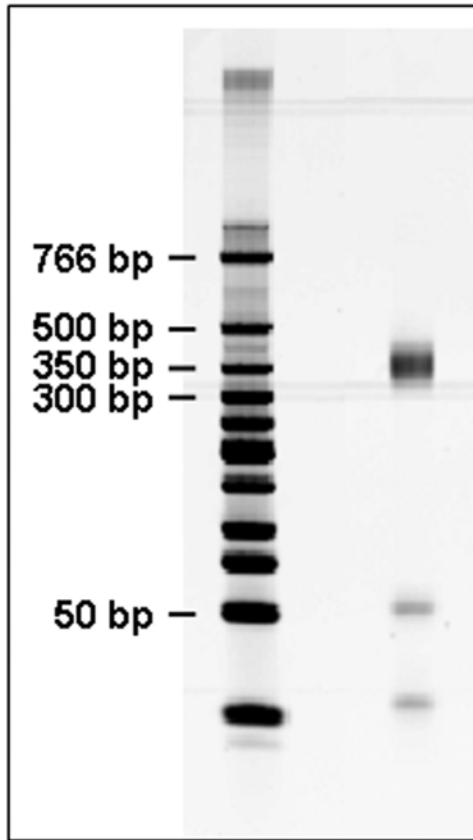


Figure 1 Library Validation Gel

This example shows an indexed 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 two bands less than 100 bp in size in the library lane on the right are primers from the enrichment PCR step and have no effect on the subsequent formation of clusters. Note that the different PCR Index Primers have different apparent sizes on such a gel.

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