

Multiplexing Sample Preparation Guide

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Introduction

This protocol explains how to prepare libraries of DNA fragments for multiplexed paired-end or single-read sequencing on the Illumina sequencing platform. You will add adapter sequences onto the ends of DNA fragments to generate the following template format:

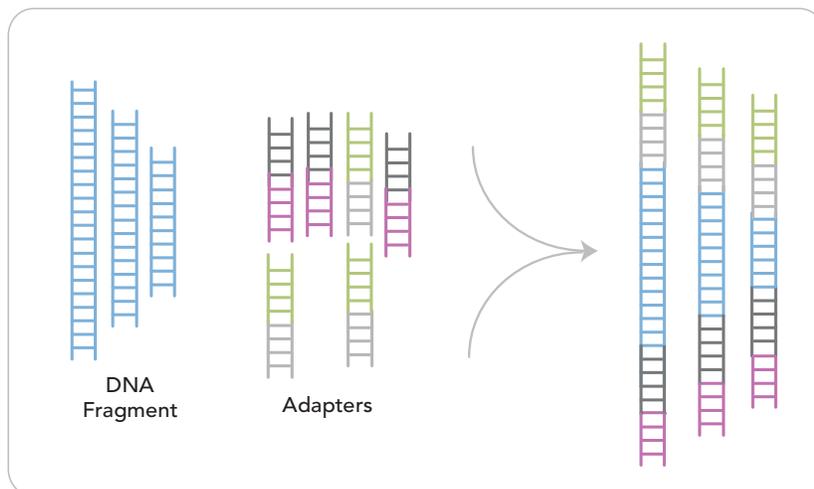


Figure 1 Fragments After Multiplexed Sample Preparation

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 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 *User-Supplied Consumables and Equipment* on page 8 for Illumina kits that contain these user-supplied consumables.

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 multiplex sequencing.



When multiplexing fewer than 12 samples, see *Low-Level Pooling* on page 32 for recommendations as to which indexes should be used together.

Sample Preparation Workflow

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

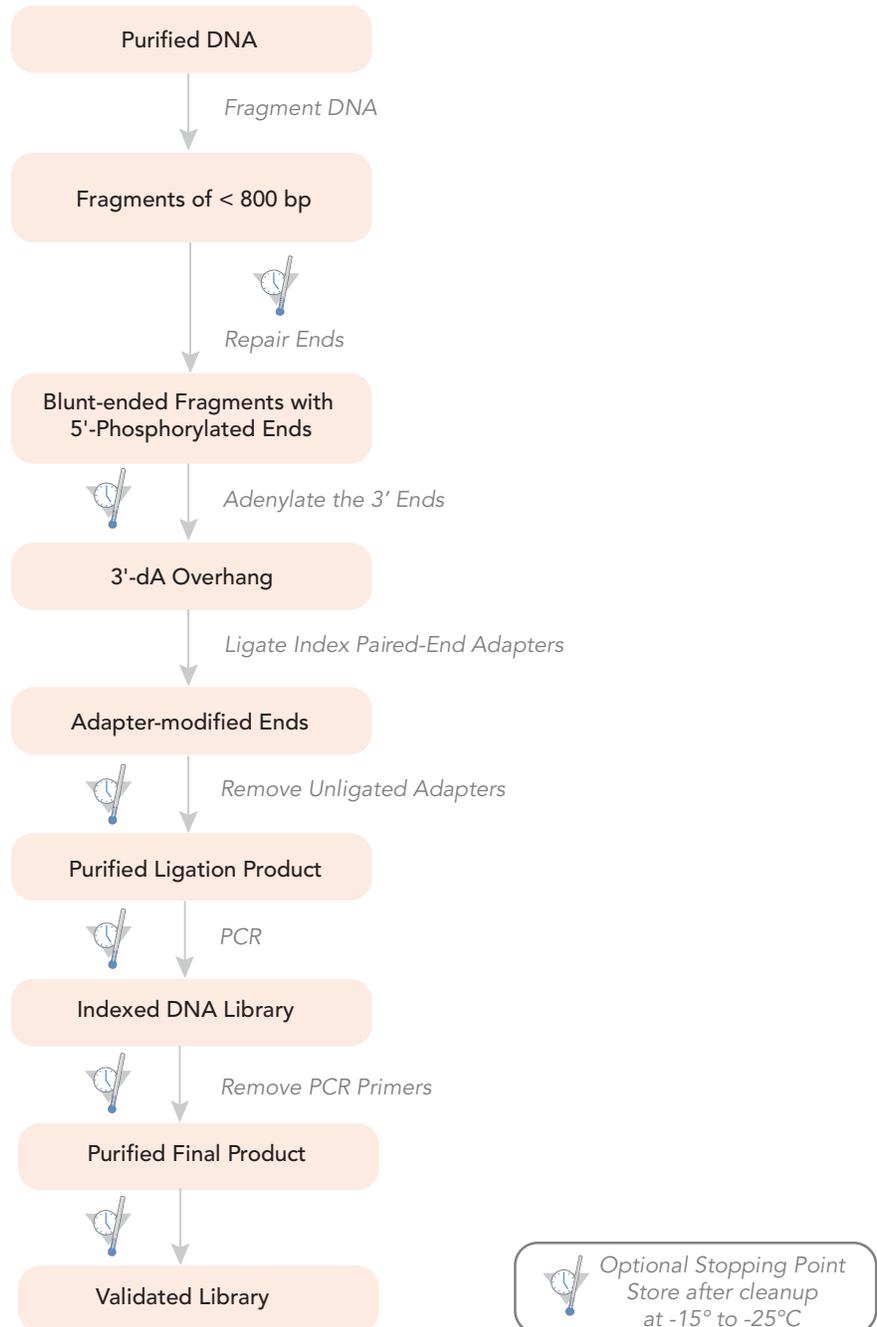


Figure 2 Multiplexing Sample Preparation Workflow

Best Practices

When preparing genomic DNA libraries for sequencing, you should always adhere to good molecular biology practices.

Liquid Handling

Good liquid handling measures are essential, particularly when quantifying libraries or diluting concentrated libraries for making clusters. Small differences in volumes ($\pm 0.5 \mu\text{l}$) can sometimes give rise to very large differences in cluster numbers ($\sim 100,000$). Small volume pipetting can also be a source of potential error in protocols that require generation of standard curves, such as PicoGreen assays or qPCR, or those that require small but precise volumes, such as the Agilent BioAnalyzer. If small volumes are unavoidable, then due diligence should be taken to ensure that pipettes are correctly calibrated. Also, care should be taken, because solutions of high molecular weight dsDNA can be viscous and not evenly dispersed, resulting in aliquot measurements that are not representative of the true concentration of the solution.

To minimize pipetting errors, especially with the small volume enzyme additions, prepare the reagents for multiple samples simultaneously. As a result, you pipette once from the reagent tubes with a larger volume, rather than many times with $1 \mu\text{l}$ volumes. Prepare a master mix of enzymes, water, buffer, etc. and aliquot this in a single pipetting movement to individual samples to standardize across multiple samples.

Potential DNA Contaminants

Incorrect DNA quantitation may result from DNA contamination, for example, by interference from superfluous nucleic acids in a sample (e.g., RNA, small nucleic acid fragments, nucleotides, single-stranded DNA), excess proteins, or other contaminating materials. DNA quality may also affect the quantity of usable DNA in a sample. For example, if the DNA is damaged (e.g., heavily nicked or containing extensive apurinic/aprimidinic sites), then many of these fragments may fail during library preparation. High molecular weight dsDNA derived from host genomes can also confound accurate quantitation. For example, bacteria artificial chromosomes (BACs) and other bacterially-derived plasmids usually contain a few percent of the chromosomal DNA from the host cells, despite the best purification efforts. These sequences may ultimately give rise to unwanted clusters on a flow cell lane. However, this contamination can be accurately quantified by analyzing aligned reads generated during sequencing against known bacterial sequences and subtracting these out. High molecular weight contamination may also be estimated prior to library preparation using qPCR assays designed to target unique chromosomal markers.

Temperature Considerations

Temperature is another important consideration for making genomic DNA libraries. Elevated temperatures should be particularly avoided in the steps preceding the adapter ligation. DNA fragments that have a high AT content are more likely to denature into single strands than GC-rich fragments, which can result in an increased probability of creating a bias in the sequencing coverage. As a general rule, libraries should be kept at temperatures below 20°C . Temperature is less of an issue after the adapters have been ligated onto the ends of the DNA, although care should be taken not to denature the library prior to the agarose gel electrophoresis process, because single-stranded DNA runs at a different position.

DNA Input Recommendations

Input DNA Quantitation

The ultimate success or failure of a library preparation strongly depends on using accurately quantified amounts of input DNA, particularly when starting quantities are $<1 \mu\text{g}$. Thus, correct quantitation of genomic DNA is essential.

Assessing DNA Quantity and Quality

Absorbance measurements at 260 nm are commonly used to quantify DNA. The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity, and values of 1.8–2.0 are considered indicative of relatively pure DNA. However, both measurements can be compromised by the presence of RNA or small nucleic acid fragments such as nucleotides. Thus, genomic DNA samples should be carefully collected to ensure that they are free of contaminants, and the most accurate spectrophotometric method available should be used to quantify the input genomic DNA.

DNA quantitation methods that rely on intercalating fluorescent dyes measure only double-stranded DNA and are less subject to excess nucleic acids. However, these methods require the preparation of calibration curves and are highly sensitive to pipetting error. Ensure that pipettes are correctly calibrated and are not used at the volume extremes of their performance specifications.

Gel electrophoresis is a powerful means for revealing the condition (even the presence or absence) of DNA in a sample. Impurities, such as detergents or proteins, can be revealed by smearing of DNA bands. RNA, which interferes with 260 nm readings, is often visible at the bottom of a gel. A ladder or smear below a band of interest may indicate nicking or other damage to DNA. Where possible, or necessary, a gel should be run to assess the condition of the DNA sample.

Consistency of Results

Given that fluorescent methods only measure double stranded DNA and 260 nm determinations measure both single and double stranded DNA, fluorescence analysis can be performed to assess the sample denaturation state (i.e. single or double stranded). Sample preparation can begin with a fixed amount of double-stranded DNA prior to fragmentation and cleanup, and thereafter, can be measured by 260 nm determination. If the DNA amount is less than expected, the amount of input DNA can be adjusted before proceeding to the end-repair steps. A further validation step can be performed by analyzing an aliquot for the presence of contaminants by electrophoresis or using an automated instrument, such as the Agilent Bioanalyzer.

Multiplexing Sample Preparation Oligonucleotide Kit Contents

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

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

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

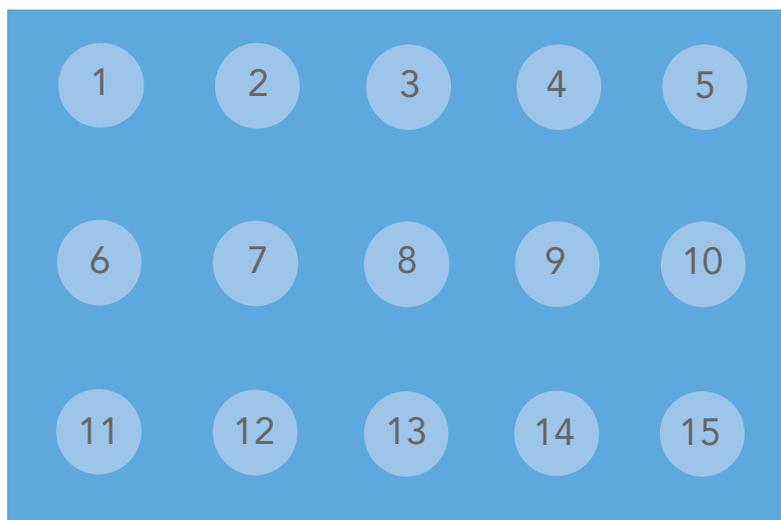


Figure 3 Multiplexing Sample Preparation Oligonucleotide Kit

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

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.

Table 1 *User-Supplied Consumables*

Consumable	Supplier
50X TAE Buffer	BIO-RAD, part # 161-0743
100 bp Redi-Load DNA ladder	Invitrogen, part # 10488-058
Certified low range ultra agarose	BIO-RAD, part # 161-3106
Compressed air source of at least 32 psi (Do not use CO ₂ which could alter the pH of the nebulizer buffer)	General lab supplier
Disposable scalpels	General lab supplier
Distilled water	General lab supplier
Ethidium Bromide	Sigma, part # E1510
Loading buffer (50 mM Tris pH 8.0, 40 mM EDTA, 40% (w/v) sucrose)	General lab supplier
MinElute PCR Purification Kit	QIAGEN, part # 28004
Paired-End Sample Prep Kit (for 10 samples) or Paired-End Sample Prep Kit (for 40 samples)	Illumina, catalog # PE-102-1001 (10 samples) Illumina, catalog # PE-102-1002 (40 samples)
Purified DNA (1–5 µg, 5 µg recommended) DNA should be as intact as possible, with an OD _{260/280} ratio of 1.8–2.0	General lab supplier
PVC tubing	Intersurgical, part # 1174-003
QIAquick Gel Extraction Kit (for 50 samples) or QIAquick Gel Extraction Kit (for 250 samples)	QIAGEN, part # 28704 (50 samples) QIAGEN, part # 28706 (250 samples)
QIAquick PCR Purification Kit	QIAGEN, part # 28104

Table 2 *Equipment Checklist*

Consumable	Supplier
Benchtop microcentrifuge	General lab supplier
Benchtop centrifuge with swing-out rotor (e.g., Sorvall Legend RT)	General lab supplier
Dark Reader transilluminator or a UV transilluminator	Clare Chemical Research, part # D195M
Electrophoresis unit	General lab supplier
Gel trays and tank	General lab supplier
Thermal cycler	General lab supplier

Fragment DNA

DNA is fragmented using a nebulization technique, which breaks up DNA into pieces less than 800 bp in minutes using a disposable device. This process generates double-stranded DNA fragments containing 3' or 5' overhangs.

A compressed air source is used to force a solution of DNA through a narrow orifice. The solution follows a cyclical route from a collection chamber via a siphon tube back to the sample reservoir and through the orifice again. After 5–6 minutes of repeated passing through the orifice, the sample is fragmented to its final size. Nebulization is a very reproducible process and produces random fragments that approximate a Poisson distribution when sequenced (Figure 4). The numbers indicate sizes, in base pairs, of an overlaid DNA marker ladder.

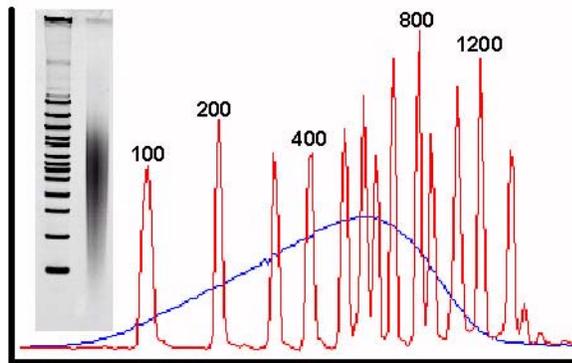


Figure 4 Fragmented DNA on Electrophoretic Gel

Alternative fragmentation methods may be more appropriate based on sample availability (see *Alternative Fragmentation Methods*).

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 μg , 5 μg recommended)
DNA should be as intact as possible, with an OD_{260/280} ratio of 1.8–2.0
- ▶ Compressed Air of at least 32 psi
Do not use CO₂ which could alter the pH of the nebulizer buffer
- ▶ PVC tubing
Dimensions: 1/4 inch ID, 3/8 inch OD, 1/16 inch wall, 1 meter length

**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 OD_{260/280} 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 and practice assembling the nebulizer before proceeding with your sample DNA.

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

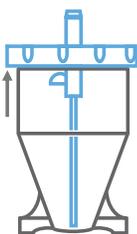


Figure 5 Remove the Nebulizer Lid

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.

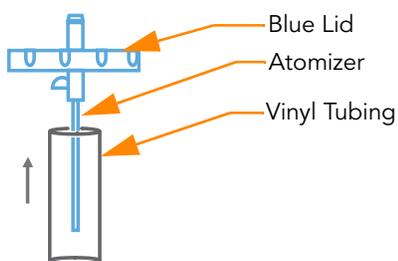


Figure 6 Assemble the Nebulizer

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.

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

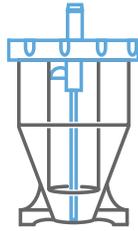


Figure 7 Replace the Nebulizer Lid

- 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.

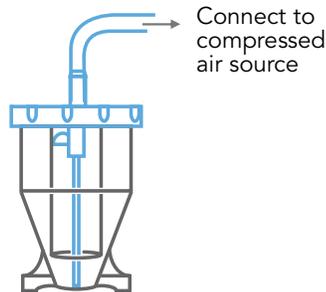


Figure 8 Connect Compressed Air

- 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 32–35 psi.
- Nebulize for 6 minutes. You may notice vapor rising from the nebulizer; this is normal. Also, the Nebulization Buffer may turn white or appear frozen.
- 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.
- 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.
- Measure the recovered volume. Typically, you should recover 400–600 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 30 μ l of QIAGEN EB.
- Perform a quality control step on the recovered DNA to ensure the presence of sufficient DNA (i.e., quantify the DNA by a 260 nm reading, or by a PicoGreen assay, bioanalyzer, or equivalent). Ensure that there is at least 0.5 μ g of DNA. If not, then fragment more DNA.



If you do not plan to proceed to *Perform End Repair* 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.

Alternative Fragmentation Methods

Genomic DNA sample preparation must generate dsDNA fragments of a narrow distribution around a desired median size. The fragmentation method should not introduce bias in the fragment sequence, should be able to recover ~100% of the DNA, and should be automatable, inexpensive, and simple to use. Illumina recommends nebulization for most cases because it meets many of these criteria. Alternative DNA fragmentation methods are outlined below:



NOTE

Illumina does not recommend these methods, but if you choose to use them, use them with caution.

Covaris

Covaris has developed an instrument that adopts ultrasound technology to fragment nucleic acids in a controlled and precise manner. DNA can be sheared in a small volume (50 μ l) without sample loss due to evaporation and without significant bias in the resulting fragments.

The Covaris instrument can achieve fragments with a smaller end-point size and a narrower range than with a nebulizer (Figure 9). However, this is only a benefit if fragment sizes of ~100 bp are desired. With the read lengths currently available using Illumina's genetic analyzers, ~100 bp fragments are not typically desired for paired-end reads. For example, a median fragment size of 300 bp (having a standard deviation of 30 bp or 10% variance) is required in the library generation protocol for paired 100 base reads. This would result in 99% of the fragments being >210 bp. Likewise, a median fragment size of 450 bp is needed for paired 150 bp reads.

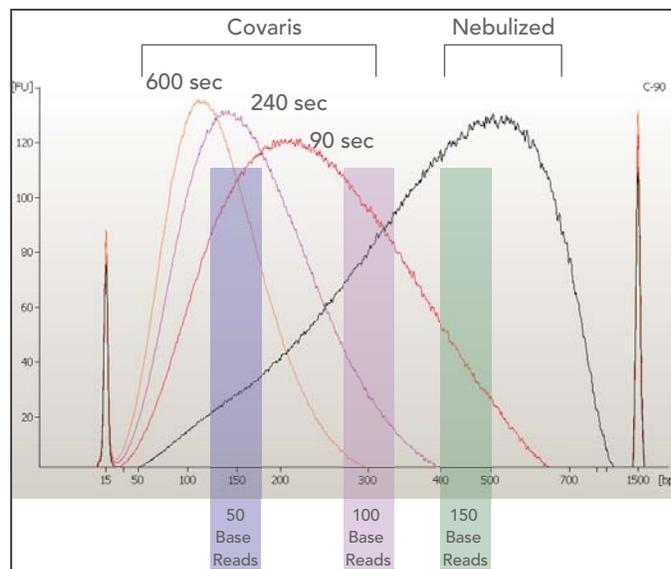


Figure 9 Distribution Of Fragment Sizes Resulting From Nebulization Or Covaris

When the Covaris instrument is used to shear DNA to a median fragment size of ≥ 200 bp and greater, the distribution of fragment sizes broadens and is comparable to that seen when using a nebulizer.

Sonication

Sonication is sometimes used as an alternative to nebulization, since sonicators are relatively common in molecular biology labs. Care must be taken to avoid raising the temperature of the DNA solution above $\sim 20^{\circ}\text{C}$. Heating small fragments with high AT content may result in denaturation. If this occurs, single stranded fragments will not ligate to the double stranded adapters and these will be under-represented in the final library. During sonication, DNA fragments are typically generated in a decreasing but broad range of fragment sizes. Therefore, sonication settings must be chosen carefully to reproducibly generate a majority of fragments in the desired size range.

A probe-based sonicator wherein the probe tip is directly immersed into the DNA sample may be used, although problems with sample heating, vaporization, and contamination may arise. Alternatively, a cup horn sonicator filled with ice and water will allow the tube containing the DNA solution to remain sealed during fragmentation.

Hydroshear

Hydroshearing fragments DNA by a similar mechanism to nebulization. A syringe pump is used to pass the DNA solution back and forth through a narrow orifice. As the liquid streams through the orifice, it becomes laminar (i.e., the center of the stream moves faster than the stream closer to the walls of the orifice) and DNA molecules are stretched and snapped at the midpoint of their length. Hydroshearing is best for generating fragments with a two-fold size range of 1,000-40,000 bp. However, it is not effective for generating fragments $< 1,000$ bp in length, and so is not suitable for generating standard Illumina single read and short-insert paired-end sequencing libraries.

Perform End Repair

DNA fragmentation by physical methods produces heterogeneous ends, comprising a mixture of 3' overhangs, 5' overhangs, and blunt ends. The overhangs will be of varying lengths and ends may or may not be phosphorylated. This step 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. In addition, the T4 PNK in this reaction phosphorylates the 5' ends of the DNA fragments.

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
- ▶ T4 DNA Polymerase
- ▶ Klenow Enzyme
- ▶ T4 PNK
- ▶ Water

User-Supplied

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

Procedure 1. Prepare the reaction mix on ice in the following order:

Reagent	Volume (μl)
Water	45
DNA Sample	30
T4 DNA Ligase Buffer with 10 mM ATP	10
10 mM dNTP Mix	4
T4 DNA Polymerase	5
Klenow Enzyme	1
T4 PNK	5
Total Volume	100

2. Mix gently, but thoroughly, and centrifuge briefly.
3. Incubate on the thermal cycler for 30 minutes at 20°C.

4. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick column, eluting in 32 μ l of QIAGEN EB.



If you do not plan to proceed to *Adenylate 3' Ends* 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.

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables Illumina-Supplied

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

- ▶ Klenow Buffer
- ▶ 1 mM dATP
- ▶ Klenow Exo -

User-Supplied

- ▶ MinElute PCR Purification Kit



NOTE

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

Procedure

1. Prepare the reaction mix on ice in the following order:

Reagent	Volume (µl)
DNA Sample	32
Klenow Buffer	5
1 mM dATP	10
Klenow Exo -	3
Total Volume	50

2. Incubate on the thermal cycler 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 µl of QIAGEN EB.

Ligate Adapters

This process ligates adapters to the ends of the DNA fragments. The reaction adds distinct sequences to the 5' and 3' ends of each strand in the genomic fragment. Later in the workflow, additional sequences are added by tailed primers during PCR. These additional sequences are necessary for library amplification on the flow cell during cluster formation. The index sequence is also added during PCR.

Consumables Illumina-Supplied

The following consumables are 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

A molar excess of adapter to fragments is used to increase the yield of adapter ligation to both ends of the DNA fragments. If the ratio is too great, then the yield of adapter dimers also increases, for example, if 1 µg or less of input DNA is used instead of 5 µg. The volume of adapter oligo mix added in the following procedure is recommended for an initial input DNA quantity of 5 µg (approximately 2.5 µg post-nebulization or 4–5 µg following covaris shearing). If 1 µg of input DNA is used, then the amount of adapter added to the reaction should be decreased to 5 µl.

1. Prepare the reaction mix on ice in the following order:

Reagent	Volume (µl)
DNA Sample	10
DNA Ligase Buffer 2X	25
Index PE Adapter Oligo Mix	10
DNA Ligase	5
Total Volume	50

2. Incubate on the thermal cycler for 15 minutes at 20°C.
3. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick MinElute column, eluting in 30 µl of QIAGEN EB.



If you do not plan to proceed to *Purify Ligation Products* 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 Ligation Products

This process purifies the products of the ligation reaction on a gel and removes unligated adapters, removes any adapters that may have ligated to one another, and selects a size-range of sequencing library appropriate for cluster generation.



NOTE

Illumina recommends that you test your electrophoresis unit in advance to ensure that you can readily resolve DNA in the range below 1000 base pairs. The DNA smear should be sufficiently resolved to enable you to excise a narrow band of a chosen size with a standard deviation as low as 5% of the median (i.e., a gel slice at 400 bp, where +/- one standard deviation is equivalent to a size range of 380–420 bp). The conditions described are typical gel electrophoresis conditions which have been validated by Illumina.

Size Selection

Illumina recommends gel electrophoresis and band excision after adapter ligation to remove excess adapter and adapter dimers and to tighten the range of fragment sizes. Ligation reaction products are separated on an agarose gel and a ~2 mm wide gel slice containing DNA of the desired size is excised.

Excision Range

Illumina suggests a 200 bp insert size target (+/- 1 standard deviation of 20 bp, i.e, a 10% variance) for read lengths of 2×75 bp or shorter. In practice, this translates to a 2 mm gel slice at ~300 bp to account for the length of the adapter sequences flanking the inserts. For read lengths of 2×100 bp or longer, Illumina recommends an insert size range of 300 bp or greater should be targeted for excision from the gel, unless you intentionally want to sequence overlapping read pairs.

Consumables

User-Supplied

- ▶ Certified Low Range Ultra Agarose
- ▶ 50X TAE Buffer
- ▶ Distilled Water
- ▶ Ethidium Bromide
- ▶ Loading Buffer
- ▶ 100 bp Redi-Load DNA Ladder
- ▶ QIAquick Gel Extraction Kit (QIAGEN, part # 28704 or 28706)

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 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 TAE according to the manufacturer's instructions. The final concentration of TAE should be 1X.
2. Add Ethidium Bromide (EtBr) after the TAE-agarose has cooled. The final concentration of EtBr should be 400 ng/ml (i.e., add 60 μ g EtBr to 150 ml of 1x TAE-agarose).
3. Cast the gel using a comb that can accommodate 56 μ l in each well. Recommended well size: 1 mm (length) x 8 mm (width) x 7 mm (height).
4. Add 3 μ l of Loading Buffer to 8 μ l of the 100 bp Redi-Load Ladder.
5. Add 10 μ l of Loading Buffer to 30 μ l of the DNA from the purified ligation reaction.
6. Load 10 μ l 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.

**NOTE**

Flanking the library on both sides with ladders may make the library excision easier.

8. Run gel at 120 V for 60 minutes (6 V/cm).
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 region of gel with a clean scalpel at approximately 400 bp using the markers as a guide.

**NOTE**

Cutting a band of 400 bp will result in an insert size of approximately 300 bp, accounting for the influence of the adapters on the gel mobility. In order to avoid contamination with adapters that have ligated to each other, Illumina recommends that the excised fraction should be 300 bp or greater.

12. Discard the scalpel to avoid sample cross-contamination.
13. Follow the instructions in the QIAquick Gel Extraction Kit to purify on one QIAquick column, eluting in 30 μ l of QIAGEN EB.



If you do not plan to proceed to *Enrich DNA Fragments* 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.

Enrich DNA Fragments

This process 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 is used to discriminate between samples after sequencing is complete.

PCR Amplification

The PCR amplification step of the protocol performs four key functions:

- ▶ **Add Sequences** - Additional sequences are added to the ends of the adapters so that the final amplified templates contain sequences to enable hybridization with primers bound to the flow cell surface for cluster generation. The index sequence is also added in order to permit sample discrimination in a multiplexed sequencing experiment.
- ▶ **Enrich Fragments** - PCR enriches for fragments that have adapters ligated on both ends. Fragments with only one or no adapters on their ends are by-products of inefficiencies in the ligation reaction. Neither species can be used to make clusters, as fragments without any adapters cannot hybridize to surface-bound primers in the flow cell, and fragments with an adapter on only one end can hybridize to surface bound primers but cannot form clusters. However, the presence of these incomplete ligation products can lead to an overestimation of the final quantity of library DNA if spectrophotometric or fluorometric quantification is performed. In contrast, these ligation products are not quantified by qPCR.
- ▶ **Enrich Templates** - PCR enriches for templates that include the non-template 'A' nucleotide added during the A-tailing step of the protocol and therefore eliminates adapter dimers. This is accomplished by using proprietary, modified primers that are completely resistant to the 3'-5' exonuclease activity of the Phusion™ polymerase (Finnzymes Oy) used for PCR. These primers reach all the way to the non-templated 'A' before the start of the genomic sequence. If the 'A' is not present (as in the case for an adapter dimer), then the terminal 'T' on the primer will mismatch and not extend.
- ▶ **Provides Material** - PCR provides enough material to enable reliable quantitation of the final library if spectrophotometric or fluorometric methods are used.

Consumables Illumina-Supplied

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

- ▶ Phusion DNA Polymerase (Finnzymes Oy)
- ▶ 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



NOTE

When multiplexing fewer than 12 samples, see *Low-Level Pooling* on page 32 for recommendations as to which indexes should be used together.

The following procedure is designed to give sufficient library after PCR to enable you to quantify your libraries by spectrophotometric methods. Quantification prior to the PCR reaction is difficult, particularly if the initial input is less than 1 µg. Therefore, the volume of adapter ligated fragments added to the PCR reaction below are based on an initial input DNA quantity of 5 µg (approximately 2.5 µg post-nebulization or 4–5 µg following covaris shearing). If the initial input DNA is 1 µg or less, then more ligated fragments should be added to the PCR reaction. If you are using 1 µg, adjust the process 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



CAUTION

To avoid sample cross-contamination, set up PCR reactions (all components except the template DNA) in a designated clean area, preferably a PCR hood with UV sterilization and positive air flow.

1. Prepare the reaction mix, in a 200 μ l thin wall PCR tube using the appropriate PCR Index Primer for each separate sample:

Reagent	Volume (μ l)
DNA	x
Ultra Pure Water	to increase DNA volume to 22
PCR primer InPE 2.0	1
PCR primer InPE 1.0	1
PCR Primer Index<#>	1
Phusion DNA Polymerase	25
Total Volume	50

2. Mix gently, but thoroughly, and centrifuge briefly.
3. Transport the PCR tubes, on ice, to the bench.
4. Amplify using the following PCR process:
 - 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. Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick MinElute column, eluting in 30 μ l of QIAGEN EB.



If you do not plan to proceed to *Purify Final Product* 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 Final Product

This process further purifies the products of the PCR ligation reaction on a gel to select a size-range of templates appropriate for subsequent cluster generation.

Consumables User-Supplied

- ▶ Certified Low Range Ultra Agarose
- ▶ 50x TAE Buffer
- ▶ Distilled Water
- ▶ Ethidium Bromide
- ▶ Loading Buffer
- ▶ 100 bp Redi-Load DNA Ladder
- ▶ QIAquick Gel Extraction Kit (QIAGEN, part # 28704 or 28706)

Procedure



CAUTION

Illumina does not recommend purifying multiple samples on a single gel due to the risk of cross-contamination between libraries. However, if you excised an additional 600 bp slice during the *Purify Ligation Products* process (page 19) and processed it alongside the 400 bp slice, it can be purified during this process in the same gel as its matching 400 bp slice, but in separate lanes of the gel.



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, and as narrow a size range as possible. Illumina recommends that a Dark Reader is used to visualize DNA on agarose gels.

1. Prepare a 150 ml, 2% agarose gel with distilled water and TAE according to the manufacturer's instructions. The final concentration of TAE should be 1X.
2. Add EtBr after the TAE-agarose has cooled. The final concentration of EtBr should be 400 ng/ml (i.e., add 60 µg EtBr to 150 ml of 1x TAE-agarose).
3. Cast the gel using a comb that can accommodate 56 µl in each well. Recommended well size: 1 mm (length) x 8 mm (width) x 7 mm (height).
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 PCR reaction.
6. Load 10 µl of the 100 bp Redi-Load Ladder onto one lane of the gel.

7. Load the entire sample onto another lane of the gel, leaving a gap of at least one empty lane between ladder and sample.



Flanking the library on both sides with ladders may make the library excision easier.

8. Run gel at 120 V for 60 minutes (6 V/cm).
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 region of gel with a clean scalpel at approximately 400 bp using the markers as a guide.



In order to avoid contamination with adapters that have ligated to each other, Illumina recommends that the excised fraction should be 300 bp or greater.

12. Discard the scalpel.
13. Follow the instructions in the QIAquick Gel Extraction Kit to purify on one QIAquick column, eluting in 30 μ l of QIAGEN EB.



If you do not plan to proceed to *Validate Library* 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.

Validate Library

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

1. Check the template size distribution by running an aliquot of the library on a gel or an Agilent Bioanalyzer.
2. If validating by gel, load 10% of the volume of the library on a gel and check that the size range is as expected: a narrow smear similar in size to the DNA excised from the gel after the ligation.
3. If using Agilent Bioanalyzer, load 1 μ l of the library on Agilent chip.

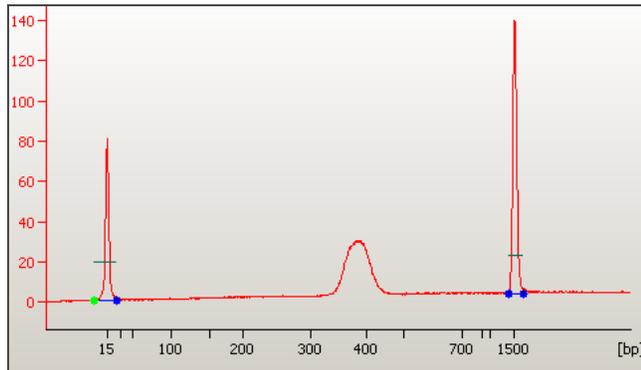


Figure 10 Paired-End Library Example

An example of a library prepared using this protocol is shown in Figure 10. The library has insert sizes ranging from 250–350 bp (i.e., a template size range of 350–450 bp).



NOTE

The sequenced Paired-End library reports the average insert size and distribution metrics in the Summary.htm output in the GERALD sub-directory of the run folder.

Library Quantitation

In order to achieve the highest quality of data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of every flow cell. This requires accurate quantitation of DNA library templates. As described for sample input quantitation, any method of measuring DNA concentration has certain advantages and potential drawbacks.

By Optical Density or Intercalating Dye

Absorbance-based quantitation can erroneously measure the presence of residual PCR primers or may not be sensitive enough to make an accurate measurement in circumstances where the final library yield is low. Measurements based on intercalating dyes such as PicoGreen may also be used, but care should be taken to minimize pipetting errors and to ensure that the library is not denatured to avoid inaccuracies.

Bioanalyzer

Automated analysis using an instrument like the Agilent Bioanalyzer can be used to quantify libraries and is an excellent alternative to traditional gel electrophoresis to determine the size of the library fragments. As with intercalating dye assays, single-stranded templates will not be detected nor contribute to the library quantitation even though these templates will form clusters. Therefore, care must be taken to ensure that the library is not denatured and that pipetting errors are minimized.

qPCR

qPCR is an alternative method of quantifying DNA that measures the relationship between the initial concentration of a template and how its concentration changes during progressing cycles of thermal amplification. As in conventional PCR, a polymerase, dNTPs, and two primers are designed to match sequences within a template. In SYBR Green-based qPCR, the amplicon quantity is measured by binding of an intercalating dye. In contrast, TaqMan-based qPCR employs a fluorescent probe that hybridizes to amplicons downstream of one of the amplification primers. During extension, the polymerase encounters the hybridized probe and removes the fluorescent moiety that then fluoresces in solution. For qPCR library quantitation, the amplification primers and TaqMan probes are designed to match sequences within the Illumina adapters. Thus, qPCR offers advantages for measuring library concentration because it will only measure templates that have adapter sequences on both ends. Moreover, qPCR sensitivity allows accurate quantitation of dilute libraries with concentrations below the threshold of detection of conventional spectrophotometric methods.

qPCR quantitates DNA of unknown concentration by comparison to a control sample of known concentration. The control sample should be a library that gives a known number of clusters when titrated on a flow cell. Although any library prepared for sequencing on the Illumina platform can be used as a control for qPCR, the control library should be as similar as possible in terms of template size, GC content, and library type (e.g., genomic DNA or ChIP-Seq) to the experimental libraries for quantitation. Control libraries can be easily prepared using the Illumina Paired-End Library Generation kit.

To correlate library concentration with cluster number, it is necessary to generate a titration flow cell for the control template. This can be achieved by preparing serial dilutions of 'template hyb' from the control library and counting the number of clusters following sequencing. A minimum of four sequencing cycles is required to achieve an accurate cluster count because Illumina's current Pipeline software uses the first four cycles to identify individual clusters in a full length sequencing run (Figure 11). A cluster titration curve for the control template should be linear to the point at which the clusters become too dense to count accurately. Once the control library has been correlated with cluster number, this library can be used as a qPCR control.

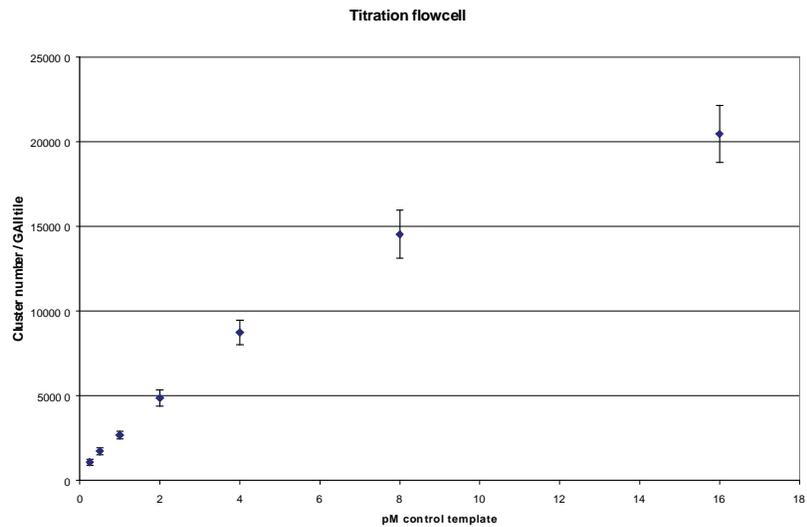


Figure 11 Titration Of A Control 300 bp Insert *E. Coli* Library

Figure 12 shows an example of the cluster numbers achieved with 21 independent *E. coli* libraries that were quantified by qPCR against a control library. All libraries, including the control library, were then hybridized to lanes of a flow cell at 10 pM concentration. All libraries gave similar cluster numbers.

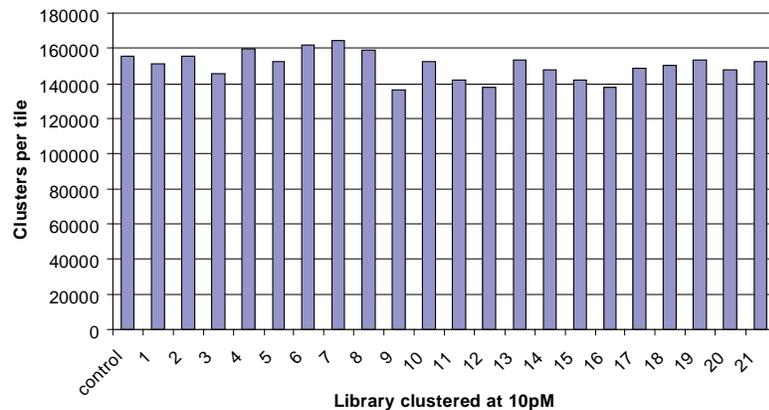


Figure 12 Example of Cluster Numbers Achieved

For more information on using qPCR to quantify libraries, reference the Illumina *qPCR Quantification Protocol Guide*.

DNA Template Storage

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.

Create Sample Sheet

The sample sheet is a file that describes the samples in each lane, including the indexes used, and is required for demultiplexing following sequencing. For instructions on using the sample sheet to direct demultiplexing, see the analysis pipeline documentation.

The sample sheet is a comma-separated values (*.csv) file that contains the sample name and related information (Figure 13). Create the sample sheet using Excel or another text editing tool that supports .csv files. Fill in your sample sheet according to the guidelines provided in this section.



NOTE

Sample sheet templates can be downloaded via <http://www.illumina.com/documents>.

	A	B	C	D	E	F	G	H	I
1	FCID	Lane	SampleID	SampleRef	Index	Description	Control	Recipe	Operator
2	FC612PV	1	sample1	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
3	FC612PV	1	sample2	E_coli	TAGCTT	desc1	N	R1	j. doe
4	FC612PV	2	sample3	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
5	FC612PV	2	sample4	CMV	CGATGT	desc1	N	R1	j. doe
6	FC612PV	2	sample5	E_coli	CTTGTA	desc1	N	R1	j. doe
7	FC612PV	3	sample6	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
8	FC612PV	3	sample7	CMV	CGATGT	desc1	N	R1	j. doe
9	FC612PV	3	sample8	E_coli	CTTGTA	desc1	N	R1	j. doe
10	FC612PV	4	sample9	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
11	FC612PV	4	sample10	CMV	CGATGT	desc1	N	R1	j. doe
12	FC612PV	4	sample11	E_coli	CTTGTA	desc1	N	R1	j. doe
13	FC612PV	5	sample12	phi	TTAGGC	desc1	N	R1	j. doe
14	FC612PV	6	sample13	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
15	FC612PV	6	sample14	CMV	CGATGT	desc1	N	R1	j. doe
16	FC612PV	6	sample15	E_coli	CTTGTA	desc1	Y	R1	j. doe
17	FC612PV	7	sample16	Potato	ATCACG	desc1	Y	R1	j. doe
18	FC612PV	7	sample17	CMV	CGATGT	desc1	Y	R1	j. doe
19	FC612PV	7	sample18	CMV	TGACCA	desc1	Y	R1	i. doe

Figure 13 Example: Sample Sheet

The sample sheet has the following fields:

Table 3 Sample Sheet Fields

Column Header	Description
FCID	The flow cell ID
Lane	A positive integer indicating the lane number (1–8)
Sample ID	The sample ID
Sample Ref	The reference sequence for the sample
Index	The index sequence or 0 for no indexing
Description	The sample description

Table 3 *Sample Sheet Fields (Continued)*

Column Header	Description
Control	Y: indicates the lane is a control lane N: indicates a sample
Recipe	The recipe used during sequencing
Operator	The name or ID of the operator



To avoid misidentifying samples, ensure that the sample IDs entered in the sample sheet correctly correspond to the DNA samples used.

Low-Level Pooling

Some sequencing experiments require the use of fewer than 12 index sequences in a lane with a high cluster density. In such cases, a careful selection of indexes is required to ensure optimum cluster discrimination by having different bases at each cycle of the index read. Illumina recommends the following sets of indexes for low-level pooling experiments.

Pool of 2 samples:

- ▶ Index #6 GCCAAT
- ▶ Index #12 CTTGTA

Pool of 3 samples:

- ▶ Index #4 TGACCA
- ▶ Index #6 GCCAAT
- ▶ Index #12 CTTGTA

Pool of 6 samples:

- ▶ Index #2 CGATGT
- ▶ Index #4 TGACCA
- ▶ Index #5 ACAGTG
- ▶ Index #6 GCCAAT
- ▶ Index #7 CAGATC
- ▶ Index #12 CTTGTA



NOTE

The control PhiX174 lane contains a single index (#3). However, it is not necessary to use a high cluster density in this control lane. Illumina recommends a raw cluster density of 160–240,000 clusters per mm².

Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 4 *Illumina General Contact Information*

Illumina Website	http://www.illumina.com
Email	techsupport@illumina.com

Table 5 *Illumina Customer Support Telephone Numbers*

Region	Contact Number
North America Toll-free	1.800.809.ILMN (1.800.809-4566)
United Kingdom Toll-free	0800.917.0041
Germany Toll-free	0800.180.8994
Netherlands Toll-free	0800.0223859
France Toll-free	0800.911850
Other European Timezones	+44.1799.534000
Other Regions and Locations	1-858-202-ILMN (1-858-202-4566)

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

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