Nextera® DNA
Sample Preparation Guide

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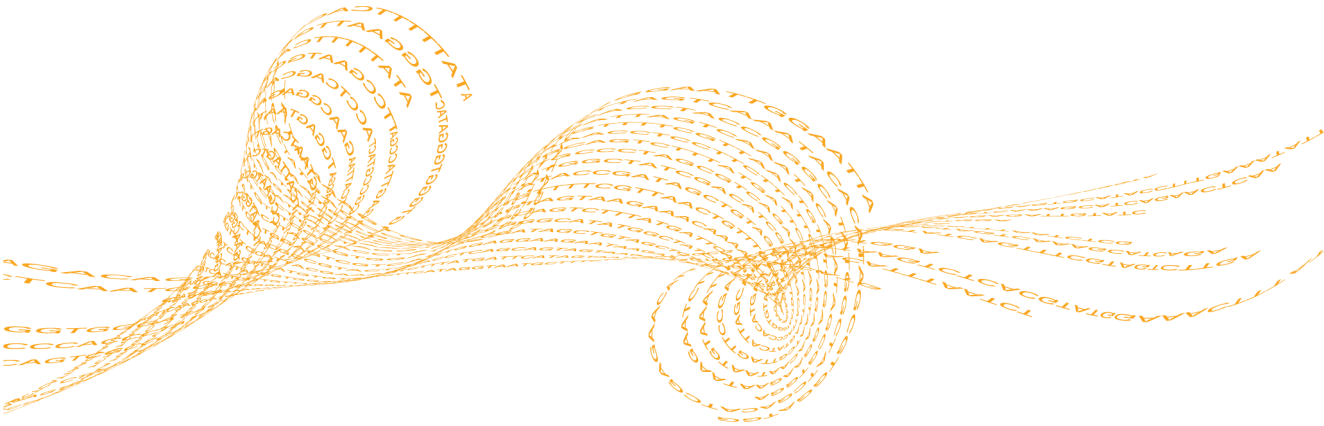

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Introduction

This protocol explains how to prepare up to 96 pooled indexed paired-end libraries from genomic DNA (gDNA) for subsequent cluster generation and DNA sequencing using the reagents provided in the Illumina Nextera®DNA Sample Preparation Kit. The goal of this protocol is to fragment and add adapter sequences onto template DNA with a single tube Nextera reaction (tagmentation) to generate multiplexed single read or paired end sequencing libraries.

The Nextera DNA Sample Preparation protocol offers:

Rapid and streamlined workflow

- Complete protocol in less than 90 minutes
- Single well enzymatic reaction both fragments and adds adapter, no mechanical fragmentation/shearing required
- Mastermixed reagents to reduce reagent containers, pipetting and hands-on time

Lowest DNA input

- Only 50 ng input DNA needed

Higher throughput

- Optimized for plate-based processing for simultaneous preparation of 96 samples
- Master-mixed reagents and automation-friendly configurations
- Volumes optimized for standard 96-well plate workflow

Higher indexing

- 96 indices available and supported on all Illumina sequencers

Table 1 Example of Applications for Different Nextera Kits

Nextera	Nextera XT
Large / complex genomes	Small genomes, amplicons, plasmids
Human genomes	PCR Amplicons (> 300 bp)*
non-human mammalian genomes (e.g. mouse, rat, bovine)	Plasmids
Plant genomes (e.g. arabidopsis, maize, rice)	Microbial Genomes (e.g. Prokaryotes, archea)
Invertebrates genomes (e.g. Drosophila)	Concatenated Amplicons
	double-stranded cDNA

* Illumina recommends > 300 bp to ensure even coverage across the length of the DNA fragment. An expected drop off in sequencing coverage about 50 bp from each distal end of a fragment may be seen. This is because the tagmentation reaction cannot add an adapter right at the distal end of a fragment. For PCR amplicon sequencing this can be easily averted by simply designing your amplicons to be ~100 bases larger than the desired insert to be sequenced.

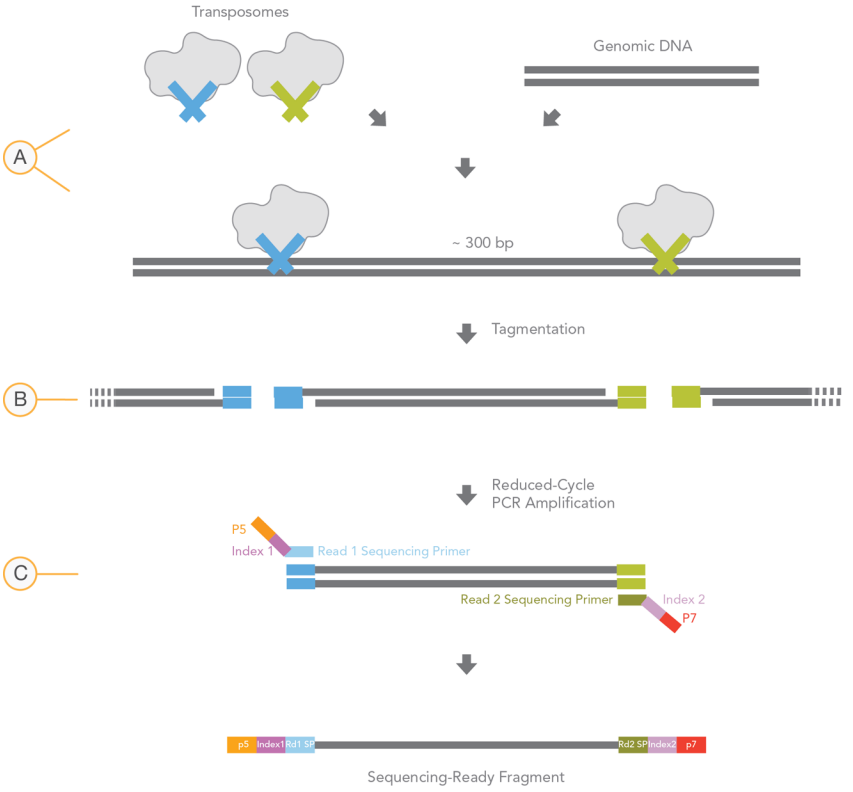
What's New

The following changes were made in this guide revision:

- Added a table showing different applications for Nextera Kits to the Introduction.
- Revised the Input DNA Quantitation section with additional details on quantitation methods. See *DNA Input Recommendations* on page 16.
- Modifications were added in *PCR Clean-Up* for 2x250 runs on the MiSeq. See *PCR Clean-Up* on page 31.
- Modifications were added in *Validate Library* for 2x250 runs on the MiSeq. See *Validate Library* on page 34.
- Changed the normalization concentration from 4nM to 2nM in the *Pool Libraries* section. See *Pool Libraries* on page 36.

How does the Nextera Assay Work?

The Nextera DNA Sample Preparation Kit uses an engineered transposome to simultaneously fragment and tag ("tagment") input DNA, adding unique adapter sequences in the process. A limited-cycle PCR reaction uses these adapter sequences to amplify the insert DNA. The PCR reaction also adds index sequences on both ends of the DNA, thus enabling dual-indexed sequencing of pooled libraries on any Illumina Sequencing System.



A Nextera XT transposome with adapters is combined with template DNA
B Tagmentation to fragment and add adapters
C Limited cycle PCR to add sequencing primer sequences and indicies

Tracking Tools

Illumina provides the following tools for sample tracking and guidance in the lab:

NOTE
You can download these documents from the Illumina website at www.illumina.com. Go to the Nextera DNA Sample Preparation support page and click the **Documentation & Literature** tab. A MyIllumina account is required.

- **Experienced User Card (EUC)** to guide you through the protocol, but with less detail than provided in this user guide. **New or less experienced users are strongly advised to follow this user guide and not the EUC.**
- **Lab Tracking Form (LTF)** to record information about library preparation such as operator name, sample and index information, start and stop times, reagent lot numbers, and barcodes.
  - Create a copy of the lab tracking form for each time you perform this protocol to prepare a library for sequencing.
  - Use it online and save it electronically or print it and fill it out manually.
- The **Illumina Experiment Manager** (IEM) can be used to create your sample sheet using a wizard-based application. The sample sheet is used to record information about your samples for later use in data analysis. The IEM guides you through the steps to create your sample sheet based on the analysis workflow for your run. The IEM provides a feature for recording parameters for your sample plate, such as sample ID, dual indices, and other parameters applicable to your 96-well plate. When using IEM for sample sheet generation, make sure you select **Adapter Trimming** when you create your sample sheet for all Illumina Sequencing Platforms. Shorter inserts can lead to sequencing into the adapter, and this feature helps filter out adapter sequence from the final sequence data. The IEM is also used to create a manifest for the PCR Amplicon analysis workflow in MiSeq Reporter. The PCR Amplicon workflow requires specifying a manifest – a list of all the targeted regions and their chromosome start and end positions. The manifest specifies regions of interest (ROIs) for the aligner and variant caller, which results in faster analysis times and visualization of results specific for only the ROIs. Note that the PCR Amplicon workflow uses a different manifest file format from the TruSeq Custom Amplicon workflow. When starting a run on MiSeq, the MiSeq software (MCS) will prompt for the appropriate

sample sheet and MiSeq Reporter will automatically analyze data based on the workflow information identified in the sample sheet.

NOTE

- You can download IEM from the Illumina website at www.illumina.com.
- IEM can be run on any Windows platform.
- For instructions on how to use the IEM application, see the *Illumina Experiment Manager User Guide* and quick reference card. Go to the Nextera DNA Sample Preparation support page and click the **Documentation & Literature** tab.
- A MyIllumina account is required for these downloads.

- When prompted to select a Sample Prep Kit in IEM, choose **Nextera**.

Documentation

Additional documentation is available for download from the Illumina website. Refer to the inside back cover of this guide for more information.

Training Videos

Illumina provides training videos to illustrate critical steps of the Nextera DNA Sample Preparation protocol. Viewing these videos is strongly recommended before starting your library preparation. To view these valuable training videos for the Nextera DNA Sample Preparation protocol, go to the the Nextera DNA Sample Preparation Kit support page and click on the **Training** tab.

Getting Started

This section describes the Nextera DNA Sample Preparation Kit contents, user-supplied consumables and equipment that you need before beginning the protocol, as well as best practices to apply during the protocol.

CAUTION

If sequencing Nextera libraries with HiSeq2000/1000, HiScanSQ, or GAIIx, you must be sure to use the TruSeq Dual Index Sequencing Primer Boxes (Single Read or Paired End, as appropriate) for all sequencing run types: non-indexed, single-indexed, and dual-indexed. **These add on kits are not required if sequencing a Nextera library with the MiSeq System.**

Nextera DNA Sample Preparation Kit

The Nextera DNA Sample Preparation Kit is packaged in 96 or 24 sample boxes and shipped on dry ice unless specified otherwise below. Each kit has a corresponding Index Kit that contains 96 or 24 indices. As soon as you receive your kit, store the kit components at the specified temperature.

96 Samples

Consumable	Catalog #
Nextera DNA Sample Preparation Kit	FC-121-1031
Nextera DNA Sample Preparation Index Kit (96 Indices, 384 Samples)	FC-121-1012

24 Samples

Consumable	Catalog #
Nextera DNA Sample Preparation Kit	FC-121-1030
Nextera DNA Sample Preparation Index Kit (24 Indices, 96 Samples)	FC-121-1011

TruSeq Index Plate Fixture Kit

It is recommended to use the index plate fixture to assist in correctly arranging the index primers during the PCR Amplification steps. Each kit contains two fixtures and can be used for both the 24-sample kit and 96-sample kit.

Consumable	Catalog #
TruSeq Index Plate Fixture Kit	FC-130-1005

96 Sample Kit Contents

Nextera DNA Sample Preparation Kit

Quantity	Acronym	Reagent Name	Storage Temperature
2	TD	Tagment DNA Buffer	-15° to -25°C
1	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	NPM	Nextera PCR Master Mix	-15° to -25°C
2	PPC	PCR Primer Cocktail	-15° to -25°C
4	RSB	Resuspension Buffer	-15° to -25°C

Nextera Index Kit

Quantity	Reagent Name	Storage Temperature
8 tubes	Index Primers, N501 to N508	-15° to -25°C
12 tubes	Index Primers, N701 to N712	-15° to -25°C

24 Sample Kit Contents

Nextera DNA Sample Preparation Kit

Quantity	Acronym	Reagent Name	Storage Temperature
1	TD	Tagment DNA Buffer	-15° to -25°C
1	TDE1	Tagment DNA Enzyme	-15° to -25°C
1	NPM	Nextera PCR Master Mix	-15° to -25°C
1	PPC	PCR Primer Cocktail	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C

Nextera Index Kit

Quantity	Reagent Name	Storage Temperature
4 tubes	Index Primers, N501 to N504	-15° to -25°C
6 tubes	Index Primers, N701 to N706	-15° to -25°C

About Indexing Reagents

CAUTION

The Nextera DNA Sample Preparation enables highly multiplexed sequencing runs. When performing an indexed sequencing run, whether single-indexed or dual-indexed, you must make sure you prepare and load the correct indexing reagents based on your library.

Indexed sequencing runs require indexing reagents for preparation of the indexing read or reads directly following Read 1. Reagent preparation requires about 20 minutes of thawing time using a water bath at room temperature. When thawed, reagents take about ten minutes to prepare.

For dual-indexing-enabled Nextera libraries, dual-indexing reagents are provided in the following two add-on kits, which can be ordered separately. Additionally, the Read 1, Read 2, and Index 1 (i7) Read sequencing primers in these add-on kits—HP10, HP11, and HP12, respectively—are compatible with non-Nextera libraries:

- TruSeq Dual Index Sequencing Primer Box, Single Read
- TruSeq Dual Index Sequencing Primer Box, Paired End

CAUTION

Nextera libraries require reagents provided in the TruSeq Dual Index Sequencing Primer Boxes (Single Read or Paired End, as appropriate) for *all* sequencing run types: non-indexed, single-indexed, and dual-indexed. Non-Nextera libraries can use reagents in these boxes, or the appropriate reagents in the TruSeq Cluster Kit (v3 or v2).

TruSeq Dual Index Sequencing Primer Box, Single Read

Kit Name	Catalog #
TruSeq Dual Index Sequencing Primer Box, Single Read	FC-121-1003

This Illumina-provided add-on kit contains the following dual-indexing reagents:

- **HP9**—Index 2 (i5) SR Sequencing Primer Mix
- **HP10**—Read 1 Sequencing Primer Mix
- **HP12**—Index 1 (i7) Sequencing Primer Mix

TruSeq Dual Index Sequencing Primer Box, Paired End

Kit Name	Catalog #
TruSeq Dual Index Sequencing Primer Box, Paired End	PE-121-1003

This Illumina-provided add-on kit contains the following dual-indexing reagents:

- **HP10**—Read 1 Sequencing Primer Mix
- **HP11**—Read 2 Sequencing Primer Mix
- **HP12**—Index 1 (i7) Sequencing Primer Mix

Consumables and Equipment

Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation. These consumables and equipment are Illumina recommended for the Nextera DNA Sample Preparation protocols.

Table 2 User-Supplied Consumables

Consumable	Supplier
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
96-well storage plates, round well, 0.8 ml (“MIDI” plate)	Fisher Scientific, part # AB-0859
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
Distilled water	General lab supplier

Consumable	Supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma Aldrich, part # E7023
Microseal 'A' film	BioRad, part # MSA-5001
Microseal 'B' adhesive seals	BioRad, part # MSB-1001
PCR grade water (for gel-free method)	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20	General lab supplier
Tween 20	Sigma, part # P7949
Ultra pure water	General lab supplier
Microseal 96-well PCR plates ("TCY" plate)	Bio-Rad, part # HSP-9601
Zymo™ Purification Kit (ZR-96 DNA Clean & Concentrator™-5)	Catalog # D4023 or D4024

Table 3 User-Supplied Equipment

Equipment	Supplier
96-well thermal cycler (with heated lid)	See table in *Thermal Cycler* section.
Magnetic stand-96	Ambion, part # AM10027
Microplate centrifuge	General lab supplier
Vortexer	General lab supplier
High Speed Micro Plate Shaker	VWR, catalog # 13500-890 (110V/120V) VWR, catalog # 14216-214 (230V)

Thermal Cycler

The following table lists the recommended settings for selected thermal cycler models. Illumina recommends that you validate any thermal cyclers not listed below if your lab has not yet performed the Nextera DNA Sample Preparation protocol.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad DNA Engine Tetrad 2	Calculated	Heated, Constant at 100°C	Polypropylene plates and tubes
MJ Research DNA Engine Tetrad	Calculated	Heated	Plate
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	Plate

Prevent PCR Product Contamination

The PCR process is commonly used in the laboratory to amplify specific DNA sequences. Unless proper laboratory hygiene is used, PCR products can contaminate reagents, instrumentation, and genomic DNA samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations.

Make sure that the lab is set up appropriately to reduce the risk of PCR product contamination:

- **Physically Separate Pre-PCR and Post-PCR Areas**
  - Physically separate laboratory space where pre-PCR processes are performed (DNA extraction, quantification, and normalization) from the laboratory space where PCR products are made and processed during the (post-PCR processes).
  - Never use the same sink to wash pre-PCR and post-PCR troughs.
  - Never share the same water purification system for pre-PCR and post-PCR processes.
  - Store all supplies used in the protocols in the pre-PCR area, and transfer to the post-PCR area as needed.
- **Use Dedicated Equipment and Supplies**

- Dedicate separate full sets of equipment and supplies (pipettes, centrifuges, oven, heat block, etc.) to pre-PCR and post-PCR lab processes, and never share between processes.
- Dedicate separate storage areas (freezers and refrigerators) to pre-PCR and post-PCR consumables.

Because the pre- and post-amplification reagents are shipped together, it is important to unpack the reagents in the pre-PCR lab area, and then move the post-amplification reagents to the proper post-PCR storage area.

Pre-PCR and Post-PCR Lab Procedures

To prevent PCR product contamination, it is important to establish lab procedures and follow best practices. Illumina recommends daily and weekly cleaning of lab areas using 0.5% Sodium Hypochlorite (10% Bleach).

CAUTION

To prevent sample or reagent degradation, make sure that all vapors from the cleaning solution have fully dissipated before beginning any processes.

Daily Cleaning of Pre-PCR Area

A daily cleaning of the pre-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps to eliminate PCR product that has entered the pre-PCR area.

Identify pre-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution before beginning any pre-PCR processes. High-risk areas might include, but are not limited to, the following items:

- Bench tops
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

Daily Cleaning of Post-PCR Area

Reducing the amount of PCR product in the post-PCR area helps reduce the risk of contamination in the pre-PCR area. Daily cleaning of the post-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps achieve this.

Identify post-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution daily. High-risk areas might include, but are not limited to, the following items:

- Thermal cyclers
- Bench space used to process amplified DNA
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

Weekly Cleaning of All Lab Areas

Once a week, perform a thorough cleaning of the pre-PCR and post-PCR areas using 0.5% Sodium Hypochlorite (10% Bleach).

- Clean all bench tops and laboratory surfaces.
- Clean all instruments that are not cleaned daily.
- Thoroughly mop lab floors.
- Make sure that personnel responsible for weekly cleaning are properly trained on prevention of PCR product contamination.

Items Fallen to the Floor

The floor is contaminated with PCR product transferred on the shoes of individuals coming from the post-PCR area; therefore, anything falling to the floor must be treated as contaminated.

- Disposable items that have fallen to the floor, such as empty tubes, pipette tips, gloves, lab coat hangers, must be discarded.
- Non-disposable items that have fallen to the floor, such as a pipette or an important sample container, must be immediately and thoroughly cleaned with a 0.5% Sodium Hypochlorite (10% Bleach) solution to remove PCR product contamination.
- Clean any lab surface that has come in contact with the contaminated item. Individuals handling anything that has fallen to the floor, disposable or non-disposable, must discard their lab gloves and put on a new pair.

DNA Input Recommendations

The Nextera DNA Sample Preparation Kit protocol is optimized for 50 ng of genomic DNA total. Illumina strongly recommends quantifying the starting genomic material.

Input DNA Quantitation

The Nextera DNA Sample Preparation protocol uses an enzymatic DNA fragmentation step and thus can be more sensitive to DNA input compared to mechanical fragmentation methods. The ultimate success of library prep strongly depends on using an accurately quantified amount of input DNA library. Therefore, the correct quantitation of the DNA library is essential.

To obtain an accurate quantification of the DNA library, it is recommended to quantify the starting DNA library using a fluorometric based method specific for duplex DNA such as the Qubit dsDNA BR Assay system. Illumina recommends using 2 µl of each DNA sample with 198 µl of the Qubit working solution for sample quantification. Methods that measure total nucleic acid content (e.g. nanodrop or other UV absorbance methods) should be avoided because common contaminants such as ssDNA, RNA, and oligos are not substrates for the Nextera DNA Sample Preparation assay.

Assessing DNA 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. This protocol is optimized for DNA with absorbance ratio values of 1.8–2.0.

Library Pooling Considerations

The Nextera DNA Sample Preparation Kit enables preparation of up to 24 or 96 libraries with unique dual indexes, referred to as index 1 (i7) and index 2 (i5). Index 1 and 2 sequences are added via PCR primers during the limited-cycle amplification during the library prep. In the case where less than the full set of 24/96 libraries will be pooled and sequenced, it is extremely important that libraries with the proper index combinations are contained in the multiplex pool. Illumina strongly recommends the following sequence of planning before the library preps begin:

1 Determine the number of libraries that will be pooled for sequencing.

2 Ensure that the pool contains the required index combinations, as described in the *Dual Indexing Principle* and *Low Plexity Index Pooling Guidelines* sections at the end of this guide. Select the index PCR primers based on the same guidelines.

3 Use the Illumina Experiment Manager to create a samplesheet which will be used during the sequencing run. This step also identifies any incorrect index combinations, allowing re-design before the library prep starts.

4 Use the Lab Tracking Form to specify the layout of all sample plates.

Acronyms

Table 4 Nextera DNA Sample Preparation Acronyms

Acronym	Definition
NAP1	Nextera Amplification Plate 1
NAP2	Nextera Amplification Plate 2
NDP	Nextera Dilution Plate
NLP	Nextera Library Plate
NPM	Nextera PCR Master Mix
NSP1	Nextera Sample Plate 1
NSP2	Nextera Sample Plate 2
NSP3	Nextera Sample Plate 3
NPP	Nextera Pooled Plate
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
TD	Tagment DNA Buffer
TDE1	Tagment DNA Enzyme

Best Practices

Adhere to the following best practices when preparing libraries for sequencing using this protocol. Several components of this kit are shipped at one temperature and stored at a warmer temperature. The components are stable at either temperature, but should be used at the warmer temperature. To avoid delay during sample preparation, each component should be stored according to the recommendations in the *Getting Started* section.

Ensuring Consistency

- **Use multichannel pipettes**—To make sure there is consistency across samples, use a multichannel pipette where possible. Calibrate pipettes periodically.
- **Pre-aliquot reagents**—To avoid unnecessary freeze-thaw cycles when performing experiments of fewer than 96 samples, Illumina recommends that you aliquot smaller volumes of reagents normally stored frozen after they are thawed for the first time.

Handling Magnetic Beads

NOTE
For instructions on viewing a video demonstration of this process see page 7.

- **Use at room temperature**—Prior to use, allow the beads to reach room temperature prior to use. Use a 25°C water bath as necessary.
- **Vortex until well-suspended**—Immediately prior to use, vortex the beads until they are well-suspended and the color appears homogeneous.
- **Mix samples thoroughly**—After adding the beads to your samples, mix thoroughly by pipetting up and down ten times. Illumina also recommends using a shaker to thoroughly mix samples.
- **Allow maximum binding**—For best results, incubate your bead/sample mixtures at room temperature for the entire duration indicated in the protocol.
- **Slowly aspirate cleared solution**—After placing the plate on the magnetic stand, wait for the solution to clear before proceeding. Keep the plate on the magnetic stand when slowly aspirating cleared solution, taking care not to disturb the separated beads.

Avoiding Cross-Contamination

- **Change tips between samples**—Always use fresh pipette tips between samples and between dispensing index primers.
- **Mix plates as directed**—Mix samples with a multichannel pipette and centrifuge the plate when indicated. Do not vortex the plates.
- **Use aerosol-resistant tips**—Using aerosol-resistant pipette tips reduces the risk of amplicon carry-over and sample-to-sample cross-contamination.

NOTE
If aerosol-resistant tips are not available, ensure careful pipetting to avoid contamination.

Washing with 80% Ethanol During PCR Clean-Up

- **Prepare fresh 80% ethanol**—Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air impacting your results.
- **Remove all ethanol from wells**—Make sure that you remove all ethanol from the bottom of the wells as it might contain residual contaminants. Use a P20 multichannel pipette to remove residual ethanol and accelerate drying.
- **Allow complete evaporation**—Allow at least ten minutes of drying time on the magnetic stand at room temperature for complete evaporation. Residual ethanol can impact the performance of subsequent reactions.

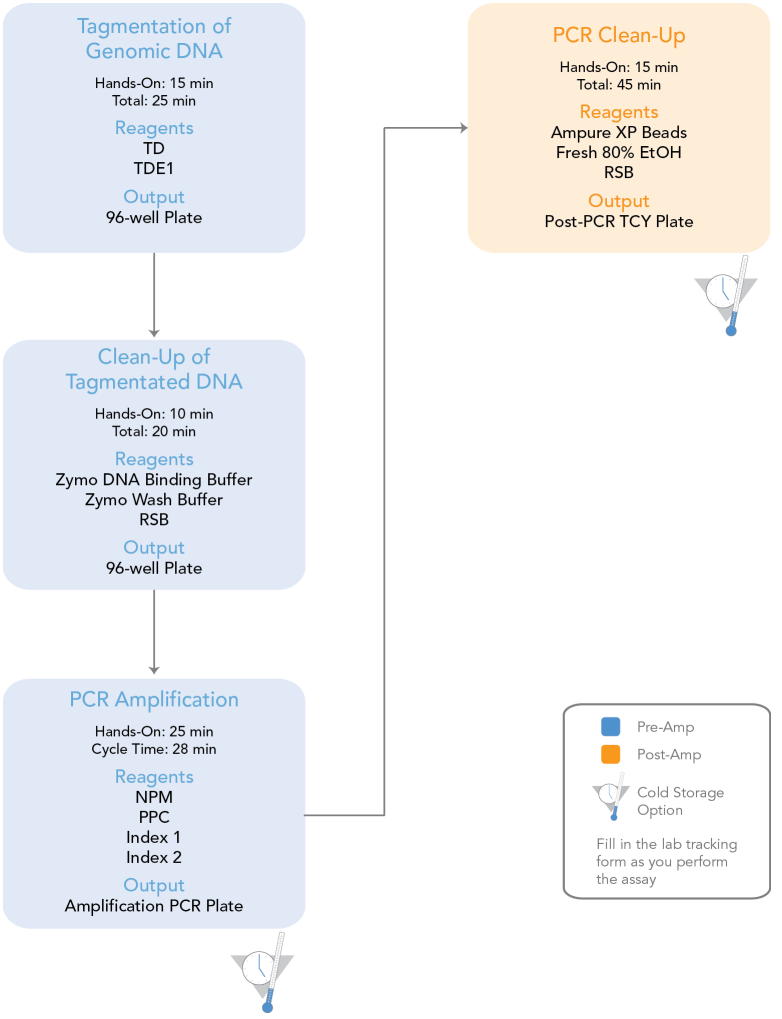
Freeze/thawing for Small Number of Samples

- Each reagent tube supplied with your assay kit contains sufficient volume to process 16 samples at once, using an 8-channel pipette and a reservoir. When processing smaller sample batches (fewer than 96 samples) using a reagent reservoir, dead volume and pipetting error losses can increase. To make sure there is an accurate reagent volume for all samples, single-pipette the reagent into each well.
- To store remaining reagent, Illumina recommends freezing aliquots, rather than repeatedly freezing and thawing the supplied reagent tubes.

Nextera DNA Sample Preparation Workflow

The following diagram illustrates the workflow using the Nextera DNA Sample Preparation Kit. Safe stopping points are marked between steps.

Figure 1 Nextera DNA Sample Preparation Workflow (For 8 samples)



Tagmentation of Genomic DNA

During this step genomic DNA is tagmented (tagged and fragmented) by the Nextera transposome. The Nextera transposome simultaneously fragments the genomic DNA and adds adapter sequences to the ends, allowing amplification by PCR in subsequent steps.

Consumables

Item	Quantity	Storage	Supplied By
TD (Tagment DNA Buffer)	1 tube	-15° to -25°C	Illumina
TDE1 (Tagment DNA Enzyme)	1 tube	-15° to -25°C	Illumina
96-well hard shell TCY plate	1 plate	Room temperature	User
Genomic DNA (2.5 ng/µl)	50 ng	-15° to -25°C	User
Microseal 'B' adhesive seal		Room temperature	User

Preparation

1 Remove the TD, TDE1, and genomic DNA from -15° to -25°C storage and thaw on ice.

NOTE
In preparation for the next step of the protocol, ensure ST buffer is at room temperature, and there are no particles or precipitate visible in the solution.

2 After thawing, ensure all reagents are adequately mixed by gently inverting the tubes 3–5 times, followed by a brief spin in a microcentrifuge.

Procedure

NOTE
Ensure the reaction is assembled in the order described for optimal kit performance. The reaction does not need to be assembled on ice.

1. Label a new 96-well TCY plate "NET1" (Nextera Enrichment Tagmentation Plate 1) with a smudge resistant pen.
2. Add 20 µl of genomic DNA at 2.5 ng/µl (50 ng total) to each sample well of the NET1 plate.
3. Add 25 µl of TD Buffer to the wells containing genomic DNA. Change tips between samples.

   NOTE
   Calculate the total volume of TD for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the NSP1 plate.

4. Add 5 µl of TDE1 to the wells containing genomic DNA and TD Buffer. Change tips between samples.

   NOTE
   Calculate the total volume of TDE1 for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the NSP1 plate.

5. Using a multichannel pipette, gently pipette up and down 10 times to mix. Change tips between samples.
6. Cover the NET1 plate with Microseal 'B'.
7. Centrifuge at 280 xg at 20°C for 1 minute.
8. Place the NET1 plate in a thermocycler and run the following program:

   NOTE
   Ensure that the thermocycler lid is heated during the incubation.

   - 55°C for 5 minutes
   - Hold at 10°C

9. Proceed to *Clean-Up of Tagmented DNA*.

Clean-Up of Tagmented DNA

The tagmented DNA is purified from the Nextera transposome. This step is critical because the Nextera transposome can bind tightly to DNA ends and will interfere with downstream processes if not removed.

Estimated Time (8 reactions)

- Hands-on: 5 minutes
- Total duration: 10 minutes

Consumables

Item	Quantity	Storage	Supplied By
RSB (Resuspension Buffer)	1 tube	-15° to -25°C	Illumina
Zymo DNA binding buffer	1 bottle	Room temperature	User
Zymo wash buffer (EtOH added)	1 bottle	Room temperature	User
Zymo Collection plate	1 plate	Room temperature	User
96-well hard shell TCY plate	1 plate		User
96-well MIDI plate	1 plate		User

Preparation

1 Remove RSB from -15° to -25°C storage and thaw at room temperature.

2 While the tagmentation reaction is in progress, perform steps 1 and 2 of the Procedure section.

Procedure

1 Label a new MIDI plate NSP2 (Nextera Sample Plate 2).

2 Add 180 µl of Zymo DNA binding buffer to each well of the NSP2 plate with a sample in the corresponding well of the NSP1 plate.

3 Transfer 50 µl from each well of NSP1 to the corresponding well of the NSP2 plate to be used in the assay. Gently pipette up and down 10 times to mix. Change tips between samples.

NOTE
A multichannel pipette may be used, and binding buffer may be poured into a trough.

4 From the Zymo Purification Kit, take the Zymo-Spin™ I-96 Plate and place it on the Collection Plate.

5 Using a multichannel pipette, transfer sample mixture from the NSP2 plate to the corresponding wells of the mounted Zymo-Spin™ I-96 Plate.

6 Centrifuge at 1,300 xg at 20°C for 2 minutes. Discard the flow-through.

NOTE
Illumina recommends the use of open-top plate holders for the centrifugation step to accommodate the plate stack.

7 Wash the Zymo-Spin™ I-96 Plate plate as follows:

a Using a multichannel pipette, add 300 µl of wash buffer from a trough to each sample well.

b Change tips between columns to avoid cross-contamination.

c Centrifuge at 1,300 xg at 20°C for 2 minutes. Discard the flow-through.

8 Repeat step 7 for a total of 2 washes.

9 Centrifuge at 1,300 xg for 2 minutes to ensure no residual wash buffer is present.

10 Take the Zymo-Spin™ I-96 Plate and place it on a new TCY plate labeled NSP (Nextera Sample Plate).

11 Add 25 µl of RSB directly to the column matrix in each well.

12 Incubate the plate for 2 minutes at room temperature.

13 Centrifuge the plate at 1,300 xg at 20°C for 2 minutes.

NOTE
(Optional) Check the products of the tagmentation reaction by loading 1 µl of undiluted Zymo eluate on a HS Bioanalyzer chip. This should produce a broad distribution of DNA fragments with a size range from ~150 bp – < 1 Kb.

PCR Setup

In this step, the purified tagmented DNA is amplified via a limited-cycle PCR program. The PCR step also adds index 1 (i7) and index 2 (i5) and sequencing, as well as common adapters (P5 and P7) required for cluster generation and sequencing. It is critical to use the full amount of recommended input DNA, as well as to not add extra cycles of PCR cycles to ensure high quality libraries that produce high-quality sequencing results.

Estimated Time (8 reactions)

- Hands-on: 10 minutes
- Spin: 30 minutes
- Total duration: 40 minutes

Consumables

Item	Quantity	Storage	Supplied By
PPM (PCR Primer Mix)	1 tube	-15° to -25°C	Illumina
Index 1 primers (N7XX)	1 tube each index	-15° to -25°C	Illumina
Index 2 primers (N5XX)	1 tube each index	-15° to -25°C	Illumina
TruSeq Index Plate Fixture			Illumina
Microseal 'A' adhesive film			User
96-well hard shell TCY plate	1 plate		User

Preparation

1 If the full set of 24/96 libraries is to be prepared for pooling and sequencing proceed to step 2. If less than a full set of libraries is pooled for sequencing, ensure that the correct index 1 (i7) and index 2 (i5) primers have been selected. See the *Dual Indexing and Low Plexity Pooling Guidelines* section at the end of the

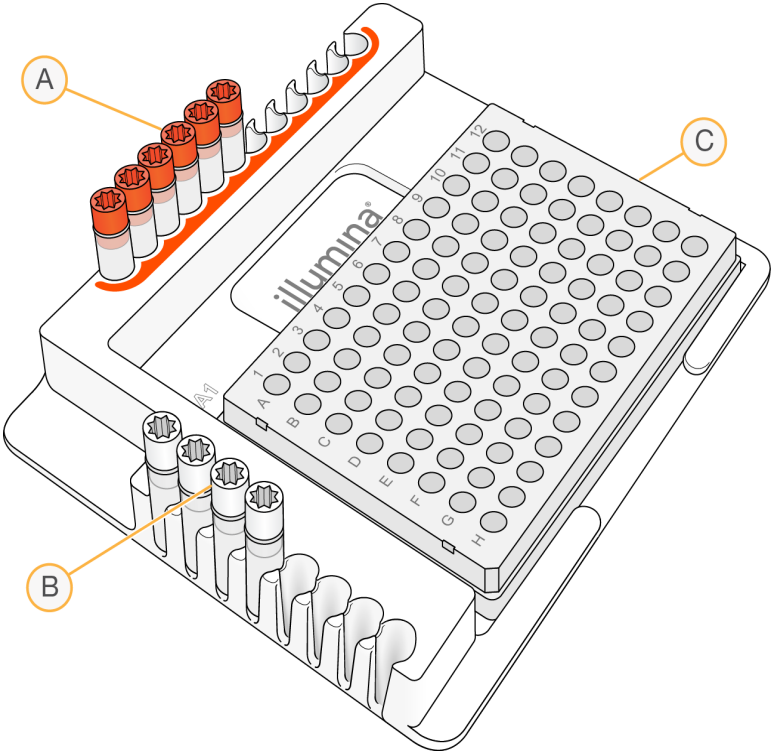
Nextera DNA Sample Preparation Guide, and use the Illumina Experiment Manager to verify that the correct index primers have been selected.

2 Remove NPM, PPC, and the index primers from -15° to -25°C storage and thaw on a bench at room temperature.
Allow approximately 20 minutes to thaw NPM, PPC, and index primers.

3 After all reagents are completely thawed, gently invert each tube 3–5 times to mix and briefly centrifuge the tubes in a microcentrifuge. Use 1.7 ml Eppendorf tubes as adapters for the microcentrifuge.

4 For 24 libraries arrange the index primers in the TruSeq Index Plate Fixture using the following arrangement:
   a Arrange index 1 (i7) primers (orange caps) in order horizontally, so N701 is in column 1 and N706 is in column 6.
   b Arrange index 2 (i5) primers (white caps) in order vertically, so N501 is in row A and N504 is in row D.
   c Record their positions on the Lab Tracking Form.

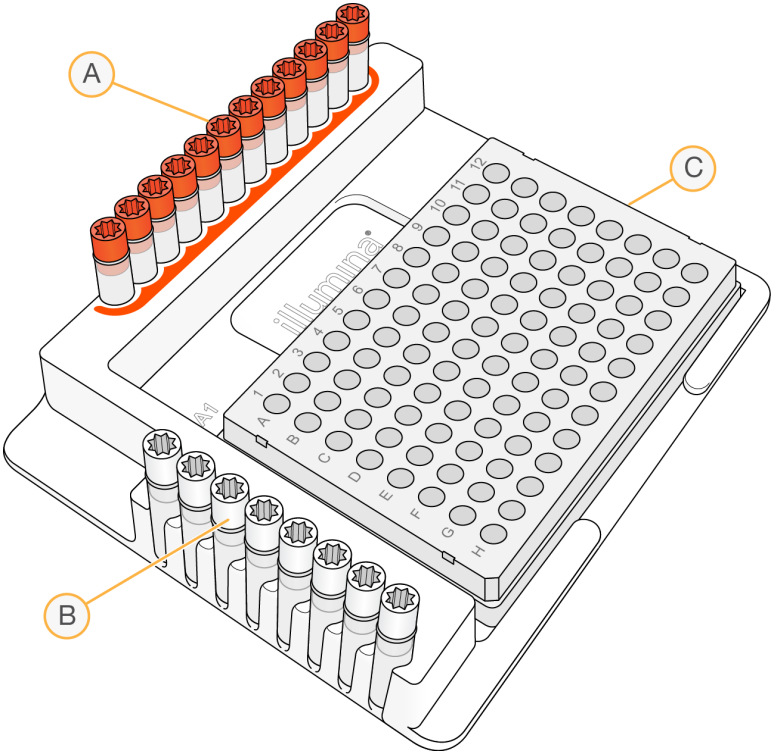
Figure 2 TruSeq Index Plate Fixture Arrangement (24 libraries)



A Index primer 1 (i7) (orange caps)
B Index primer 2 (i5) (white caps)
C NAP1 plate

5 For 96 libraries arrange the index primers in the TruSeq Index Plate Fixture using the following arrangement:
   a Arrange index 1 (i7) primer tubes (orange caps) in order horizontally, so that N701 is in column 1 and N712 is in column 12.
   b Arrange index 2 (i5) primers (white caps) in order vertically, so that N501 is in row A and N508 is in row H.
   c Record their positions on the lab tracking form.

Figure 3 TruSeq Index Plate Fixture (96 libraries)



A Index primer 1 (i7) (orange caps)
B Index primer 2 (i5) (white caps)
C NAP1 plate

6 Label a new 96-well microplate NAP1 (Nextera Amplification Plate 1).

7 Using a multichannel pipette, add 5 µl of index 2 primers (white caps) to each column of the NAP1 plate. Changing tips between columns is required to avoid cross-contamination.

8 Using a multichannel pipette, add 5 µl of index 1 primers (orange caps) to each row of the NAP1 plate. ***Tips must be changed after each row to avoid index cross-contamination.***

9 To avoid index cross-contamination, discard the original *white* caps and apply new *white* caps provided in the kit.

10 To avoid index cross-contamination, discard the original *orange* caps and apply new *orange* caps provided in the kit. Remove all the index primer tubes from the working area.

Procedure

1 Add 15 µl of NPM to each well of the NAP1 plate containing index primers. Change tips between samples.

NOTE
Calculate the total volume of NPM for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the NAP1 plate.

2 Add 5 µl PPC to each well containing index primers and NPM. Change tips between samples.

NOTE
Calculate the total volume of PPC for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the NAP1 plate.

3 Using a multichannel P20 pipette set to 20 µl:
- Transfer 20 µl of purified tagmented DNA from the NSP3 plate to the corresponding well on the NAP1 plate.
- Gently pipette up and down 3–5 times to thoroughly combine the DNA with the PCR mix. Change tips between samples to avoid index and sample cross contamination.

4 Cover the plate with Microseal 'A' and seal with a rubber roller.

5 Centrifuge at 280 xg at 20°C for 1 minute.

6 Transfer the NAP1 plate to the post-amplification area.

7 Perform PCR using the following program on a thermal cycler:

NOTE
Ensure that the thermocycler lid is heated during the incubation.

- 72°C for 3 minutes

- 98°C for 30 seconds
- 5 cycles of:
  - 98°C for 10 seconds
  - 63°C for 30 seconds
  - 72°C for 3 minutes
- Hold at 10°C

SAFE STOPPING POINT

If you do not plan to immediately proceed to *PCR Clean-Up* following the completion of PCR, the plate can remain on the thermal cycler overnight, or you can store it at 2° to 8°C up to two days.

PCR Clean-Up

This step uses AMPure XP beads to purify the library DNA, and provides a size selection step that removes very short library fragments from the population.

NOTE
For instructions on viewing a video demonstration of this process see page 7.

Estimated Time (8 reactions)

- Hands-on: 15 minutes
- Total duration: 30 minutes

Consumables

Item	Quantity	Storage	Supplied By
RSB (Resuspension Buffer)	1 tube	-15° to -25°C	Illumina
AMPure XP beads		2° to 8°C	User
80% Ethanol, freshly-prepared			User
96-well MIDI plates	1 plate		User
96-well TCY plates	1 plate		User

Preparation

NOTE
Please review the **Best Practices** section at the beginning of this protocol regarding the handling of magnetic beads and washing with 80% ethanol during the PCR clean-up.

1 Bring the AMPure XP beads to room temperature.

2 Prepare fresh 80% ethanol from absolute ethanol.

NOTE
Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air impacting your results.

Procedure

1 Centrifuge the NAP1 plate at 280 xg for 1 min (20˚C) to collect condensation.

2 Label a new MIDI plate **NAP2** (Nextera Amplification Plate 2).

3 Using a multichannel pipette set to 50 µl, transfer the PCR product from the NAP1 plate to the NAP2 plate. Change tips between samples.

4 Vortex the AMPure XP beads for 30 seconds to ensure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough.

5 Using a multichannel pipette, add 30 µl of AMPure XP beads to each well of the NAP2 plate.
For 2x250 runs on the MiSeq, add 25 µl of AMPure XP beads to each well of the NAP2 plate.

6 Gently pipette mix up and down 10 times.

NOTE
Alternatively the solution can be mixed by shaking the NAP2 plate on a microplate shaker at 1,800 rpm for 2 minutes.

7 Incubate at room temperature without shaking for 5 minutes.

8 Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.

9 With the NAP2 plate on the magnetic stand, use a multichannel pipette to carefully remove and discard the supernatant. Change tips between samples.

NOTE
If any beads are inadvertently aspirated into the tips, dispense the beads back to the plate and let the plate rest on the magnet for 2 minutes and confirm that the supernatant has cleared.

10 With the NAP2 plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
   a Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well. You should not resuspend the beads at this time.

b Incubate the plate on the magnetic stand for 30 seconds or until the supernatant appears clear.
c Carefully remove and discard the supernatant.

11 With the NAP2 plate on the magnetic stand, perform a second ethanol wash as follows:
a Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
b Incubate the plate on the magnetic stand for 30 seconds or until the supernatant appears clear.
c Carefully remove and discard the supernatant.
d Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.

12 With the NAP2 plate still on the magnetic stand, allow the beads to air-dry for 15 minutes.

13 Remove the NAP2 plate from the magnetic stand. Using a multichannel pipette, add 32.5 µl of RSB to each well of the NAP2 plate.

14 Gently pipette mix up and down 10 times, changing tips after each column.

NOTE
Alternatively the solution can be mixed by shaking the NAP2 plate on a microplate shaker at 1,800 rpm for 2 minutes.

15 Incubate at room temperature for 2 minutes.

16 Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

17 Label a new TCY plate **NLP** (Nextera Library Plate).

18 Using a multichannel pipette, carefully transfer 30 µl of the supernatant from the NAP2 plate to the NLP plate. Change tips between samples to avoid cross-contamination.

Validate Library

Illumina strongly recommends performing the following procedures for quality control analysis on your sample library and quantification of the DNA library templates.

Quantify Libraries

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. Quantify your libraries using a dsDNA-specific fluorescent dye method, such as Qubit or picogreen. Based on an average library size of 500bp, convert the library concentration using the formula 1ng/ul = 3nM.

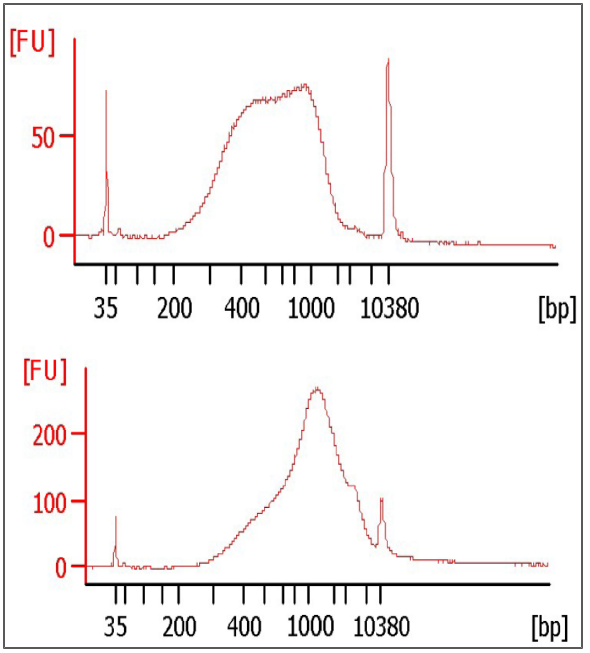
Quality Control

It is recommended to check the size distribution for some/all of libraries by running 1 µl of 1:3 diluted library on an Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip. Figure 4 shows example traces of successfully sequenced libraries. Typical libraries show a broad size distribution from ~250 bp to 1000 bp, as in the top panel. A wide variety of libraries can be sequenced, with average fragment sizes as small as 250 bp to as large as 1000-1500 bp. For larger libraries (example in bottom panel), the DNA concentration used for clustering may need to be adjusted (see table). Libraries with average size greater than 1000 bp may require clustering at several concentrations to achieve optimal density.

The modified *PCR Clean-Up* step for 2x250 runs on the MiSeq results in libraries with larger average size. It is recommended to adjust the DNA concentration conversion formula to 1ng/µl = 1.5 nM. Clustering at several different DNA concentrations may be required to achieve optimal cluster density.

Library Size from Bioanalyzer in bp	Conversion Factor for ng/µl > nM	DNA Concentration for Cluster Generation
250	1 ng/µl = 6 nM	6–12 pM
500	1 ng/µl = 3 nM	6–12 pM
1000–1500	1 ng/µl = 1.5 nM	12–20 pM

Figure 4 Successful Human Genomic DNA Library Size Distributions Sequenced on HiSeq



Pool Libraries

This process describes how to prepare DNA templates that will be applied to cluster generation. Multiplexed DNA libraries are normalized to 2nM in the Nextera Dilution Plate and then pooled in equal volumes in the Nextera Pooled Plate. Non-multiplexed DNA libraries are normalized to 2nM in the Nextera Dilution Plate without pooling.

Consumables

Item	Quantity	Storage	Supplied By
NDP (Nextera Dilution Plate) barcode label	1		Illumina
NPP (Nextera Pooled Plate) barcode label (for multiplexing only)	1		Illumina
Microseal 'B' adhesive film			User
Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20		-15° to -25°C	User
96-well MIDI plate	2 plates		User

Preparation

- Apply a NDP barcode label to a new 96-well MIDI plate.
- Apply a NPP barcode label to a new 96-well MIDI plate (for multiplexing only).
- Remove the NLP plate from -15° to -25°C storage, if it was stored at the conclusion of *PCR Clean-Up*, and let stand to thaw at room temperature.
  - Briefly centrifuge the thawed NLP plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed NLP plate.

Make NDP

1 Transfer 10 µl of sample library from each well of the NLP plate to the corresponding well of the new MIDI plate labeled with the NDP barcode. Change the tip after each sample.

2 Normalize the concentration of sample library in each well of NDP plate to 2nM using Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20.

NOTE
Depending on the yield quantification data of each sample library, the final volume in the NDP plate may vary from 10–100 µl.

3 Mix the NDP plate as follows:

a Seal the NDP plate with a Microseal 'B' adhesive seal.

b Shake the NDP plate on a microplate shaker at 1,000 rpm for 2 minutes.

c Centrifuge the NDP plate to 280 xg for 1 minute.

d Remove the adhesive seal from the NDP plate.

4 Depending on the type of library you want to generate, do one of the following:

- For non-multiplexed paired-end libraries, the protocol stops here. Do one of the following:
  - Proceed to cluster generation. See the *Illumina Cluster Generation User Guide*.
  - Seal the NDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.
- For multiplexed paired-end libraries, proceed to *Make NPP*.

Make NPP (for multiplexing only)

NOTE
Do not make a NPP plate if there is no pooling.

1 Using a multichannel pipette, transfer 5 µl of each sample in column 1 of the NDP plate to column 1 of the NPP plate.

2 Change tips and transfer 5 µl of each sample in column 2 of the NDP plate to column 1 of the NPP plate.

3 Repeat step 2 for as many times as there are remaining columns in the NDP plate. Change tips between columns. The result will be a NPP plate with pooled samples in column 1.

NOTE
Keep track of which sample goes into which well, to avoid pooling two samples with the same index.

4 Combine the contents of each well of column 1 into well A2 of the NPP plate, for the final pool.

5 Mix the NPP plate as follows:
   a Seal the NPP plate with a Microseal 'B' adhesive seal.
   b Shake the NPP plate on a microplate shaker at 1,800 rpm for 2 minutes.

6 Do one of the following:
   - Proceed to cluster generation. See the *Illumina Cluster Generation User Guide*.
   - Seal the NPP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.

Dual Indexing Principle

The dual indexing strategy uses two 8 base indices, Index 1 (i7) adjacent to the P7 sequence, and Index 2 (i5) adjacent to the P5 sequence. Dual indexing is enabled by adding a unique Index 1 (i7) and Index 2 (i5) to each sample from 12 different Index 1 (i7) adapters (N701–N712) and 8 different Index 2 (i5) adapters (N501–N508) for the 96 sample Nextera Index Kit (FC-121–1012), and 6 different Index 1 (i7) adapters (N701–N706) and 4 different Index 2 (i5) adapters (N501–N504) for the 24 sample Nextera Index Kit (FC-121–1011). In the Index adapter name, the N refers to Nextera sample preparation, 7 or 5 refers to Index 1 (i7) or Index 2 (i5), respectively, and 01–12 refers to the Index number. A list of index sequences is provided for generating sample sheets to demultiplex the samples:

Index 1 (i7)	Sequence	Index 2 (i5)	Sequence
N701	TAAGGCGA	N501	TAGATCGC
N702	CGTACTAG	N502	CTCTCTAT
N703	AGGCAGAA	N503	TATCCTCT
N704	TCCTGAGC	N504	AGAGTAGA
N705	GGACTCCT	N505	GTAAGGAG
N706	TAGGCATG	N506	ACTGCATA
N707	CTCTCTAC	N507	AAGGAGTA
N708	CAGAGAGG	N508	CTAAGCCT
N709	GCTACGCT		
N710	CGAGGCTG		
N711	AAGAGGCA		
N712	GTAGAGGA		

Low Plexity Pooling Guidelines

Illumina uses a green laser to sequence G/T and a red laser to sequence A/C. At each cycle at least one of two nucleotides for each color channel need to be read to ensure proper registration. It is important to maintain color balance for each base of the index read being sequenced, otherwise index read sequencing could fail due to registration failure. If you choose the dual index sequencing workflow always use at least two unique and compatible barcodes for each index (index 1 and index 2). The following tables illustrate possible pooling strategies:

Table 5 Libraries Pooled: 6 or fewer; Sequencing Workflow: Single Index

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
1-plex (no pooling)	Any Index 1 adapter	Any Index 2 adapter
2-plex	• [option 1] N702 and N701 • [option 2] N702 and N704	
3-plex	• [option 1] N701, N702, and N704 • [option 2] N703, N705, and N706	
4- or 5-plex	• [option 1] N701, N702, N704, and any other Index 1 adapter • [option 2] N703, N705, N706, and any other Index 1 adapter	
6-plex	N701, N702, N703, N704, N705, and N706	

Table 6 Sequencing Workflow: Single or Dual Index

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
7–12 plex, Dual Index	• [option 1] N701, N702, N704, and any other Index 1 adapter (as needed)	• [option 1] N501 and N502 • [option 2] N503 and N504 • [option 3] N505 and N506

Plex	Index 1 (i7) Selection	Index 2 (i5) Selection
	• [option 2] N703, N705, N706, and any other Index 1 adapter (as needed)	
7–12 plex, Single Index (96 sample Nextera Index adapter kit)	• N701–N706 and any other Index 1 adapter (as needed)	• Any Index 2 (i5) adapter
Greater than 12-plex	N701, N702, N703, N704, N705, N706, and any other Index 1 adapter	• [option 1] N501, N502, and any other Index 2 adapter (as needed) • [option 2] N503, N504, and any other Index 2 adapter (as needed) • [option 3] N505, N506, and any other Index 2 adapter (as needed)

These represent only some of the acceptable combinations. Alternatively, please check the real sequences of each index in the table above to make sure each base position will have signal in both color channels for the index read:

Good				Bad			
Index 1		Index 2		Index 1		Index 2	
705	GGACTCCT	503	TATCCTCT	705	GGACTCCT	502	CTCTCTAT
706	TAGGCATG	503	TATCCTCT	706	TAGGCATG	502	CTCTCTAT
701	TAAGGCGA	504	AGAGTAGA	701	TAAGGCGA	503	TATCCTCT
702	CGTACTAG	504	AGAGTAGA	702	CGTACTAG	503	TATCCTCT
	√√√√√√√√		√√√√√√√√		√√√√√√√√		√√√√xxxx

√=signal in both color
x=signal missing in one color channel

Notes

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 7 Illumina General Contact Information

Illumina Website	www.illumina.com
Email	techsupport@illumina.com

Table 8 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at www.illumina.com/msds.

Product Documentation

Additional product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support and select a product. A MyIllumina login is required. To register for a MyIllumina account, please visit my.illumina.com/Account/Register.

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