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Nextera XT DNA Library Prep

Product Documentation

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Overview

This guide explains how to prepare up to 384 dual-indexed paired-end libraries from DNA using the Nextera XT DNA Library Prep workflow.

The Nextera XT workflow:

- Uses tagmentation, an enzymatic reaction, to fragment DNA and add partial adapter sequences in only 15 minutes.
- Reduces reagent containers, pipetting, and hands-on time using master mixed reagents.
- Requires only 1 ng input DNA.
- Supports genomes that are less than 5 Mb.

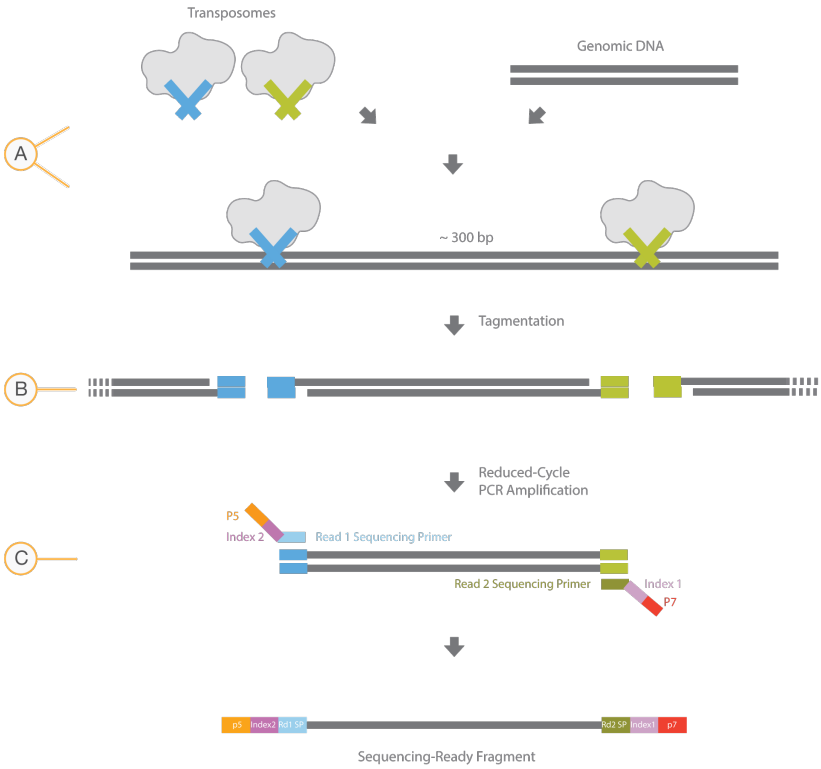
Table 1 Example Applications

Nextera XT (FC-131-1024, FC-131-1096)	Illumina DNA Prep (20018704, 20018705)
Small genomes, amplicons, plasmids	Human genomes, large or complex genomes
PCR amplicons (> 300 bp)*	Small genomes, microbial genomes, plasmids, PCR amplicons (> 150 bp)
Plasmids	Nonhuman mammalian genomes (eg, mouse, rat, bovine)
Microbial genomes (eg, Prokaryotes, Archaea)	Plant genomes (eg, Arabidopsis, maize, rice)
Concatenated amplicons	Invertebrate genomes (eg, Drosophila)
Double-stranded cDNA	–
Single-cell RNA-Seq	–

* Using > 300 bp amplicon size ensures even coverage across the length of the DNA fragment. For more information, refer to *PCR Amplicons* on page 3.

How the Nextera XT Assay Works

The Nextera XT DNA Library Prep uses an engineered transposome to tagment genomic DNA, which is a process that fragments DNA and then tags the DNA with adapter sequences in one step. Limited-cycle PCR uses the adapters to amplify the insert DNA. The PCR step also adds index adapter sequences on both ends of the DNA, which enables dual-indexed sequencing of pooled libraries on Illumina sequencing platforms.



A. Nextera XT transposome with adapters combined with template DNA
B. Tagmentation to fragment and add adapters
C. Limited-cycle PCR to add index adapter sequences

DNA Input Recommendations

The Nextera XT protocol is optimized for 1 ng of input DNA. Quantify the starting material before preparing libraries.

Assess DNA purity to make sure that the initial DNA sample does not contain > 1 mM EDTA and is free of organic contaminants, such as phenol and ethanol. These substances can interfere with the Nextera XT tagmentation reaction and result in assay failure.

Input DNA Quantification

The enzymatic DNA fragmentation used for this protocol is more sensitive to DNA input compared to mechanical fragmentation. Success depends on accurate quantification of input DNA.

Use a fluorometric-based method to quantify input DNA. For example, if you use the Qubit dsDNA BR Assay system, use 2 µl of each DNA sample with 198 µl of the Qubit working solution. Avoid methods that measure total nucleic acid, such as NanoDrop or other UV absorbance methods.

Assess DNA Purity

UV absorbance is a common method used for assessing the purity of a DNA sample. The ratio of absorbance at 260 nm to absorbance at 280 nm provides an indication of sample purity. This protocol is optimized for DNA with 260/280 absorbance ratio values of 1.8–2.0, which indicates a pure DNA sample.

For a secondary indication of sample purity, use the ratio of absorbance at 260 nm to absorbance at 230 nm. Target a 260/230 ratio of 2.0–2.2. Values outside this range indicate the presence of contaminants. For a complete list of contaminants, including sources, avoidance, and effects on the library preparation, refer to *Nextera XT Library Prep: Tips and Troubleshooting (Pub. No. 770-2015-015)*.

Dilute the starting material in 10 mM Tris-HCl, pH 7.5–8.5. Incomplete tagmentation caused by contaminants can cause library preparation failure, poor clustering, or low quality sequencing results.

PCR Amplicons

When starting with PCR amplicons, the PCR amplicon must be > 300 bp. The standard clean-up protocol depletes libraries < 500 bp. Therefore, Illumina recommends that amplicons < 500 bp undergo a 1.8 x Illumina Purification Beads volume normal ratio to supernatant during *Clean Up Libraries* on page 14. Shorter amplicons can otherwise be lost during the library cleanup step.

Tagmentation cannot add an adapter directly to the distal end of a fragment, so a drop in sequencing coverage of ~50 bp from each distal end is expected. To ensure sufficient coverage of the amplicon target region, design primers to extend beyond the target region by 50 bp per end.

Consumables & Equipment

The protocol described in this guide assumes that you have reviewed the contents of this section, confirmed protocol contents, and obtained all required consumables and equipment.

Illumina-Supplied Consumables

Completing the Nextera XT protocol requires library prep reagents and index adapters.

Component	Kit Options	Catalog #
Library prep reagents	Nextera XT DNA Library Preparation Kit (24 Samples)	FC-131-1024
	Nextera XT DNA Library Preparation Kit (96 Samples)	FC-131-1096

Component	Kit Options	Catalog #
Index adapters	IDT for Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)	20027213
	IDT for Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples)	20027214
	IDT for Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples)	20042666
	IDT for Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples)	20042667
	IDT for Illumina Nextera DNA Unique Dual Indexes Set C (96 Indexes, 96 Samples)	20027215
	IDT for Illumina Nextera DNA Unique Dual Indexes Set D (96 Indexes, 96 Samples)	20027216
	Nextera XT Index Kit v2 Set A (96 Indexes, 384 Samples)	FC-131-2001
	Nextera XT Index Kit v2 Set B (96 Indexes, 384 Samples)	FC-131-2002
	Nextera XT Index Kit v2 Set C (96 Indexes, 384 Samples)	FC-131-2003
	Nextera XT Index Kit v2 Set D (96 Indexes, 384 Samples)	FC-131-2004
	Nextera XT Index Kit (24 Indexes, 96 Samples)	FC-131-1001
Accessory Products for Nextera XT	Illumina Purification Bead, 100 ml	20060057
	Illumina Purification Bead, 400 ml	20060058

Nextera XT Library Prep Kit Contents

Box 1 of 2

These reagents are shipped at -25°C to -15°C. Promptly store reagents at the indicated tube temperature to ensure proper performance.

Tube Quantity		Acronym	Reagent Name	Storage Temperature
24 Samples	96 Samples			
1	1	ATM	Amplicon Tagment Mix	-25°C to -15°C
1	2	TD	Tagment DNA Buffer	-25°C to -15°C
1	1	NPM	Nextera PCR Master Mix	-25°C to -15°C
1	4	RSB	Resuspension Buffer	-25°C to -15°C
1	1	LNA1	Library Normalization Additives 1	-25°C to -15°C
1	2	LNW1	Library Normalization Wash 1	2°C to 8°C

Box 2 of 2

These reagents are shipped at -25°C to -15°C. Promptly store reagents at the indicated tube temperature to ensure proper performance.

Tube Quantity		Acronym	Reagent Name	Storage Temperature
24 Samples	96 Samples			
1	1	NT	Neutralize Tagment Buffer	Room temperature
1	1	LNB1	Library Normalization Beads 1	2°C to 8°C
1	1	LNS1	Library Normalization Storage Buffer 1	Room temperature

Index Kit Contents

For index adapter sequences, refer to *Illumina Adapter Sequences*.

IDT for Illumina Nextera DNA UD Indexes (96 Indexes, 96 Samples) Plate Format, Store at -25°C to -15°C

Quantity	Description
1	96 Dual Adapter Index Plate

Nextera XT Index Kit v2 (96 indexes, 384 Samples), Store at -25°C to -15°C

Set	Quantity	Description
A	8 tubes	Index Primers, S502, S503, S505–S508, S510, and S511
	12 tubes	Index Primers, N701–N707, N710–N712, N714, and N715
B	8 tubes	Index Adapters: S502, S503, S505–S508, S510, and S511
	12 tubes	Index Adapters: N716, N718–N724, and N726–N729
C	8 tubes	Index Adapters: S513, S515–S518, and S520–S522
	12 tubes	Index Adapters: N701–N707, N710–N712, N714, and N715
D	8 tubes	Index Adapters: S513, S515–S518, and S520–S522
	12 tubes	Index Adapters: N716, N718–N724, and N726–N729

Index Adapter Replacement Caps, Store at 15°C to 30°C

Quantity	Description
1 bag	i7 Index Tube Caps, Orange
1 bag	i5 Index Tube Caps, White

Nextera XT Index Kit (24 Indexes, 96 Samples), Store at -25°C to -15°C

Quantity	Description
4 tubes	Index Adapters: S502–S504 and S517
6 tubes	Index Adapters: N701–N706

Index Adapter Replacement Caps, Store at 15°C to 30°C

Quantity	Description
1 bag	i7 Index Tube Caps, Orange
1 bag	i5 Index Tube Caps, White

TruSeq Index Plate Fixture Kit, Store at Room Temperature (Optional)

Each TruSeq Index Plate Fixture Kit contains two fixtures to help arrange index primers before dispensing to a 96-well plate during library amplification. The fixture pairs with both the 24-sample kit and 96-sample kit.

Quantity	Description
2	TruSeq Index Plate Fixture

User-Supplied Consumables & Equipment

Make sure that you have the required consumables and equipment before starting the protocol.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Consumables

Consumable	Supplier
10 µl pipette tips	General lab supplier
200 µl pipette tips	General lab supplier
1000 µl pipette tips	General lab supplier
96-well storage plates, round well, 0.8 ml (MIDI plate)	Fisher Scientific, catalog # AB-0859
Illumina Purification Beads	Illumina, 1 x 100 ml, catalog # 20060057 Illumina, 4 x 100 ml, catalog # 20060058
Distilled water	General lab supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, product # E7023
Microseal 'A' film	Bio-Rad, catalog # MSA-5001
Microseal 'B' adhesive seals	Bio-Rad, catalog # MSB-1001
Microseal 'F' foil seals	Bio-Rad, catalog # MSF-1001
NaOH 1 N, pH > 12.5, molecular biology grade	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, catalog # 89094-658
Ultrapure water	General lab supplier
Hard-Shell 96-well PCR plates	Bio-Rad, catalog # HSP-9601

Equipment

Equipment	Supplier
10 µl multichannel pipettes	General lab supplier

Equipment	Supplier
10 µl single channel pipettes	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
High-Speed microplate shaker	VWR, catalog # 13500-890 (110 V/120 V) VWR, catalog # 14216-214 (230 V)
Magnetic stand-96	Thermo Fisher Scientific, catalog # AM10027
Microplate centrifuge	General lab supplier
Vortexer	General lab supplier

Thermal Cyclers

Use the following recommended settings for selected thermal cycler models. Before performing library prep, validate any thermal cyclers not listed.

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

Signatures

FAS/FSE	Customer

Protocol

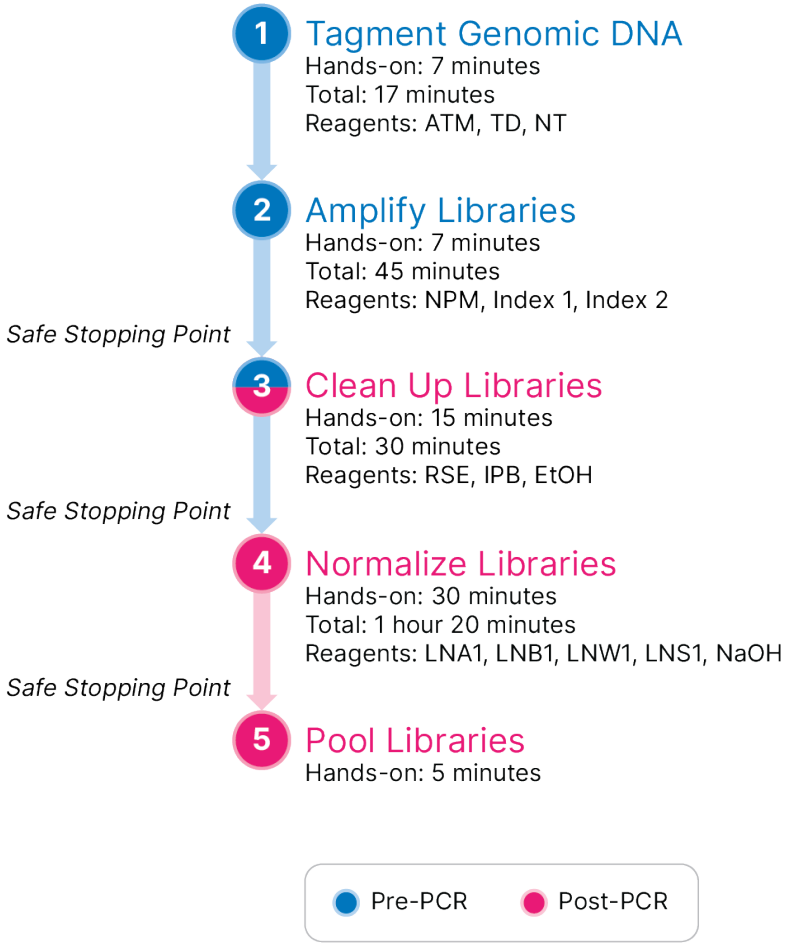
This section describes the Nextera XT DNA protocol.

- Review the planned complete sequencing workflow, from sample through analysis, to ensure compatibility of products and experiment parameters.
- Before proceeding, confirm kit contents and make sure that you have the required components, equipment, and consumables. This protocol requires library prep reagents and index adapters. Index adapters are sold separately. Refer to *Illumina-Supplied Consumables* on page 3.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.

Library Prep Workflow

The following diagram illustrates the Nextera XT DNA Library Prep workflow. Safe stopping points are marked between steps.

Time estimates are based on processing 8 samples.



Tips and Techniques

Safe Stopping Point

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples or reagent master mixes, change tips between ***each sample***.
- When adding index adapters with a multichannel pipette, change tips between ***each row*** or ***each column***. If using a single channel pipette, change tips between each sample.
- [Tubes] Open only one index adapter tube at a time to prevent misplacing caps. Remove unused index adapter tubes from the working area.

Sealing the Plate

- Always seal the 96-well plate with the adhesive seal using a rubber roller to cover the plate before the following steps in the protocol:
  - Shaking steps
  - Thermal cycling steps
  - Centrifuge steps
- Microseal 'A' adhesive film is used for thermal cycling steps to prevent evaporation.
- Microseal 'B' adhesive seals are effective at -40°C to 110°C and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' seals for thermal cycling or short-term storage.
- Microseal ‘F’ foil seals are effective at temperatures down to -70°C and are suitable for storing the 96-well plates containing the final libraries long term.

IPB 100 ml Bottle Resuspension

Manually mix the bead reagent container by manual inversion. Inversion mixing constitutes turning a container upside down and then returning it to its upright position.

1. Mix the bead reagent by inversion, at a rate of at least a single inversion per second.
2. Rotate bottle 90 degrees every 30s, and continually inverting for a total of 2 minutes.
3. After this time, visually inspect the inside of the container for any solid material still adhering to the walls.
4. If container inner walls are free of any residual material, bead reagent can be considered fully resuspended.
5. If reagent is still found on the inner walls, repeat the mixing step for additional 1 minute.

6. Repeat visual inspection of the container walls to ensure complete mixing. If necessary, repeat the mixing step (5).

Preparing IDT for Illumina DNA/RNA Unique Dual (UD) Indexes Plate

- Prepare IDT for Illumina DNA/RNA UD Indexes as follows.
- Nextera XT is compatible with IDT for Illumina DNA/RNA Unique Dual (UD), IDT for Illumina Nextera DNA Unique Dual (UD), and Nextera DNA Combinatorial Dual (CD) Indexes.
- Pipette slowly to minimize foaming.
- Each index plate is for single use only.
- IDT for Illumina DNA/RNA UD Indexes use 10 base pair index codes that differ from Nextera XT, and Nextera XT v2 indexes, which use eight base pair index codes. Confirm that your sequencing system is configured for 10 base pair index codes.
- Centrifuge at 1000 × g for 1 minute to settle liquid away from the seal.
- **[< 96 samples]** Pierce the foil seal on the index adapter plate using a new pipette tip for each well for only the number of samples being processed.
- **[96 samples]** Align a new Eppendorf 96-well PCR plate above the index adapter plate and press down to puncture the foil seal on all 96 wells. Press down slowly to avoid tipping the volume over.
- Discard the empty Eppendorf plate used to puncture the foil seal.

Tagment Genomic DNA

This step uses the Nextera XT transposome to tagment gDNA, which is a process that fragments and tags DNA with adapter sequences.

Consumables

- ATM (Amplicon Tagment Mix)
- TD (Tagment DNA Buffer)
- NT (Neutralize Tagment Buffer)
- 96-well PCR plate
- Microseal 'B' adhesive seals

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
ATM	-25°C to -15°C	Thaw on ice. Invert the thawed tubes 3–5 times, and then centrifuge briefly.
TD	-25°C to -15°C	Thaw on ice. Invert the thawed tubes 3–5 times, and then centrifuge briefly.
NT	15°C to 30°C	Check for precipitates. If present, vortex until all particulates are resuspended.

2. Save the following TAG program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 55°C for 5 minutes
   - Hold at 10°C

Procedure

1. Add the following volumes ***in the order listed*** to each well of a new 96-well PCR plate.
   - TD (10 µl)
   - 1 ng DNA (5 µl)
2. Pipette to mix.
3. Add 5 µl ATM to each well.
4. Pipette 10 times to mix, and then seal the plate.
5. Centrifuge at 280 × g at 20°C for 1 minute.
6. Place on the preprogrammed thermal cycler and run the TAG program. When the program reaches 10°C, ***immediately*** proceed to step 7 because the transposome is still active.
7. Add 5 µl NT to each well.
8. Pipette 10 times to mix, and then seal the plate.
9. Centrifuge at 280 × g at 20°C for 1 minute.
10. Incubate at room temperature for 5 minutes.

Amplify Libraries

This step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds the Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing cluster generation. To confirm indexes selected for low plexity pooling have the appropriate color balance, refer to the *Index Adapters Pooling Guide*.

Index adapter tubes or plates are ordered separately from the library prep components. For a list of compatible index adapters for use with this protocol, refer to *Illumina-Supplied Consumables* on page 3.

Consumables

- NPM (Nextera PCR Master Mix)
- Index adapters (tubes or plates)
- Microseal 'A' adhesive film

About Reagents

- Index adapter plates
  - A well may contain > 10 µl index adapters.
  - Do not add samples to the index adapter plate.
  - Each well of the index plate is single use only.
- Index adapter tubes
  - Open only one index adapter tube at a time to prevent misplacing caps. Alternatively, use fresh caps after opening each tube.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
Index adapters	-25°C to -15°C	Thaw at room temperature. [Tubes] Vortex to mix, and then centrifuge briefly. [Plates] Spin briefly before use.
NPM	-25°C to -15°C	Thaw on ice for 20 minutes.

2. Save the following NXT PCR program on a thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 72°C for 3 minutes
   - 95°C for 30 seconds
   - 12 cycles of:
     - 95°C for 10 seconds
     - 55°C for 30 seconds
     - 72°C for 30 seconds
   - 72°C for 5 minutes
   - Hold at 10°C

Procedure

1. Add the following index adapter volumes per sample according to your index adapter kit type.

Index Adapter Kit Type	Volume of Index Adapter per Sample
Index adapter tubes	5 µl i7 adapter 5 µl i5 adapter
Index adapter plate	10 µl prepared i7 and i5 index adapters

2. Add 15 µl NPM to each well.
3. Pipette 10 times to mix, and then seal the plate.
4. Centrifuge at 280 × g at 20°C for 1 minute.
5. Place on the preprogrammed thermal cycler and run the NXT PCR program.

SAFE STOPPING POINT

If you are stopping, seal the plate, and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Clean Up Libraries

This step uses single-sided bead purification to purify amplified libraries.

Consumables

- IPB (Illumina Purification Beads)
- EtOH (Freshly prepared 80% ethanol)
- RSB (Resuspension Buffer)
- 96-well 0.8 ml polypropylene deepwell storage plate (MIDI plate) (2)
- 96-well PCR plate
- Microseal 'B' adhesive seal
- Microseal 'F' foil seals
- Nuclease-free water

About Reagents

- Illumina Purification Beads
  - Must be at room temperature before use.
  - Resuspend before each use.
  - Resuspend frequently to make sure that beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
IPB	15°C to 30°C	Resuspend IPB beads.
RSB	-25°C to -15°C	Thaw and bring to room temperature. Vortex to mix. RSB can be stored at 2°C to 8°C after the initial thaw.

2. Prepare fresh 80% EtOH from absolute ethanol.

Procedure

1. Centrifuge at 280 × g at 20°C for 1 minute to collect contents at the bottom of the well.
2. Transfer 50 µl supernatant from each well of the PCR plate to corresponding wells of a new MIDI plate.

   The ratio of supernatant to volume of IPB is 3:2. If you transfer less than 50 µl supernatant, adjust the volume of IPB accordingly.

3. If you are using standard DNA input, add 30 µl IPB to each well containing supernatant.
4. If you are using small PCR amplicon sample input, add the IPB volume according to your input size.

Input Size (bp)	IPB Recommendation	IPB Volume (µl)
300–500	1.8x IPB	90
> 500	0.6x IPB (0.5x IPB for ≥ 2 x 250 cycles)	30 (25 µl for ≥ 2 x 250 cycles)

5. Seal the plate, and then use a plate shaker at 1800 rpm for 2 minutes.
6. Incubate at room temperature for 5 minutes.
7. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
8. Without disturbing the beads, remove and discard all supernatant.
9. Wash two times with 200 µl 80% EtOH as follows.
   a. With the plate on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
   b. Incubate for 30 seconds.
   c. Without disturbing the beads, remove and discard all supernatant.
10. Use a 20 µl pipette to remove and discard residual EtOH.
11. Air-dry on the magnetic stand for 15 minutes.
12. Remove from the magnetic stand.
13. Add 52.5 µl RSB to the beads.
14. Seal the plate, and then use a plate shaker at 1800 rpm for 2 minutes.

15. Incubate at room temperature for 2 minutes.
16. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
17. Transfer 50 µl supernatant to a new 96-well PCR plate.

SAFE STOPPING POINT

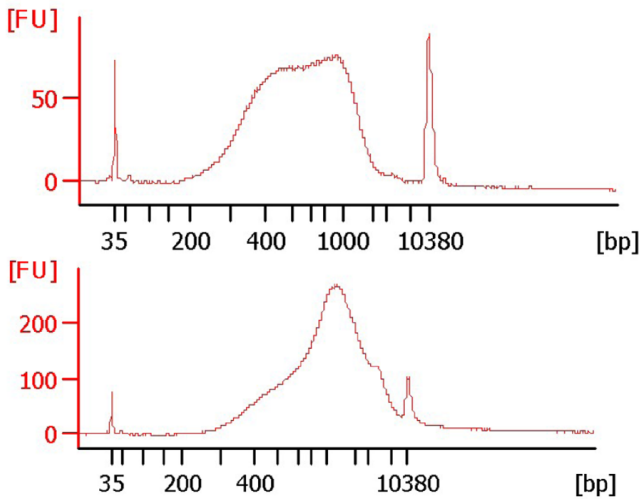
If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal and store at -25°C to -15°C for up to 7 days.

Check Library Quality

Run 1 µl undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA kit.

Typical libraries show a broad size distribution of ~250–1000 bp, as shown in the top panel. Various libraries can be sequenced with average fragment sizes as small as 250 bp or as large as 1500 bp.

Figure 1 Example Bioanalyzer Trace



Quality Metrics

Two factors can cause cluster density fluctuations in libraries prepared with the Nextera XT DNA Library Prep:

- An average sample size that is too large or too small after tagmentation.
- A final sample concentration that is too low due to a low yield when starting the bead-based normalization step.

To troubleshoot fluctuations in cluster density, consider checking library size and library concentration. For more information, refer to *Nextera XT Library Prep: Tips and Troubleshooting (Pub. No. 770-2015-015)*.

Check Library Size

Larger molecules cluster less efficiently than smaller molecules. If the fragment size after tagmentation is larger than expected, low cluster numbers are possible. The inverse is also true. The average expected library size after tagmentation is between 400 bp and 1.2 kb.

Check the library size with a high sensitivity Bioanalyzer trace after the PCR cleanup step. Look for a long low plateau. Alternatively, PCR-amplify the library with qPCR primers and run the product on an agarose gel. The sequence for these primers is available in the *Sequencing Library qPCR Quantification Guide (document # 11322363)*.

- **Short libraries indicate too little input DNA**—Requantify the input DNA with a fluorometric method. Start with 10%–25% more input DNA. If the library peak is below 400 bp and you want to continue with this library, dilute the library further.
- **Long libraries indicate too much input DNA or the presence of inhibitors**—Start with less input DNA, make sure that the input DNA is free from inhibitors, and repeat the quantification step.

For more information on library dilution, refer to the denature and dilute libraries guide for your sequencing system.

Check Library Concentration

Bead-based normalization is most efficient when the library yield after amplification is 10–15 nM, or higher. Measure library concentration using high sensitivity dsDNA Qubit after library cleanup, and measure library size with a Bioanalyzer to calculate molarity.

If you are starting with high-quality DNA and see low yield after library cleanup, there are possible issues with IPB cleanup or the amplification step. If results show either condition, confirm proper storage of the PCR master mix at -25°C to -15°C in a no-frost freezer. Confirm minimal freeze-thaw cycles.

Normalize Libraries

This step normalizes the quantity of each library made with Nextera XT Index v2 or Nextera XT Index Kits to ensure more equal library representation in the pooled library.

Do not follow the normalization protocol and instead use *Dilute Libraries to the Starting Concentration* on page 20 for manual normalization:

- If you are using IDT for Illumina Nextera UD Indexes.
- If the final library yield is < 10 nM.
- If your sequencing system uses onboard denaturation.

Consumables

- LNA1 (Library Normalization Additives 1)

- LNB1 (Library Normalization Beads 1)
- LNW1 (Library Normalization Wash 1)
- LNS1 (Library Normalization Storage Buffer 1)
- 0.1 N NaOH (fewer than 7 days old) (3 ml per 96 samples)
- 96-well 0.8 ml polypropylene deep-well storage plate (MIDI plate)
- 96-well PCR plate
- 15 ml conical tube
- Microseal 'B' adhesive seals

About Reagents

- Vortex LNA1 vigorously to make sure that all precipitates have dissolved. Inspect in front of a light.
- Vortex LNB1 vigorously, with intermittent inversion (at least 1 minute). Repeat until all beads are resuspended and no beads are present at the bottom of the tube when it is inverted.
- Always use a wide-bore pipette tip for LNA1.
- Mix only the required amounts of LNA1 and LNB1 for the current experiment. Store the remaining LNA1 and LNB1 separately at the recommended temperatures.
- Aspirate and dispense beads slowly due to the viscosity of the solution.

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, refer to the SDS at support.illumina.com/sds.html.

Preparation

Prepare the following consumables:

Item	Storage	Instructions
LNA1	-25°C to -15°C	Prepare under a fume hood. Bring to room temperature. Use a 20°C to 25°C water bath as needed.
LNB1	2°C to 8°C	Bring to room temperature. Use a 20°C to 25°C water bath as needed.
LNW1	2°C to 8°C	Bring to room temperature. Use a 20°C to 25°C water bath as needed.

Item	Storage	Instructions
LNS1	Room temperature	Keep at room temperature.

Procedure

1. Transfer 20 µl supernatant from each well of the PCR plate to the corresponding well of a new MIDI plate.
2. Combine the following volumes in a 15 ml conical tube to prepare the LN master mix. Multiply each volume by the number of samples being processed.
   - LNA1 (46 µl)
   - LNB1 (8 µl)

   Reagent overage is included in the volume to ensure accurate pipetting.
3. Pipette 10 times to mix.
4. Pour the LN master mix into a trough.
5. Use a 200 µl multichannel pipette to transfer 45 µl LN master mix to each well.
6. Seal the plate, and then use a plate shaker at 1800 rpm for 30 minutes.
7. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
8. Without disturbing the beads, remove and discard all supernatant.
9. Wash two times with 45 µl LNW1 as follows.
   a. Add 45 µl LNW1 to each well.
   b. Seal the plate, and then use a plate shaker at 1800 rpm for 5 minutes.
   c. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
   d. Without disturbing the beads, remove and discard all supernatant.
10. Add 30 µl 0.1 N NaOH to each well.
11. Seal the plate, and then use a plate shaker at 1800 rpm for 5 minutes.
12. Add 30 µl LNS1 to each well of a new 96-well PCR plate labeled SGP.
13. After the 5 minute elution completes, make sure that all samples in the MIDI plate are resuspended. If they are not, resuspend as follows.
    a. Pipette 10 times to mix or lightly tap the sample plate on the bench.
    b. Seal the plate, and then use a plate shaker at 1800 rpm for 5 minutes.
14. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
15. Transfer 30 µl supernatant from each well of the MIDI plate to the corresponding well of the SGP plate.
16. Seal the sample plate, and then centrifuge at 1000 × g for 1 minute.

At this point, the libraries are single-stranded DNA, which resolves poorly on an agarose gel or Bioanalyzer chip. For quality control, use the double-stranded DNA saved from step 17 of the cleanup procedure.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal and store at -25°C to -15°C for up to 7 days.

Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system and is the first step in a serial dilution. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

Use this procedure when the *Normalize Libraries* on page 17 protocol is not followed.

For sequencing, Illumina recommends the read lengths indicated on the Nextera XT DNA Library Prep compatible products support page. If you would like additional overlapped reads, raw coverage, or adjusted IPB recommendations for ≥ 2 x 250 cycles, you can sequence up to 2 x 250 or 2 x 300, but it is not required.

IDT for Illumina DNA/RNA UD Indexes uses 10 base pair index codes that differ from the Nextera XT and Nextera XT v2 indexes, which use eight base pair index codes. This change in base pair index codes can require adjustments to your sequencing run set up.

Procedure

1. Calculate the molarity value of the library or pooled libraries using the following formula.
   - For libraries qualified on a Bioanalyzer or other trace instrument, use the average size obtained for the library.
   - If a trace instrument is not available, use 600 bp as the average library size.

$$\frac{\text{concentration in ng/}\mu\text{l}}{660\ \text{g/mol} \times \text{average library size in bp}} \times 10^6 = \text{Molarity (nM)}$$

2. Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system.
3. Dilute libraries using RSB:
   - **Libraries quantified as a multiplexed library pool**—Dilute the pool to the starting concentration for your system.
   - **Libraries quantified individually**—Dilute each library to the starting concentration for your system.

   Add 10 µl of each diluted library to a tube to create a multiplexed library pool.

4. Follow the denature and dilute instructions for your system to dilute to the final loading concentration.
   - For the iSeq 100 System, refer to the system guide for dilution instructions (libraries are automatically denatured).
   - For the NovaSeq 6000 System, refer to the system guide for pool and denature instructions.
   - For the HiSeq 4000 and HiSeq 3000 Systems, refer to the cBot 2 or cBot system guide for reagent preparation instructions.
   - For all other systems, refer to the denature and dilute libraries guide.

   Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

Resources & References

Additional Resources

The following resources provide instructions and guidelines for using the prepared libraries. Refer to the Illumina support page for additional information.

- Compatible products and requirements for recording sample information, sequencing libraries, and analyzing data.
- Questions and answers about using the kit.
- Training videos about the kit and courses for related products and subjects.
- The latest versions of the kit documentation.

Table 2 Additional Recommended Resources

Resource	Description
Custom Protocol Selector	A tool for generating end-to-end instructions tailored to your library prep method, run parameters, and analysis method, with options to refine the level of detail.
Index Adapters Pooling Guide	Provides pooling guidelines and dual-index strategies for using the 10-base pair IDT for Illumina DNA/RNA UD Indexes or 8-base pair Nextera XT and Nextera XT v2 Indexes with the Nextera XT DNA Library Prep kit.
Illumina Adapter Sequences	Provides the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.
IDT for Illumina DNA/RNA UD Indexes support page	Provides information about IDT for Illumina DNA/RNA Unique Dual (UD) Indexes.

Revision History

Document	Date	Description of Change
Document # 15031942 v07	April 2023	Clarified Nextera XT DNA Library Prep workflow overview. Corrected normalize libraries procedure reagent name LNB1. Clarified instructions for calculating molarity. Clarified instructions for manual normalization. Removed statement pertaining to incompatible TruSeq v3 primers on discontinued HiSeq 2500 System. Updated kit options and catalog numbers. Updated list of additional resources. Removed references to formalin-fixed paraffin-embedded (FFPE) applications not supported by the workflow. Removed list of acronyms. Added HTML format.
Document # 15031942 v06	August 2021	Add IPB bead resuspension section to tips and techniques Replaced AMPure XP with IPB Bead Replaced Vortex verbiage with resuspend Changed storage temperature and instructions in Clean Up Libraries preparation. IPB bead added to consumables and acronyms sections

Document	Date	Description of Change
Document # 15031942 v05	May 2019	Added information on IDT® for Illumina®–Nextera™ UD Indexes sets A, B, C, and D, including kit contents, preparation procedures, and additional resources. Removed plate layout information. Removed the Pool Libraries section and moved the Check Library Quality section before the Normalize Libraries section. Revised Additional Resources to provide more clarity on the resources available. Revised language throughout document to provide consistency across other Nextera library preparation reference guides. Added protocol for diluting libraries to the starting concentration. Removed obsolesced Nextera XT Index Kit (96 Indexes, 384 Samples) (# FC-131-1002) from Kit Contents.
Document # 15031942 v04	January 2019	Added information on reviewing sequencing workflows to ensure compatibility with library prep methods.
Document # 15031942 v03	February 2018	Updated the normalize libraries procedure to indicate that shaking samples after the five-minute elution is necessary only if samples are not resuspended. Reorganized kit contents information, including renaming some sections to match kit labeling and identify storage temperature. Corrected the diagram that shows how the Nextera XT assay works to clarify each transposome dimer has two of the same adapter color.

Document	Date	Description of Change
Document # 15031942 v02	April 2017	Added the following information: • Supported genome size of < 5 Mb. • The ratio of absorbance that indicates contaminants. • Recommendations for PCR amplicons. • AMPure XP bead recommendations for runs ≥ 2 × 250 cycles. • Reagent and library volumes in the PCR plate after the tagmentation and amplification steps. • Beckman Coulter Genomics item # A63880 for Agencourt AMPure XP, 5 ml. • Illumina catalog # PE-121-1003 and # FC-121-1003 for the TruSeq Dual Index Sequencing Primer Box. Added the following technical notes to the list of additional resources: • *Best Practices for Standard and Bead-Based Normalization in Nextera XT DNA Library Preparation Kits (Pub. No. 470-2016-007)* • *Nextera XT Library Prep: Tips and Troubleshooting (Pub. No. 770-2015-015)* Consolidated steps in the pool libraries procedure. Identified the NaOH consumable as molecular biology grade. Specified the use of molecular-grade water or 10 mM Tris-HCl, pH 7.5–8.5 to dilute starting material for DNA quality assessment. Specified proceeding immediately when tagmentation is complete so that neutralization occurs while the transposome is active. Specified a thaw time of 20 minutes for NPM (Nextera PCR Master Mix). Updated the normalize libraries procedure to apply to various sample numbers, not only 96. Updated TCY plate to Hard-Shell 96-well PCR plate, skirted. Updated magnetic stand supplier to Thermo Fisher Scientific. Corrected the catalog numbers for Nextera kits provided in the introduction. Corrected the illustration showing how the Nextera assay works.

Document	Date	Description of Change
Document # 15031942 v01	January 2016	Updated design of workflow diagram. Renamed and combined some procedures as needed to improve continuity. Simplified consumables information at the beginning of each section. Revised step-by-step instructions to be more succinct. Removed reference to obsolete Experienced User Cards and added reference to the Custom Protocol Selector. Clarified AMPure XP bead recommendation for nonamplicon applications. See Clean Up Libraries. Added information about normalizing low yield libraries. See Normalize Libraries. Corrected index adapter labels on the assay diagram.
15031942 Rev. E	January 2015	Corrected kit contents for Nextera XT DNA Library Preparation Index Kit v2 Set A (FC-131-2001) to include index N715.

Document	Date	Description of Change
15031942 Rev. D	September 2014	Added info for new index kits that enable preparation of up to 384 indexed paired-end libraries. Updated DNA Input Recommendations for diluting starting material and the potential results of incomplete tagmentation. Added new Nextera XT Quality Metrics with new information on how to troubleshoot fluctuations in cluster density. Removed Dual Indexing Principle and Low Plexity Pooling Guidelines sections. This information can be found in the Nextera Low-Plex Pooling Guidelines Tech Note on the Nextera XT DNA Library Prep support page. References to read lengths on the MiSeq were updated for v3 chemistry. Added instructions for alternate tip if processing fewer than 24 samples while transferring LNB1 beads in Library Normalization. Added NaOH 1N pH > 12.5 to the Consumables and Equipment list as a user-supplied consumable. Removed Tween 20 from Consumables and Equipment list. Consumable not used in protocol.

Document	Date	Description of Change
15031942 Rev. C	October 2012	Modifications were added in *PCR Clean-Up* for 2x300 runs on the MiSeq. New section for clustering samples on the HiSeq, HiScanSQ, and GAIIx. See Clustering Samples for HiSeq, HiScanSQ, and GAIIx. The *Dual Indexing Principle* section listed incorrect catalog numbers for the Nextera XT Index kits. The correct catalog numbers are now listed. Emphasized making sure the NT (Neutralize Tagment Buffer) and LNS1 (Library Normalization Storage Buffer 1) reagents are at room temperature before use in the protocol. Removed reference to Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20 from the User-Supplied Consumables table because it is not used in this library preparation.
15031942 Rev. B	July 2012	Emphasized making sure the NT (Neutralize Tagment Buffer) and LNS1 (Library Normalization Storage Buffer 1) reagents are at room temperature before use in the protocol. Removed reference to Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20 from the User-Supplied Consumables table because it is not used in this library preparation.
15031942 Rev. A	May 2012	Initial release.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

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

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download from support.illumina.com.

Illumina, Inc.
5200 Illumina Way
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com

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