Nextera® XT DNA
Sample Preparation Guide

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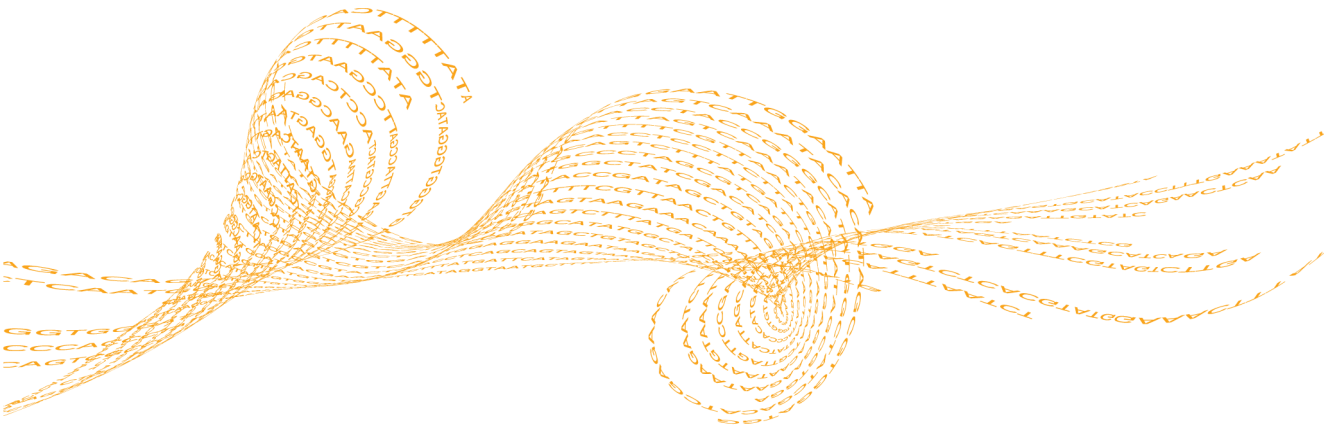

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Introduction

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

The Nextera XT DNA Sample Preparation protocol offers:

Sequencing's fastest and easiest preparation

- Single well enzymatic reaction both fragments and adds adapter in only 15 minutes, no mechanical fragmentation/shearing required
- Master mixed reagents to reduce reagent containers, pipetting and hands-on time
- Innovative sample normalization that eliminates the need for library quantification prior to sample pooling and sequencing

Lowest DNA input

- Only 1 ng input DNA needed

Single kit for many applications

- Easily prepare amplicons, small genomes, and plasmids
- Fastest method to prepare libraries for any Illumina sequencer

Flexible throughput

- 96 indices available and supported on all Illumina sequencers
- Master mixed reagents and automation-friendly configurations

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Table 1 Example of Applications for Different Nextera Kits

Nextera (FC-131-1031)	Nextera XT (FC-131-1096)
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. This enzymatic clipping of PCR primers avoids wasted sequencing output on non-informative bases that do not contain genomic inserts. If you wish to sequence the genomic loci contained within a PCR primer, simply design 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:

- Modifications were added in *PCR Clean-Up* for 2x250 runs on the MiSeq.
- New section for clustering samples on the HiSeq, HiScanSQ, and GAIIx. See *Clustering Nextera XT Samples for HiSeq, HiScanSQ, and GAIIx* on page 42.
- The *Dual Indexing Principle* section listed incorrect catalog numbers for the Nextera XT Index kits. The correct catalog numbers are now listed.
- Added emphasis on 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 10mM, pH8.5 with 0.1% Tween 20* from the User-Supplied Consumables table because it is not used in this sample preparation.

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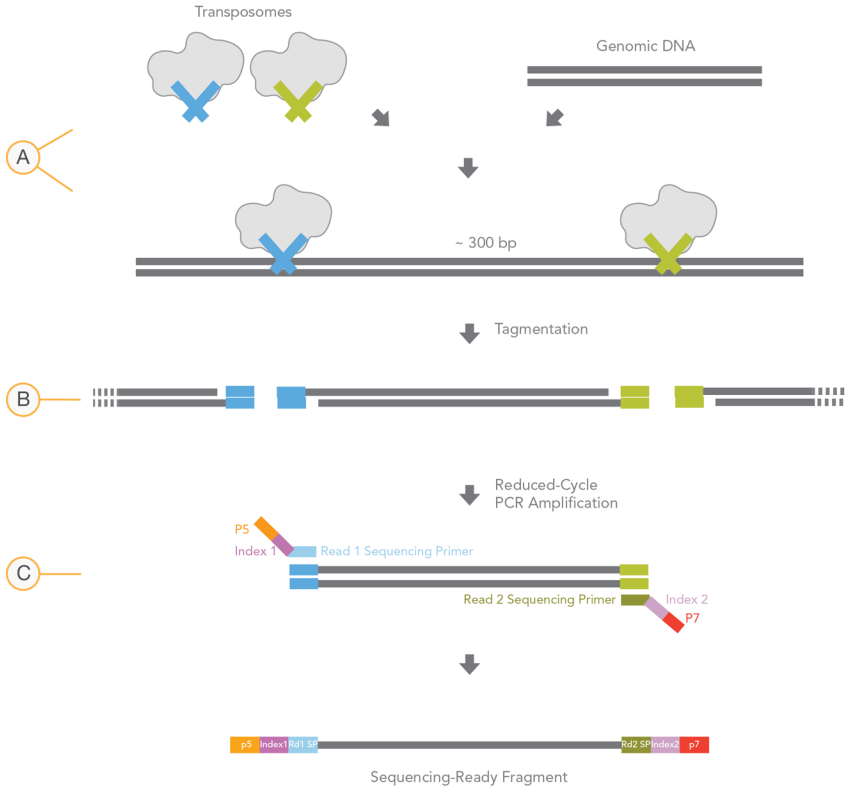
How does the Nextera XT Assay Work?

The Nextera XT 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

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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 XT 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

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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 XT 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 XT**.

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

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Getting Started

This section describes the Nextera XT 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 XT 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 XT library with the MiSeq System.**

Nextera XT DNA Sample Preparation Kit

The Nextera XT 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.

NOTE
Certain components of the kit should be stored at a different temperature than the temperature at which they are shipped. As soon as you receive your kit, store the kit components at the specified temperature.

96 Samples

Consumable	Catalog #
Nextera XT DNA Sample Preparation Kit	FC-131-1096
Nextera XT DNA Sample Preparation Index Kit (96 Indices, 384 Samples)	FC-131-1002

24 Samples

Consumable	Catalog #
Nextera XT DNA Sample Preparation Kit	FC-131-1024
Nextera XT DNA Sample Preparation Index Kit (24 Indices, 96 Samples)	FC-131-1001

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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 (FC-131-1096)

Nextera XT DNA Sample Preparation Kit

- Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
1	ATM	Amplicon Tagment Mix, 96 RXN	-15° to -25°C
2	TD	Tagment DNA Buffer	-15° to -25°C
1	NPM	Nextera PCR Master Mix	-15° to -25°C
4	RSB	Resuspension Buffer	-15° to -25°C
1	LNA1	Library Normalization Additives 1	-15° to -25°C
2	LNW1	Library Normalization Wash 1	2° to 8°C
1	HT1	Hybridization Buffer	-15° to -25°C

- Box 2

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

Nextera XT DNA Sample Preparation Index Kit

Quantity	Reagent Name	Storage Temperature
8 tubes	Index Primers, S501 to S508	-15° to -25°C
12 tubes	Index Primers, N701 to N712	-15° to -25°C

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24 Sample Kit Contents (FC-131-1024)

Nextera XT DNA Sample Preparation Kit

- Box 1

Quantity	Acronym	Reagent Name	Storage Temperature
1	ATM	Amplicon Tagment Mix, 24 RXN	-15° to -25°C
1	TD	Tagment DNA Buffer	-15° to -25°C
1	NPM	Nextera PCR Master Mix	-15° to -25°C
1	RSB	Resuspension Buffer	-15° to -25°C
1	LNA1	Library Normalization Additives 1	-15° to -25°C
1	LNW1	Library Normalization Wash 1	2° to 8°C
1	HT1	Hybridization Buffer	-15° to -25°C

- Box 2

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

Nextera XT DNA Sample Preparation Index Kit

Quantity	Reagent Name	Storage Temperature
4 tubes	Index Primers, S501 to S504	-15° to -25°C
6 tubes	Index Primers, N701 to N706	-15° to -25°C

About Indexing Reagents (GA/HiSeq platforms only)

CAUTION
The Nextera XT 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

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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 XT 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 XT libraries:

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

CAUTION
Nextera XT libraries require reagents provided in the TruSeq Dual Index Sequencing Primer Boxes for *all* sequencing run types: non-indexed, single-indexed, and dual-indexed. This kit is available in a paired-end and a single-read format based on the type of flowcell being used. For example, single-read run on a paired-end flowcell would need the Paired-End TruSeq Dual Index Sequencing Primer Kit, because the flowcell itself is paired-end. Non-Nextera XT libraries can also use reagents in these boxes, or the appropriate reagents in the TruSeq Cluster Kit (v3 or v2).

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 XT DNA Sample Preparation protocols.

Table 2 User-Supplied Consumables

Consumable	Supplier
10 µl pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
1000 µl pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier

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Consumable	Supplier
1000 µl single channel pipettes	General lab supplier
200 µl 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
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
Tween 20	Sigma, part # P7949
Ultra pure water	General lab supplier
Microseal 96-well PCR plates (“TCY” plate)	Bio-Rad, part # HSP-9601

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Table 3 User-Supplied Equipment

Equipment	Supplier
96-well thermal cycler (with heated lid)	See table in *Thermal Cycler* section.
Heat Block for 1.5 ml centrifuge tubes	General lab supplier
High Speed Micro Plate Shaker	VWR, catalog # 13500-890 (110V/120V) VWR, catalog # 14216-214 (230V)
Magnetic stand-96	Ambion, part # AM10027
Microplate centrifuge	General lab supplier
Vortexer	General lab supplier

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 XT 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.

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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

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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.

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- 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.

Library Pooling Considerations

The Nextera XT 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.

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Acronyms

Table 4 Nextera XT DNA Sample Preparation Acronyms

Acronym	Definition
ATM	Amplicon Tagment Mix
CAA	Clean Amplified Plate
CAN	Clean Amplified NTA Plate
DAL	Diluted Amplicon Libraries
HT1	Hybridization Buffer
LNA1	Library Normalization Additives 1
LNB1	Library Normalization Beads 1
LNS1	Library Normalization Storage Buffer 1
LNW1	Library Normalization Wash 1
LNP	Library Normalization Plate
NT	Neutralize Tagment Buffer
NPM	Nextera PCR Master Mix
NTA	Nextera XT Tagment Amplicon Plate
PAL	Pooled Amplicon Library
RSB	Resuspension Buffer
SGP	Storage Plate
TD	Tagment DNA Buffer

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DNA Input Recommendations

The Nextera XT DNA Sample Preparation Kit protocol is optimized for 1 ng of input DNA total. Illumina strongly recommends quantifying the starting genomic material.

Input DNA Quantitation

Nextera XT DNA Sample Preparation library preps use an enzymatic DNA fragmentation step and thus can be more sensitive to DNA input compared to mechanical fragmentation methods. The ultimate success of the assay 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 XT assay.

Assessing DNA Quality

UV absorbance is a commonly used method to assess the quality of a DNA sample. 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.

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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.

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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.

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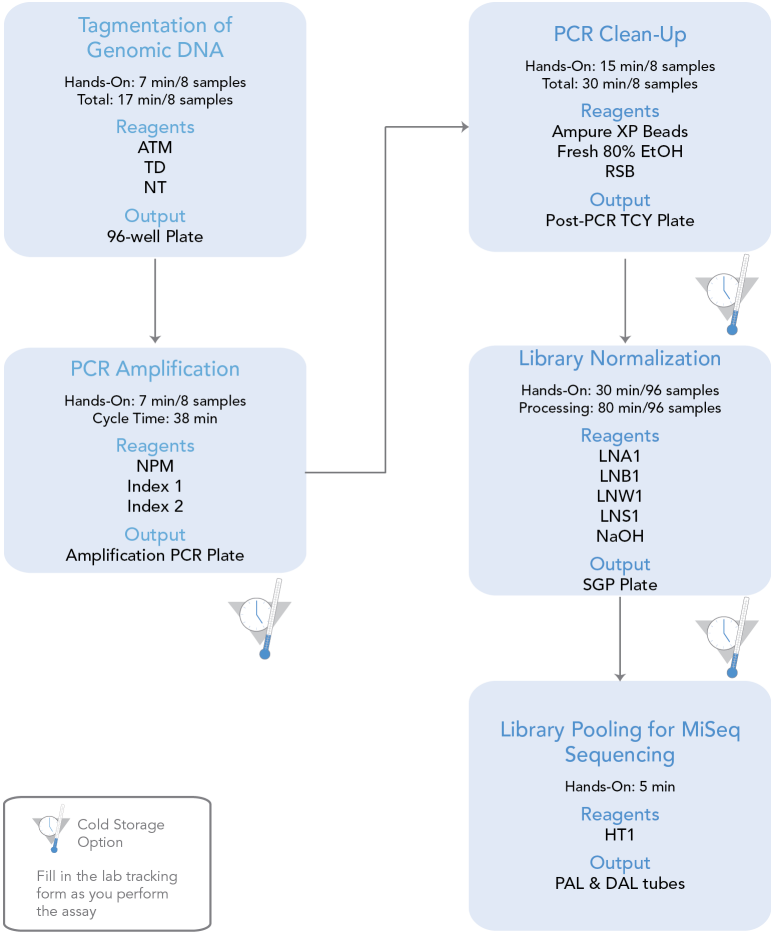
Nextera XT DNA Sample Preparation Workflow

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

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



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Tagmentation of Input DNA

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

Estimated Time (8 reactions)

- Hands-on: 7 minutes
- Total duration: 17 minutes

Consumables

Item	Quantity	Storage	Supplied By
ATM (Amplicon Tagment Mix)	1 tube	-15° to -25°C	Illumina
TD (Tagment DNA Buffer)	1 tube	-15° to -25°C	Illumina
NT (Neutralize Tagment Buffer)	1 tube	Room temperature	Illumina
Input DNA (0.2 ng/µl)		-15° to -25°C	User
96-well hard shell TCY plate	1 plate		User
Microseal 'B' adhesive film			User

Preparation

1. Remove the ATM, TD, and input DNA from -15° to -25°C storage and thaw on ice.
2. Ensure that NT is at room temperature. Visually inspect NT to ensure there is no precipitate. If there is precipitate, vortex until all particulates are resuspended.
3. After thawing, ensure all reagents are adequately mixed by gently inverting the tubes 3–5 times, followed by a brief spin in a microcentrifuge.

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Make NTA

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 **NTA** (Nextera XT Tagment Amplicon Plate).

2 Add 10 µl of TD Buffer to each well to be used in this assay. 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 **NTA** plate.

3 Add 5 µl of input DNA at 0.2 ng/µl (1 ng total) to each sample well of the **NTA** plate.

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

NOTE
Calculate the total volume of ATM 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 **NTA** plate.

5 Using a multichannel pipette, gently pipette up and down 5 times to mix. Change tips between samples.

6 Cover the **NTA** plate with Microseal 'B'.

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

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

8 Place the **NTA** 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 Once the sample reaches 10°C proceed immediately to *Neutralize NTA*.

IDT Exhibit 1057.0023

Neutralize NTA

NOTE
Calculate the total volume of NT 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 **NTA** plate.

1 Carefully remove the Microseal “B” seal and add 5 µl of NT Buffer to each well of the **NTA** plate. Change tips between samples..

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

2 Using a multichannel pipette, gently pipette up and down 5 times to mix. Change tips between samples.

3 Cover the **NTA** plate with Microseal 'B'.

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

5 Place the **NTA** plate at room temperature for 5 minutes.

IDT Exhibit 1057.0024

PCR Amplification

In this step, the tagmented DNA is amplified via a limited-cycle PCR program. The PCR step also adds index 1 (i7) and index 2 (i5) and sequences required for cluster formation. 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 libraries that produce high-quality sequencing results.

Estimated Time (8 reactions)

- Hands-on: 7 minutes
- Cycle time: 38 minutes
- Total duration: 45 minutes

Consumables

Item	Quantity	Storage	Supplied By
NPM (Nextera PCR Master Mix)	1 tube	-15° to -25°C	Illumina
Index 1 primers (N7XX)	1 tube each index	-15° to -25°C	Illumina
Index 2 primers (S5XX)	1 tube each index	-15° to -25°C	Illumina
TruSeq Index Plate Fixture			Illumina
Microseal 'A' film			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 XT Sample Preparation Guide*, and use the Illumina Experiment Manager to verify that the correct index primers have been selected.

IDT Exhibit 1057.0025

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

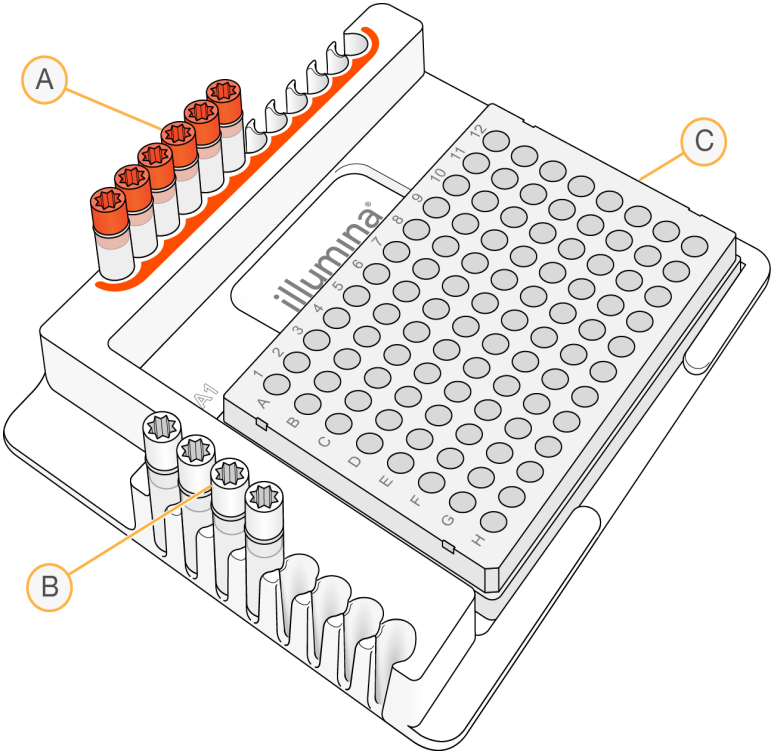
2 Remove NPM and the index primers from -15° to -25°C storage and thaw on a bench at room temperature.
Allow approximately 20 minutes to thaw NPM and index primers.

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 S501 is in row A and S504 is in row D.
   c Record their positions on the Lab Tracking Form.

IDT Exhibit 1057.0026

Figure 2 TruSeq Index Plate Fixture Arrangement (24 libraries)

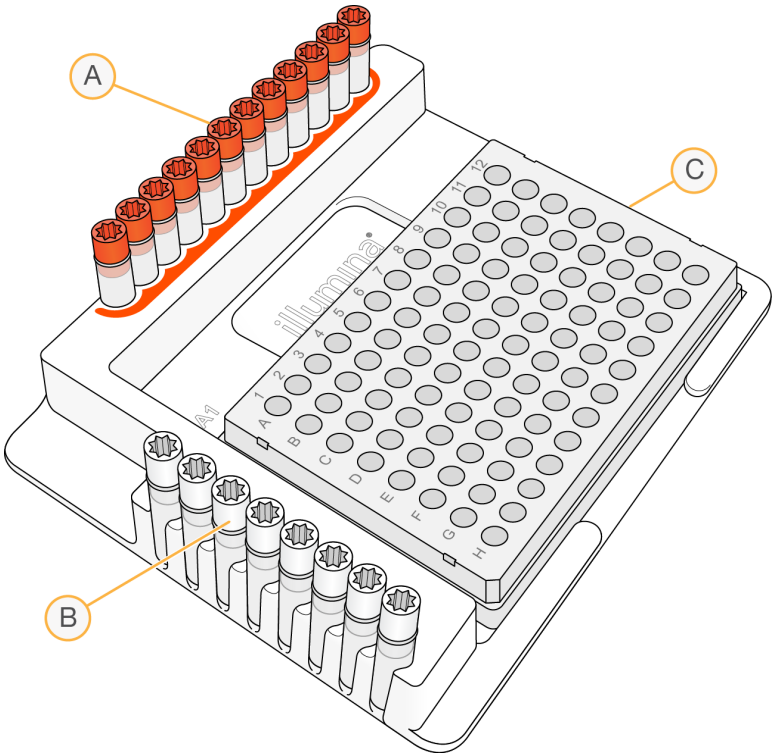


A Index primer 1 (i7) (orange caps)
B Index primer 2 (i5) (white caps)
C NAP1 plate

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 S501 is in row A and S508 is in row H.
   c Record their positions on the lab tracking form.

IDT Exhibit 1057.0027

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

Amplify NTA

1 Place the **NTA** plate in the TruSeq Index Plate Fixture.

2 Add 15 µl of NPM to each well of the **NTA** 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 **NTA** plate.

IDT Exhibit 1057.0028

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

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

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

6 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.

7 Using a multichannel pipette, gently pipette up and down 3 to 5 times to mix. Change tips between samples to avoid index and sample cross contamination.

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

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

10 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
- 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

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 for up to two days.

IDT Exhibit 1057.0029

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.

IDT Exhibit 1057.0030

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

Make CAN

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

2 Label a new MIDI plate **CAA** (Clean Amplified Plate).

3 Using a multichannel pipette set to 50 µl, transfer the PCR product from the **NTA** plate to the **CAA** 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 **CAA** plate.
For 2x250 runs on the MiSeq, add 25 µl of AMPure XP beads to each well of the **CAA** plate.
Smaller amplicon inputs into Nextera XT preps typically yield smaller insert size ranges. To maximize recovery of smaller fragments out of the SPRI cleanup we recommend the following conditions:

Size of Largest Amplicon in Pool	AMPure XP Recommendation	AMPure XP Volume
< 300 bp	1.8x AMPure XP*	90 µl
300 – 500 bp	1.8x AMPure XP	90 µl
> 500 bp	0.6x AMPure XP (0.5x AmpureXP for 2x250 runs on the MiSeq)	30 µl (25 µl for 2x250 runs on the MiSeq)

* 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. This enzymatic clipping of PCR primers avoids wasted sequencing output on non-informative bases that do not contain genomic inserts. If you wish to sequence the genomic loci contained within a PCR primer, simply design your amplicons to be ~100 bases larger than the desired insert to be sequenced.

IDT Exhibit 1057.0031

6 Gently pipette mix up and down 10 times.

> NOTE
> Alternatively the solution can be mixed by shaking the **CAA** 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 **CAA** 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 **CAA** 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.
   c Carefully remove and discard the supernatant.

11 With the **CAA** 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.
   c Carefully remove and discard the supernatant.
   d Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.

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

13 Remove the **CAA** plate from the magnetic stand. Using a multichannel pipette, add 52.5 µl of RSB to each well of the **CAA** plate.

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

IDT Exhibit 1057.0032

NOTE
Alternatively the solution can be mixed by shaking the **CAA** 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 **CAN** (Clean Amplified NTA Plate).

18 Using a multichannel pipette, carefully transfer 50 µl of the supernatant from the **CAA** plate to the **CAN** plate. Change tips between samples to avoid cross-contamination.

SAFE STOPPING POINT
If you do not plan to immediately proceed to *Library Normalization* following the completion of *PCR Clean-up*, seal the **CAN** plate with Microseal "B" adhesive seal and store it at -15° to -25°C for up to a week.

IDT Exhibit 1057.0033

Library Normalization

This process normalizes the quantity of each library to ensure more equal library representation in your pooled sample.

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

Estimated Time (96 reactions)

- Total duration: 1 hour 20 minutes
- Hands-on: 30 minutes

Consumables

Item	Quantity	Storage	Supplied By
LNA1 (Library Normalization Additives 1)	1 tube	-15° to -25°C	Illumina
LNB1 (Library Normalization Beads 1)	1 tube	2° to 8°C	Illumina
LNW1 (Library Normalization Wash 1)	2 tubes	2° to 8°C	Illumina
LNS1 (Library Normalization Storage Buffer 1)	1 tube	Room temperature	Illumina
0.1 N NaOH (less than one week old)	3 ml per 96 samples		User
96-well MIDI plate	1 plate		User
96-well TCY plate	1 plate		User
15 ml conical tube	1 tube		User

WARNING
This set of reagents contains formamide, an aliphatic amide that is a probable

IDT Exhibit 1057.0034

reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.

For more information, see the MSDS for this kit, at http://www.illumina.com/msds.

Preparation

NOTE
Illumina recommends performing the LNA1 preparation step under a fume hood.

1 Remove LNA1 from -15° to -25°C storage and bring to room temperature. Use a 20° to 25°C water bath as needed.

NOTE
LNA1 might form visible precipitates or crystals. Before use, vortex vigorously, and then hold the tube in front of a light and visually inspect to make sure that all precipitate has completely dissolved.

2 Remove LNB1 and LNW1 from 2° to 8°C storage and bring to room temperature. Use a 20° to 25°C water bath as needed.

3 Vigorously vortex LNB1 for at least 1 minute with intermittent inversion until the beads are well-resuspended and no pellet is found at the bottom of the tube when the tube is inverted.

4 Ensure that LNS1 is at room temperature before use.

Elute LNP

1 Label a new MIDI plate **LNP** (Library Normalization Plate).

2 Using a P20 multichannel pipette and fine tips, carefully transfer 20 µl of the supernatant from the **CAN** plate to the **LNP** plate. Change tips between samples to avoid cross-contamination.

3 For 96 samples, add 4.4 ml of LNA1 to a fresh 15 ml conical tube.

4 Use a P1000 pipette set to 1000 µl to resuspend LNB1 thoroughly by pipetting up and down 15–20 times, until the bead pellet at the bottom is completely resuspended.

IDT Exhibit 1057.0035

NOTE
It is extremely critical to completely resuspend the LNB1 bead pellet at the bottom of the tube. The use of a P1000 ensures that the beads are homogeneously resuspended and that there is no bead mass at the bottom of the tube. This is essential for achieving consistent cluster density on the flow cell.

5 Immediately after LNB1 is thoroughly resuspended, use a P1000 pipette to transfer 800 µl of LNB1 to the 15 ml conical tube containing LNA1. Mix well by inverting the tube 15–20 times. The resulting LNA1/LNB1 bead mix is enough for 96 samples. Pour the bead mix into a trough and use it immediately in the next step.

NOTE
If you do not plan to use full tubes for 96 samples, a P1000 set to 1000 µl is still required to resuspend the beads completely in step 2. Mix only the required amounts of LNA1 and LNB1 for the current experiment. Never use a P200 pipette to handle LNB1. You must store the remaining LNA1 and LNB1 separately at their respective recommended temperatures. To preserve stability, LNB1 beads should never be frozen or mixed with LNA1 if not used immediately.

6 Using a multichannel pipette, add 45 µl of the combined LNA1/LNB1 to each well of the **LNP** plate containing libraries. Changing tips between columns is not required if you use care to avoid cross-contamination.

7 Seal the **LNP** plate with Microseal 'B'.

8 Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 30 minutes.

NOTE
This 30 minute incubation is critical for proper library normalization. Incubations of greater or less than 30 minutes may affect library representation and cluster density.

9 Place the plate on a magnetic stand for 2 minutes and confirm that the supernatant has cleared.

10 With the **LNP** plate on the magnetic stand, using a multichannel pipette set to 80 µl carefully remove and discard the supernatant in an appropriate hazardous waste container.

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

IDT Exhibit 1057.0036

11 Remove the **LNP** plate from the magnetic stand and wash the beads with LNW1 as follows:
   a Using a multichannel pipette, add 45 µl of LNW1 to each sample well. Changing tips between columns is not required if you use care to avoid cross-contamination.
   b Seal the **LNP** plate with Microseal 'B'.
   c Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.
   d Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
   e Carefully remove and discard the supernatant in an appropriate hazardous waste container.

12 Remove the **LNP** plate from the magnetic stand and repeat the wash with LNW1 as follows:
   a Using a multichannel pipette, add 45 µl of LNW1 to each well. Changing tips between columns is not required if you use care to avoid cross-contamination.
   b Seal the **LNP** plate with Microseal 'B'.
   c Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.
   d Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
   e Carefully remove and discard the supernatant in an appropriate hazardous waste container.

13 Remove the **LNP** plate from the magnetic stand and add 30 µl of 0.1 N NaOH (less than a week old) to each well to elute the sample.

14 Seal the **LNP** plate with Microseal 'B'.

15 Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.

16 During the 5 minute elution, apply the **SGP** (StoraGe Plate) barcode plate sticker to a new 96-well PCR plate.

17 Add 30 µl LNS1 to each well to be used in the **SGP** plate.

18 After the 5 minute elution, ensure all samples in the **LNP** plate are completely resuspended. If the samples are not completely resuspended, gently pipette those samples up and down or lightly tap the plate on the bench to resuspend the beads, then shake for another 5 minutes.

IDT Exhibit 1057.0037

19 Place the **LNP** plate on the magnetic stand for 2 minutes or until the supernatant appears clear.

20 Using a multichannel pipette set to 30 µl, transfer the supernatant from the **LNP** plate to the **SGP** plate. Change tips between samples to avoid cross-contamination.

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.

21 Seal the **SGP** plate with Microseal 'B' and then centrifuge at 1,000 xg for 1 minute.

NOTE
The final library pool consists of single-stranded DNA, which will not resolve well on an agarose gel or Bioanalyzer chip. qPCR can be used for quality control if desired. For more information, please see the *Sequencing Library qPCR Quantification Guide*.

SAFE STOPPING POINT
If you do not plan to proceed to Library Pooling and MiSeq Sample Loading following the completion of Library Normalization, store the sealed **SGP** plate at -15° to -25°C.

IDT Exhibit 1057.0038

Library Pooling and MiSeq Sample Loading

In preparation for cluster generation and sequencing, equal volumes of normalized library are combined, diluted in hybridization buffer, and heat denatured prior to MiSeq sequencing.

Estimated Time (8 reactions)

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

Consumables

Item	Quantity	Storage	Supplied By
HT1 (Hybridization buffer)	1 tube	-15° to -25°C	Illumina
MiSeq reagent cartridge	1 cartridge	-15° to -25°C	Illumina
Eppendorf tubes (screwcap recommended)	2 tubes		User
PCR eight-tube strip	1		User
2.5 L Ice bucket	1		User

Preparation

1. Set a heat block suitable for 1.5 ml centrifuge tubes to 96°C.
2. Remove a MiSeq reagent cartridge from -15 to -25°C storage and thaw at room temperature.
3. In an ice bucket, prepare an ice-water bath by combining 3 parts ice and 1 part water.

IDT Exhibit 1057.0039

Make DAL

NOTE
Fresh **DAL** should be prepared for each use.

1. If the **SGP** plate was stored frozen, thaw the **SGP** plate at room temperature.
2. Centrifuge the **SGP** plate at 1,000 xg for 1 minute at 20°C to collect condensation.
3. If the **SGP** plate was stored frozen, using a P200 multichannel pipette, mix each library to be sequenced by pipetting up and down 3–5 times. Change tips between samples.
4. Using a P20 multichannel pipette, transfer 5 µl of each library to be sequenced from the **SGP** plate, column by column, to a PCR eight-tube strip. Change tips after each column to avoid sample cross-contamination.
5. Label a fresh Eppendorf tube **PAL** (Pooled Amplicon Library).
6. Combine and transfer the contents of the PCR eight-tube strip into the PAL tube. Mix PAL well.
7. Label a fresh Eppendorf tube **DAL** (Diluted Amplicon Library).
8. Add 576 µl of HT1 to the **DAL** tube.
9. Transfer 24 µl of **PAL** to the **DAL** tube containing HT1. Using the same tip, pipette up and down 3–5 times to rinse the tip and ensure complete transfer.

NOTE
The recommended volumes for diluting **PAL** with HT1 represents a 25-fold dilution. This dilution ratio was established by using the recommended equipment (e.g. plate shaker calibrated for shaking speed) and following the normalization procedure strictly under typical laboratory conditions (e.g. 20°–25°C). If cluster density is found to be too high or too low, you may change this dilution ratio to better suit the equipment, temperature, and user handling in your laboratory after validation.

10. Mix **DAL** by vortexing the tube at top speed.
11. Using a heat block, incubate the **DAL** tube at 96°C for 2 minutes.
12. After the incubation, invert **DAL** 1–2 times to mix and immediately place in the ice-water bath.
13. Keep **DAL** in the ice-water bath for 5 minutes.

IDT Exhibit 1057.0040

14 Load **DAL** into a thawed MiSeq reagent cartridge into the **Load Samples** reservoir.

NOTE
It is required to perform this heat denaturation step immediately before loading **DAL** into the MiSeq reagent cartridge to ensure efficient template loading on the MiSeq flowcell.

15 Store the **PAL** tube and sealed **SGP** plate at -15° to -25°C for up to a week.

16 Sequence your library as indicated in the *MiSeq System User Guide*.

IDT Exhibit 1057.0041

Clustering Nextera XT Samples for HiSeq, HiScanSQ, and GAIIx

In preparation for cluster generation and sequencing, equal volumes of normalized library are combined, diluted in hybridization buffer, and heat denatured prior to MiSeq sequencing.

Estimated Time (8 reactions)

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

Consumables

Item	Quantity	Storage	Supplied By
HT1 (Hybridization buffer)	1 tube	-15° to -25°C	Illumina
Eppendorf tubes (screwcap recommended)	2 tubes		User
PCR eight-tube strip	2		User

Make DAL

NOTE
Fresh **DAL** should be prepared for each use.

1. If the **SGP** plate was stored frozen, thaw the **SGP** plate at room temperature.
2. Centrifuge the **SGP** plate at 1,000 xg for 1 minute at 20°C to collect condensation.
3. If the **SGP** plate was stored frozen, using a P200 multichannel pipette, mix each library to be sequenced by pipetting up and down 3–5 times. Change tips between samples.
4. Using a P20 multichannel pipette, transfer 5 µl of each library to be sequenced from the **SGP** plate, column by column, to a PCR eight-tube strip. Change tips after each column to avoid sample cross-contamination.

IDT Exhibit 1057.0042

5 Label a fresh Eppendorf tube **PAL** (Pooled Amplicon Library).

6 Combine and transfer the contents of the PCR eight-tube strip into the PAL tube. Mix PAL well.

7 Label a fresh Eppendorf tube **DAL** (Diluted Amplicon Library).

8 Add 585 µl of HT1 to the **DAL** tube.

9 Transfer 15 µl of **PAL** to the **DAL** tube containing HT1. Using the same tip, pipette up and down 3–5 times to rinse the tip and ensure complete transfer.

NOTE
The recommended volumes for diluting **PAL** with HT1 represents a 40-fold dilution. This dilution ratio was established by using the recommended equipment (e.g. plate shaker calibrated for shaking speed) and following the normalization procedure strictly under typical laboratory conditions (e.g. 20°–25°C). If cluster density is found to be too high or too low, you may change this dilution ratio to better suit the equipment, temperature, and user handling in your laboratory after validation.

10 Mix **DAL** by vortexing the tube at top speed.

11 Transfer 120 µl of the **DAL** tube into each well of the PCR eight-tube strip that will be loaded onto the cBot for clustering.

NOTE
It is not required to perform heat denaturation prior to cBot loading, since the clustering process includes a heat denaturation step.

12 Store the **PAL** tube and sealed **SGP** plate at -15° to -25°C for up to a week.

13 Cluster your library as indicated in the *cBot User Guide*.

IDT Exhibit 1057.0043

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 (S501–S508) for the 96 sample Nextera XT Index Kit (FC-131–1002), and 6 different Index 1 (i7) adapters (N701–N706) and 4 different Index 2 (i5) adapters (N501–N504) for the 24 sample Nextera XT Index Kit (FC-131–1001). In the Index adapter name, the N refers to Nextera XT 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	S501	TAGATCGC
N702	CGTACTAG	S502	CTCTCTAT
N703	AGGCAGAA	S503	TATCCTCT
N704	TCCTGAGC	S504	AGAGTAGA
N705	GGACTCCT	S505	GTAAGGAG
N706	TAGGCATG	S506	ACTGCATA
N707	CTCTCTAC	S507	AAGGAGTA
N708	CAGAGAGG	S508	CTAAGCCT
N709	GCTACGCT		
N710	CGAGGCTG		
N711	AAGAGGCA		
N712	GTAGAGGA		

IDT Exhibit 1057.0044

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] S501 and S502 • [option 2] S503 and S504 • [option 3] S505 and S506

IDT Exhibit 1057.0045

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] S501, S502, and any other Index 2 adapter (as needed) • [option 2] S503, S504, and any other Index 2 adapter (as needed) • [option 3] S505, S506, 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

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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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