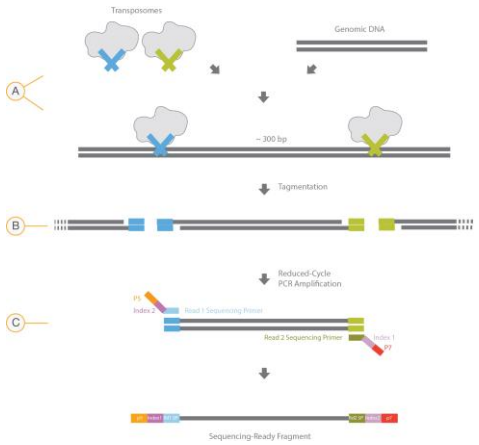
Nextera XT Library Prep: Tips and Troubleshooting

Sample Input: Success Relies on Quality and Quantity

Sample input is the most important factor in a successful Nextera XT library prep. The first step in library prep is the tagmentation reaction, which involves the transposon cleaving and tagging of the double-stranded DNA with a universal overhang. The following figure illustrates the process.



A. Nextera XT transposome with partial adapters is combined with template DNA Call out.
B. Tagmentation to fragment and add partial adapters
C. Limited cycle PCR to add sequencing primer sequences and indexes

How well the sample is tagmented determines the success of the library prep. The Nextera XT library preparation kit is optimized for 1 ng double-stranded genomic DNA. Quantify the input DNA with a fluorometric method, such as PicoGreen or Qubit. Avoid using methods that quantify total nucleic acid, such as NanoDrop or other UV absorbance methods.

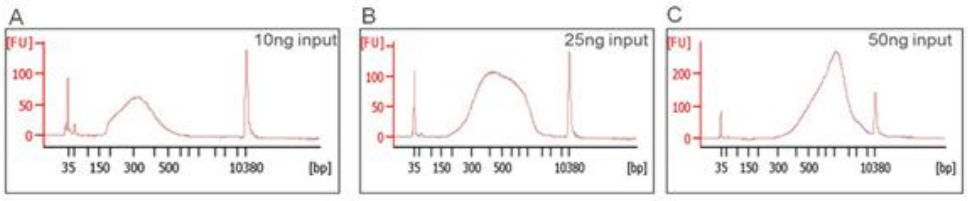
The transposons are end point, in that they cleave double-stranded DNA one time only and then the step is complete. Therefore, successful tagmentation is highly dependent on mass.

- Using > 1 ng can lead to undertagmentation of the sample.
- Using < 1 ng can lead to overtagmentation of the sample.

Undertagmentation

Final libraries above 1.2–1.5 kb average size, including adapters, do not cluster on the flow cell. Larger libraries do not cluster as efficiently as smaller libraries. An ideal library size ranges from 200 bp to 1 kb. See the *Nextera Library Validation and Cluster Density Optimization Technical Note* to learn more about sequencing larger libraries and how to calculate appropriate loading concentrations based on average library length.

Some projects might benefit from shorter or longer insert sizes. An Illumina experiment using the Nextera DNA library preparation kit showed that using less DNA input than the recommended 50 ng, led to decreased average library insert size with minimal effect on sequencing run quality and diversity in the final analysis.



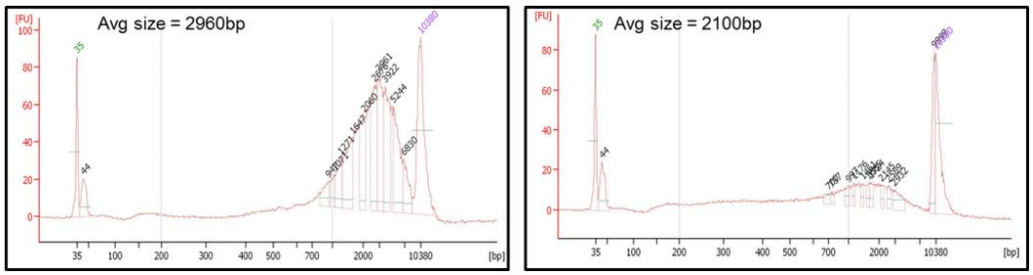
DNA Input	Median insert size: On Bioanalyzer	Median insert size: Sequenced	Mean Quality Metrics: Pass Filter	Align	Mismatch	>Q30	A+T Dropout	G+C Dropout	Diverse Reads	Duplicate Read Pairs
10ng*	180bp	N/A*	92%	64%*	0.50%	92%	not calculated			
25ng	330bp	230bp	93%	82%	0.50%	91%	4%	0%	2.53E+09	6.50%
50ng	1kb	346bp	92%	86%	0.60%	90%	6%	0%	2.67E+09	5%

A. 10 ng input library sequenced in triplicate
B. 25 ng input library sequenced in triplicate
C. 50 ng input library sequenced in triplicate

*A decrease in % align was observed in the run using 10 ng input due to significant sequencing into the adapter on this short-insert library. Therefore, the 10 ng input data set was not analyzed for drop out or diversity rates. Use shorter sequencing reads to mitigate this effect.

All libraries were normalized to produce a cluster density of ~800K/mm^2, and then sequenced using 2 x 100 bp reads on a HiSeq in high output mode.

Another cause of undertagmentation is enzymatic inhibitors in the input sample. Chelators such as EDTA, salts, and polysaccharides can inhibit the enzymatic activity of the transposons and lead to reduced final yield, failure of library prep, or low and uneven coverage when analyzed bioinformatically.



These traces represent 2 Nextera libraries with undertagmented profiles. Most of the DNA in these libraries is greater than 1.5 kb in length, which does not efficiently form clusters on the flow cell.

Inhibitors of Enzymatic Reactions

Several factors can impair the performance of the Nextera enzyme:

- Proteins can coat DNA, preventing enzyme binding to the substrate.
- Sequestration of enzyme cofactors by EDTA can negatively affect enzyme function.
- Proteinases, detergents, and phenol can degrade the enzyme.
- Changes in ionic strength and pH caused by chemicals left over from library preparation.

Best Practices

- Use careful sample handling with extraction protocols optimized to purify inhibitor-free nucleic acids.
- Apply accurate quantitation approaches to detect inhibitors in the isolated DNA samples.
- Illumina recommends UV spectrophotometry for quality assessment and fluorometric-based methods like Qubit or PicoGreen for DNA quantitation.
- When eluting or resuspending nucleic acids with water, make sure that the pH is 7.0–8.5.
- In the absence of a buffering agent, store samples at -25°C to -15°C to prevent degradation. Samples can also be stored in 10 mM Tris-HCl pH 8.5 with no EDTA.
- Make single-use aliquots of the input sample to prevent cross-contamination and avoid repeated freeze-thaw cycles.

What If I Suspect or Detect Contaminants in my DNA Samples?

Assess the purity of nucleic acids in solutions using UV spectrophotometry.

Substance	Absorbance (nm)	Purity indicator
Nucleic acids	260 nm	A260:280 > 1.8 DNA
Proteins	280 nm	A260:280 > 2.0 RNA
Organics	230 nm	A260:230 > 2.0 DNA/RNA

If the sample has contaminants that might affect downstream enzymatic reactions, additional purification steps or centrifugal filtration/concentration can help remove contaminants.

List of Common Contaminants from Library Preparation Protocols

The following list includes the most common inhibitors that originate from library preparation.

Carryover	Inhibitory Effect	Likely Source	Methods to Minimize Inhibition
EDTA	Chelation of metal ions	TE buffer	Reduce the concentration of EDTA to 0.1 mM in the TE buffer or use Tris-HCl (10 mM) or water to bring DNA in solution.
Alcohols	Enzyme precipitation and denaturation	Ethanol, isopropanol, isoamyl alcohol	Dry pellet and resuspend.
Excess salts	Template blocking	KCl, NaCl, CsCl, NaAc	Wash with 70% ethanol or use silica-based purification.
Chaotropic salts	Enzyme denaturation	Guanidinium chloride; magnesium chloride; urea	Wash with 70% ethanol or use silica-based purification.
Phenol:chloroform	Enzyme denaturation	Organic carryover	Use PVP, PVP/ammonium acetate. Add 1.2% citric acid during the DNA extraction step.
Polysaccharides	Template blocking	Dextrans, glycans	High salt precipitation, CTAB buffer, chloroform extraction, pectinase, cellulose, hemicellulose, or α-amylase digestion.
Protein	Template blocking	BSA, immunoglobins, PEG	Use SDS, CTAB or guanidinium buffers, Proteinase K.

Carryover	Inhibitory Effect	Likely Source	Methods to Minimize Inhibition
Fat	Template blocking	Glycerol	Lipase or hexane treatment and chloroform extraction.
Detergents/DDT	Enzyme precipitation and denaturation	Sodium deoxycholate, sarakosyl, SDS, Nonidet P-40, Tween 20, Triton X-100, N-octyl glucoside	Wash with 70% ethanol.
Proteases	Protein degradation	Proteinase K, sample handling	Phenol:chloroform extraction, followed by EtOH precipitation.
Nucleases	Template degradation	Sample handling, restriction enzymes, micrococcal nuclease, S1	Use B-ME, EGTA, or SDS during protein precipitation.
Exogenous DNA/RNA	Template competition	Carryover	DNase I for DNA removal; RNase A for RNA, and RNA:DNA hybrid removal.
Carriers	Template blocking or competition	RNA, heparin, glycogen	Only use carriers that do not serve as template or block the template such as linear acrylamide, N- or P- carriers.
Agarose	Template blocking	Gel extractions	Use a spin column with chaotropic salt buffer; dialysis.
Excess metal ions	Reduce oligo specificity	Mg++ from PCR buffer	Dialysis against PBS (pH 7.4), phenol:chloroform extraction, followed by EtOH precipitation.

See the *Nextera Library Validation and Cluster Density Optimization Technical Note* for help in addressing quantification and sample loading questions when sequencing Nextera and Nextera XT libraries with varying Bioanalyzer traces.

Overtagmentation

Samples that have been overtagmented by the transposons can lead to reduced library yield, which can result in coverage dropout.

Causes of overtagmentation include:

- Inaccurate quantification that results in < 1 ng for the tagmentation step
- Use of FFPE or degraded DNA samples
- Use of smaller sized amplicons

Transposons require at least 300 bp of genomic space to sit down on the DNA. For this reason, FFPE, degraded samples, and small amplicons are not supported.

Using Amplicons

- If adapting the Nextera XT protocol for amplicons, use amplicons ≥ 300 bp in length.
- For full coverage of the entire amplicon region, make sure that primers have ≥ 50 bases upstream and downstream of your target of interest.
- Having the additional 50 bases upstream and downstream accounts for low coverage that might occur at the distal ends of the amplicon.

Quality Control

The Nextera XT protocol includes optional quality control (QC) steps. The first QC step occurs after tagmentation and the other after PCR cleanup.

After Tagmentation

- Run 1 µl of library on a Bioanalyzer using a high-sensitivity DNA chip. The yield or amplitude of that trace might be low, but expect the library to have a broad distribution between 200 bp and 1.5 kb, with most of the fragments < 1 kb.
- Alternatively, the sample can be run on the Bioanalyzer after the PCR step and before bead cleanup, especially if you see little to no peaks after tagmentation. Checking libraries at this point confirms that libraries are greater than 200 bp and will be retained during the subsequent bead clean ups.
- When comparing traces post-tagmentation and post-PCR traces, you might notice a slight increase in library size due to the addition of a full length index sequence. Dual indexed adapters add ~135 bp in length to the final library.

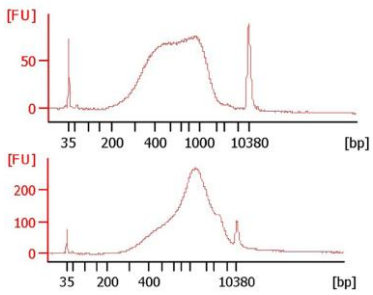
After PCR Cleanup

During PCR Cleanup, fragments < 200 bp are discarded, which removes any residual adapter and primer dimers.

Run 1 µl of library on a Bioanalyzer using a high-sensitivity DNA chip to check library size. Alternatively, PCR amplify the library using adapter primers and run on an agarose gel to visualize the presence of library.

Adapter primer sequences can be found on page 6 of the *Sequencing Library Quantification Guide (part# 11322363).*

The following traces represent examples of a successful final library.



These traces represent examples of a successful final library.

Results can vary due to the enzymatic shearing steps used in Nextera preps. As long as you have a distribution of sample from approximately 200 bp to 1.5 kb, you can proceed with sequencing.

Library Quantification

The best method for quantifying the final library depends on which stopping point is used in the protocol. The Nextera XT protocol provides 2 methods for preparing final libraries.

Manual Quantification and Normalization

After the PCR cleanup step, the library is dsDNA and can be quantified using a fluorometric method, such as Qubit or PicoGreen.

- Bioanalyzer traces or qPCR are not acceptable methods for quantifying Nextera libraries. Although a Bioanalyzer trace is a good method for assessing final library size, it is not accurate for quantification due to a wide sample size distribution.
- qPCR is appropriate only for libraries with a very narrow size range. A qPCR standard is not available for the large size distribution of the final library.

Quantify samples with Qubit or PicoGreen, and skip the bead-based normalization steps of the protocol. Denature and dilute the samples before loading onto the flow cell. At least 2–4 nM of final library is required to denature and dilute libraries in preparation for sequencing.

Bead-Based Normalization

Optionally, qualify samples for normalization suitability. Use a Bioanalyzer trace to verify final library size, use Qubit or PicoGreen to verify quantity, and then convert ng/µl to nM.

- If the concentration is > 15 nM, proceed to bead-based normalization.
- If the concentration is < 15 nM, skip the bead-based normalization step, pool libraries to a 1:1 ratio, and then denature as described in *Preparing Libraries for Sequencing on the MiSeq (part # 15039740)*.

After bead-based normalization, the libraries are single-stranded after elution from the beads and are not visible on a Bioanalyzer trace.

Storing Bead-Based Normalized Libraries

Because the bead-based normalized libraries are single-stranded after elution from the beads, the libraries are not stable, are more susceptible to degradation, and can stick to the walls of the tube. After bead-based normalization, only store libraries up to 1 week at -25°C to -15°C.

To ensure that all library strands are denatured, perform a quick heat denaturation of the libraries immediately before loading them onto the MiSeq cartridge, as follows:

1. Incubate the denatured and diluted library at 96°C for 2 minutes.
2. After the heat incubation, invert the tube 1–2 times to mix.
3. Quickly move the library to an ice water bath for 5 minutes. The quick cooling step helps to lock the sample in its single-stranded form.
4. Proceed immediately to cluster generation.

For longer term storage of the library, stop immediately after PCR cleanup when the libraries are still double-stranded, and store the SGP plate at -25°C to -15°C. Aliquot as needed to avoid multiple freeze-thaw cycles.

See the Nextera XT Support Page for additional resources, training, and documentation.

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