FAQs

NxSeq® UltraLow DNA Library Kit, 12 Reactions and NxSeq® Single Indexing Kits, Sets A and B

1. How much DNA should I start with when performing enzymatic fragmentation of my samples?

Since the enzymatic fragmentation reaction mix is used directly in the End-repair/A-tailing Reaction (no cleanup or buffer exchanges), there is no loss of DNA. So you should use between 50 pg and 75 ng of DNA in your fragmentation reaction for which the library prep kit is optimized.

2. What is the minimal DNA concentration needed prior to starting the enzymatic fragmentation reaction?

As stated above, you can use a minimum of 50 pg of DNA in your enzymatic fragmentation reaction. Since the maximal volume of input DNA is 12.3 µL, your DNA must be at a concentration ≥4.1 pg/µL.

3. What is the best method for quantification of genomic DNA?

We recommend using the Qubit for quantification of DNA. However, genomic DNA is difficult to quantify due to its viscosity and the difficulty in accurately pipetting small quantities. We recommend re-quantification of your DNA after shearing, if possible, to get accurate information.

4. How do I measure 4.1 pg/µL DNA samples?

You can't using fluorescent dsDNA binding dye systems such as Qubit® with such low levels of DNA. If you want to use this very low amount of DNA, you'll most likely be guessing at the input amount. qPCR quantitation is another option if your sample is amenable to that type of quantitation (e.g. a single species). Fortunately, after PCR amplification, you will have a chance to verify that you produced a high quality library by analyzing final library size on the Bioanalyzer and quantitating library yield by Qubit prior to starting your Illumina sequencing run.

5. Are libraries constructed with the NxSeq UltraLow DNA Library Kit and Indexing Kits compatible with other sequencers besides Illumina?

No. This kit produces libraries that are only compatible with Illumina sequencers.

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6. Why do you add Elution Buffer after the Ligation Step in the protocol before the first Cleanup Step? What am I eluting?

Don't worry! You're not eluting anything. The Elution Buffer is simply a buffered solution that is mainly used for eluting the DNA from the AMPure beads, and in this case, it is added just after ligation to increase the volume and dilute the ligation reaction buffer so it's less viscous. The addition of this buffer actually helps with the Cleanup Step before PCR and improves the removal of any adaptor dimers that formed during the Ligation Step.

7. Can I use standard P5 and P7 PCR amplification primers to amplify my libraries after ligation?

No. During the ligation step, you are adding our Universal Adaptor to the ends of the fragments. This adaptor does not contain any index/barcode information or the sequences (P5 and P7 regions) necessary to capture these fragments on the Illumina sequencers. You <u>must use our Single (or Dual) Indexing Primers</u> to add both the appropriate index information and P5 and P7 sequences necessary for sample identification and sequencing on the Illumina instruments.

8. What is the structure of the final library fragments? How do they compare to the libraries made using other kits?

After ligation with the Universal Adaptor and PCR amplification with one of our NxSeq® Single Indexing Primer Sets, the final library fragments are completely double-stranded and have the same sequence structure as Illumina libraries made with TruSeq LT Adaptors and amplified with standard P5 and P7 primers.

9. Can I use other adaptors and/or indexing primers with your library prep kit?

Yes. After the End-repair/A-tailing Step, the DNA fragments are A-tailed (single 3' A overhang). These fragments are compatible with any adaptors that have a compatible T-overhang. Barcoded adaptors (e.g. TruSeq adaptors) can be ligated to the A-tailed fragments and the resulting library can be amplified with standard P5/P7 primers. However, our Universal Adaptor is optimized for ligation efficiency, and the use of other adaptors may decrease ligation efficiency and produce lower quality libraries. Also note that other PCR indexing primers may not be compatible with our Universal Adaptor, and conversely, other adaptors may not be compatible with our NxSeq Single Indexing Primer Sets. For optimal results, we recommend using our NxSeq Single Indexing Kits with the NxSeq UltraLow DNA Library Kit.

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10. Are there any good stopping points in the protocol?

Yes. If you need to stop, we recommend stopping at the steps listed below. When stopping at one of these points, always store your DNA at -20°C.

- a. After mechanical shearing or enzymatic fragmentation.
- b. After cleanup of your ligated DNA and before PCR amplification.
- c. After completing the PCR amplification reaction and before the next cleanup step.
- d. After cleanup of the PCR amplified DNA and before starting the size selection.
- e. After library prep is fully completed before starting the sequencing run. However, we recommend not storing the final libraries longer than 7 days at -20°C.

11. Are libraries made with this kit compatible with all Illumina sequencers?

Yes, including the HiSeq X Ten and X Five Sequencers.

12. Are the Single Index sequences the same as the Illumina TruSeq LT Adaptor sequences?

Yes. When setting up a sample sheet in Illumina Experiment Manager, select TruSeq LT from the drop down menu. Use A001-A027 when entering sample information, since these indices correspond to the same numbers used for the indices used in the NxSeq Single Indexing Kits. E.g. Illumina A001 and A027 = Lucigen 1 and 27, respectively.

13. Can I interchange the adaptors in the NxSeq® UltraLow DNA Library Kit with the adaptors in the NxSeq® AmpFREE Low DNA Library Kit?

Yes. After the End-repair/A-tailing Step, the DNA fragments are A-tailed (single 3' A overhang). These fragments are compatible with any adaptors that have a compatible T-overhang. Barcoded adaptors, including our NxSeq Adaptors (Cat. No. 14300-1, 14400-1) can be ligated to the A-tailed fragments and the resulting libraries can be amplified with standard P5/P7 primers. However, our Universal Adaptor in the NxSeq® UltraLow Kit are optimized for ligation efficiency, and the use of other adaptors may decrease ligation efficiency and produce lower quality libraries.

Also note that other PCR indexing primers may not be compatible with our Universal Adaptor, and conversely, other adaptors may not be compatible with our NxSeq® Single Indexing Primer Sets. For optimal results, we recommend using our NxSeq Single Indexing Kits with the NxSeq UltraLow DNA Library Kit.

Do not use the Universal Adaptor with standard P5/P7 PCR primers. The Universal Adaptor must be amplified with the indexed primer sets included in our kits.

14. Can you use this kit with ancient or short DNA fragments?

Yes, however the protocol must be modified for fragments between 30 bp and 150 bp. Please contact our Technical Support Team for protocol guidance.

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15. Can I use other magnetic beads besides the AMPure XP beads?

Our Size Selection protocol has been optimized with room temperature AMPure XP beads. Beads from other vendors might have different sizing properties and must be optimized prior to use in library construction.

16. When would I use the Double Bead Size Selection protocol? Is the cleanup step required if I select this protocol?

Double size Selection is advised when the size range/distribution of your library is too large for efficient sequencing. Library fragments larger than 700 bp will not sequence efficiently due to the limitations of bridge amplification on the flow cell. With double size selection you can adjust your library size range to match your data analysis needs. See Appendix D in the NxSeq® UltraLow Kit manual for instruction on double size selection.

Yes, the cleanup step removes salts and proteins from the PCR reaction. If these 'contaminants' are not removed prior to size selection, your size selection protocol will result in slightly smaller inserts.

17. When should I use a single index primer vs a dual index primer?

Single indexed primer sets can be used for pooling 1 to 24 libraries. Dual indexed primer sets can be used for pooling 1 to 96 libraries.

In addition, you can use a limited set of the dual index primers (Cat. No. 15300-1) to create libraries with unique P5 and P7 indices on each end, which will help prevent index hopping and misassignment of library reads to the wrong sample. To accomplish this goal, combine each P5 primer (Primers 501 – 508) with 8 unique P7 primers (e.g. Primers 701 – 708) to amplify 8 libraries with these completely unique dual indices. This approach can be repeated with the 501-508 primers combined with different unique 701-712 series primers in order to eventually use the entire dual indexing kit.

18. And can I pool libraries prepared with a both single- and dual-indexed libraries?

For most Illumina sequencing platforms, a mixture of single-and dual-indexed adapters is supported with minor modifications to the Illumina sample sheet. Note that dual indexing is not compatible with parallel Illumina platforms such as HiSeq X Ten, and a single/dual index mixture cannot be used with these platforms. Please contact Lucigen Tech Support for additional information regarding pooling single- and dual-indexed libraries.

19. How can I more accurately QC my libraries prior to sequencing?

In certain cases, Qubit and Bioanalyzer QC of your final library might not be sufficient for sequencing. qPCR and/or Nano runs can be used to more precisely determine the desired cluster density.

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20. How do I avoid sample mix-ups and cross contamination when using samples in individual or strip tubes?

Pre-label all tubes that you will need to complete your libraries. Arrange the tubes in a rack so that all tubes are in order. Double-check the identity of a tube prior to transferring your sample or adding to a sample. Always use barrier tips to prevent cross-contamination and never re-use a tip. Work in a filtered PCR hood when possible, and always set up your PCR reaction in a filtered PCR hood.

21. Why do I have to amplify my library for at least 5 cycles?

After ligation of the Universal Adaptor to your DNA fragments, your library will lack indices and the P5/P7 flow cell-binding sequences. Amplification with the provided indexed primer sets will add the index and the P5/P7 flow cell-binding sequences to the amplified fragments. After amplification for 5 cycles, your library will have all necessary sequences on ~94% of the fragments and 8 cycles will provide over ~99% complete sequences. See our workflow figure on the NxSeq® UltraLow DNA Library Kit webpage for a graphical representation of how the adaptor-ligated fragments are completed using PCR to add the indices and P5/P7 flow cell-binding sequences. Note that when using only 4 cycles of PCR with 75 ng of starting input DNA, your library will have ~85% complete, sequenceable fragments.

22. Can I adjust the PCR annealing/extension temperature from 72°C to 68°C?

Yes, the optimal range for the annealing/extension step is from 68°C to 72°C.

23. What are the possible sources of duplicates in my sequencing data?

Duplicate copies of sequences in your library can occur in several forms including: 1) biological duplicates; 2) PCR duplicates; 3) optical duplicates and 4) exclusion amplification duplicates (ExAmp). Biological duplicates occur when you extract DNA from multiple cells/organisms and your DNA sample contains multiple genomes (copies). For example, a standard library (2 ng/µL in 20 µL) made from human genomic DNA can contain the equivalent of 5,714 genomes which translates into 5,714 potential exact copies of each ~300 bp insert; an *E. coli* K12 library can contain over 7.8 M exact copies of each 300 bp insert. So as the size of the genome decreases, the number of potential biological duplicates increases with the same starting amount of DNA.

PCR duplicates are produced at each round of amplification; with each amplification cycle, you produce more duplicates. In an unbiased system (adaptor ligation/PCR) with 100% efficiency, PCR duplicates will not cause problems if you have adequate starting DNA that is not limiting for the sequencer used. PCR duplication rates are also affected by the number of samples multiplexed per lane, the number of reads analyzed per sample, and the capacity of the sequencer used. The figure below illustrates many of these effects. In this experiment, libraries were made from 50 pg, 500 pg or 75 ng of *E. coli* genomic DNA. The samples were pooled with other samples and run on a MiSeq with 2 x 150 bp chemistry. Different numbers of reads were sampled (indicated) from 100K up to 5.4M depending on the library, and the percent of CLC (software package) mapped duplicates was determined. As shown, even with 50 pg of input in this case, the maximal number of duplicates observed was 6%. Importantly and as expected, as the number of reads sampled decreased, the percentage of duplicates per library also decreased.

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Number of Reads and Library Input Amount Affect PCR Duplicate Rates
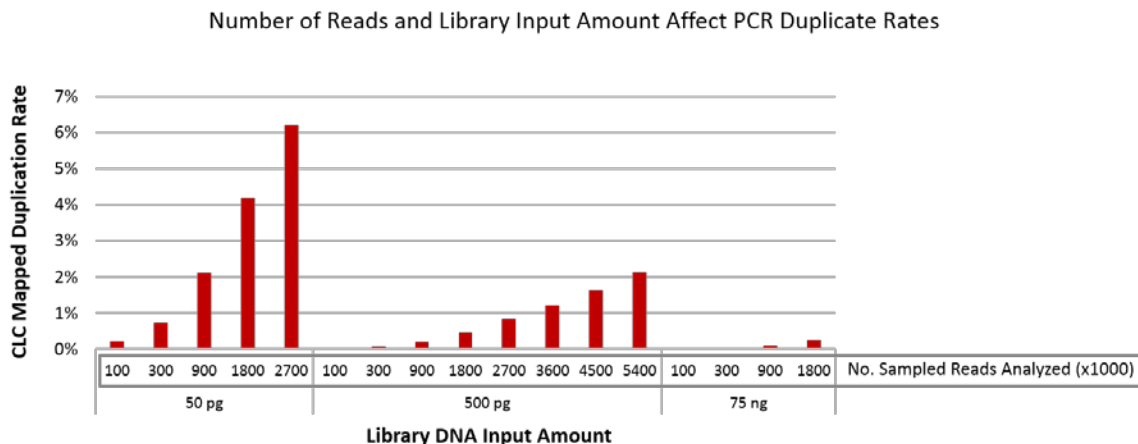
We do not recommend making and analyzing human gDNA libraries made from only 50 pg (8 genome equivalents) of starting DNA followed by deep sequencing on a HiSeq instrument. In this case, the theoretical number of unique starting DNA fragments is significantly lower than the number of possible clusters on the instrument. So in order to fill the lane of the flow cell with fragments from a single library (or a few), you must load a large number of duplicated library fragments and the duplication rates will be extremely high (80% or more). Importantly, the quality of these libraries are quite good, but the high duplication rate is unavoidable as discussed due to the number of unique fragments in the starting sample and the high instrument capacity. This duplication rate can be lowered by multiplexing, which in effect reduces the number of reads sampled per library (as shown above).

Unfortunately, all PCR polymerases and buffer systems have some bias and variable efficiencies. So it is important to use a PCR system, like the one in the NxSeq UltraLow DNA Library Kit, which is designed to produce minimal bias. PCR duplicates can also be minimized by using sufficient DNA so that only a very few cycles of PCR are required to produce a sequenceable library (4-5 cycles). However, if you do not have sufficient input DNA, you will have to run more PCR cycles and your data will have an increased number of duplicates (although remember that the sample complexity, extent of multiplexing, number of reads and sequencer used also have a dramatic impact on the number of observed PCR duplicates) which could interfere with analysis. Data for duplicate *E. coli* K12 libraries made with DNA input amounts ranging from 25 pg to 100 ng show that, although average genome coverage is similar for all libraries, duplicates are higher for the 25 pg library (2.5%) compared to the 250 pg libraries (0.15%) and the 75 ng libraries (0.02%) when analyzed on a MiSeq.

Optical duplicates are a problem for HiSeq 2500/MiSeq/NextSeq data. They result when large clusters are called as two separate clusters, or from re-clustering of the original library molecule when it is released from polymerase copying.

Exclusion Amplification duplicates (ExAmp) arise on Illumina systems using patterned flow cells such as HiSeqX, HiSeq 3000/4000, and the NovaSeq. They result from seeding of additional nanowells by the original library molecule when it is released from polymerase copying and diffuses to a nearby well. This problem may be exacerbated by multiplexing of libraries with different insert sizes and made with different library kits. For best results, you should multiplex only libraries with similar sized inserts made with the same kit.

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24. Why are the mapped, paired-end reads from my library smaller than the expected size, based on the size of my input DNA?

Illumina sequencing platforms are biased toward smaller insert sizes due in part to bridge amplification efficiencies and hybridization kinetics on the sequencer. When you pool libraries with different insert sizes, the smaller insert libraries will have more sequence reads than the larger libraries. Even when sequencing a single library, the resulting sequence will be biased toward smaller inserts and the final mapped size will be smaller than the input library size as shown in Table 1 below. Illumina does not support, and strongly advises against, running libraries prepared with different library prep kits in the same lane of a flow cell because of differences in final libraries from prep to prep.

Library Insert Sizes are Smaller than Expected when Sequencing on an Illumina Instrument

Sample	1 Sheared DNA Peak Size (Starting Insert Size)	2 Expected Library Size w/Ligated Universal Adaptor (+60 bp)	3 Pre-PCR Bead Ratio Used for Cleanup after Ligation Step	4 Post-PCR Bead Ratio Used for Cleanup	5 Post-PCR Bead Ratio Used for Size Selection	6 Final Library Peak Size (Bioanalzyer)	7 Expected Final Insert Length (Final Library Size minus 120 bp Adaptor)	8 Observed Final Insert Length Based on Sequencing Results	9 Difference Between Expected and Observed Insert Lengths
A	148 bp	208 bp	1.0 x	1.0 x	0.80 x	311 bp	191 bp	181 bp	-10 bp
B	225 bp	285 bp	0.80 x	1.0 x	0.75 x	376 bp	256 bp	238 bp	-18 bp
C	305 bp	365 bp	0.80 x	1.0 x	0.66 x	483 bp	363 bp	298 bp	-65 bp
D	410 bp	470 bp	0.80 x	1.0 x	0.63 x	544 bp	424 bp	332 bp	-92 bp
E	493 bp	553 bp	0.80 x	1.0 x	0.61 x	594 bp	474 bp	364 bp	-110 bp
F	532 bp	592 bp	0.80 x	1.0 x	0.58 x	661 bp	541 bp	391 bp	-150 bp

Table 1. Expected vs. Observed Library Insert Sizes Based on Starting DNA Peak Size and AMPure XP Bead Ratios Used in Protocol. Six sets of duplicate NxSeq® UltraLow DNA Libraries (A – F) were prepped according the User Manual using DNA samples fragmented to the indicated peak sizes (Column 1) and each library was processed using the AMPure XP bead to sample ratios for the various library prep steps indicated in Columns 3 – 5. Based on the size (Column 6) of the final amplified, cleaned up and size selected libraries, the Expected Final Insert Size of each library is shown in Column 7 followed by the Observed Final Insert Size based on the sequencing of each library(Column 8). As the Expected Library Insert Size increased, the difference between the Expected and Observed Insert Sizes increased indicating the preference of the Illumina Sequencer for smaller inserts (Column 9).

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