Lucigen®
Simplifying Genomics

NxSeq® UltraLow DNA Library Kit, 12 Reactions

Illumina-compatible

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Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the reagents supplied by the user are of the highest quality. Please follow the instructions carefully and contact our technical service representatives if additional information is necessary. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

Lucigen Technical Support
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Product Guarantee: Lucigen guarantees that this product will perform as specified for one year from the date of shipment.

Product Description

The NxSeq® UltraLow DNA Library Kit supplies the buffers and enzymes needed to make high efficiency DNA fragment libraries for whole genome next generation sequencing on Illumina sequencers. This kit is optimized for the production of DNA fragment libraries starting with 50 pg to 75 ng of sheared/fragmented DNA. This kit may be used in other applications such as FFPE, ChIP and exome sequencing where PCR amplified libraries are required, but it has not been tested extensively in these applications. Note, the number of PCR cycles required for amplification is dependent on the starting

amount of the ultralow input sheared DNA. Also, the number of observed PCR duplicates sequenced is dependent on sample complexity, number of multiplexed samples, and the model of Illumina sequencer used.

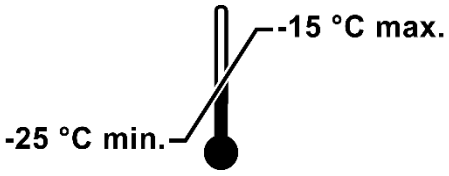
This User Manual couples the NxSeq® UltraLow DNA Library Kit, 12 Reactions with the NxSeq® Single Indexing Kits for lower throughput sequencing experiments. For higher throughput sequencing experiments (e.g. 96 library preps in a 96 well plate), we recommend using the NxSeq® UltraLow DNA Library Kit, 96 Reactions (Cat. No. 15096-1) with the NxSeq® HT Dual Indexing Kit (Cat. No. 15300-1). If you prefer to use dual indices with the NxSeq® UltraLow DNA Library Kit, 12 Reactions, please follow the amplification protocol outlined in the NxSeq® UltraLow DNA Library Kit, 96 Reactions User Manual and adapt it to the single tube reaction format.

Product Designations and Kit Components

Product	Kit Size	Catalog Number	Reagent Description	Part Numbers	Volume	Cap Identifier
NxSeq® UltraLow DNA Library Kit, 12 Reactions	12 Reactions	15012-1	Enzyme Mix	F833962-1	100 µL	EM
			2X Buffer	F883396-4	300 µL	2XB
			Ligase	F832792-4	50 µL	LIG
			Elution Buffer	F882705-1	3 mL	none
			2X PCR Master Mix	F833963-1	300 µL	MM
NxSeq® Single Indexing Kit, Set A	48 Reactions (12 x 4 rxn)	15100-1	NxSeq® Universal Adaptor	F813785-1	144 µL	UA
			Adaptor Dilution Buffer	F883786-1	3 mL	none
			Primer Mix - Index 1	F813066-2	20 µL	1
			Primer Mix - Index 2	F813067-2	20 µL	2
			Primer Mix - Index 3	F813068-2	20 µL	3
			Primer Mix - Index 4	F813069-2	20 µL	4
			Primer Mix - Index 5	F813070-2	20 µL	5
			Primer Mix - Index 6	F813071-2	20 µL	6
			Primer Mix - Index 7	F813072-2	20 µL	7
			Primer Mix - Index 8	F813073-2	20 µL	8
			Primer Mix - Index 9	F813074-2	20 µL	9
			Primer Mix - Index 10	F813075-2	20 µL	10
			Primer Mix - Index 11	F813076-2	20 µL	11
			Primer Mix - Index 12	F813077-3	20 µL	12
NxSeq® Single Indexing Kit, Set B	48 Reactions (12 x 4 rxn)	15200-1	NxSeq® Universal Adaptor	F813785-1	144 µL	UA
			Adaptor Dilution Buffer	F883786-1	3 mL	none
			Primer Mix - Index 13	F813964-1	20 µL	13
			Primer Mix - Index 14	F813965-1	20 µL	14
			Primer Mix - Index 15	F813966-1	20 µL	15
			Primer Mix - Index 16	F813967-1	20 µL	16
			Primer Mix - Index 18	F813968-1	20 µL	18
			Primer Mix - Index 19	F813969-1	20 µL	19
			Primer Mix - Index 20	F813970-1	20 µL	20
			Primer Mix - Index 21	F813971-1	20 µL	21
			Primer Mix - Index 22	F813972-1	20 µL	22
			Primer Mix - Index 23	F813973-1	20 µL	23
			Primer Mix - Index 25	F813974-1	20 µL	25
			Primer Mix - Index 27	F813975-1	20 µL	27

Components & Storage Conditions

Store all kits and components at -20 ºC



Additional Materials and Equipment Needed

Material and Equipment Needed	Vendor
Agencourt AMPure® XP Beads	Beckman Coulter (Cat. #A63880 or A63881)
Ethanol (high purity)	Various
Nuclease-Free Water	Thermo Fisher Scientific (Cat. #AM9938)
Magnetic rack	Various
0.2 mL thin wall PCR tubes	Various
1.5 mL DNA LoBind Tubes	Eppendorf (Cat. #022431021)
Qubit® dsDNA HS Assay Kit	Thermo Fisher Scientific (Cat. # Q32851 or Q32854)
Qubit® Fluorometer	Thermo Fisher Scientific
Bioanalyzer High Sensitivity Kit	Agilent Technologies (Cat. #5067-4626)
2100 Bioanalyzer	Agilent Technologies
8 microTUBE-15 AFA Beads Strip V2	Covaris (Cat. #520159)
PCR Thermocycler	Various
Minifuge	Various
LE220 Focused-ultrasonicator or similar	Covaris (Cat. #LE220)
Bioruptor® Ultrasonicator	Diagenode
dsDNA Shearase™ Plus	Zymo Research (Cat.# E2018-50)

Protocol

DNA Shearing/Fragmentation

Notes:

- DNA must be mechanically sheared or enzymatically fragmented to produce appropriately sized DNA fragments prior to end-repair/A-tailing. This protocol is written for mechanically sheared DNA that is 300-500 bp, and enzymatically sheared DNA that is 100-300 bp. If you are using DNA fragments outside these ranges, please contact Lucigen Tech Support for additional bead cleanup and size selection guidelines. If the starting DNA is already fragmented to the appropriate size and purity, start at the End-repair/A-tailing step (p.7).
- Choose one option for generating appropriately sized DNA fragments prior to library preparation - Mechanical Shearing or Enzymatic Fragmentation - and follow instructions below.
- **DNA must be quantified using a fluorometric method (Qubit or PicoGreen® fluorescent dyes)**. Fluorometric methods provide an accurate measurement of dsDNA only; in contrast to UV-based methods (including NanoDrop™ Instruments), which measure all nucleic acids including ssDNA, RNA, and free nucleotides.
- **DNA used must be free of contaminating RNA (No RNA visible on a gel) and resuspended in Low TE** (0.1 mM EDTA; 10 mM Tris pH 8).

Mechanical Shearing (Option 1)

1. Mechanically shear your DNA sample(s) such that the peak centers around 300 bp (or to your desired fragment size) when analyzed on a Bioanalyzer. If 50 pg to 75 ng of input DNA is used in the following shearing protocol, it is not necessary to shear more DNA than needed since the entire contents of the shearing tube can be transferred to the End-repair/A-tailing Reaction. Recommendations for shearing to 300 bp are provided.

<table>
<tr><th>Shearing System</th><th>Recommendations</th></tr>
<tr><td>LE220 Covaris Focused-ultrasonicator</td><td>

Use an 8 microTUBE strip according to manufacturer's recommendations for shearing to 300 bp
or
Use an 8 microTUBE-15 AFA Beads Strip v2 using Covaris settings for LE220:*
<table>
<tr><td>Target Size (bp of peak)</td><td>300</td></tr>
<tr><td>Peak Incident Power (W)</td><td>180</td></tr>
<tr><td>Duty Factor (%)</td><td>18</td></tr>
<tr><td>Cycles/Burst</td><td>50</td></tr>
<tr><td>Treatment Time (s)</td><td>90</td></tr>
<tr><td>Temperature (°C)</td><td>20</td></tr>
<tr><td>Water Level</td><td>4</td></tr>
<tr><td>Sample Volume (µL)</td><td>15</td></tr>
</table>

Include Y-dithering in sample treatment using the following steps:

Y Dither (mm) – 5
X-Y Dither Speed (mm/sec) – 20
Both X Dither (mm) and X-Y Dwell (sec) should be set to 0.

</td></tr>
<tr><td>Other Covaris Focused-utrasonicator Models</td><td>Follow manufacturer's recommendations for shearing to 300 bp.</td></tr>
<tr><td>Diagenode Bioruptor Ultrasonicator</td><td>Follow manufacturer's recommendations for shearing to 300 bp.</td></tr>
</table>

Optional: If the sheared DNA is not at the minimum required concentration and volume, ≥50 pg in ≤17 µL or ≥2.94 pg/µL with a minimum of 17 µL, use Agencourt AMPure XP Beads (at a 1.8X ratio; e.g. add 180 µL beads to 100 µL sample) to concentrate the sheared DNA to obtain the volume and concentration necessary to proceed to the next step.

It is not necessary to purify samples sheared with the 8 microTUBE-15 Strip v2 after shearing. The 15 µL of sheared DNA can be transferred directly to the End-repair/A-tailing Reaction. However, this step can be added as a cleanup/buffer exchange step if the DNA (i) is in a buffer with >0.1 mM EDTA or (ii) contains potential inhibitors of the downstream enzymatic steps.

Enzymatic Fragmentation (Option 2)

Notes:

- The following Lucigen protocol utilizes a modified version of the dsDNA Shearase Plus Enzyme protocol (Zymo Research, Cat.# E2018-50) to prepare 50 pg to 75 ng of fragmented DNA. Do not use the vendor's kit protocol, but **follow this optimized protocol** to avoid over- or under-digestion.
- Do not use EDTA to stop the digestion reaction; use the 65°C heat-kill step in this protocol.
- This enzymatic fragmentation protocol produces final libraries with mean fragment sizes of about 350 bp with ~200 bp inserts. (See Figure 4, p. 12).

Fragmentation Reaction

1. Set up the following reaction on ice. Add the following components to a 0.2 mL PCR tube in order:

Volume (µL)	Component
X	DNA (50 pg – 75 ng in ≤ 12.3 µL Low TE buffer)
12.3 - X	Nuclease-free Water
3.4	5X Shearase™ Plus Reaction Buffer
1.3	dsDNA Shearase™ Plus Enzyme
17	**Total**

2. Mix gently by pipetting up and down 10 times.
3. Spin briefly to collect material in the bottom of the tube.
4. Place tube(s) in a thermocycler preheated to 42°C and incubate as follows:

Step	Temperature	Time (min)
1	42°C	12.5
2	65°C	10
3	4°C	Hold

5. The enzymatically fragmented DNA is now ready for the End-repair/A-tailing step. Proceed directly to End-repair/A-tailing step (p.7). **Note that an AMPure bead cleanup or size selection prior to starting the End-repair/A-tailing Reaction is not required nor recommended for the libraries sheared enzymatically.**

Analysis

1. If enough sheared/fragmented DNA is available, analyze 1 µL on a Bioanalyzer High Sensitivity Chip to determine size distribution. Be aware that you must run ≥0.5 ng of DNA on a High Sensitivity Chip in order to detect it. See Figures 1 and 2 for typical Bioanalyzer traces after shearing and enzymatic fragmentation.

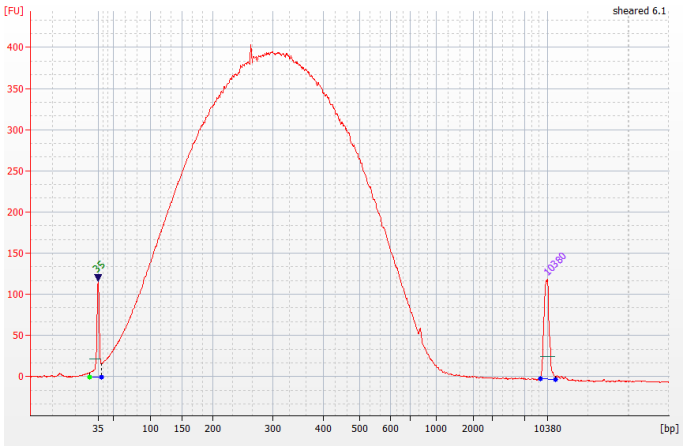


Figure 1. Typical Bioanalyzer trace for gDNA sheared to 300 bp using a Covaris Focused-ultrasonicator.

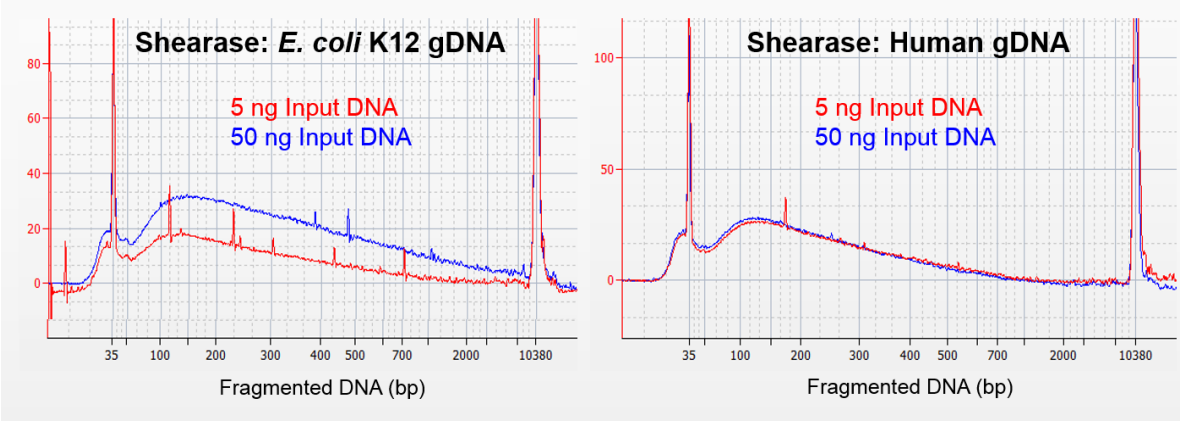


Figure 2. Typical Bioanalyzer traces for gDNA sheared enzymatically.

Library Preparation

Notes:

- Thaw all reagents on ice.
- Vortex the 2X Buffer and Adaptor Dilution Buffer for 20 seconds each and the 2X PCR Master Mix for 5 seconds before use. Please ensure that the 2X Buffer is completely mixed.
- Equilibrate AMPure XP Beads to room temperature for at least 30 minutes before use.
- Pipette viscous reagents (Enzyme Mix, Ligase, and AMPure XP beads) slowly throughout the workflow.
- Use Eppendorf LoBind tubes when possible (strongly recommended).
- Prepare 5 mL of fresh 70% ethanol solution per library preparation.

End-repair/A-tailing Reaction

1. Add the following components to a 0.2 mL PCR tube in order:

Volume (µL)	Component
17 - X	Nuclease-Free Water
25	2X Buffer (2XB)
X	Sheared/fragmented DNA (50 pg – 75 ng)
8	Enzyme Mix (EM)
50	**Total**

2. Mix gently by pipetting up and down 10 times.
3. Spin briefly to collect material in the bottom of the tube.
4. Place tube(s) in a thermocycler and incubate as follows with a heated lid set at 80°C:

Step	Temperature	Time (min)
1	25°C	20
2	72°C	20
3	4°C	Hold

Adaptor Ligation Reaction

1. Dilute the NxSeq® Universal Adaptor with Adaptor Dilution Buffer* based on the amount of DNA added to each End-repair/A-tailing reaction as outlined in the following table:

End-repair/A-tailing Reaction DNA Input	Final Adaptor Dilution	Adaptor Volume (µL)	Adaptor Dilution Buffer* Volume (µL)
50 pg – 250 pg	1:60	1	59
251pg – 1 ng	1:30	1	29
2 ng – 75 ng	No dilution	-	-

***** For optimal results, Adaptors must be diluted with the provided Adaptor Dilution Buffer

2. To each of the End-repair/A-tailing Reactions (50 µL), add the following volumes of diluted NxSeq Universal Adaptor and Ligase in the order shown at room temperature.

Volume (µL)	Reagent
50	End-repair/A-tailing Reaction
3	NxSeq® Universal Adaptor (UA)
4	Ligase (LIG)
57	**Total**

3. Mix gently by pipetting up and down 10 times.
4. Spin briefly to collect material in the bottom of the tube.
5. Place tube(s) in a thermocycler and incubate at 25°C for 30 minutes.
6. Proceed to required Cleanup Step.

Cleanup (Required)

Notes: See Appendix C: Bead Cleanup for an illustration of this workflow.

1. Vortex the room temperature AMPure XP Beads to resuspend them.
2. Transfer the contents of each adaptor ligation reaction tube (57 µL) to a 1.5 mL LoBind Tube.
3. Perform all of the following steps at room temperature.
4. Add 43 µL of Elution Buffer to each sample to increase sample volume and improve cleanup.
5. Add the following amounts of AMPure XP beads to each sample:

   Mechanically sheared library: Add 80 µL of AMPure XP Beads.

   Enzymatically fragmented library: Add 100 µL of AMPure XP Beads.
6. Mix gently by pipetting up and down 10 times.
7. Spin briefly to collect material in the bottom of the tube.
8. Incubate at room temperature for 5 minutes; do not use a magnetic rack.
9. Place the tube in a magnetic rack for 5 minutes (until the supernatant becomes clear).
10. With the tube in the magnetic rack, gently remove the supernatant with a pipette and discard.
11. Wash the beads by adding 750 µL of 70% ethanol to the tube. Wait 30 seconds, remove the ethanol with a pipette and discard the ethanol.

12. Repeat the previous wash step (11).
13. Remove the tube from the magnetic rack, spin briefly to collect any residual ethanol in the bottom of the tube and then place the tube back into the magnetic rack.
14. Keeping the tube in the magnetic rack, carefully remove any remaining ethanol and then air-dry the bead pellet for 5 minutes.
15. With the tube in the magnetic track, add 22 µL of Elution Buffer.
16. Remove the tube from the magnetic rack.
17. Mix the beads and the buffer gently by pipetting up and down 10 times (Do not vortex).
18. Incubate at 37°C for 5 minutes; do not use a magnetic rack during the incubation.
19. Place the tube in a magnetic rack for 5 minutes (until the supernatant becomes clear).
20. Transfer 20 µL of supernatant containing your cleaned fragment library to a new PCR tube.

PCR Amplification (Required)

1. For each cleaned fragment library, mix the following components in a PCR tube being certain to use different indices for libraries that will be pooled for sequencing:

Volume (µL)	Component
20	Bead-cleaned library
25	2X PCR Master Mix (MM)
5	NxSeq® Single Indexed Primer Mix[1, 2]
50	**Total**

[1] See *Appendix A: Index Primer Sequences* for details on Primer Index Sequences.

[2] See *Appendix B: Library Pooling Guidelines* for information on pooling indexed libraries prior to sequencing.

2. Place each tube in the thermocycler and cycle as follows:

Step	Temperature	Time	Cycles
1	98°C	30 seconds	
2	**98°C**	**10 seconds**	**Repeat Steps 2 – 3 X** cycles**
3	**72°C**	**75 seconds**	
5	65°C	5 minutes	
6	4°C	Hold	

****Recommended PCR Cycle Numbers Based on Input Amount**

Starting DNA Input Amount	Number of Cycles (X)
50 pg – 100pg	16
101 pg – 250 pg	15
251 pg – 500 pg	14
501 pg – 750 pg	13
751 pg – 1 ng	12
2 ng – 10 ng	8
11 ng – 25 ng	7
26 ng – 50 ng	6
51 ng – 75 ng	5

PCR Cleanup (Required)

Notes:

- See *Appendix C: Bead Cleanup* for a detailed illustration of this workflow.

1. Vortex the room temperature AMPure XP Beads to resuspend them.
2. Transfer the contents (50 µL) of each PCR reaction tube to a 1.5 mL LoBind Tube.
3. Perform all of the following steps at room temperature.
4. Add 50 µL of AMPure XP Beads to the 1.5 mL tube.
5. Mix gently by pipetting up and down 10 times.
6. Spin briefly to collect material in the bottom of the tube.
7. Incubate at room temperature for 5 minutes; do not use a magnetic rack.
8. Place the tube in a magnetic rack for 5 minutes (until the supernatant becomes clear).
9. With the tube in the magnetic rack, gently remove the supernatant with a pipette and discard.
10. Wash the beads by adding 750 µL of 70% ethanol to the tube. Wait 30 seconds, remove the ethanol with a pipette and discard the ethanol.
11. Repeat the previous wash step (10).
12. Remove the tube from the magnetic rack, spin briefly to collect any residual ethanol in the bottom of the tube and then place the tube back into the magnetic rack.
13. Keeping the tube in the magnetic rack, carefully remove any remaining ethanol and then air-dry the bead pellet for 5 minutes.
14. With the tube in the magnetic track, add 102 µL of Elution Buffer.
15. Remove the tube from the magnetic rack.
16. Mix the beads and the buffer gently by pipetting up and down 10 times. (Do not vortex)
17. Incubate at 37°C for 5 minutes; do not use a magnetic rack during the incubation.
18. Place the tube in a magnetic rack for 5 minutes (until the supernatant becomes clear).
19. Transfer 100 µL of supernatant containing the cleaned, PCR amplified library to a new 1.5 mL LoBind Tube and proceed to Size Selection.

Size Selection (Required)

Notes:

- If your experiment requires dual size selection at both lower and upper ends, see *Appendix D: Double Bead Size Selection* for instructions or contact Lucigen Technical Support.
- The following size selection protocol is specific to the fragmentation method used; the size selection method for enzymatically fragmented DNA libraries is different than mechanically sheared DNA libraries due to the smaller size of the fragments generated by enzymatic fragmentation (see below).

1. Vortex the room temperature AMPure XP Beads to resuspend them.
2. Perform all of the following steps at room temperature.
3. Add the following amounts of AMPure XP beads to each bead-cleaned library:

Mechanically sheared library: Add 75 µL of AMPure XP Beads to 100 µL of a bead-cleaned library.

Enzymatically fragmented library: Add 100 µL of AMPure XP Beads to 100 µL of a bead-cleaned library.

4. Mix gently by pipetting up and down10 times.
5. Spin briefly to collect material in the bottom of the tube.
6. Incubate at room temperature for 5 minutes; do not use a magnetic rack.
7. Place the tube in a magnetic rack for 5 minutes (until the supernatant becomes clear).
8. With the tube in the magnetic rack, gently remove the supernatant with a pipette and discard.
9. Wash the beads by adding 750 µL of 70% ethanol to the tube. Wait 30 seconds, remove the ethanol with a pipette and discard the ethanol.
10. Repeat the previous wash step (9).
11. Remove the tube from the magnetic rack, spin briefly to collect any residual ethanol in the bottom of the tube and then place the tube back into the magnetic rack.
12. Keep the tube in the magnetic rack and carefully remove any remaining ethanol, then air-dry the bead pellet for 5 minutes.
13. With the tube in the magnetic track, add 22 µL of Elution Buffer.
14. Remove the tube from the magnetic rack.
15. Mix the beads and the buffer gently by pipetting up and down 10 times being certain to completely resuspend the beads in the Elution Buffer (Do not vortex).
16. Incubate at 37°C for 5 minutes; do not use a magnetic rack during the incubation.
17. Place the tube in a magnetic rack for 5 minutes (until the supernatant becomes clear).
18. Transfer 20 µL of supernatant containing your final PCR amplified library to a new 1.5 mL tube.
19. Store the finished library at -20°C or proceed to analysis and sequencing. We recommend sequencing your final libraries within 7 days.

Analysis

1. Quantify the size-selected library with a Qubit Fluorometer, following the manufacturer's instructions.
2. Analyze 1 µL of the library on a Bioanalyzer High Sensitivity Chip to determine size distribution. Ideally, the peak should center on 420 bp for a 300 bp insert (mechanically sheared). See Figure 3 (below) for typical Bioanalyzer trace of library generated using DNA initially sheared to 350 bp. See Figure 4 (below) for library generated using enzymatically fragmented DNA (~200 bp inserts).

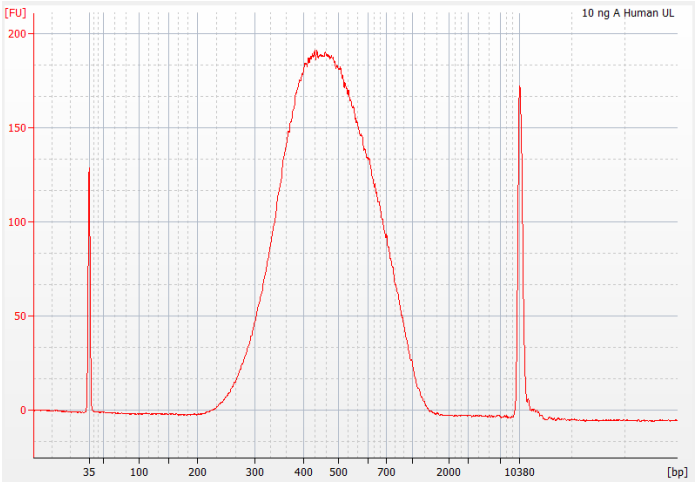


Figure 3. Typical Bioanalyzer trace for library generated using input gDNA sheared mechanically to 350 bp.

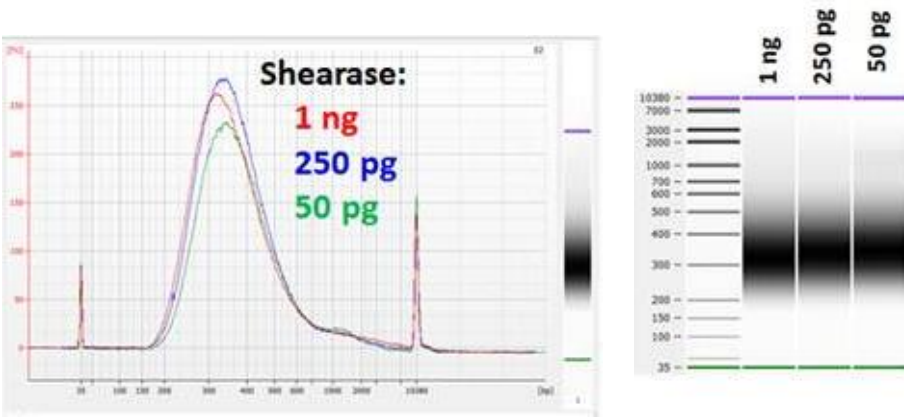


Figure 4. Typical Bioanalyzer traces for final libraries generated using input gDNA sheared enzymatically.

Sequencing

- Final libraries are compatible with and can be run on any Illumina sequencer.
- Lucigen recommends using PhiX Control v3 library (Illumina, FC-110-3001) as a control for Illumina sequencing runs.
- See *Appendix B: Library Pooling Guidelines* for information on pooling requirements prior to sequencing.

Appendix A: Single Index Sequences (Cat. No. 15100-1, 15200-1)

The 6 base primer index sequences are listed below.

PCR Primer Indices	Index Sequence
Primer Mix - Index 1	ATCACG
Primer Mix - Index 2	CGATGT
Primer Mix - Index 3	TTAGGC
Primer Mix - Index 4	TGACCA
Primer Mix - Index 5	ACAGTG
Primer Mix - Index 6	GCCAAT
Primer Mix - Index 7	CAGATC
Primer Mix - Index 8	ACTTGA
Primer Mix - Index 9	GATCAG
Primer Mix - Index 10	TAGCTT
Primer Mix - Index 11	GGCTAC
Primer Mix - Index 12	CTTGTA
Primer Mix - Index 13	AGTCAA
Primer Mix - Index 14	AGTTCC
Primer Mix - Index 15	ATGTCA
Primer Mix - Index 16	CCGTCC
Primer Mix - Index 18	GTCCGC
Primer Mix - Index 19	GTGAAA
Primer Mix - Index 20	GTGGCC
Primer Mix - Index 21	GTTTCG
Primer Mix - Index 22	CGTACG
Primer Mix - Index 23	GAGTGG
Primer Mix - Index 25	ACTGAT
Primer Mix - Index 27	ATTCCT

Appendix B: Library Pooling Guidelines

It is important to use compatible indices when pooling to maintain color balance of each base of the index sequencing read. For proper imaging on the Illumina platform, at least one base needs to be read in the green laser channel (G or T) and one in the red laser channel (A or C). Creating this signal balance can be difficult when pooling low numbers of samples, and using incompatible indices could result in image registration failure and ultimately run failure. The following table provides recommendations on which indices to use when pooling 2 to 4 libraries. When pooling ≥5 libraries, any combination of indices will provide the proper red/green laser channel balance.

Pooling Strategies for 2 to 5 samples Using the NxSeq Single Indexing Kits, Set A or Set B

Number of Samples to Pool	Option	Index Combinations to Use from the NxSeq® Single Indexing Kit, Set A	Index Combinations to Use from the NxSeq® Single Indexing Kit, Set B
2	1	Index 6 and Index 12	Index 18 and Index 25
3	1	Index 6, 12 and 5	Index 13, Index 18 and Index 23
	2	2-plex option with any other index	2-plex option with any other index
4	1	Index 1, Index 8, Index 10 & Index 11	Index 13, Index 14, Index 16 & Index 18
	2	3-plex option with any other index	3-plex option with any other index
≥5	Many	Any	Any

Libraries should be pooled in equimolar amounts. If multiplexing, normalize and pool samples according to the appropriate Illumina Platform User's Manual.

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 sequences correspond with the PCR Primer Index sequences used in the NxSeq Single Indexing Kits.

Appendix C: Bead Cleanup

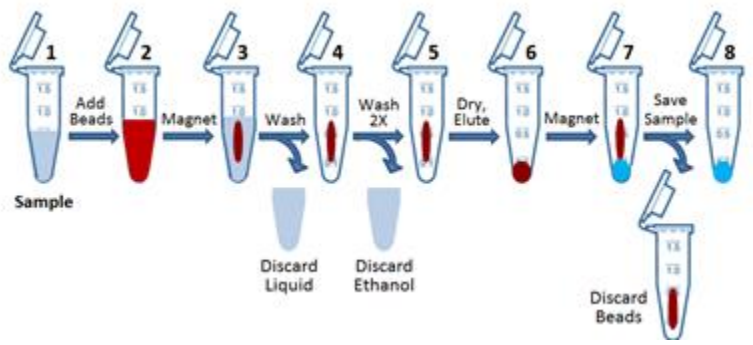


Figure 5. Bead cleanup workflow. 1) Sample DNA; 2) Add bead solution to sample and mix, incubate 5 min; 3) Place tube on magnetic rack; 4) Discard liquid and Wash 2x with 70% ethanol; 5) Dry beads for 5 min; 6) Add Elution Buffer (EB), remove from magnet, mix and incubate at 37°C for 5 minutes; 7) Place tube on magnetic rack after incubation; 8) Transfer the liquid containing the cleaned-up DNA to new tube and discard beads. The bead solution must be equilibrated to room temperature prior to use to assure accurate pipetting.

Appendix D. Double Bead Size Selection (Upper & Lower Ends)

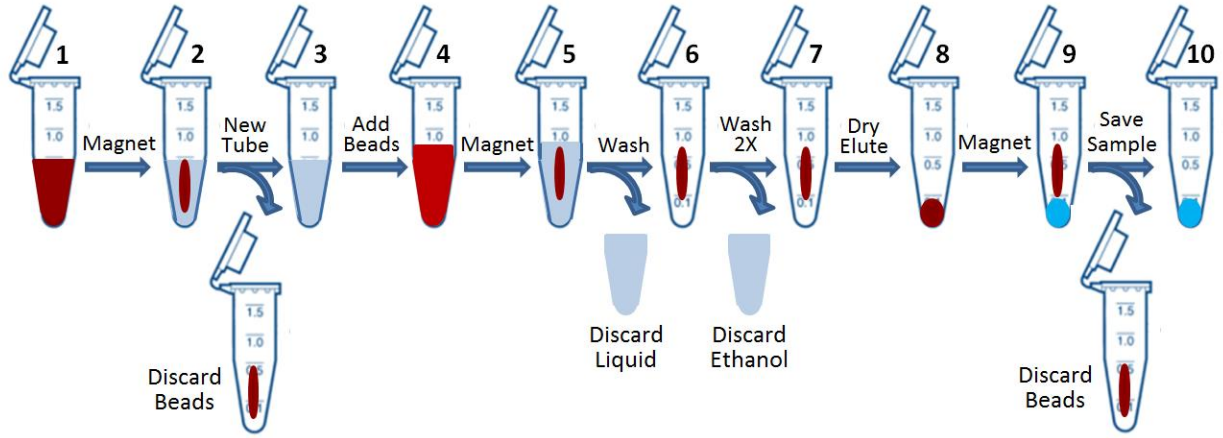


Figure 6. Size selection workflow. Briefly: 1) Add bead solution to sample and mix; 2) Place tube on magnetic rack; 3) Transfer liquid to new tube and discard tube with beads; 4) Add second volume of bead solution and mix; 5) Place on magnetic rack; 6) Discard liquid and Wash 2x with 70% ethanol; 7) Dry beads; 8) Add Elution Buffer (EB) and remove from magnet. Incubate at 37°C for 5 minutes and mix; 9) Place tube on magnetic rack; 10) Transfer liquid to new tube and discard beads. The bead solution must be equilibrated to room temperature prior to use to assure accurate pipetting.

Double bead size selection is based on the concentration of Polyethylene glycol (PEG) and sodium chloride (NaCl) in the bead solution. A higher concentration will bind both small and large fragments while a lower concentration will only bind large fragments. When a low amount of the bead solution is added to the DNA sample (“low concentration”), large DNA fragments will bind to the beads and be

removed when the bead pellet is discarded in the first size selection. By adding a second aliquot of bead solution to the saved supernatant, the concentration of PEG and NaCl will increase and allow binding of the desired range of DNA fragments up to the size removed by the first step. Smaller, contaminating DNA fragments will not bind and will be removed when the beads are washed.

For example, if your optimal library size range for sequencing is between 300 and 800 bp, add 0.53X volume of bead solution to your sample. As shown in Figure 7, this amount of beads will bind fragments of 800 bp and larger for removal. When 0.24X* volume of bead solution is added to the saved supernatant, the buffer concentration will be increased to 0.77X beads and buffer (0.77X – 0.53X = 0.24X beads) and will bind the remaining DNA fragments between 300 and 800 bp. DNA fragments smaller than 300 bp will be removed when the beads are washed. (X = the original sample volume).

*Note: As the percentage of beads in your sample decreases, your sample recovery will also decrease due to DNA binding kinetics. For example, the addition of ≤0.24X beads to your sample will result in 50-90% loss of sample. By adding clean, dry beads (no PEG or NaCl) to your sample along with the 0.24X beads, you will increase sample recovery. To clean the beads, add 100 µL beads to a 1.5 mL tube and place on a magnetic stand. Remove the supernatant and wash the beads 2X with 70% ethanol. Briefly spin the tube and place it on the magnetic rack; remove the excess ethanol and allow it to dry for 5 minutes. Remove the tube with washed beads from the magnetic rack and transfer your sample into it, after you have added the 0.24X beads, and mix by pipetting. Continue with the double size selection protocol by incubating your sample for 5 minutes (off the magnetic rack).

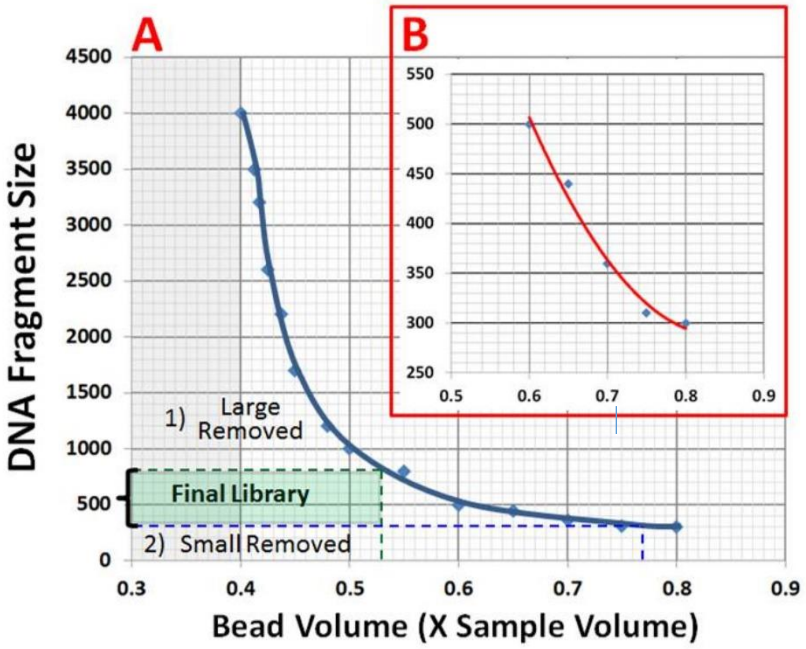


Figure 7. Effect of bead solution volume on fragment size removal. When selecting the optimal bead volume for library size selection, use these graphs (A or B) to determine how much bead volume to add in order to remove DNA fragments smaller than your final desired fragment size. The inserted graph (B) is an enlarged view of the smaller DNA fragments (300 to 500 bp) that are most commonly used. The bead solution volumes used to 1) remove ≥ 800 bp fragments (---) and 2) ≤ 300 bp fragments (---) in a double bead size selection of a 300-800 bp final library is depicted in (A) as described in more detail in above the text.

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