NxSeq® AmpFREE Low DNA Library Kit

Illumina Compatible

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE

Lucigen Corporation 2905 Parmenter St, Middleton, WI 53562 USA
Toll Free: (888) 575-9695 | (608) 831-9011 | FAX: (608) 831-9012
lucigen@lucigen.com www.lucigen.com

MA163 12Dec2018

Table of Contents

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
Email: techsupport@lucigen.com
Phone: (888) 575-9695

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

Product Description

The NxSeq® AmpFREE Low DNA Library Kit supplies the buffers and enzymes needed to make high efficiency, DNA fragment libraries for whole genome sequencing on Illumina sequencers. This kit is optimized for sheared/fragmented DNA input ranging from 75 ng to 1 µg.

Product Designations and Kit Components

Product	Kit Size	Catalog Number	Reagent Description	Part Numbers	Volume	Cap Identifier
NxSeq® AmpFREE Low DNA Library Kit	12 Reactions	14000-1	Enzyme Mix	F833397-4	100 µL	EM
			2X Buffer	F883396-4	300 µL	2XB
			Ligase	F832792-4	48 µL	LIG
			Elution Buffer	F882705-6	2 x 1.6 mL	EB
NxSeq® AmpFREE Low DNA Library Kit	48 Reactions	14000-2	Enzyme Mix	F833397-7	2 x 230 µL	EM
			2X Buffer	F883396-7	2 x 700 µL	2XB
			Ligase	F832792-7	2 x 120 µL	LIG
			Elution Buffer	F882705-7	2 x 6 mL	Not labeled
NxSeq® Adaptors, Box 1	12 x 4 Reactions	14300-1	Adaptor 1	F823501-1	12 µL	1
			Adaptor 2	F823502-1	12 µL	2
			Adaptor 3	F823503-1	12 µL	3
			Adaptor 4	F823504-1	12 µL	4
			Adaptor 5	F823505-1	12 µL	5
			Adaptor 6	F823506-1	12 µL	6
			Adaptor 7	F823507-1	12 µL	7
			Adaptor 8	F823508-1	12 µL	8
			Adaptor 9	F823509-1	12 µL	9
			Adaptor 10	F823510-1	12 µL	10
			Adaptor 11	F823511-1	12 µL	11
			Adaptor 12	F823512-1	12 µL	12
NxSeq® Adaptors, Box 2	12 x 4 Reactions	14400-1	Adaptor 13	F823513-1	12 µL	13
			Adaptor 14	F823514-1	12 µL	14
			Adaptor 15	F823515-1	12 µL	15
			Adaptor 16	F823516-1	12 µL	16
			Adaptor 17	F823517-1	12 µL	17
			Adaptor 18	F823518-1	12 µL	18
			Adaptor 19	F823519-1	12 µL	19
			Adaptor 20	F823520-1	12 µL	20
			Adaptor 21	F823521-1	12 µL	21
			Adaptor 22	F823522-1	12 µL	22
			Adaptor 23	F823523-1	12 µL	23
			Adaptor 24	F823524-1	12 µL	24

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	Ambion (Cat. #AM993)
Magnetic rack	Various
0.2 mL thin wall PCR tubes	Various
1.5 mL Eppendorf DNA LoBind Microcentrifuge tubes	Eppendorf (Cat. #22431021)
Qubit® dsDNA HS Assay Kit	Life Technologies (Cat. # Q32854)
Bioanalyzer High Sensitivity kit	Agilent Technologies (Cat. #5067-4626)
8 microTUBE strip	Covaris (Cat. #520053)
8 microTUBE-15 AFA Beads Strip V2	Covaris (Cat. #520159)
PCR Thermocycler	Various
2100 Bioanalyzer	Agilent Technologies
Qubit Fluorometer	Life Technologies
Minifuge	Various
Focused-ultrasonicator	Covaris (Cat. #LE220)
Bioruptor ultrasonicator	Diagenode

Protocol

Shear Genomic DNA

1. Shear gDNA such that the peak centers around 300 - 500 bp when analyzed on a Bioanalyzer.

Notes:

- This protocol is optimized for DNA fragments that have been mechanically sheared to 300-500bp. If you are using DNA fragments outside these ranges, please contact Lucigen Technical 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.6)
- **gDNA must be quantified using a fluorometric method (Qubit or PicoGreen).** Fluorometric methods provide an accurate measurement of dsDNA. In contrast, UV-based methods (including Nanodrop) measure all nucleotides, including ssDNA, RNA, and free nucleotides.
- **gDNA used must be free of contaminating RNA (no RNA visible on a gel).**
- **gDNA must be re-suspended in Low TE** (0.1 mM EDTA; 10 mM Tris pH 8) or water.

<table>
<tr><th>Shearing System</th><th>Options</th></tr>
<tr><td>LE220 Covaris Focused-Ultrasonicator</td><td>

8 microTUBE strip using manufacturer's recommendations for shearing to 300 - 500 bp, or
8 microTUBE-15 AFA Beads Strip v2 using Covaris settings for LE220:
<table>
<tr><td>Target BP (Peak)</td><td>300</td><td>500</td></tr>
<tr><td>Peak Incident Power (W)</td><td>180</td><td>180</td></tr>
<tr><td>Duty Factor (%)</td><td>18</td><td>16</td></tr>
<tr><td>Cycles/Burst</td><td>50</td><td>50</td></tr>
<tr><td>Treatment time (s)</td><td>90</td><td>70</td></tr>
<tr><td>Temperature (°C)</td><td>20</td><td>20</td></tr>
<tr><td>Water Level</td><td>4</td><td>4</td></tr>
<tr><td>Sample Volume (µL)</td><td>15</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 ultrasonicator Models</td><td>Follow manufacturer's recommendations for shearing to 300 - 500 bp.</td></tr>
<tr><td>Diagenode Bioruptor ultrasonicator</td><td>Follow manufacturer's recommendations for shearing to 300 - 500 bp.</td></tr>
</table>

Optional: If the sheared DNA is not at the minimum required concentration and volume (≥4.4 ng/µL in 17µL), use Agencourt AMPure XP Beads to concentrate the sheared DNA to obtain the volume and concentration necessary to proceed to the next step. Add the Agencourt AMPure XP Beads at a 1.8X ratio; e.g., add 180 µL beads to 100 µL DNA samples.

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, bead purification can be added as a cleanup/buffer exchange step if the DNA is in buffer with >0.1 mM EDTA

Optional/Safe Stopping Point. Mechanically sheared DNA can be stored at -20 °C in a 1.5mL Eppendorf LoBind tube.

Analysis

1. Analyze 1 µL of the sheared DNA on a Bioanalyzer High Sensitivity Chip to determine the size distribution. See Figures 1 and 2 below for typical Bioanalyzer traces.

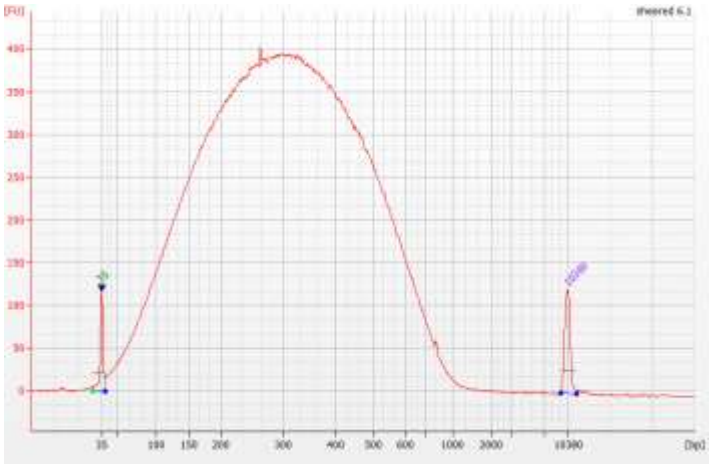


Figure 1: Typical Bioanalyzer trace for gDNA sheared to 300 bp.

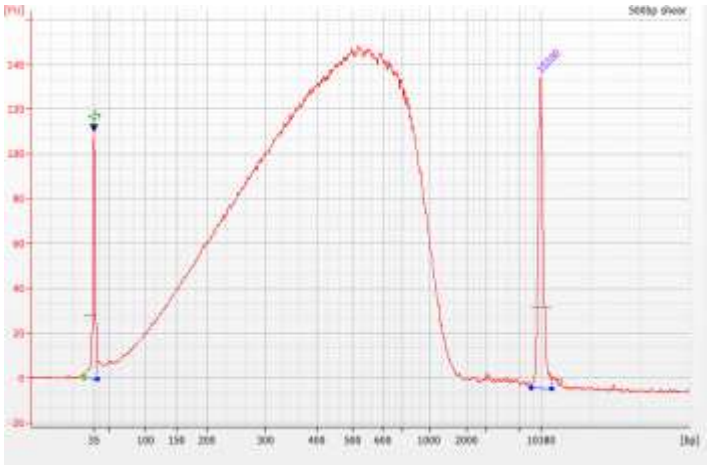


Figure 2: Typical Bioanalyzer trace for gDNA sheared to 500 bp.

Important Library Preparation Recommendations

- Thaw Elution Buffer (EB) and store at room temperature.
- Thaw all other reagents at room temperature and place on ice during library preparation. Store at -20 °C after library preparation.
- Vortex the 2X Buffer for 20 seconds and ensure it is completely mixed.
- Equilibrate AMPure XP Beads to room temperature for at least 30 minutes before use.
- Pipette viscous reagents [Enzyme Mix (EM), Ligase (LIG), and AMPure XP beads] slowly throughout the workflow.
- Use Eppendorf LoBind tubes whenever possible (strongly recommended).
- Prepare 3.5 mL of fresh 70% ethanol solution per library preparation.
- See Appendix A: Multichannel Library Preparation for recommendations on generating eight or more libraries using multichannel pipettes.

End Repair, A-Tailing Reaction

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

Volume (µL)	Component
17-X	Nuclease Free water to 17 µL
25.0	2X Buffer (2XB)
8.0	Enzyme Mix (EM)
X	Sheared/fragmented DNA (75ng – 1µg)
50	**Total**

2. Mix by gently 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 with 80 °C heated lid and incubate as follows:

Step	Temperature	Time
1	25 °C	20 minutes
2	72 °C	20 minutes
3	4 °C	Hold

Adaptor Ligation Reaction

1. Add the following reagents in the order shown to each End-repair, A-tailing reaction tube:

Reagent	Volume (µL)
Lucigen Adaptor at 15 µM[1]	3.0
Ligase (LIG)	4.0
Total	**57**

2. See Appendices B and C for information on Lucigen adaptors and pooling options.
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. Immediately proceed to required Cleanup step.

Cleanup (Required step)

Notes:

- See Appendix D: Bead Cleanup for a detailed description of this workflow.
- Both Cleanup and Size Selection steps are required.
- Perform all of the following steps at room temperature.

1. Vortex the room temperature-equilibrated AMPure XP beads to resuspend them.
2. Transfer the contents of each adaptor ligation reaction tube to a 1.5 mL LoBind tube.
3. Add 57 µL of AMPure XP Beads to the adaptor ligation reaction in the 1.5 mL tube.
4. Mix gently by pipetting up and down 10 times.
5. Spin briefly to collect any droplets to the bottom of the tube. (Do not pellet the beads).
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 ethanol wash step (Step 9).
11. Spin briefly to collect any residual ethanol in the bottom of the tube and place the tube back into the magnetic rack.
12. Keep the tube in the magnetic rack and carefully remove any remaining ethanol. Air-dry the bead pellet for 5 minutes.
13. Remove the tube from the magnetic rack.
14. Add 102 µL of Elution Buffer (EB).
15. Mix the beads and the buffer gently by pipetting up and down 10 times. 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 100 µL of supernatant to a new 1.5 mL LoBind tube.

Optional/Safe Stopping Point. Bead-cleaned samples can be stored at -20 °C in a 1.5mL LoBind tube.

Size Selection (Required)

1. Vortex the room temperature-equilibrated AMPure XP beads to resuspend them.
2. Add 75 µL of AMPure XP beads to 100 µL of a 300-bp insert bead-cleaned library;

   or, add 65 µL of AMPure XP beads to 100 µL of a 500-bp insert bead-cleaned library.
3. Mix gently by pipetting up and down 10 times.
4. Spin briefly to collect droplets to the bottom of the tube. Do not pellet the beads.
5. Incubate at room temperature for 5 minutes; do not use a magnetic rack.
6. Place the tube in a magnetic rack for 5 minutes (until the supernatant becomes clear).
7. With the tube in the magnetic rack, gently remove the supernatant with a pipette and discard.
8. 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.
9. Repeat the ethanol wash step (Step 8).
10. Spin briefly to collect any residual ethanol at the bottom of the tube and place the tube back into the magnetic rack.
11. Keep the tube in the magnetic rack and carefully remove any remaining ethanol, then air-dry the bead pellet for 5 minutes.
12. Remove the tube from the magnetic rack.
13. Add 22 µL of Elution Buffer (EB).
14. 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.

15. Incubate at 37 °C for 5 minutes; do not use a magnetic rack during the incubation.
16. Place the tube in a magnetic rack for 5 minutes (until the supernatant becomes clear).
17. Transfer 20 µL of supernatant to a new 1.5 mL tube.
18. Store the finished library at -20 °C or proceed to analysis and sequencing. It is recommended to proceed to sequencing within 7 days after library completion.

Optional/Safe Stopping Point. Size-selected samples can be stored at -20 °C in a 1.5mL LoBind tube.

Analysis

1. Analyze 1 µL of the library on a Bioanalyzer High Sensitivity Chip to determine size distribution. After ligation of the adaptors, the peak should center at 500 bp for a 300-bp insert library and at 800 bp for a 500-bp insert library. See figures 3 and 4 for typical Bioanalyzer traces of libraries generated using gDNA sheared to 300 bp and 500 bp.

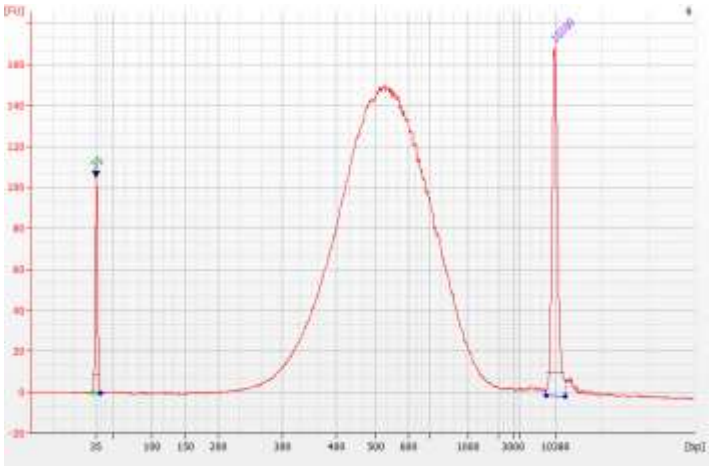


Figure 3: Typical Bioanalyzer trace for library generated using gDNA sheared to 300 bp.

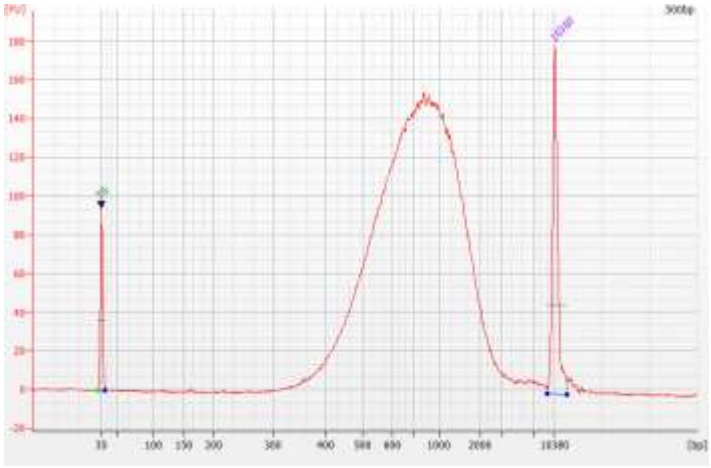


Figure 4: Typical Bioanalyzer trace for library generated using gDNA sheared to 500 bp.

2. Quantify the size-selected library with a Qubit Fluorometer, following the manufacturer's instructions.

Notes:

- If size selection is necessary at both lower and upper ends, see Appendix E: Double Bead Size Selection for instructions.
- If library yield is insufficient for sequencing, the library may be amplified. See Appendix F: Optional PCR Amplification for recommendations using KAPA HiFi Hot Start Ready Mix PCR Kit (Catalog # KK2602). Other Hi Fidelity PCR systems can be used.

Sequencing

- Generated size-selected libraries may be sequenced on any Illumina sequencer.
- We recommend quantifying the libraries using qPCR to ensure efficient loading on Illumina platforms.
- Lucigen recommends using PhiX Control v3 library (Illumina, FC-110-3001) as a control for Illumina sequencing runs.
- See Appendix G: Sample Sheet Instructions for information on using Illumina Experiment Manager to sequence libraries generated with Lucigen adaptors.

Appendix A: Multichannel Library Preparation for 48 Libraries

This kit provides enough reagents to prepare 48 libraries at one time. Scale reagent volumes appropriately if making less than 48 libraries.

Material and Equipment Needed	Vendor
PCR strip tubes, 0.2 mL	Various
15 mL and 50 mL Reagent Reservoir	Various
1.5 mL and 5 mL Microcentrifuge tubes	Various
96 Well PCR LoBind Plate	Eppendorf (Cat. # 0030129504)
Heat-resistant Adhesive Plate Seals (foil)	Various
Magnetic stand-96 for plates, or Dynamag 96 Side Magnet for strip tubes and plates, or Agencourt SPRIplate 96R-Ring Super Magnet Plate	Ambion (Cat.# AM10027) Thermo Fisher Scientific (Cat.# 12331D) Beckman Coulter (Cat.# A32782)
Pipet-Lite Multi Pipette 20XLS+ and corresponding tips	Rainin (Cat.# 17013808 or 17013803) or equivalent
Pipet-Lite Multi Pipette 200XLS+ and corresponding tips	Rainin (Cat.#17013810 or 17013805) or equivalent
20 µL, 200 µL and 1000 µL Single Channel Pipettes	Various
Benchtop plate centrifuge*	Various

* Note: Common salad spinners can be adapted for use as a low-speed plate centrifuge.

End Repair, A-Tailing Reaction

1. Add 17 µL of each sheared DNA (75 ng – 1 µg) to a well of a 96-well PCR LoBind plate.
2. Prepare a master mix using a single channel pipette with the following components in a 5 mL tube on ice:

Component	Volume Per Reaction	Volume for 48 Reactions (µL)
2X Buffer (2XB)	25	1400
Enzyme Mix (EM)	8	448
Total	**33**	**1848**

Note: Volumes listed for 48-reactions master mix include additional volume to accommodate pipetting.

3. Mix gently by pipetting up and down 10 times.
4. Aliquot 230 µL of master mix into each well of an 8-well strip tube (to serve as a reservoir).
5. Use an 8-channel pipette to aliquot 33 µL of master mix into the 1st column of 8 wells each containing 17 µL of DNA. Pipette up and down 5 times to mix each reaction. Discard the tips and reload multichannel pipette with new tips.
6. Repeat step 5 five more times to add master mix to each DNA-containing well.
7. Seal the plate with heat-resistant adhesive plate seal.
8. Spin briefly in a benchtop centrifuge to collect material in the bottom of the plate.
9. Place plate in a thermocycler with an 80 °C heated lid and incubate according to the following parameters.

Step	Temperature	Time
1	25 °C	20 minutes
2	72 °C	20 minutes
3	4 °C	Hold

Adaptor Ligation Reaction

1. Briefly spin plate in a benchtop centrifuge to collect material.
2. Aliquot 28 µL of Ligase (LIG) using a single channel pipette into each well of an 8-well strip tube (to serve as a reservoir).
3. Using an 8-channel pipette, aliquot 4 µL of Ligase into the 1st column of 8 wells containing the End-repair/A-tailing reaction. Discard tips and reload multichannel pipette with new tips.
4. Repeat Step 3 five more times to add Ligase to each reaction well.
5. Using a single-channel pipette, add 3 µL of the appropriate Indexed Adaptor to each well containing End-repaired/A-tailed DNA reaction.

   Note: See Appendixes B and C for information on Lucigen Adaptors and pooling options.
6. Mix gently by pipetting up and down 10 times with a 200-µL multichannel pipette set at 40 µL, using fresh tips for each row.
7. Seal the plate with an adhesive plate seal.
8. Spin briefly using a benchtop centrifuge to collect material in the bottom of the plate.
9. Place the plate in a thermocycler and incubate at 25 °C for 30 minutes.

Bead Cleanup

1. Briefly spin the plate in a benchtop centrifuge to collect material.
2. Prepare 60 mL of fresh 70% ethanol solution, and pour 30 mL into a 50-mL reagent reservoir.
3. Vortex the room temperature-equilibrated AMPure XP beads to resuspend them.
4. Pour 3.6 mL of AMPure XP beads into a 15-mL reagent reservoir.
5. Using an 8-channel, 200 µL multichannel pipette, add 57 µL of AMPure XP Beads to the first column of the plate and mix 10 times with the pipette. Continue by repeating this step with the next 5 columns.
6. Incubate on the bench at room temperature for 5 minutes.
7. Place the plate in a magnetic plate for 5 minutes (until the supernatant becomes clear).
8. Wash the beads with 70% ethanol as follows. Wash the beads in each column one at a time to prevent over drying of the beads:
    a. Set an 8-channel, 200 µL multichannel pipette to 200 µL. With the plate in the magnetic plate (beads attracted to sides of wells), carefully aspirate the supernatant from one column and discard the tips
    b. Using fresh tips, carefully add 200 µL of 70% ethanol to the column without disturbing the beads. Discard the tips and proceed to the next row.
    c. Repeat 8a-b until all the columns of the plate contain 70% ethanol (1st wash)
    d. Using fresh tips, carefully aspirate the first ethanol wash from the first column and discard the tips
    e. Using fresh tips carefully add 200 µL of 70% ethanol to the first column without disturbing the beads and discard the tips.
    f. Repeat Steps 8 d-e until all the rows of the plate contain 70% ethanol (2nd wash)
9. Using an 8-channel, 200 µL multichannel pipette with fresh tips for each column, aspirate the ethanol from all columns.
10. Using an 8-channel, 20 µL multichannel pipette, remove any remaining ethanol, and let the bead pellets air dry for 5 minutes (no more) while still on the magnetic plate.
11. Pour at least 6.5 mL of Elution Buffer (EB) into a 15-mL reagent reservoir.

    Important: Keep unused Elution Buffer (EB) in reagent reservoir for use in Step 11 of Size Selection.
12. Remove the plate from the magnetic plate.
13. Add 105 µL of Elution Buffer (EB) to each well using an 8-channel, 200 µL multichannel pipette.
14. Mix the beads and the buffer gently by pipetting up and down with a multichannel pipette at least 10 times or until thoroughly mixed. Use fresh tips for each column. Do not vortex.
15. Incubate at 37 °C for 5 minutes; do not use a magnetic plate during this incubation.
16. Place the reaction plate on a magnetic plate for 5 minutes (until the supernatant becomes clear).
17. Being careful not to disturb the beads, transfer 100 µL of supernatant into a new plate.

Optional/Safe Stopping Point. Bead-cleaned samples can be stored at -20 °C in a 96-Well PCR LoBind plate.

Size Selection

1. Vortex the room temperature-equilibrated AMPure XP beads to resuspend them.
2. Pour at least 3.6 mL of AMPure XP beads into a 15-mL reagent reservoir.
3. Pour 30 mL of fresh 70% ethanol solution into a 50-mL reagent reservoir.
4. Using an 8-channel, 200µL multichannel pipette, add 75 µL of AMPure XP beads for a 300-bp insert library or 65 µL of AMPure XP beads for a 500-bp insert library to the bead-cleaned libraries. Use fresh tips for each column of the plate.
5. Mix gently by pipetting up and down 10 times or until thoroughly mixed.
6. Incubate on the bench at room temperature for 5 minutes.
7. Place the plate onto a magnetic plate for 5 minutes (until the supernatant becomes clear).
8. Wash the beads with 70% ethanol as follows. Wash the beads in each column one at a time to prevent over drying of the beads:
    a. Set an 8-channel, 200 µL multichannel pipette to 200 µL. With the plate in the magnetic plate (beads attracted to sides of wells), carefully aspirate the supernatant from one column and discard the tips
    b. Using fresh tips, carefully add 200 µL of 70% ethanol to the column without disturbing the beads. Discard the tips and proceed to the next row.
    c. Repeat 8a-b until all the columns of the plate contain 70% ethanol (1st wash)
    d. Using fresh tips, carefully aspirate the first ethanol wash from the first column and discard the tips
    e. Using fresh tips, carefully add 200 µL of 70% ethanol to the first column without disturbing the beads and discard the tips.
    f. Repeat Steps 8 d-e until all the rows of the plate contain 70% ethanol (2nd wash)
9. Using an 8-channel, 200 µL multichannel pipette with fresh tips for each column, aspirate the ethanol from all columns.
10. Using an 8-channel, 20 µL multichannel pipette, remove any remaining ethanol, and let the bead pellets air dry for 5 minutes (no more) while still on the magnetic plate.
11. Add 25 µL of Elution Buffer (EB) from the reagent reservoir used during Cleanup to each well using an 8-channel, 200 µL multichannel pipette.
12. Remove the plate from the magnetic plate.
13. Mix the beads and the buffer gently by pipetting up and down with a multichannel pipette at least 10 times or until thoroughly mixed. (Do not vortex, use fresh tips in each row.)
14. Incubate at 37 °C for 5 minutes; do not use a magnetic plate during the incubation.
15. Place the plate on a magnetic plate for 5 minutes (until the supernatant becomes clear).
16. Transfer 20 µL of supernatant to a new plate, being careful not to disturb the beads.
17. Proceed to Analysis as outlined on page 8 of this manual.

Optional/Safe Stopping Point. Size selected samples can be stored at -20 °C in a 96-Well PCR LoBind plate.

Note: Upon thawing of a plate stored at -20 °C, we recommend thorough mixing of each library by pipetting each sample up and down 5-7 times using a multichannel pipette set at 15 µL. Use new pipet tips for each sample to avoid cross-contamination of the libraries.

Appendix B. Index Sequences

The index sequences contained in each adaptor are listed below. If using an 8-base index, use the full sequence listed below. If using a 6-base index, use the first 6 bases (underlined) listed below.

Box 1 Adaptors	Index Sequence
Adaptor 1	AACGTGAT
Adaptor 2	CAGATCTG
Adaptor 3	GTACGCAA
Adaptor 4	TATCAGCA
Adaptor 5	TCTTCACA
Adaptor 6	CTAAGGTG
Adaptor 7	GAGTTAGT
Adaptor 8	AAGGTACA
Adaptor 9	ACGCTCGA
Adaptor 10	AGATCGCA
Adaptor 11	ATCCTGTA
Adaptor 12	GACTAGTA

Box 2 Adaptors	Index Sequence
Adaptor 13	ATGCCTAA
Adaptor 14	CATCAAGT
Adaptor 15	AGTACAAG
Adaptor 16	GAATCTGA
Adaptor 17	GATAGACA
Adaptor 18	GCTCGGTA
Adaptor 19	GTCTGTCA
Adaptor 20	TCCGTCTA
Adaptor 21	TGAAGAGA
Adaptor 22	AACGCTTA
Adaptor 23	ACGTATCA
Adaptor 24	AGTCACTA

Note: The NxSeq® AmpFREE Low DNA Library Kit is also compatible with Illumina TruSeq adaptors.

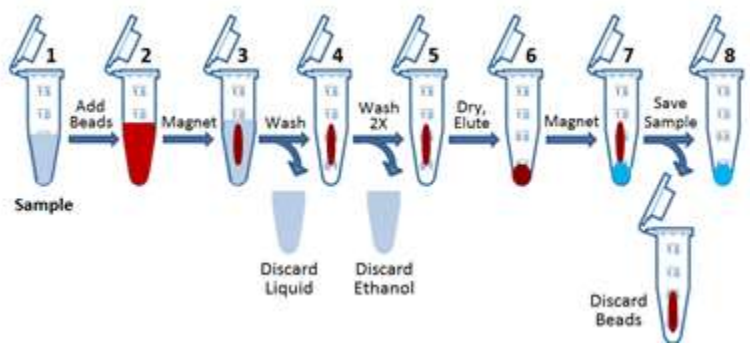
Appendix C: Adaptor Pooling Guidelines

Single-indexed pooling strategies for 2-4 samples

Number of Pooled Libraries	Option	Box 1	Box 2
2		Adaptor 5 and 6	Adaptor 14 and 19
3	1	Adaptor 5, 6, and 10	Adaptor 14, 17, and 19
	2	5 and 6 with any other	14 and 19 with any other
4	1	Adaptor 4, 5, 6 and 10	Adaptor 14, 17, 19 and 23
	2	5, 6 and 10 with any other	14, 17, and 19 with any other
>4 samples		Any Adaptors from Set 1	Any Adaptors from Set 2

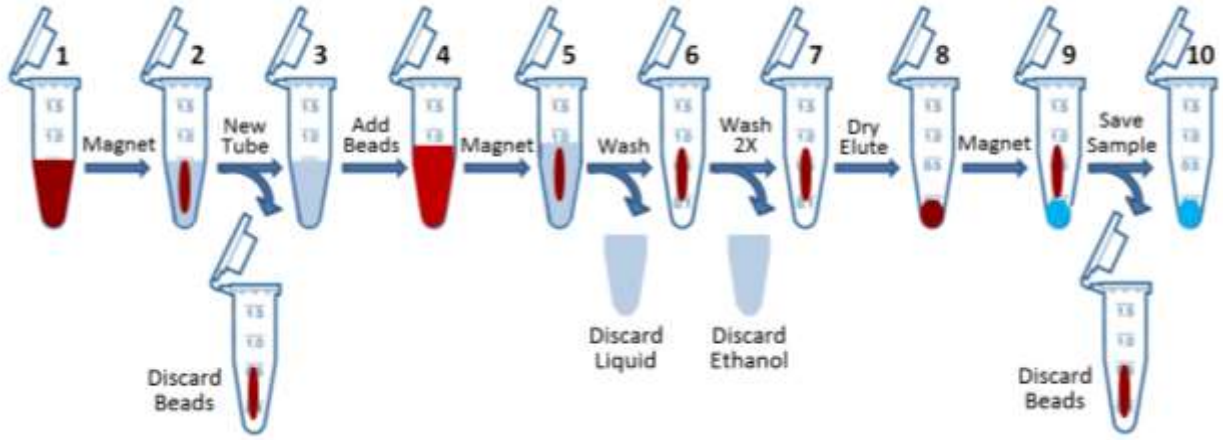
Note: The NxSeq® AmpFREE Low DNA Library Kit is also compatible with Illumina TruSeq adaptors. If pooling Lucigen adaptors with TruSeq adaptors, Lucigen adaptor #2 should not be pooled with TruSeq adaptor #7.

Appendix D: Bead Clean Up



Bead Clean Up. 1) Sample DNA; 2) Add beads 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, and mix. Incubate at 37 °C for 5 minutes; 7) Place tube on magnetic rack after incubation; 8) Transfer liquid to new tube and discard beads.

Appendix E. Double Bead Size Selection (Upper & Lower ends)

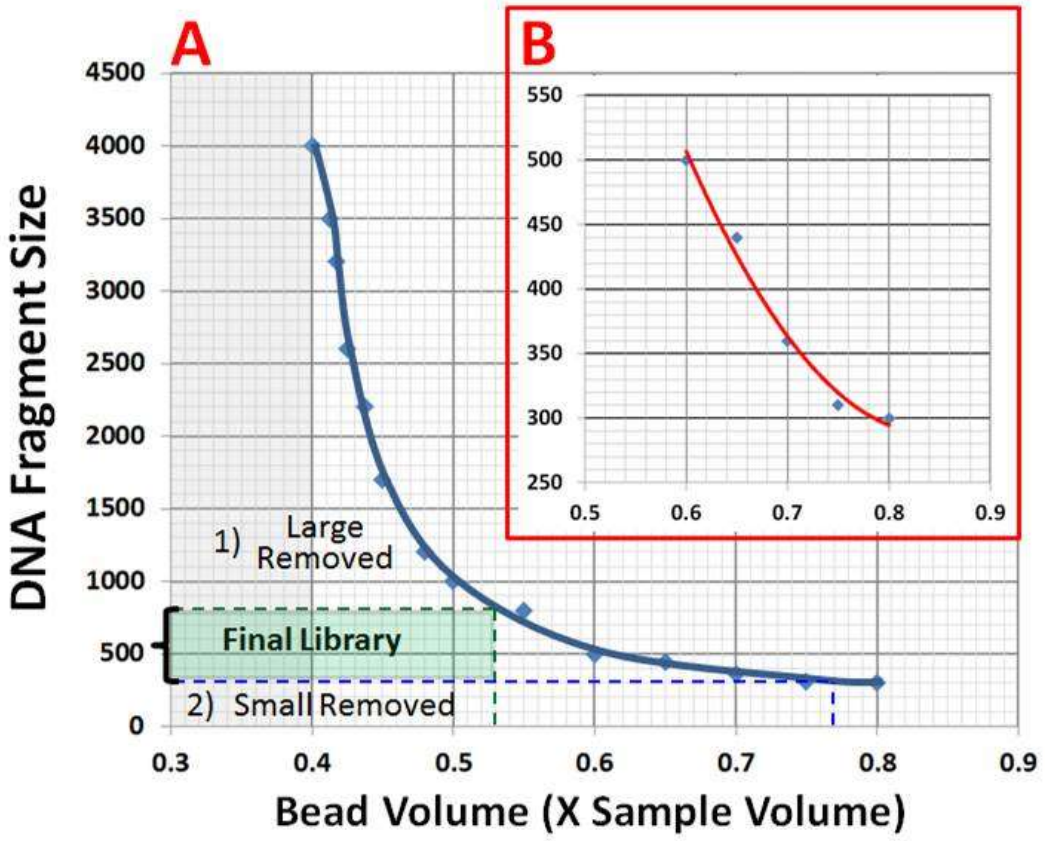


Double Size Selection Workflow. 1) Add beads to sample and mix; 2) Place tube on magnetic rack; 3) Transfer liquid to new tube and discard tube with beads; 4) Add second volume of beads and mix; 5) Place on magnetic rack; 6) Discard liquid and Wash 2x with 70% ethanol; 7) Dry beads; remove from magnet 8) Add Elution Buffer (EB), and mix. Incubate at 37 °C for 5 minutes; 9) Place tube on magnetic rack; 10) Transfer liquid to new tube and discard beads.

Bead size selection is based on the concentration of Polyethylene glycol (PEG) and sodium chloride (NaCl) in the bead buffer solution. A higher concentration will bind both small and large fragments, whereas a lower concentration will only allow binding of larger fragments. When a small amount of beads and buffer are added to your sample, large DNA fragments will bind to the beads. These large contaminating DNA fragments will be discarded with the beads. By adding a second aliquot of beads and buffer to your sample, the concentration of PEG and NaCl will increase and allow binding of the

desired range of smaller DNA fragments. DNA fragments that are smaller than the selected size range will not bind and will be removed when the beads are washed.

For example, if your optimal library size range for sequencing is between 400 and 900 bp, add 0.6X volume of beads and buffer to your sample. This amount of beads will bind fragments of 900 bp and larger for removal. When an additional 0.2X volume of beads and buffer is added to the saved supernatant, the buffer concentration will be increased to 0.8X beads and buffer (0.6X + 0.2X = 0.8X beads) and will bind DNA fragments between 400 and 900 bp. DNA fragments smaller than 400 bp will be removed when the beads are washed (See Figure below). (X = the original sample volume).



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.

Appendix F: Optional PCR Amplification

Amplification

1. Mix the following components in a PCR tube.

Volume (µL)	Component
20	Bead-cleaned ligated library
25	KAPA HiFi HotStart ReadyMix PCR Kit (catalog # KK2602).
5	Illumina-compatible Primers (e.g. P5/P7 Primers)
50	**Total**

2. Place the tube in the thermocycler and cycle according to the following:

Step	Temperature	Time
1	95 °C	3 minutes
2	98 °C	20 seconds
3	65 °C	15 seconds
4	72 °C	1 minute
5	Repeat steps 2-4 for 5-7 additional cycles	
6	72 °C	10 minutes
7	4 °C	Hold

Cleanup

Notes:

- See Appendix D: Bead Cleanup for a detailed description of this workflow.
- If libraries were size selected before PCR, perform two cleanup steps following instructions below; or
- If libraries were not size selected before PCR, perform cleanup and size selection step following instructions below
- Perform all of the following steps at room temperature.

1. Prepare fresh 70% ethanol solution (~3.5 mL needed per library).
2. Vortex the room temperature-equilibrated AMPure XP beads to resuspend them.
3. Transfer the amplification reaction to a 1.5 mL tube.
4. Add 50 µL of AMPure XP Beads to the amplification reaction in the 1.5 mL tube (see Appendix D: Bead Clean Up for a description of this step).
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, 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 ethanol wash step (Step 10).

12. Spin briefly to collect residual ethanol in the bottom of the tube. Place the tube back into the magnetic rack.
13. Remove any remaining ethanol and air-dry the bead pellet for 5 minutes while still in the magnetic rack.
14. Remove the tube from the magnetic rack.
15. Add 52 µL of Elution Buffer (EB) if the library was size selected before PCR;

    or, add 102 µL of Elution Buffer (EB) if the library was not size selected before PCR.
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 50 µL or 100 µL of supernatant into a new 1.5 mL tube.
20. Repeat steps 4-14 to proceed with a second cleanup if the library was size selected before PCR;

    or, follow Size Selection Steps on pages 8-9 if the library was not size selected before PCR.
21. Add 22 µL of Elution Buffer (EB).
22. Mix the beads and the buffer gently by pipetting up and down 10 times. (Do not vortex.)
23. Incubate at 37 °C for 5 minutes; do not use a magnetic rack during the incubation.
24. Place the tube in a magnetic rack for 5 minutes (until the supernatant becomes clear).
25. Transfer 20 µL of supernatant into a new 1.5 mL tube.

Appendix G: Sample Sheet Instructions

A Sample Sheet is required for running a sequencing run on an Illumina platform. This sheet is useful for demultiplexing during the run. A Sample Sheet is available on the Lucigen website for editing as needed. A completed Example NxSeq AmpFREE Low DNA Sample Sheet (pdf file) is also available on the Lucigen website as a reference.

Documents and Website Links:

NxSeq AmpFREE Low DNA Sample Sheet:
http://www.lucigen.com/docs/sequencing/NxSeq_AmpFREE_Low_DNA_Sample_Sheet.csv

Example NxSeq AmpFREE Low DNA Sample Sheet:
http://www.lucigen.com/docs/sequencing/Example_NxSeq_AmpFREE_Low_DNA_Sample_Sheet.pdf

Lucigen Index Sequences:
http://www.lucigen.com/docs/sequencing/Lucigen_Index_Sequences.xlsx

Sample Sheet Instructions:

1. Download NxSeq AmpFREE Low DNA Sample Sheet from the Lucigen website.
2. Make the following edits:
    a. Change Date
    b. Change Investigator Name
    c. Change Experiment Name

d. Change Application to reflect the appropriate platform being used.
    i. MiSeq: FASTQ Only
    ii. NextSeq 500: NextSeq FASTQ Only
    iii. HiSeq 2000, 2500, 4000: HiSeq FASTQ Only
e. Change Reads to reflect the chemistry being used for the run. For example, the reference sheet is set up for a 2x150 bp paired end run.
f. Change Data. Note: Refer to the Example NxSeq AmpFREE Low DNA Sample Sheet for correct column placement. Index names and sequences can be found in the Lucigen Index Sequence document.
    i. Add sample names to the appropriate column
    ii. Add adaptor index numbers to the appropriate column
    iii. Add adaptor index sequences

3. All other items in the Sample Sheet should remain unchanged.
4. Save file as a .csv format. Transfer to an Illumina platform.
5. Contact Illumina Technical Support at techsupport@illumina.com if experiencing difficulty with Sample Sheet formatting.

Notice of Limited Label License, Copyright, Patents, Warranties, Disclaimers and Trademarks

A complete list of trademarks, registered trademarks, limited label license, etc. held by Lucigen Corp. can be found at http://www.lucigen.com/Legal-Information.html.