Lucigen®
Simplifying Genomics

# NxSeq® AmpFREE Low DNA Library Kit

## Illumina Compatible


FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE

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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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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

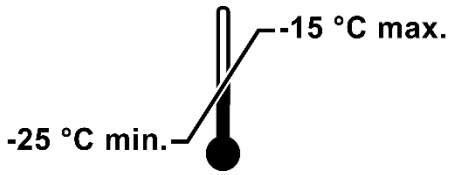
The NxSeq® AmpFREE Low DNA Library Kit supplies the buffers and enzymes needed to make high efficiency, next generation sequencing fragment libraries.

## 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 °C**



## 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 |
| 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) |
| Bioruptorµtrasonicator | Diagenode |

## Protocol

### Shear Genomic DNA

**Notes**:

- **gDNA must be quantified using a fluorometric method (Qubit or PicoGreen).** Fluorometric methods provide an accurate measurement of dsDNA only; in contrast to UV-based methods (including Nanodrop) which measure all nucleotides including ssDNA, RNA, and free nucleotides.
- **gDNA used must be free of contaminating RNA.**
- **gDNA must be re-suspended in Low TE** (0.1 mM EDTA; 10 mM Tris pH 8).

1. Shear gDNA such that the peak centers around 300 bp or 500 bp when analyzed on a Bioanalyzer depending on the desired size of your fragment library.

<table>
<tr><th>Shearing System</th><th>Options</th></tr>
<tr><td>LE220 Covaris Focused-Ultrasonicator</td><td>
<ul>
<li>8 microTUBE strip using manufacturer's recommendations for shearing to 300 bp or 500 bp or</li>
<li>8 microTUBE-15 AFA Beads Strip v2 using Covaris settings for LE220:*
<table>
<tr><th>Target BP (Peak)</th><th>300</th><th>500</th></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>
<ul>
<li>Include Y-dithering in sample treatment using the following steps:
<ul>
<li>Y Dither (mm) – 5</li>
<li>X-Y Dither Speed (mm/sec) - 20</li>
<li>Both X Dither (mm) and X-Y Dwell (sec) should be set to 0.</li>
</ul></li>
</ul></li>
</ul>
</td></tr>
<tr><td>Other Covarisµµtrasonicator Models</td><td>Follow manufacturer's recommendations for shearing to 300 bp or 500 bp.</td></tr>
<tr><td>Diagenode Bioruptor ultrasonicator</td><td>Follow manufacturer's recommendations for shearing to 300 bp or 500 bp.</td></tr>
</table>

2. Use Agencourt AMPure XP Beads to clean up the gDNA and obtain the volume necessary to proceed to the next step. The minimum DNA amount needed to proceed is 75 ng in a maximum volume of 17 µL.

   Note: It is not necessary to purify samples sheared with the 8 microTUBE-15 Strip v2 after shearing.

## Analysis

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

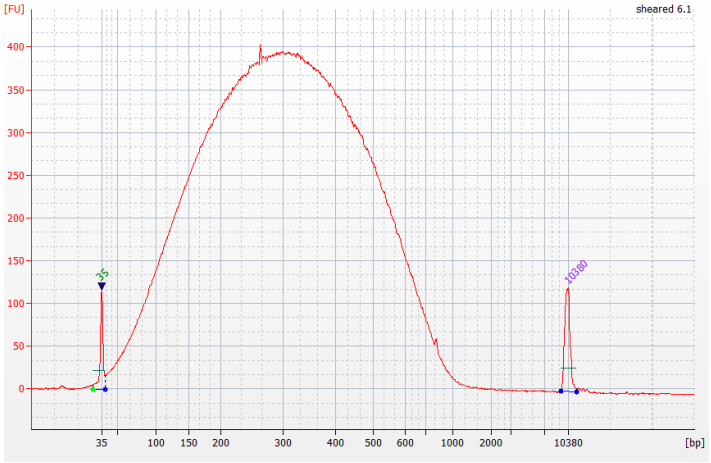



**Figure 1:** Typical Bioanalyzer trace for gDNA sheared to 300 bp.

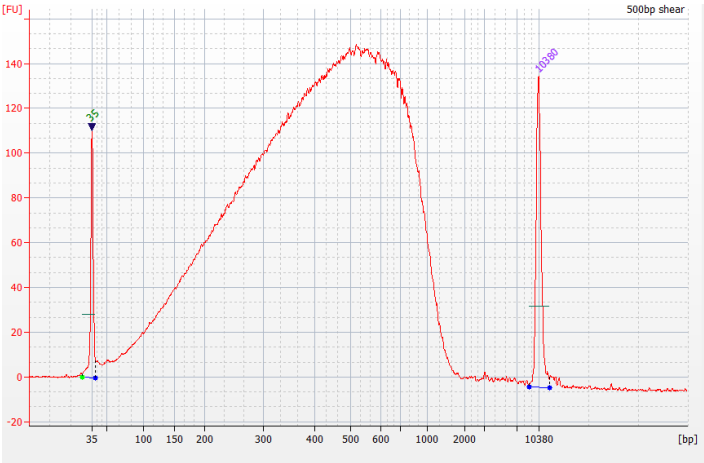



**Figure 2:** Typical Bioanalyzer trace for gDNA sheared to 500 bp.

## Important Library Preparation Recommendations

- Thaw all ingredients on ice.
- 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 “Lo Bind” tubes (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 order:

| Volume (µL) | Component |
|---|---|
| X | purified, fragmented DNA (75 ng – 1 µg) |
| Y | Nuclease-free water to 17 µL |
| 25.0 | 2X Buffer (2XB) |
| 8.0 | 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 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. 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** |

[1.] See Appendices B and C for information on Lucigen adaptors and pooling options.

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 at 25 °C for 30 minutes.
5. Proceed to Clean Up

## Clean Up (Required step)

**Notes**:

- See Appendix D: Bead Clean Up for a detailed description of this workflow.
- Both Clean Up and Size Selection steps are required.

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 “Lo-Bind” tube.
3. Perform all of the following steps at room temperature.
4. Add 102 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. Spin briefly to collect any residual ethanol in the bottom of the tube and place the tube back into the magnetic rack.
13. Keep the tube in the magnetic rack and carefully remove any remaining ethanol and then air-dry the bead pellet for 5 minutes.
14. With the tube in the magnetic track, add 202 µL of Elution Buffer (EB).
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 200 µL of supernatant to a new 1.5 mL “Lo-Bind” tube.

## Size Selection (Required step)

1. Vortex the room temperature-equilibrated AMPure XP beads to resuspend them.
2. Perform all of the following steps at room temperature.
3. Add 150 µL of AMPure XP beads to 200 µL of a 300 bp insert bead-cleaned library or add 130 µL of AMPure XP beads to 200 µL of a 500 bp insert bead-cleaned
4. Mix gently by pipetting up and down 10 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 into 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. 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, then air-dry the bead pellet for 5 minutes.
13. With the tube in the magnetic track, add 22 µL of Elution Buffer (EB).
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 to a new 1.5 mL tube.
19. 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.

## Analysis

1. Analyze 1 µL of the library on a Bioanalyzer High Sensitivity Chip to determine size distribution. Ideally, the peak should center on 500 bp for a 300 bp insert library and 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.

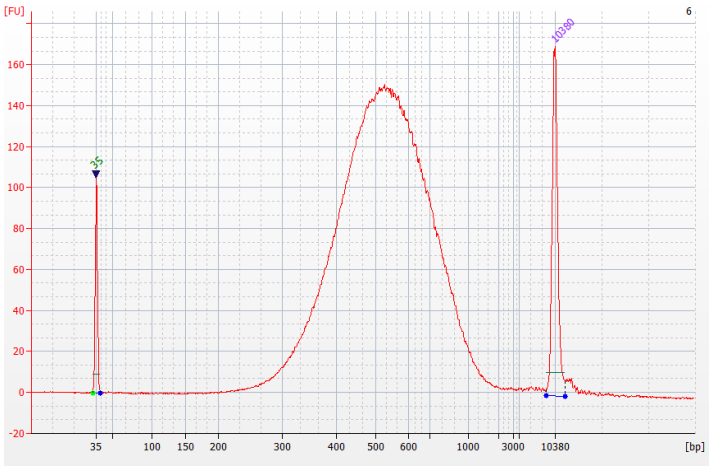



**Figure 3:** Typical Bioanalyzer trace for library generated using gDNA sheared to 300 bp.

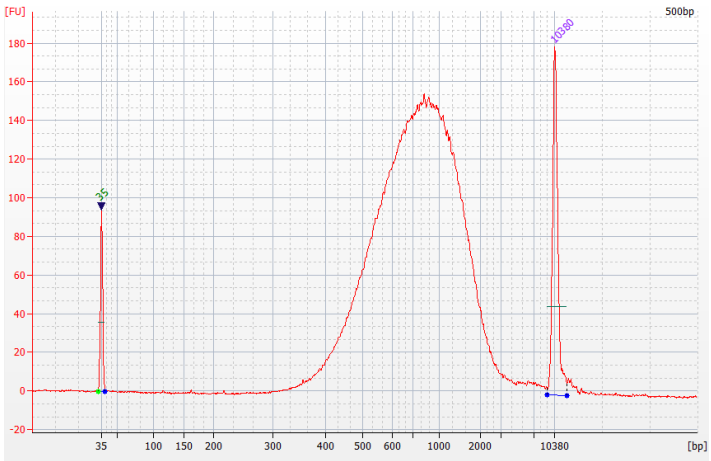



**Figure 4:** Typical Bioanalyzer trace for library generated using gDNA sheared to 500 bp.

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 HotStart ReadyMix PCR Kit (Catalog # KK2602).

# Sequencing

- Generated size-selected libraries may be sequenced on the Illumina HiSeq and MiSeq platforms.
- Lucigen recommends using PhiX Sequencing Control according to the recommendations provided by Illumina for the sequencer being used.
- 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 |
|---|---|
| 0.2 mL strip tubes | Various |
| 15 mL and 50 mL Reagent Reservoirs | Various |
| 1.5 mL and 2 mL Microcentrifuge tubes | Various |
| 96 well PCR low bind plates | Bio-Rad MLL9601 or Eppendorf 0030129504 |
| Adhesive Plate Seals | Various |
| Magnetic stand-96 for plates, or<br>Dynamag 96 Side Magnet for strip tubes and plates, or<br>Agencourt SPRIplate 96R-Ring Super Magnet Plate | Ambion AM10027<br>Thermo Fisher 12331D<br>Beckman Coulter A32782 |
| Rainin Pipet-Lite Multi Pipette 20XLS+ and corresponding tips | 17013808 or 17013803 or equivalent |
| Rainin Pipet-Lite Multi Pipette 200XLS+ and corresponding tips | 17013810 or 17013805 or equivalent |
| 20 µL, 200 µL and 1000 µL Single Channel Pipettes | Various |

## End Repair, A-Tailing Reaction

1. Remove Elution Buffer and thaw at room temperature. The Elution Buffer can be stored at room temperature.
2. Add 17 µL of each sheared DNA (75 ng – 1 µg) to a well of a 96-well PCR plate.
3. Prepare a master mix using a single channel pipette with the following components in a 2 mL tube on ice:

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

**Note**: Volumes listed for 48 reactions include additional volume to accommodate pipetting.

4. Mix gently by pipetting up and down 10 times.
5. Spin briefly in a benchtop centrifuge to collect material in the bottom of the tube.
6. Aliquot 230 µL of master mix into each well of an 8-well strip tube (acting as a reservoir).
7. Use an 8-channel 200 µL multichannel pipette to aliquot 33 µL of master mix into the 1st set 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.
8. Repeat step 6 five more times until master mix is added to each DNA-containing well.
9. Seal the plate with adhesive plate seal.
10. Spin briefly in a benchtop centrifuge to collect material in the bottom of the plate.
11. Place plate in a thermocycler with a 100 °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 (acting as a reservoir).
3. Using an 8-channel, 20 µL multichannel pipette, aliquot 4 µL of Ligase into the 1st set 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 until Ligase has been added to each reaction well.
5. Then using a single channel pipette, add 3 µL of the appropriate Indexed Adaptor to each well containing End-repaired/A-tailed gDNA 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 Clean Up

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. Perform all the following steps at room temperature.
6. Using an 8-channel, 200 µL multichannel pipette, add 57 µL of AMPure XP Beads to each well of the plate containing the ligated DNA. If necessary, change tips between rows.
7. Mix gently by pipetting up and down 10 times or until thoroughly mixed.
8. Incubate on the bench at room temperature for 5 minutes.
9. Place the plate in a magnetic plate for 5 minutes (until the supernatant becomes clear).
10. Set a 200 µL multichannel pipette to 110 µL. With the plate in the magnetic plate, carefully remove the supernatant with the multichannel pipette and discard. Again, use fresh tips between rows.

11. Using an 8-channel, 200 µL multichannel pipette, wash the beads by carefully adding 200 µL of 70% ethanol to each row of the plate without disturbing the beads. Wait 30 seconds at room temperature, remove the ethanol by pipetting and discard the ethanol.
12. Repeat the previous step (11.) to complete a total of two ethanol washes.
13. Using an 8-channel, 20 µL multichannel pipette, remove any remaining ethanol and let the bead pellets air dry for 5 minutes while still in the magnetic plate.
14. 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 15 of Size Selection.
15. With the plate still in the magnetic plate, add 102.5 µL of Elution Buffer (EB) to each well using a 200 µL multichannel pipette.
16. Remove the plate from the magnetic plate.
17. Mix the beads and the buffer gently by pipetting up and down 10 times using a multichannel pipette or until thoroughly mixed. (Do not vortex, use fresh tips for each row.)
18. Incubate at room temperature for 10 minutes; do not use a magnetic plate during this incubation.
19. Place the reaction plate on a magnetic plate for 5 minutes (until the supernatant becomes clear).
20. Being careful not to disturb the beads, transfer 100 µL of supernatant into a new 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. Perform all of the following steps at room temperature.
5. Using an 8-channel, 200 µL 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 row of the plate.
6. Mix gently by pipetting up and down 10 times or until thoroughly mixed.
7. Incubate on the bench at room temperature for 5 minutes.
8. Place the plate onto a magnetic plate for 5 minutes (until the supernatant becomes clear).
9. Set a 200 µL multichannel pipette to 170 µL. With the reaction plate in the magnetic plate, carefully remove the supernatant with a multichannel pipette and discard. Again, use fresh tips between rows.
10. Using an 8-channel, 200 µL multichannel pipette, wash the beads by carefully adding 200 µL of 70% ethanol to each row of the plate without disturbing the beads.
11. Wait 30 seconds at room temperature.
12. Remove the ethanol by pipetting and discard the ethanol.
13. Repeat steps 9-13 to complete a total of two ethanol washes.
14. Use an 8-channel, 20 µL multichannel pipette to remove any remaining ethanol and let the bead pellets to air dry for 5 minutes while still in the magnetic plate.

15. With the plate in the magnetic rack, add the 25.5 µL of Elution Buffer (EB) from the reagent reservoir used during Clean Up to each well using a multichannel pipette.
16. Remove the plate from the magnetic rack.
17. Mix the beads and the buffer gently by pipetting up and down 10 times or until thoroughly mixed using a multichannel pipette. (Do not vortex, use fresh tips in each row.)
18. Incubate at room temperature for 10 minutes; do not use a magnetic plate during the incubation.
19. Place the plate on a magnetic plate for 5 minutes (until the supernatant becomes clear).
20. Transfer 20 µL of supernatant to a new plate being careful not to disturb the beads.
21. Proceed to Analysis as outlined on page 8 of this manual.

## 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 | Box 2 Adaptors | Index Sequence |
|---|---|---|---|
| Adaptor 1 | AACGTGAT | Adaptor 13 | ATGCCTAA |
| Adaptor 2 | CAGATCTG | Adaptor 14 | CATCAAGT |
| Adaptor 3 | GTACGCAA | Adaptor 15 | AGTACAAG |
| Adaptor 4 | TATCAGCA | Adaptor 16 | GAATCTGA |
| Adaptor 5 | TCTTCACA | Adaptor 17 | GATAGACA |
| Adaptor 6 | CTAAGGTG | Adaptor 18 | GCTCGGTA |
| Adaptor 7 | GAGTTAGT | Adaptor 19 | GTCTGTCA |
| Adaptor 8 | AAGGTACA | Adaptor 20 | TCCGTCTA |
| Adaptor 9 | ACGCTCGA | Adaptor 21 | TGAAGAGA |
| Adaptor 10 | AGATCGCA | Adaptor 22 | AACGCTTA |
| Adaptor 11 | ATCCTGTA | Adaptor 23 | ACGTATCA |
| Adaptor 12 | GACTAGTA | Adaptor 24 | AGTCACTA |

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

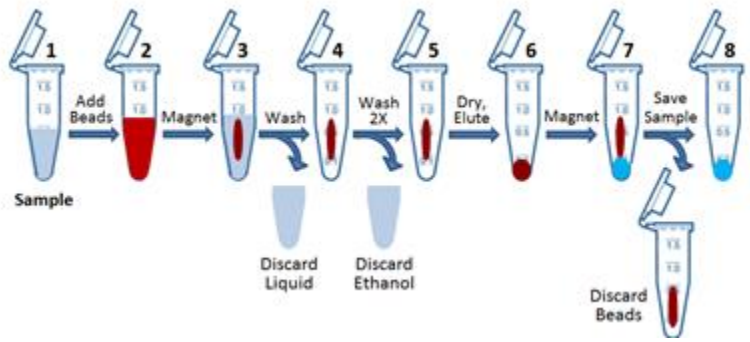
## Appendix C: Adaptor Pooling Guidelines

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 |

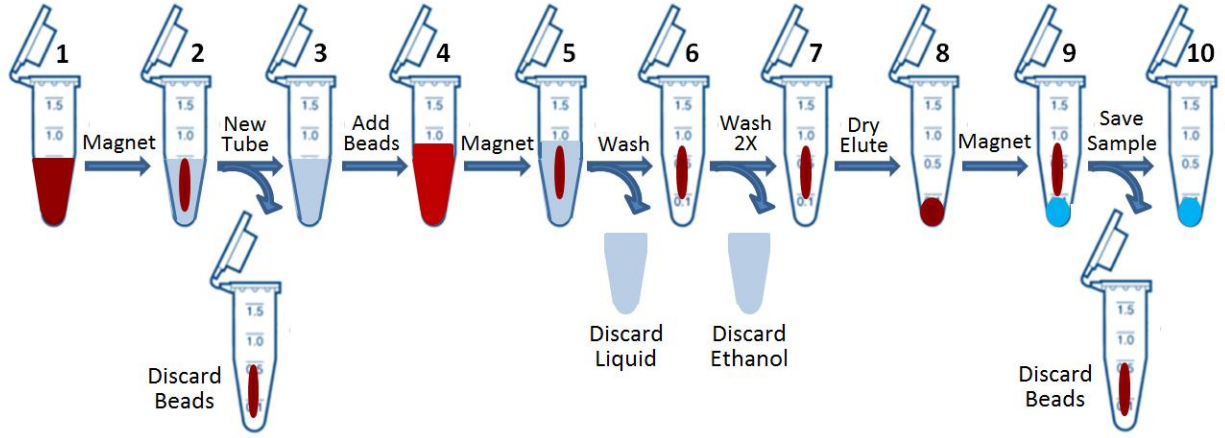
**Note**: The NxSeq® AmpFREE Low DNA Library Kit is compatible with Illumina TruSeq adaptors. If pooling Lucigen adaptors with TruSeq adaptors, Lucigen adaptor #2 should not be pooled with TruSeq adaptor #7.

## 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, mix and incubate at 37 °C for 5 minutes; 7) Place tube on magnetic rack after incubation; 8) Transfer liquid to new tube and discard beads.

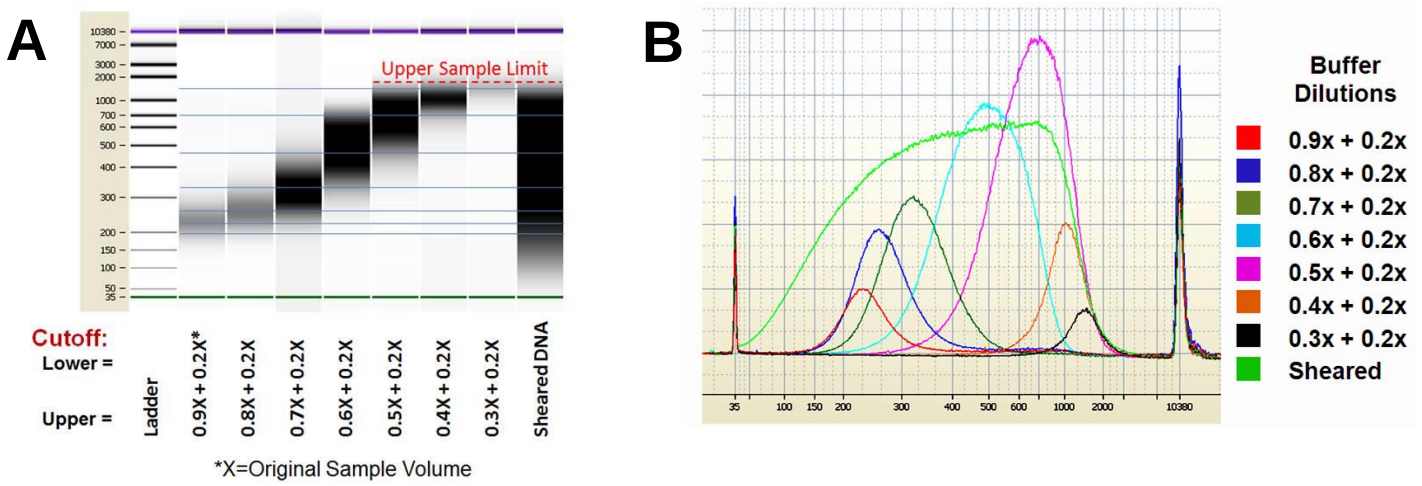
## Appendix E. Double Bead Size Selection (Upper & Lower ends)




**Size Selection Workflow** Briefly: 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; 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.

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 while 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 and when these beads are discarded, the 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 DNA fragments. Smaller, contaminating DNA fragments will not bind and will be removed when the beads are washed.

If your optimal library size range for sequencing is between 400 and 900 bp for example, 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 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 (0.8X – 0.6X = 0.2X 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. (X = the original sample volume).




**Size Selection with AMPure XP Magnetic Beads**. Analysis of size selected DNA with Agilent 2100 Bioanalyzer High Sensitivity Chip. **A)** Gel view of samples that were size selected with varying ratios of beads. **B)** Electropherogram view of data. Briefly, a lower concentration of buffer and beads is used to bind larger DNA fragments to the beads, which are then discarded. Addition of a second volume of buffer and beads to the sample allows for the binding of the desired, smaller DNA fragments. After the Ethanol wash, the DNA is eluted from the beads with TE or water.

# 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 parameters.

| 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 |

**Clean Up**

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. Perform all of the following steps at room temperature.
5. Add 90 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).
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, 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. Spin briefly to collect residue ethanol in the bottom of the tube and place the tube back into the magnetic rack.
14. Remove any remaining ethanol and air-dry the bead pellet for 5 minutes while still in the magnetic rack.
15. With the tube in the magnetic rack, add 202 µL of Elution Buffer (EB).
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 200 µL of supernatant into a new 1.5 mL tube.

**Size Selection**

1. Vortex the room temperature-equilibrated AMPure XP beads to resuspend them.
2. Perform all of the following steps at room temperature.
3. Add 150 µL of AMPure XP beads to the bead-cleaned, amplified library.
4. Mix gently by pipetting up and down 10 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 into a magnetic rack until the supernatant becomes clear (~5 minutes).
8. With the tube in the magnetic rack, 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. Spin briefly to collect material in the bottom of the tube and place the tube back into the magnetic rack.
12. Remove any remaining ethanol and air-dry the bead pellet 5 minutes while still in the magnetic rack.
13. With the tube in the magnetic track, add the 22 µL Elution Buffer (EB).
14. Remove the tube from the magnetic rack.
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 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://lucigen.com/docs/sequencing/NxSeq_AmpFREE_Low_DNA_Sample_Sheet

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

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

**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-refer to the Example NxSeq AmpFREE Low DNA Sample Sheet for correct column placement. Note: Index names and sequences can be found in Lucigen Index Sequence document.
        i. Add sample names to the appropriate column
        ii. Add adapter index numbers to the appropriate column
        iii. Add adapter 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.

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