Lucigen®
Simplifying Genomics

NxSeq® UltraLow DNA Library Kit, 96 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.

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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, 96 Reactions 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. Please note that the number of PCR cycles required for amplification is dependent

on the starting amount of the sheared, input 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.

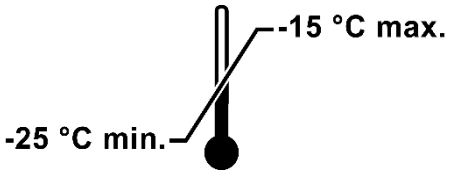
This User Manual couples the NxSeq® UltraLow DNA Library Kit, 96 Reactions with the NxSeq® HT Dual Indexing Kit for high throughput sequencing experiments, and the protocol is optimized for library construction in 96-well plates. For lower throughput sequencing experiments, we recommend using the NxSeq® UltraLow DNA Library Kit, 12 Reactions (Cat. No. 15012-1) with the NxSeq® Single Indexing Kits (Cat. No. 15100-1, 15200-1). If you prefer to use NxSeq® HT Dual Indexing Kit with the NxSeq® UltraLow DNA Library Kit, 12 Reactions, please follow the amplification protocol outlined in this manual and adapt it to the single tube reaction format in the NxSeq® UltraLow DNA Library Kit, 12 Reactions User Manual (MA166).

Product Designations and Kit Components

Product	Kit Size	Catalog Number	Reagent Description	Part Numbers	Volume	Cap Identifier
NxSeq® Ultra Low DNA Library Kit	96 Reactions	15096-1	Enzyme Mix	F833962-2	1040 µL	EM
			2X Buffer	F883396-5	3.25 mL	none
			Ligase	F832792-2	540 µL	LIG
			Elution Buffer	F882705-2	24.5 mL	none
			PCR Master Mix	F833963-2	2.76 mL	none
NxSeq® HT Dual Indexing Kit	96 Reactions	15300-1	Adaptor Dilution Buffer	F813785-2	6 mL	none
			NxSeq® Universal Adaptor	F883786-2	360 µL	UA
			Primer 501	F72501-1	30 µL	Orange
			Primer 502	F72502-1	30 µL	Orange
			Primer 503	F72503-1	30 µL	Orange
			Primer 504	F72504-1	30 µL	Orange
			Primer 505	F72505-1	30 µL	Orange
			Primer 506	F72506-1	30 µL	Orange
			Primer 507	F72507-1	30 µL	Orange
			Primer 508	F72508-1	30 µL	Orange
			Primer 701	F72701-1	20 µL	Blue
			Primer 702	F72702-1	20 µL	Blue
			Primer 703	F72703-1	20 µL	Blue
			Primer 704	F72704-1	20 µL	Blue
			Primer 705	F72705-1	20 µL	Blue
			Primer 706	F72706-1	20 µL	Blue
			Primer 707	F72707-1	20 µL	Blue
			Primer 708	F72708-1	20 µL	Blue
			Primer 709	F72709-1	20 µL	Blue
			Primer 710	F72710-1	20 µL	Blue
			Primer 711	F72711-1	20 µL	Blue
			Primer 712	F72712-1	20 µL	Blue

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)
PCR strip tubes, 0.2 mL	Various
96-Well PCR Low Bind Plate	Eppendorf (Cat. # 0030129504)
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
PCR Thermocycler	Various
Minifuge	Various
LE220 Focused-ultrasonicator (optional)	Covaris (Cat. # LE220)
8 microTUBE-15 AFA Beads Strip V2	Covaris (Cat. # 520159)
dsDNA Shearase™ Plus (optional)	Zymo Research (Cat. # E2018-50)
0.8 mL Storage Plate (96-well) (optional)	Thermo Fisher Scientific (Cat. # AB-0765)
TruSeq Index Plate Fixture Kit (optional)	Illumina (Cat. # FC-130-1005)
UNIPLATE Collection and Analysis Microplate, 96 well plate (optional)	VWR (Cat. #28317-472)
0.5 ml PCR tubes (optional)	VWR (Cat. # PCR-05-C)
25 mL Reagent Reservoir (optional)	VistaLab Technologies (Cat. #3054-1002)
Adhesive Plate Seals	Various
Magnetic Stand-96, or DynaMag™-96 Side Magnet, 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 tips	Rainin (Cat. # 17013808 or 17013803) or equivalent
Pipet-Lite Multi Pipette 200XLS+ and tips	Rainin (Cat. # 17013810 or 17013805) or equivalent
20 µL, 200 µL and 1000 µL Single Channel Pipettes	Various

DNA Shearing and Fragmentation Protocols

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.
- The mechanical shearing protocol (Covaris) provided is based on using the 15 µL volume strip shearing tubes (8 microTUBE-15 AFA Beads Strip V2). If you prefer to use a different shearing tube/plate, following manufacturer's recommendations and our DNA input requirements.
- **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 samples 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 using 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 µltrasonicator 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.

Optional/Safe Stopping Point. Mechanically sheared DNA can be stored at -20C° in a 96-Well PCR Low Bind Plate.

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 Zymo Research protocol provided with the kit, 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.
- The enzymatic fragmentation protocol produces final libraries with peak sizes ranging from 200 - 300 bp with inserts of approximately 80 - 200 bp. See Figure 5 (p. 18) for a Bioanalyzer trace of a typical library.

Fragmentation Reaction

1. Set up the following reaction on ice. Add the following components to a 96-Well PCR Low Bind Plate in order:

Volume per Well (µL)	Component
12.3	DNA (50 pg – 75 ng of genomic DNA in Low TE buffer)
3.4	5X Shearase™ Plus Reaction Buffer
1.3	dsDNA Shearase™ Plus Enzyme
17	**Total**

2. Add 12.3 µL of genomic DNA (50 pg – 75 ng) to a well of a 96-Well PCR Low Bind Plate. Keep this plate on ice.
3. Aliquot 37 µL of 5X Shearase™ Plus Reaction Buffer into each tube of a 12-tube 0.2 mL PCR strip tube, which acts as a reservoir, and keep the 5X Shearase™ Plus Reaction Buffer on ice while dispensing.
4. Using a 12-channel, 20 µL multichannel pipette, dispense 3.4 µL of the 5X Shearase™ Plus Reaction Buffer into each well of the 1st row (12 wells) of the 96-Well PCR Low Bind Plate.
5. Repeat Step 4 seven times using new tips each time until the 5X Shearase™ Plus Reaction Buffer is completed dispensed across the plate.
6. Aliquot 20 µL of dsDNA Shearase™ Plus Enzyme into each tube of a 12-tube 0.2 mL PCR strip tube, which acts as a reservoir, and keep the dsDNA Shearase™ Plus Enzyme on ice while dispensing.
7. Using a 12-channel, 20 µL multichannel pipette, dispense 1.3 µL of the dsDNA Shearase™ Plus Enzyme into each well of the 1st row (12 wells) of the 96-Well PCR Low Bind Plate.
8. Repeat Step 7 seven times using new tips each time until the dsDNA Shearase™ Plus Enzyme is completed dispensed across the plate.
9. Mix gently by pipetting up and down 10 times.
10. Spin briefly to collect material in the bottom of the tube.

11. Place the plate in a thermocycler preheated to 42°C and incubate as follows:

Step	Temperature	Time
1	42°C	12.5 minutes
2	65°C	10 minutes
3	4 °C	Hold

12. Fragmented DNA is ready for the End Repair, A-tailing step. Proceed directly to End Repair/A-tailing and follow the library construction protocol**.**

Analysis

1. If enough sheared/fragmented gDNA is available, analyze 1 µL on a Bioanalyzer High Sensitivity Chip to determine size distribution. Be aware that very low DNA amounts are not detectable on a High Sensitivity Chip. See Figures 1 and 2 for typical Bioanalyzer traces.

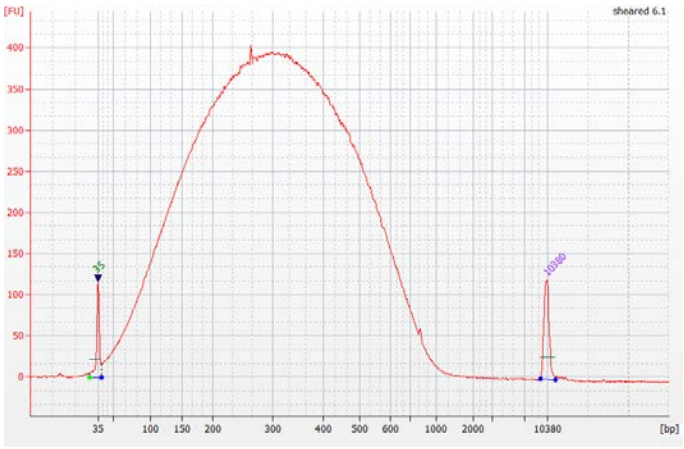


Figure 1: Typical Bioanalyzer trace for gDNA sheared to 300 bp using a Covaris Focused-ultrasonicator.

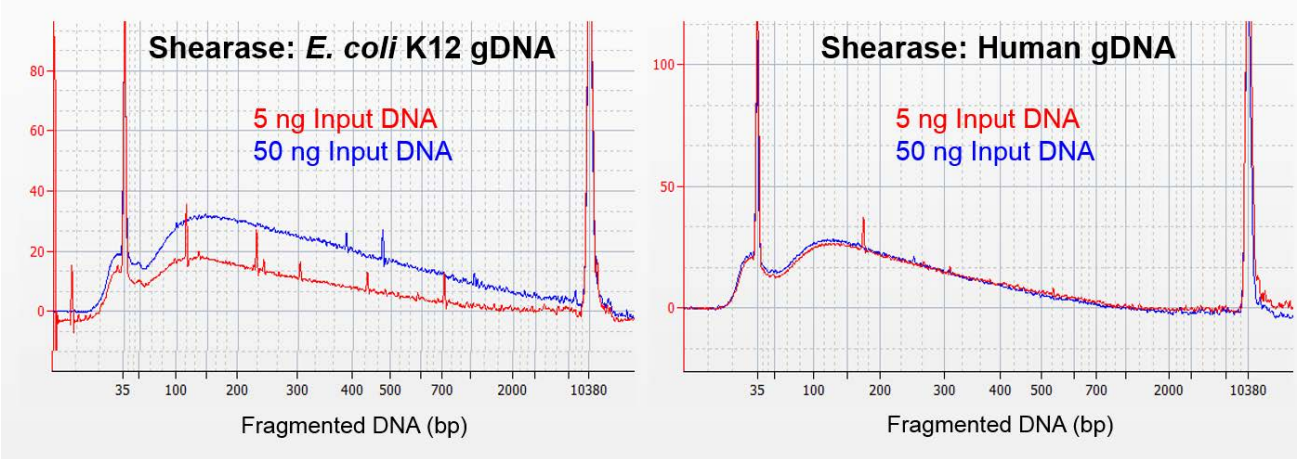


Figure 2: Typical Bioanalyzer traces for gDNA sheared enzymatically.

96 Libraries Multichannel Library Preparation Protocol

This kit provides enough reagents to prepare 96 libraries at one time in a 96-Well PCR Low Bind Plate. Scale reagent volumes appropriately if making fewer than 96 libraries.

Important Library Preparation Recommendations

- **This library preparation protocol is written assuming that a single DNA input amount is used for all 96 libraries. If using different input amounts, adjustments may be required to the amount of Universal Adaptor and number of PCR cycles during amplification**
- Thaw and store Elution Buffer (EB) at room temperature; thaw all other reagents at room temperature but then place on ice during library preparation.
- 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.
- Follow the recommended pipetting directions when provided in the various tables to ensure you have enough volume to dispense across the entire plate.
- Equilibrate AMPure XP Beads to room temperature for at least 30 minutes before use.
- Pipette viscous reagents (2X Buffer, Enzyme Mix, Ligase, and AMPure XP beads) slowly throughout the workflow.
- Prepare 150 mL of fresh 70% ethanol solution per 96 reactions for bead washing.
- Take extra precautions when working with 96-well plates to avoid cross-contaminating samples. Avoid splashing, drips and aerosols particularly when adding DNA, reagents and removing the adhesive plate seals.
- The use of fresh tips is required at multiple steps to avoid cross-contamination.

End-repair/A-Tailing Reaction

1. Add 17 µL of each sheared DNA (50 pg – 75 ng) to each well of a 96-well PCR Low Bind Plate. Keep this plate on your benchtop at room temperature.
2. Prepare a master mix using a single channel pipette with the following components in a 15-mL polypropylene tube on ice:

Volume Per Reaction (µL)	Volume for 96 Reactions Plus Overfill (µL)	Component
25	**3250** (3 x 1000 µL + 2 x 125 µL)	2X Buffer (2XB)
8	**1040** (1 x 1000 µL + 1 x 40 µL)	Enzyme Mix (EM)
33	**4290**	**Total**

Note: Volumes listed for 96 reactions include 30% additional volume to accommodate pipetting losses. Use a P1000 for mL volumes and a P200 for smaller volumes to achieve total.

3. Mix gently by pipetting up and down 10 times with a 1000 µL single channel pipette.
4. Spin briefly in a benchtop centrifuge to collect material in the bottom of the tube. Store on ice until use.
5. Aliquot 330 µL of master mix into each (12) 0.5 ml PCR tubes, which will act as a reservoir. Arrange tubes in 2 mL UNIPLATE Collection and Analysis Microplate, (96-well), which will serve as a rack. (Alternatively, a row of 0.8 mL Storage Plate (96-well) or a 25 mL Reagent Reservoir (VistaLab) could be used as a reservoir.) This End-repair/A-tailing master mix is stable at room temperature and does not need to be kept cool during dispensing.
6. Use a 12-channel 200 µL multichannel pipette to dispense 33 µL of End-repair/A-tailing master mix into each well of the 1st row (12 wells) of the 96-well PCR plate containing the 17 µL of input DNA. Immediately pipette gently up and down 10 times to mix each

reaction, avoid creating air bubbles. Discard the tips and reload multichannel pipette with new tips for each row.

7. Repeat Step 6 seven times until master mix is added to each DNA-containing well, using a new set of pipette tips each time.
8. Seal the plate with an adhesive plate seal.
9. Spin briefly in a benchtop centrifuge to collect material in the bottom of the plate.
10. Place the 96-well plate in a thermocycler with 80°C heated lid and incubate according to the following parameters:

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

Adaptor Ligation Reaction

1. Briefly spin the plate in a benchtop centrifuge to collect material in the bottom of the wells. Carefully remove the adhesive plate seal.
2. If necessary, diluted the NxSeq® Universal Adaptor as needed depending on the starting input DNA amount (See Appendix A for guidelines on adaptor dilution).
3. Aliquot 30 µL of diluted Adaptor into each tube of a 12-tube 0.2 mL PCR strip tube, which acts as a reservoir, and keep the Adaptor at room temperature while dispensing.
4. Using a 12-channel, 20 µL multichannel pipette, dispense 3 µL of the NxSeq® Universal Adaptor into each well of the 1st row (12 wells) of the 96-well PCR plate.
5. Repeat Step 4 seven times using new tips each time until the Adaptor is completed dispensed across the plate.
6. Aliquot 45 µL of Ligase (LIG) using a single channel pipette into each tube of a 12-tube 0.2 mL PCR strip tube, which acts as a reservoir, and then keep the dispensed Ligase at room temperature while dispensing.
7. Using a 12-channel, 20 µL multichannel pipette, dispense 4 µL of Ligase into each well of the 1st row (12 wells) of the 96-well PCR plate. The total volume per well is now 57 µL.
8. Repeat Step 7 seven times using new tips each time until the Ligase has been added to each well of the 96-well plate.
9. Using a 12-channel 200 µL multichannel set at 40 uL, mix each row of the plate gently by pipetting up and down 10 times, using fresh tips for each row. Avoid introducing air bubbles.
10. Seal the plate with an adhesive plate seal.
11. Spin briefly using a benchtop centrifuge to collect material in the bottom of the plate.
12. Place the plate in a thermocycler and incubate at 25 °C for 30 minutes.

PCR Plate Setup – Dual Index Primer Dispensing

Notes:

- We recommend setting up the PCR Plate while the Ligation reaction is incubating.
- Each set of NxSeq® Dual Index Primes contains an inert dye to help visualize pipetting across the 96-well PCR plate. The dyes do not interfere with downstream sequencing.
- The 500 Series NxSeq® Indexing Primers contain a yellow/orange dye.
- The 700 Series NxSeq® Indexing Primers contain a blue dye.
- Once both primer sets are correctly aliquoted into the wells of the 96-well PCR plate, each primer mix will become green indicating that a 500 Series and 700 Series Primers were correctly added to each well.
- Figure 3 illustrates the recommended layout and dispensing process for aliquoting the 700 and 500 Series Primers into the 96-well PCR plate.
- We strongly recommend using all of the NxSeq® Dual Indexing Primers in a single experiment to avoid possible cross-contamination of the indices. However, if you do not plan on using all the primers in a single plate/experiment and want to reuse them, do not discard the caps as directed below. After removing the caps, keep them in order so they can be added back to the same original primer tube to avoid cross contamination of the indices.
- If using a custom primer order, be sure to record the arrangement used.

1. Thaw each of the Dual Index Primers (501-508, 701-712) at room temperature. Vortex the thawed Primer tubes and briefly centrifuge to settle contents (empty 1.5 mL micro tubes can be used as carriers in a standard microfuge rotor).
2. **Refer to Figure 3 below for an overview of 96-well PCR plate and primer tube setup** using the (12) 700 Series Primers (blue caps) and the (8) 500 Series Primers (orange caps). We recommend using the Illumina Dual Index Setup Rack; alternatively, use two 2 mL UNIPLATE Collection and Analysis Microplates, (96-well) – one to hold the (8) 501-508 Primers and another to hold the (12) 701-712 Primers. Place the plate containing the 500 Series Primers to the left of the 96-Well PCR Low Bind Plate and the 700 Series Primer plate across the top of the 96-well PCR plate to aid in dispensing each set of primers across the 96-well PCR plate.

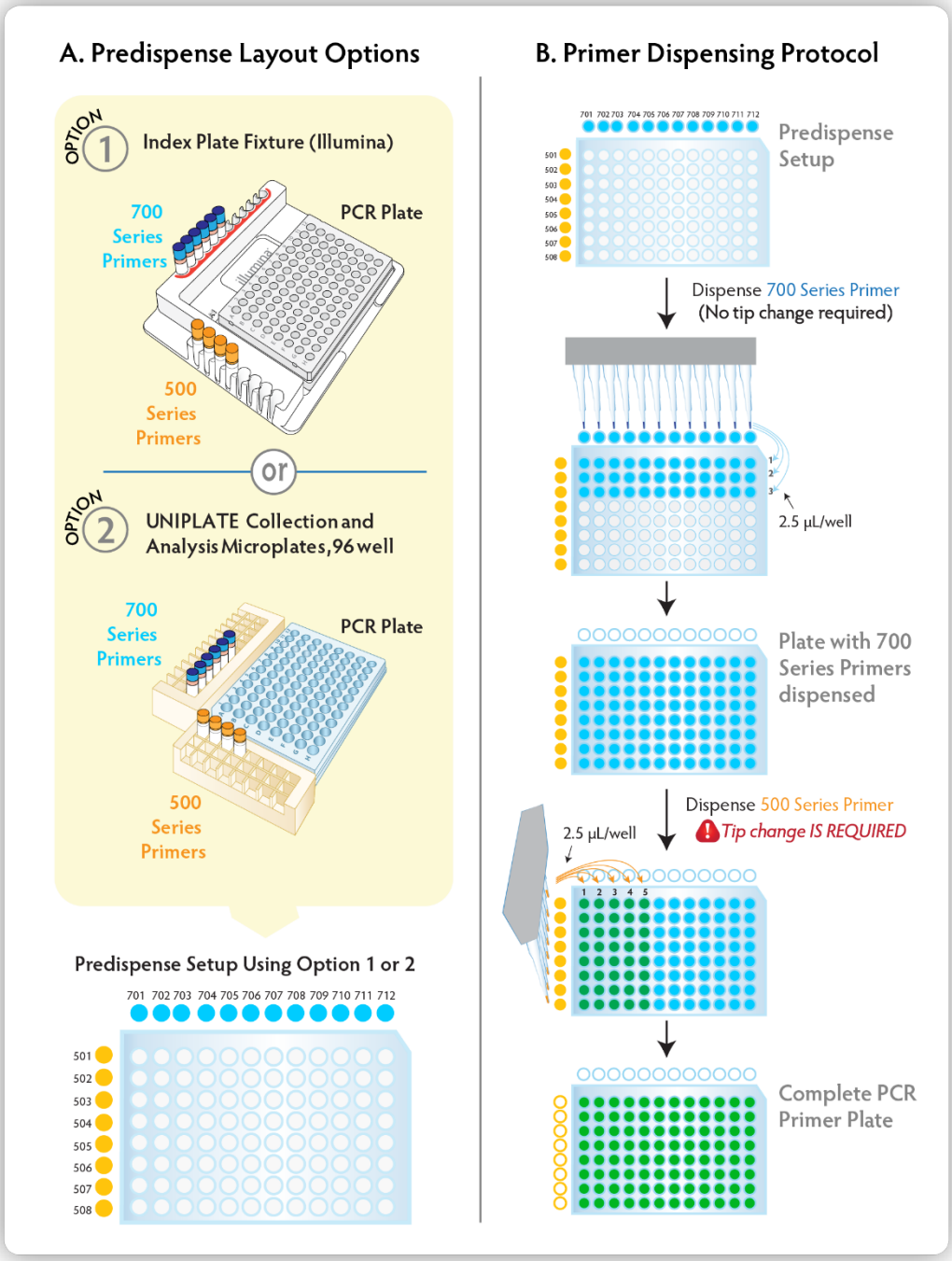


Figure 3: Plate and primer tube setup options and recommended dispensing protocol. Two different options for managing the position of the 96-well PCR Low Bind Plate and the 500 and 700 Series Primers for plate dispensing are illustrated in Panel A. Panel B illustrates the recommended primer dispensing protocol once the plate and primers are set up.

3. Remove and discard caps from the (12) 700 Series Primer tubes, and place them in your rack of choice in the order illustrated in Figure 3.

4. Using a 12-channel, 20 µL multichannel pipette, dispense 2.5 µL of 700 Series primers per well in the first row (12 wells) of a new 96-Well PCR Low Bind Plate. (It is OK to use a single set of tips for this set of dispenses).
5. Repeat Step 4 seven more times until the 700 Series Primers are added to all rows.
6. Visually check the plate for blue color in each well to verify correct pipetting into every well.
7. Discard the 700 Series Primer tubes. Change to a fresh pair of gloves before proceeding.
8. Remove and discard caps from the (8) 500 Series Primer tubes, and ensure tubes are in numerical order.
   Note: If using a custom primer order, be sure to record the arrangement used.
9. Using an 8-channel, 20 µL multichannel pipette, dispense 2.5 µL 500 Series Primers per well in the first column (8 wells) of the plate. Discard tips and reload multichannel pipette with new tips.
10. Repeat Step 9 eleven times until the 500 Series Primers are added to all columns of the plate.
11. Visually check the plate for a green color in each well to verify correct pipetting across the plate.
12. Discard the 500 Series Primer tubes. Change to a fresh pair of gloves and place a cover over the plate to reduce evaporation.
13. Keep the PCR Primer Plate on ice until ready to use after the ligated DNA samples have completed Bead Cleanup (next section).

Post-Ligation Cleanup (Required)

Notes:

- Ensure AMPure XP Beads, Elution Buffer (EB) and fresh 70% ethanol are all equilibrated to room temperature before proceeding with Cleanup.
- Perform all the following Cleanup steps at room temperature.
- Depending upon the magnetic plate used, the location of the AMPure XL bead pellet after drying may vary. Be certain to wet each pellet with Elution Buffer and verify that each pellet is thoroughly resuspended after repeated pipetting for all of the bead elution steps (Post-Ligation Cleanup, PCR Cleanup and Size Selection).

1. Briefly spin the ligation plate in a benchtop centrifuge to collect material in the wells. Carefully remove the adhesive plate seal taking care to avoid splashing of plate contents.
2. Vortex the AMPure XP beads to completely resuspend the beads.
3. Pour 5 mL of Elution Buffer (EB) into a new reagent reservoir.
4. Using a 12-channel, 200 µL multichannel pipette, add 43 µL Elution Buffer (EB) to the wells in each row (12 wells) of the plate. Use fresh tips for each row to avoid cross-contamination.
5. Pour 11 mL of AMPure XP Beads into a new reagent reservoir.
6. Using a 12-channel, 200 µL multichannel pipette, add the following volume of beads to each row:

   Mechanically sheared library: Add 80 µL of AMPure XP Beads **OR**

   Enzymatically sheared library: Add 100 µL of AMPure XP Beads

   and pipet up and down 10 times to mix, and then discard tips. Avoid introducing air bubbles. Reload multichannel pipette with fresh tips.
7. Repeat Step 6 seven more times to complete dispensing of the AMPure XP Beads across the entire plate.

8. Incubate the plate 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. Pour 40 mL of fresh 70% ethanol into a new reagent reservoir.
11. Wash the beads with 70% Ethanol as follows. Wash the beads in each row one at a time to prevent over drying of the beads:
    a. Set a 12-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 row and discard the tips.
    b. Using fresh tips, carefully add 200 µL of 70% ethanol to the row without disturbing the beads. Discard the tips and proceed to the next row.
    c. Repeat 11a-b until all the rows of the plate contain 70% ethanol (1st wash).
    d. Using fresh tips, carefully aspirate the first ethanol wash from the first row and discard the tips.
    e. Using fresh tips, carefully add 200 µL of 70% ethanol to the first row without disturbing the beads and discard the tips.
    f. Repeat Steps 11d-e until all the rows of the plate contain 70% ethanol (2nd wash).
12. Using a 12-channel, 200 µL multichannel pipette with fresh tips for each row, aspirate the ethanol from all rows.
13. Using a 12-channel, 20 µL multichannel pipette with fresh tips for each row, remove any residual ethanol that collects in the bottom of the wells. Start a 5 minute timer at the aspiration of the first row. Let each row of pellets air dry for 5 minutes (no more) while still in the magnetic plate.
14. Pour 3 mL of Elution Buffer (EB) into a new reagent reservoir.
15. Remove the plate from the magnetic plate.
16. Using a 12-channel, 200 µL multichannel pipette, add 25 µL of Elution Buffer (EB) per well of each row and thoroughly resuspend the AMPure XP Beads in the Elution Buffer by pipetting up and down 10 times (Do not vortex).
17. Repeat Step 16 seven times using fresh tips for each row until all rows are resuspended.
18. Incubate the plate at 37C° for 5 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. Using a 12-channel, 20 µL multichannel pipette and being careful not to disturb the beads, transfer 20 µL of supernatant from the 1st row to the 1st row of the **prepared PCR Primer Plate** (containing Dual Index Primers) generated in the previous section. Using fresh tips for each row, continue transferring the supernatants from each row to corresponding row of the new PCR Primer Plate.

Optional/Safe Stopping Point. Cleaned, Ligated DNA can be stored at -20°C in 96-Well PCR Low Bind Plate.

PCR Amplification (Required)

1. Aliquot 230 µL of the PCR Master Mix (MM) using a single channel pipette into each tube of a 12-tube 0.2 mL PCR strip tube, which acts as a reservoir.

2. Using a 12-channel, 200 µL multichannel pipette, add 25 µL of Master Mix (MM) per well to the first row of the **PCR Plate containing the bead-cleaned libraries from step 20 above**. Gently pipette up and down 10 times to mix. Discard the tips and reload multichannel pipette with fresh tips.
3. Repeat Step 2 seven times until the PCR Master Mix has been added to all rows of the plate.
4. Carefully apply an adhesive plate seal and briefly spin the plate in a benchtop centrifuge to collect material in the bottom of the wells.
5. Place the plate in a thermocycler and cycle as follows:

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

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

Starting DNA Input Amount	Number of Cycles (X)
50 pg – 100 pg	17
101 pg – 250 pg	15
251 pg – 500 pg	14
501 pg – 750 pg	13
751 pg – 1 ng	12
2 ng – 10 ng	9
11 ng – 25 ng	7
26 ng – 50 ng	6
51 ng – 75 ng	5

Optional/Safe Stopping Point. PCR Amplified DNA can be stored at -20°C in 96-Well PCR Low Bind Plate.

PCR Cleanup (Required)

Notes:

- Ensure AMPure XP Beads, Elution Buffer (EB) and fresh 70% ethanol are all equilabrated to room temperature before proceeding with PCR Cleanup.
- Perform the following PCR Cleanup steps at room temperature unless specified otherwise.

1. Briefly spin the plate in a benchtop centrifuge to collect material in the wells. Carefully remove the adhesive plate seal to avoid splashing and cross-contamination.
2. Vortex the AMPure XP Beads to completely resuspend the beads.
3. **For mechanically sheared DNA only** (for enzymatically fragmented DNA, skip to Step 4):
    a. Pour 2.5 mL of Elution Buffer (EB) into a new reagent reservoir
    b. Using a 12-channel, 200 µL multichannel pipette, add 20 µL Elution Buffer to the wells in each row (12 wells) of the plate. Use fresh tips for each row to avoid cross-contamination.
4. Pour 6 mL of AMPure XP Beads into a new reagent reservoir.
5. Using a 12-channel, 200 µL multichannel pipette, add 50 µL of AMPure XP Beads to each well in the first row (12 wells) of the plate; pipet up and down 10X to mix, and then discard tips. Avoid introducing air bubbles. Reload multichannel pipette with fresh tips.

6. Repeat Step 5 seven more times to complete dispensing of the AMPure XP Beads across the entire plate.
7. Incubate the plate on the bench at room temperature for 5 minutes.
8. Pour 40 mL of fresh 70% ethanol into a new reagent reservoir.
9. Place the plate in a magnetic plate for 5 minutes (until the supernatant becomes clear).
10. Wash the beads with 70% Ethanol as follows. Wash the beads in each row one at a time to prevent over drying of the beads:
    a. Set a 12-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 row and discard.Discard the tips.
    b. Using fresh tips, carefully add 200 µL of 70% ethanol to the row without disturbing the beads. Discard the tips and proceed to the next row.
    c. Repeat 10a-b until all the rows of the plate contain 70% ethanol (1st wash).
    d. Using fresh tips, carefully aspirate the first ethanol wash from the first row and discard the tips.
    e. Using fresh tips, carefully add 200 µL of 70% ethanol to the first row without disturbing the beads and discard the tips.
    f. Repeat Steps 10d-e until all the rows of the plate contain 70% ethanol (2nd wash).
11. Using a 12-channel, 200 µL multichannel pipette with fresh tips for each row, aspirate the ethanol from all rows.
12. Using a 12-channel, 20 µL multichannel pipette with fresh tips for each row, remove any residual ethanol that collects in the bottom of the wells. Start a 5 minute timer after the aspiration of the first row. Let each row of pellets air dry for 5 minutes (no more) while still in the magnetic plate.
13. Pour at 11 mL of Elution Buffer (EB) into a new reagent reservoir.
14. Remove the plate from the magnetic plate.
15. Using a 12-channel, 200 µL multichannel pipette, add 105 µL of Elution Buffer (EB) per well of each row and thoroughly resuspend the AMPure XP Beads in the Elution Buffer by pipetting up and down 10 times. (Do not vortex).
16. Repeat Step 15 seven times using fresh tips for each row until all rows are resuspended.
17. Incubate at 37°C for 5 minutes; do not use a magnetic plate during this incubation.
18. Place the reaction plate on a magnetic plate for 5 minutes (until the supernatant becomes clear).
19. Using a 12-channel, 200 µL multichannel pipette and being careful not to disturb the beads, transfer 100 µL of supernatant from the 1st row to the 1st row of a new 96-well PCR Low Bind Plate. Using fresh tips for each row, continue transferring the supernatents from each row to corresponding row of the new PCR Low Bind Plate.
20. Proceed directly to Size Selection.

Optional/Safe Stopping Point. DNA can be stored at -20°C in 96-Well PCR Low Bind Plate after PCR Cleanup Step.

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).
- Ensure AMPure XP Beads, Elution Buffer (EB) and fresh 70% ethanol are all equilibrated to room temperature before proceeding with Size Selection.
- Perform all the following Size Selection steps at room temperature unless specified otherwise.

1. Vortex the room temperature-equilibrated AMPure XP Beads to resuspend them.
2. Pour 11 mL of AMPure XP beads into a new reagent reservoir.
3. Using a 12-channel, 200 µL multichannel pipette, add the following volume of beads to each row:

   Mechanically sheared library: Add 70 µL of AMPure XP Beads **OR**

   Enzymatically sheared library: Add 100 µL of AMPure XP Beads

   and pipet up and down 10 times to mix, and then discard tips. Avoid introducing air bubbles. Reload multichannel pipette with fresh tips.
4. Repeat Step 3 seven more times to complete dispensing of the AMPure XP Beads across the entire plate.
5. Incubate the plate on the bench at room temperature for 5 minutes.
6. Place the plate onto a magnetic plate for 5 minutes (until the supernatant becomes clear).
7. Pour 40 mL of fresh 70% ethanol solution into a 50-mL reagent reservoir.
8. Wash the beads with 70% Ethanol as follows. Wash the beads in each row one at a time to prevent over drying of the beads:
   a. Set a 12-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 row and discard the tips.
   b. Using fresh tips, carefully add 200 µL of 70% ethanol to the row without disturbing the beads. Discard the tips and proceed to the next row.
   c. Repeat 8a-b until all the rows of the plate contain 70% ethanol (1st wash).
   d. Using fresh tips, carefully aspirate the first ethanol wash from the first row and discard the tips.
   e. Using fresh tips, carefully add 200 µL of 70% ethanol to the first row without disturbing the beads and discard the tips.
   f. Repeat Steps 8d-e until all the rows of the plate contain 70% ethanol (2nd wash).
9. Using a 12-channel, 200 µL multichannel pipette with fresh tips for each row, aspirate the ethanol from all rows.
10. Using a 12-channel, 20 µL multichannel pipette with fresh tips for each row, remove any residual ethanol that collects in the bottom of the wells. Start a 5 minute timer after the aspiration of the first row. Let each row of pellets air dry for 5 minutes (no more) while still in the magnetic plate.

11. Pour 3 mL of Elution Buffer (EB) into a reagent reservoir.
12. Remove the plate from the magnetic plate.
13. Using a 12-channel, 200 µL multichannel pipette, add 25 µL of Elution Buffer (EB) per well of each row and thoroughly resuspend the AMPure XP Beads in the Elution Buffer by pipetting up and down 10 times. (Do not vortex).
14. Repeat Step 13 seven times using fresh tips for each row until all rows are resuspended.
15. Incubate the plate 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 clears).
17. Using a 12-channel, 20 µL multichannel pipette and being careful not to disturb the beads, transfer 20 µL of supernatant from the 1st row to the 1st row of a new 96-well PCR Low Bind Plate. Using fresh tips for each row, continue transferring the supernatants from each row to corresponding row of the new PCR plate.
18. Proceed to Analysis or cover plate with an adhesive plate seal and store at -20°C.

    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 20 µL multichannel pipette set at 15 µL. Use new pipet tips for each sample to avoid cross-contamination of the libraries.

Analysis

1. Quantify the size-selected libraries with a Qubit Fluorometer, following the manufacturer's instructions.
2. Analyze 1 µL of final libraries on a Bioanalyzer High Sensitivity Chip to determine size distribution. Ideally, the peak should center on 420 bp for a 300 bp mechanically sheared insert. See Figure 4 for a typical Bioanalyzer trace of a library generated using gDNA initially sheared to 300 bp and Figure 5 for a typical trace of a library generated with enzymatically fragmented gDNA. The enzymatic fragmented library peak should center around 200-300 bp.

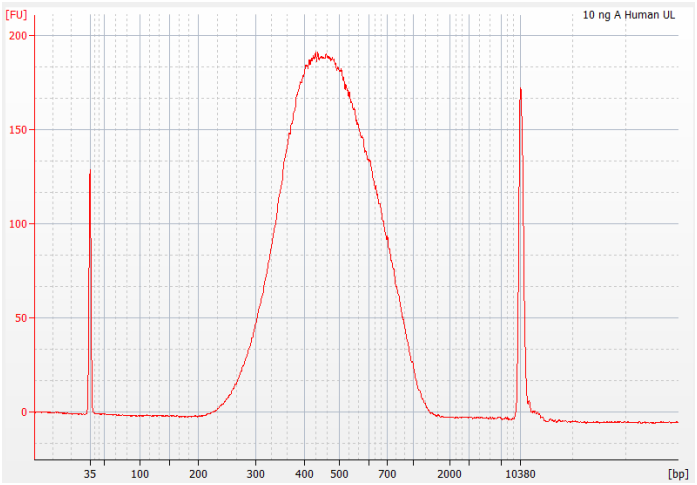


Figure 4: Typical Bioanalyzer trace for library generated using input gDNA sheared mechanically to 300 bp.

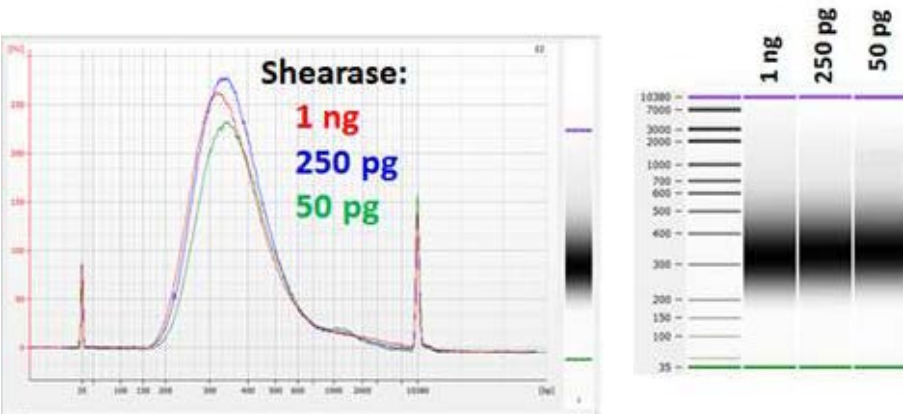


Figure 5: Typical Bioanalyzer trace for final libraries generated using input gDNA sheared enzymatically.

Sequencing

- Final libraries are compatible with Illumina sequencers and can be run on any Illumina platform.
- Lucigen recommends using PhiX Sequencing Control according to the recommendations provided by Illumina for the sequencer being used.

Appendix A: NxSeq® Universal Adaptor Dilution Guidelines

This table describes the dilution of the Universal Adaptor based on the starting amount of DNA and provides enough diluted adaptor for one full 96-well plate. If you are making libraries using different amounts of input DNA that require different dilutions of Universal Adaptor, please scale down the final volumes of diluted Universal Adaptor as necessary.

For optimal results, the Universal Adaptor must be diluted in the provided Adaptor Dilution Buffer.

DNA input	Adapter Dilution	Adapter Volume (µL)	Dilution Buffer Volume (µL)
50 pg – 250 pg	1:60	6.5	383.5
251 pg – 1 ng	1:30	13	377
2 ng – 75 ng	No dilution	-	-

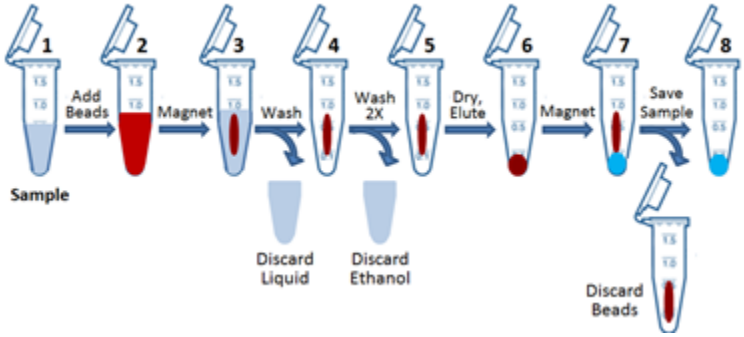
Appendix B: Index Sequences

The 8 base primer dual index sequences are listed below.

PCR Primer	Index Sequence
Primer 501	TATAGCCT
Primer 502	ATAGAGGC
Primer 503	CCTATCCT
Primer 504	GGCTCTGA
Primer 505	AGGCGAAG
Primer 506	TAATCTTA
Primer 507	CAGGACGT
Primer 508	GTACTGAC
Primer 701	ATTACTCG
Primer 702	TCCGGAGA

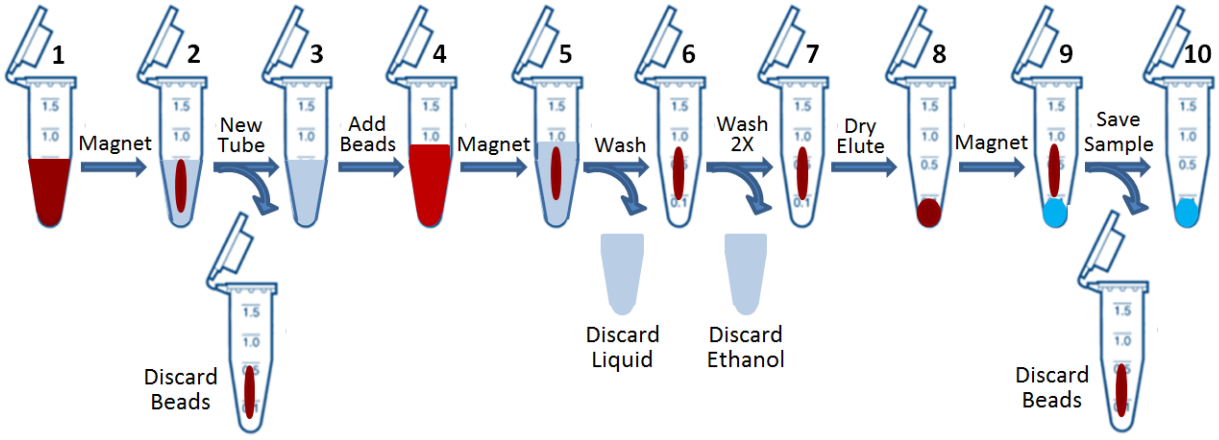
PCR Primer	Index Sequence
Primer 703	CGCTCATT
Primer 704	GAGATTCC
Primer 705	ATTCAGAA
Primer 706	GAATTCGT
Primer 707	CTGAAGCT
Primer 708	TAATGCGC
Primer 709	CGGCTATG
Primer 710	TCCGCGAA
Primer 711	TCTCGCGC
Primer 712	AGCGATAG

Appendix C: Bead Cleanup Overview



Bead cleanup workflow illustrated for a single tube. 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 D. Double Bead Size Selection (Upper & Lower ends) Overview



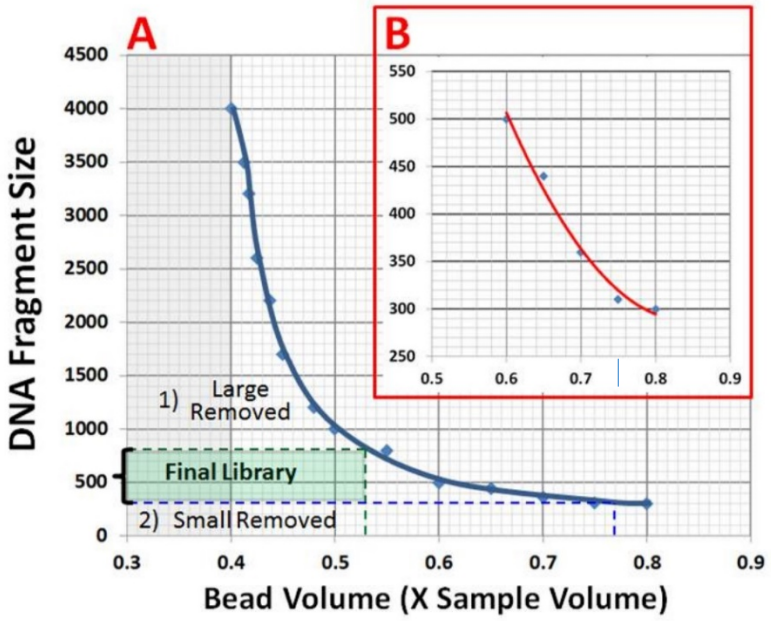
Size Selection Workflow Briefly: 1) Add beads to sample and mix (the bead solution must be equilibrated to room temperature prior to use to assure accurate pipetting) ; 2) Place tube on magnetic

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.

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).



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.